12-2022

# Effect of Metal Ions and Temperature on Stability of Thiamine Determined by HPLC

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EFFECT OF METAL IONS AND TEMPERATURE ON STABILITY OF THIAMINE
DETERMINED BY HPLC

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Food, Nutrition, and Culinary Sciences

by
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December 2022

Accepted by:
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ABSTRACT

Thiamine degradation occurs during storage and transportation for short and long periods due to the exposure to several factors, such as heat, oxidation-reduction reactions, and alkali. In this study, the effects of four metal ions (i.e., $Cu^+$, $Cu^{2+}$, $Fe^{2+}$, and $Fe^{3+}$) on thiamine stability in aqueous solutions at three temperatures (i.e., 25, 40, and 55°C) for the storage time of 7 days were discussed. Thiamine degradation was found to follow the first-order kinetic reaction, and the degradation rate could also be estimated. The factors in influencing thiamine stability included pH values, temperatures, and chemical properties of metal chlorides.

High-performance liquid chromatography (HPLC) equipped with an Eclipse XDB C18 column (4.6 mm x 150 mm, 5 $\mu$m) was used to analyze thiamine contents. The method was operated in a gradient elution program, which comprised solvents A (0.1M ammonium acetate was adjusted to pH = 5.8 by 0.1% acetic acid) and solvent B (acetonitrile in HPLC grade) at the flow rate of 1 mL min$^{-1}$ up to 5 mins. The column was maintained at 30°C, and the UV detector was set at a wavelength of 254 nm. The pH values of the samples were also monitored during the entire storage time.

At 25°C, the highest loss of thiamine (64.00%) was in 50 mg L$^{-1}$ of CuCl solution, while the lowest loss of thiamine (78.34%) was in 50 mg L$^{-1}$ of $FeCl_3$ solution. At 40°C, the highest loss of thiamine (59.76%) was in 50 mg L$^{-1}$ of $FeCl_2$ solution, while the lowest loss of thiamine (91.48%) was in 50 mg L$^{-1}$ of $FeCl_3$ solution. At 55°C, the highest loss of thiamine (61.94%) was in 50 mg L$^{-1}$ of CuCl solution, while the lowest loss of thiamine (95.98%) was in 50 mg L$^{-1}$ of $FeCl_3$ solution.

DEDICATION

I would like to dedicate this thesis to my parents, Yan Jyi Huang and Li Chuan Huang, my brother Jhong Yan Huang, and my grandmother. Without their endless love, support, and encouragement, all the research and writing work would not have been finished during this long journey.

# ACKNOWLEDGMENTS

Firstly, I would like to send my sincere gratitude and appreciation to my advisor, Dr. Feng Chen, for offering me an opportunity to study at Clemson University and guiding me to finish my research project in his lab. With his immense knowledge, endless patience, and continuous encouragement, I could complete the MS degree study and the challenging thesis work. Besides my major advisor, I would like to thank my committee members, Dr. Kurt Young and Dr. Jun Luo, for their insightful opinions, helpful guidance, and encouragement through my research study.

My sincere thankfulness also goes to my senior lab mate, Jack Huang, for teaching me how to operate HPLC and other equipment and brainstorming the research with me. His valuable experiences in Food Chemistry and Analysis strengthen my research. During my time at Clemson, I was enjoyable and made friends with many students. I want to thank them for accompanying me through good times and hard times.

Finally, my deepest gratitude goes to my family in Taiwan. I am grateful for the unconditional support and the considerable sacrifices that they made on my behalf. I also want to thank all my sincere friends in Taiwan with whom I can share my life. This long journey at Clemson would not have been possible and wonderful if not with all of them.

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CHAPTER ONE

LITERATURE REVIEW

## 1.1 Introduction of vitamins

Vitamins can be divided into water-soluble vitamins and fat-soluble vitamins, respectively. The former can be obtained from various foods, and their excess amounts are excreted from humans' bodies, while the latter can be stored in fatty tissues and livers in humans' bodies (Stevens, 2021).

Water-soluble vitamins are organic compounds, which can often be obtained from fruits, vegetables, and beverages (Vállez-Gomis et al., 2021). Water-soluble vitamins consist of B vitamins, which include thiamin ($B_1$), riboflavin ($B_2$), niacin ($B_3$), pantothenic acid ($B_5$), pyridoxine ($B_6$), biotin ($B_7$), folate ($B_9$), and cyanocobalamin ($B_{12}$), and vitamin C. They often act as coenzymes involved in many biochemical reactions, such as energy metabolism, biosynthesis of amino acids, simple sugars and fatty acids, DNA synthesis, and antioxidant reactions (Yaman et al., 2021). Although nutritious foods contain abundant vitamins, vitamins are subject to loss during food processing, such as cleaning, cooking, and frying, and may be degraded in storage. The following factors, including pH value, temperature, moisture content, presence of oxygen, and exposure to light, have significant impacts on the stability of vitamins. Among the water-soluble vitamins, thiamin ($V_{B1}$), folate ($V_{B9}$), and vitamin C are subject to degradation, while niacin ($V_{B3}$) and biotin ($V_{B7}$) are relatively stable (Ball, 2004).

Although deficiencies of B vitamins are not common globally, their deficiencies can lead to minor or severe symptoms. Because humans cannot synthesize B vitamins by themselves, sufficient B vitamins must be obtained through the diet. Fortunately, plants can serve as the

primary sources of dietary essentials for humans since plants can synthesize most vitamins (Ball, 2004).

In comparison, fat-soluble vitamins include vitamins A, D, E, and K, which, after their absorption and circulation in the human body, can be transported to the adipose tissues or the liver for storage and use (Stevens, 2021). The fat-soluble vitamins are involved in the body for maintaining homeostasis. For example, vitamin A is essential for vision, immune function, and regulation of cell growth. Retinol, one of the vitamin A family, which is used to treat vitamin A deficiency, is available in liver, flesh foods, eggs, and fortified milk (Kohlmeier, 2015). Vitamin D can be synthesized in the skin by exposure to sunlight and controls the regulation and distribution of calcium and phosphorus in the bones and cells. The good dietary sources of vitamin D include fatty fish and fortified milk (Kohlmeier, 2015; Stevens, 2021). Vitamin E is a natural antioxidant with anti-inflammatory functions, which can protect cells from damage caused by free radicals in the body. The primary form of vitamin E in the plasma is alpha-tocopherol (Stevens, 2021). Wheat germ, sunflower oils, and nuts are excellent dietary sources of vitamin E. Vitamin K can combine with specific proteins, playing an essential role in blood coagulation, counteracting the calcification of arteries and other soft tissues, promoting the mineralization of bone, and regulating cell division. The absorption of vitamin K requires interaction within the pancreas, bile, and fat components (Stevens, 2021). Cooked spinach, kale, and other green leafy vegetables are helpful in achieving an adequate intake of 120 µg for adult men and 90 µg for adult women (Kohlmeier, 2015). For example, a single serving of one cup (67 g) of chopped raw kale contains 472.22 g of vitamin K, which is much more than the required amounts of around 100 µg for mature adults.

1.1.1 Bioaccessibility of vitamins

Bioaccessibility of nutrients means the bioactive chemicals are released from the food substances before they are absorbed by the small intestines. The bioaccessible amounts of water-soluble vitamins can fluctuate in the gastrointestinal tract depending on the conditions of various pH, temperature, their connection with polypeptides or polysaccharides bonds, and the presence of enzymes and metal ions. Bioavailability of vitamins refers to the number of vitamins ingested in the intestine. It depends on the chemical form and physical state of the vitamin in the foods. Therefore, food matrix can affect the absorption of vitamins (Uğur et al., 2020; Yamen et al., 2021; Ball, 2006).

However, some factors may negatively affect the bioaccessibility of vitamins. For example, ascorbic acid (vitamin C) is easily degraded during digestion because of the existence of oxygen and alkaline pH in small intestines (Pathy, 2018). Exposure to light, canning, cooking, and metal (e.g., copper and iron) ions can also lead to significant losses of vitamin C (EI-Ishaq and Obirinakem, 2015). Besides, folates (vitamin $B_9$) and folic acid are susceptible to photochemical degradation. Other factors, such as decreasing pH, increasing temperatures, adding sodium nitrates, and the presence of metal ions ($Fe^{2+}$), cause the loss of folates, too (Yamen et al., 2021). In addition, the folate contents would reduce during cereal processing, such as milling, debranning, soaking, and cooking. As a result, the populations in West Africa who primarily rely on cereal-based fermented foods would be subject to a risk of folate deficiency (Bationo et al., 2020).

1.1.2 Dietary Reference Intakes (DRIs) of water-soluble vitamins

Dietary References Intakes (DRIs) are reference values that are quantitative estimates of nutrient intakes to plan and assess diets for healthy people (Yates et al., 1998), which are often represented by recommended dietary allowance (RDA) and adequate intake (AI) (IOM, 1998; Yates et al., 1998). **Table 1.1** shows the RDA and AI values of water-soluble vitamins. For

example, the RDAs of vitamin C are 75 mg for adult females and 90 mg for adult males, and the RDAs of thiamine (vitamin $B_1$) are between 1.1 and 1.4 mg per day for adult males and females during pregnancy and lactation (IOM, 1998; IOM, 2000). In comparison, the RDI values of water-soluble vitamins are shown in **Table 1.2**, which also lists the Tolerable Upper Intake Levels (Uls) that represent the maximum amounts of nutrients that can be consumed by human individuals daily without any adverse health effects (IOM, 1998). For example, the daily Uls of niacin and vitamin $B_6$ for adults are between 30-35 mg and between 80-100 mg, respectively. However, the Uls for thiamine, riboflavin, pantothenic acid, biotin, and vitamin $B_{12}$ are unavailable due to a lack of data on adverse effects on males and females of different ages (IOM, 1998; IOM, 2000).

### 1.2 Thiamine (vitamin $B_1$)

Thiamine, also known as vitamin $B_1$, is one of the water-soluble vitamins. This micronutrient is commonly available in the human diet because many natural foods and fortified food products contain thiamine. The natural sources of thiamine include sunflower seed, nuts, pulses, legumes, oat, brown rice, whole grain rye, cauliflower, potatoes, asparagus, flax, liver, egg, and meats (Shrivas et al., 2018). Especially, yeast and cereal are rich in thiamine for humans (Jakobsen, 2008). Thiamine often serves as a coenzyme for the metabolism of carbohydrates and branched-chain amino acids, thus playing an essential role in digestion, the nervous system, and muscle contraction in human bodies (Voelker et al., 2018).

1.2.1 The history of thiamine

In 1890, Eijkman, a Dutch medical officer in Java, discovered chickens fed with polished rice induced polyneuritis, similar to the human beriberi. He concluded that adding rice bran as a

supplement to the avian diet could prevent the disease. After a couple of years, Eijkman's successor, Gerrit Grijns, who was also a Dutch researcher, extracted a water-soluble chemical from rice bran, which was considered as a preventive factor for polyneuritis. He concluded that a dietary lack of an essential nutrient could contribute to beriberi (Ball, 2004). In addition, Jansen and Donath, two Dutch chemists, successfully isolated vitamin $B_1$ in crystalline form from rice bran extracts (Jansen and Donath., 1926). By 1936, Robert R. Williams had illustrated the structure of vitamin $B_1$ and accomplished its chemical synthesis (Williams., 1938). In the early 1930s, Sir Rudolph Peters and his colleagues discovered the essential role of thiamine in pyruvate metabolism and the strong association of thiamine with the science of oxidative metabolism (Peters., 1936; Lonsdale and Marrs., 2017). Further, Lohmann and Schuster discovered that dephosphorylated thiamine derivative (thiamine diphosphate, TDP) was an active coenzyme form of thiamine for the oxidative decarboxylation of pyruvate (Lohmann and Schuster., 1937; Ball., 2004).

1.2.2 Nomenclature and structure

Vitamin $B_1$ is a nonspecific generic term. Instead, the name thiamine, or individual phosphates of thiamine, is used as a specific term. Total thiamine means the sum of thiamine and its phosphates (Ball, 2004). The thiamine molecule comprises substituted pyrimidine (2,5-dimethyl-6-aminopyrimidine) and thiazole moieties (4-methyl-5-hydroxy ethyl thiazole) linked by a methylene bridge (**Figure 1.1**). Thiamin is rapidly phosphorylated upon entry into the cell (Combs and McClung, 2017). Except for its free form, three forms of thiamine exist, including thiamine monophosphate (TMP), thiamine diphosphate (TDP), and thiamine triphosphate (TTP), respectively (**Figure 1.2**).

Firstly, thiamine monophosphate (TMP) appears to be biologically inactive, and it might not only be the final product of the biosynthesis of free thiamine in organisms such as yeasts but also

be a product of TDP degradation in animal tissues (Ball, 2004; Bettendorff and Wins, 2009; Schmidt et al., 2017). Secondly, thiamine diphosphate (TDP), also named thiamine pyrophosphate (TPP), is the most abundant thiamine compound in most eukaryotic tissues, and it is a metabolically active form sometimes referred to as cocarboxylase since it performs as a coenzyme in carbohydrate metabolism, the pentose-phosphate pathway, and the tricarboxylic acid cycle (Frank, Leeper and Luisi, 2007). Thirdly, thiamine triphosphate (TTP) is regarded as a messenger molecule rather than an enzymatic cofactor. In one study, it was suggested TTP might be a signal produced to respond to the bacteria's changes in the nutritional environment (Bettendorff and Wins, 2009; Schmidt et al., 2017). **Figure 1.3** shows the metabolic activation of thiamine in peripheral tissues (Combs and McClung, 2017).

Over 90% of thiamine is phosphorylated in most animal tissues. Adult humans store only 30 – 50 mg of TDP in skeletal muscle, heart, brain, liver, and kidneys. TDP is the primary form of intracellular thiamine, accounting for 70% to 90% of total thiamine, while TMP and free thiamine usually account for 5% to 15%. Thiamine in the phosphorylated form can be hydrolyzed to free thiamine in the intestinal lumen by various phosphatases, such as alkaline phosphatase (Yamen et al., 2021). In addition, pigs have relatively high tissue storage of thiamine. Pig skeletal muscle and chicken skeletal white muscle comprise 70% – 80% TTP of the total thiamine present (Ball, 2004).

1.2.3 Physical and chemical properties

Thiamine is sensitive to heat, alkali, oxygen, radiation, sulfites, and the food matrix (Schmidt et al., 2017). Firstly, mild oxidation can yield thiamine disulfide, while more dynamic oxidation with alkaline potassium hexacyanoferrate (III) ($K_3Fe(CN)_6$) can produce the biologically inactive thiochrome (Ball, 2006). Thiochrome (THC) is a product of thiamine oxidation with strong fluorescence. It can be degraded after exposure to UV light to form oxodihydrothiochrome

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(ODTHC), another fluorescent compound. The photolysis of THC is affected by pH, and an increase in pH can improve the reaction rate (Anwar et al., 2020). **Figure 1.4** shows the scheme of thiamine to thiochrome during oxidation (Yamabe, Tsuchida and Yamazaki, 2021). Secondly, when it comes to the reaction between thiamine and sulfites, the latter can attack the methylene bridge and cleave the bond of the former. Thus, sulfites in food preservatives that are used to preserve the color of various food products, such as juice, dehydrated fruits and vegetables, and canned green beans and peas (Ball, 2006), may affect the stability of thiamine (Kaur et al., 2019).

### 1.2.4 Stability and degradation

#### 1.2.4.1 Temperature, pH value, and moisture

Thiamine is one of the water-soluble vitamins and heat-labile at high temperatures. Its loss varies widely according to different thermal processing and type of foods (Lešková et al., 2006). For example, thiamine contents in grain-cereal products, such as barley, buckwheat groats, and millet, were found to decrease during roasting. Its content in roasted buckwheat groats was lower by about 60% than in unroasted buckwheat groats (Lebiedzińska and Szefer, 2006). Compared to whole grain, thiamine is significantly lower in the processed cereal products. For example, the dark-colored malts contained markedly lower levels of thiamine after the roasting process up to 230 °C in 80 mins, and the same authors found the most thermal destruction occurred within the first half of the process, which was at around 150 °C (Hucker, Wakeling and Vriesekoop, 2012). In another study, the thiamine contents in four canned vegetable products, including white asparagus, lentils, peeled tomatoes, and mushrooms, decreased during the canning process at 116°C for at least 25 minutes, from the lowest retention at 51% DW (dry weight basis) in mushrooms to the highest retention at 74% DW in white asparagus (Martín-Belloso and Llanos-Barriobero, 2001). Similarly, the thermal losses of thiamine frequently occur in meat products

during the cooking process, such as boiling, braising, roasting, and stewing. Stewing can cause the highest thiamine loss in meat, around 43-65% (Farrer, 1955).

In addition, pH is a crucial factor in affecting the stability of thiamine. Thiamine is stable in acidic conditions between pH 2.0 to 4.0. On the contrary, an alkaline environment would promote the loss of thiamine. For example, baking powder can destroy over 50% of the original thiamine content in flour during making a cake (Ball, 2006). The presence of water also negatively affects the stability of thiamine in the solid-state. The rates of thiamine degradation would increase under conditions with higher relative humidity or water activity (Schmidt et al., 2017). For instance, it was found that both thiamine mononitrate and thiamine hydrochloride in a semolina dough system were less stable at $a_w$ of 0.86 than at $a_w$ of 0.58 (Labuza and Kamman, 1982).

1.2.4.2 Reducing sugars and amino acids

The concentration and types of reducing sugars were found to be able to affect the thiamine stability too. One study showed xylose (pentose) led to the fastest rate of thiamine destruction, followed by glucose (hexose) and maltose (reducing disaccharide). The possible reason for this phenomenon is that pentoses are more reactive than hexoses and reducing disaccharides because the amounts of open chain structures are in the following order: pentose > hexose > reducing disaccharides (Doyon and Smyrl, 1983; deMan et al., 2018). Similarly, amino acids can influence the stability of thiamine. A study showed that cystine acted to catalyze thiamine degradation rather than stabilize thiamine, with a nonlinearity of increase in the thiamine degradation with the rising titer of cystine, for which the calculated catalytic constant was 2.08However, tyrosine did not exhibit catalytic activity in the decomposition of thiamine (McIntire and Frost, 1944; Windheuser and Higuchi, 1963).

1.2.5 Thiamine salts

Thiamine mononitrate (TMN) and thiamine hydrochloride (THCl) are two salt forms served as food additives. **Figure 1.5** shows the structures of TMN and THCl. Thiamine mononitrate (TMN) is a mono-salt with the presence of one nitrate anion, with a molecular formula $C_{12}H_{17}N_4OS \bullet NO_3$. Its activation energy is 26.3 kcal/ mol. Due to its low hygroscopicity, TMN is generally used to fortify dry food products. In comparison, THCl is a salt with two chlorides, with a molecular formula $C_{12}H_{17}ClN_4OS \bullet HCl$. Its activation energy is 22.4 kcal/ mol. Due to higher free energy from the crystalline salt form, THCl has a high solubility in water, and is commonly added in liquid applications or beverage products. **Table 1.3** shows the properties of two thiamine salts in the solid state (Labuza and Kamman, 1982; Hiatt et al., 2008; Voelker et al., 2018). Thiamine salts dissociate in solution to become the thiamine cations and corresponding anions. According to the study, the range of pH values in different concentrations of TMN solutions was from 5.36 to 6.96, while the range of pH values in THCl was from 1.12 to 3.59. Therefore, the thiamine degradation in THCl was slower than in the TMN solutions since thiamine is stable in an acidic environment (Voelker et al., 2018).

1.2.6 Deficiency, toxicity, and supplementation

1.2.6.1 Deficiency

Lack of proper food consumption and disturbance in absorption and metabolic processes, such as biotransformation and excretion, may cause thiamine deficiency, particularly occurring in developing countries. Although rare cases of thiamine deficiency arise in developed countries because the fortified foods with thiamine are widely available, thiamine deficiency may also be found in people with alcoholism, people who strictly follow weight-loss diets, and people who avoid consuming fortified grain products, including the patients with Celiac disease (Voelker et al., 2018). On the other hand, people who stick to a diet high in unfortified grains such as polished

rice are more likely subject to thiamine deficiency because the milling process causes the significant loss of vitamin $B_1$ even though cereal and cereal products are rich in the vitamin. (Ball, 2004; Voelker et al., 2018). Thus, thiamine enrichment in white flour, breakfast cereals, and polished rice is typical in several countries (Ball, 2004).

Thiamine deficiency causes a disturbance in carbohydrate metabolism and then leads to damage to mitochondria and increased oxidative stress. It is strongly related to brain disorders and other neurodegenerative disorders associated with alcohol consumption (Gonçalves, Soldi and Portari, 2020). **Table 1.4** shows general signs of thiamine deficiency. Many signs are nonspecific, familiar, and frequently overlooked in the early phases of deficiency, including anorexia, weight loss, muscle weakness, mental changes, fatigue, insomnia, irritability, and other neurological indicators. Serious diseases might happen in patients with a long-term thiamine deficiency (Lonsdale and Marrs, 2017; Combs and McClung, 2017), such as beriberi and Wernicke-Korsakoff syndrome (Ball., 2004; Combs and McClung, 2017; Voelker et al., 2018).

1.2.6.2 Toxicity

Thiamine is generally well-tolerated, and it is non-toxic by the oral route because an excess of ingested thiamine can be promptly excreted through the urine (Ball, 2004). Thiamine has no tolerable upper intake levels because no evidence shows adverse effects on humans (Strohm et al., 2016). Therapeutic doses as great as 300 mg/day are used therapeutically to treat the disease of thiamine deficiency, such as beriberi and Wernicke–Korsakoff syndrome, without adverse reactions. However, greater doses have produced allergic reactions, headaches, convulsions, weakness, paralysis, and cardiac arrhythmia (Combs and McClung, 2017). Moreover, it has been reported that a sizeable parental dose of thiamine administered for a long time led to clinical manifestation and death in some cases (Cumming, 1981).

### 1.2.6.3 Supplementation

Patients with thiamine deficiency, such as dry beriberi and wet beriberi, require thiamine supplementation. **Table 1.5** shows the recommended dosages in several cases. In dry beriberi, the daily dose of thiamine ranges from 10-20 mg for two weeks for mild neuropathy and up to 20-30 mg for several weeks for more severe neuropathy. Patients should take 100 mg per day for several days in wet beriberi. Thiamine supplementation should be considered prophylactically before the occurrence of symptoms. Patients at risk for developing thiamine deficiency should take 100 mg of thiamine three times daily, and patients in cases of proven deficiency should take 200 mg of thiamine three times daily (DiNicolantonio et al., 2013).

The daily recommended daily allowance (RDA) is 1.1-1.2 mg. The requirement for thiamine varied with the proportion of carbohydrates in the diet since thiamine pyrophosphate (TPP) is essential as a coenzyme for the released energy from carbohydrates. Furthermore, people should increase their intake during high muscular activity, pregnancy, lactation, protracted fever, and hyperthyroidism. (Ball, 2004).

### 1.2.7 Physiology

### 1.2.7.1 Absorption and transport

Phosphorylated dietary forms of thiamine release free thiamine through the gut, and the free thiamine is absorbed by two mechanisms: active transport and passive diffusion. Active transport occurs at low luminal concentrations (lower than 2 µM), and it is located in the apical brush border of the mucosal epithelium. Passive diffusion occurs at higher concentrations, such as a 2.5 mg dose for a person.

When entering the cells, thiamine is promptly phosphorylated. Besides, most thiamine is present in phosphorylated forms in the intestinal mucosa, while thiamine is mainly present in the

free (non-phosphorylated) monovalent cation in the serosal side intestine. The movement of thiamine goes with the phosphorylation and dephosphorylation through the mucosal cell. Most thiamine in serum is non-specifically bound to protein, chiefly albumin, and 90% of the total thiamine in the blood is contained in erythrocytes. Therefore, 70-90% of intracellular thiamine is thiamine diphosphate (TDP), 90% of which is bound to proteins (Combs and McClung, 2017).

On the other hand, ethanol reduces the absorption of thiamine and impairs the metabolism of thiamine, and interrupts several metabolic processes associated with TDP, which is the biologically active form of thiamine (Rindi, Imarisio and Patrini, 1986; Gonçalves, Soldi and Portari, 2020; Yaman et al., 2021).

The anti-thiamine activity also negatively affects the absorption of thiamine. Anti-thiamine factors, such as caffeic acid, chlorogenic acid, tannin, and quercetin in tea, coffee, blueberries, black currants, beetroot, red cabbage, and Brussel sprouts, can reduce thiamine bioavailability during food storage, preparation, and gastrointestinal tracts. (Yaman et al., 2021). Therefore, it is recommended to delay consuming the phenolic compounds, such as drinking tea or chewing fermented tea leaves after meals. However, ascorbic acid could inhibit thiamine degradation caused by polyphenols. Besides, consuming foods with high levels of ascorbic acid could also solve the issue of thiamine deficiency (Vimokesant et al., 1982).

1.2.7.2 Metabolism and excretion

After the absorption, thiamine is carried by the portal blood to the liver. Both thiamine monophosphate and non-phosphorylated thiamine combined with plasma proteins circulate in the bloodstream. **Figure 1.6** shows the chemical reactions in varied conversions of thiamine during the post-absorptive metabolism. Firstly, thiamine diphosphate (TDP) is formed by thiamine diphosphokinase in the liver and other tissues (see Equation (a) in **Table 1.6**). TDP and inorganic

phosphate (PPi) are produced from the hydrolysis of thiamine triphosphate (TTP) by thiamine triphosphatase in nerve tissue (see Equation (b) in **Table 1.6**). Secondly, the TTP is formed from TDP in two ways: by adenylate kinases in the cytosol of skeletal muscle and by a chemiosmotic mechanism, respectively. TTP is converted from some TDP by thiamine pyrophosphate-ATP phosphoryl transferase in the brain and other nervous tissues (see Equation (c) in **Table 1.6**). Thirdly, thiamine monophosphate (TMP) and inorganic phosphate (PPi) are produced from some small amounts of TDP (see Equation (d) in **Table 1.6**). The hydrolysis of TMP is to yield free thiamine by thiamine monophosphatase in many tissues (see Equation (e) in **Table 1.6**). Due to the relatively high turnover rate and low storage capacity, a continuous dietary intake of vitamin $B_1$ is necessary for human beings (Ball, 2004; Combs and McClung, 2017).

When it comes to excretion, the excess of thiamine and the small amounts of their metabolites are rapidly excreted in the urine, mainly as free thiamine and TMP. Other small amounts of metabolites include TPP, thiochrome, and thiamine-containing peptides. The thiamine excretion exceeds 100 µg/ day, while urinary excretion in patients with thiamine deficiency is less than 25 µg/ day (Combs and McClung, 2017). There is no direct evidence of reabsorption by the kidney, but negligible amounts of thiamine can be excreted in the bile. A significant thiamine loss may occur through sweating in tropical countries (Ball, 2004).

1.2.8 Traditional methods for analysis of thiamine

There are several analytical methods for thiamine determination, such as chromatographic separation, fluorescence methods, and bio-assay. In the colorimetric assay, thiamine is reacted with various reagents and produces a visible color change. The formation of colored products redshifts the maximum absorbance out of the UV range and could prevent many interferences while improving the molar extinction coefficient. The challenges in the method include instability of

reagents and the reaction products, lengthy procedures during extraction, reaction with interfering chemicals, and low intensity of colored species (Edwards et al., 2017). In the fluorescence method, thiamine is oxidized to the blue fluorescent product, thiochrome. When using an HPLC-fluorescence detector, oxidation to thiochrome is accomplished either by pre-column or post-column derivatization with reagents. This method can be used for the quantification of different phosphate forms of thiamine, but HPLC with fluorescence detector is less commonly available (Edwards et al., 2017). In microbiological assays, thiamine is indirectly measured as a function of microbial growth. For example, parallel yeast (S. cerevisiae) assay was used to assess the content of thiamine and its biosynthetic intermediates in the model plant samples (Strobbe et al., 2022). However, this method was laborious, time-consuming, nonspecific, and sensitive for testing the different vitamers of thiamine.

## 1.3 Introduction of high-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) is an effective tool for determining various compounds, such as thermolabile, polar, and high molecular weight chemicals. It is used as a separation method for isolating target substances from coexisting components. **Figure 1.6** shows an example of the configuration of HPLC system (Shimadzu Instruments, 2017), which usually consists of a pump, degasser, automatic or manual injector, column, and detector.

### 1.3.1 HPLC configuration

An HPLC pump is responsible for maintaining the operating pressure and elution at a stable flow rate. The composition of an eluent, also known as the mobile phase, can be prepared from various types of solutions, such as distilled water, methanol, acetonitrile, and phosphate. The proportion of eluents can be adjusted in an isocratic or gradient mode. The HPLC instruments can

develop up to 6000 psi, which is about 400 Bar of pressure. Moreover, the pressure in new models of HPLC extends to higher levels, such as up to 9500 psi in the Arc HPLC system from Waters and up to 600 bar (around 8702 psi) in the 1220 Infinity II LC system from Agilent Technologies. With the pumps with higher pressure, the columns with smaller particle sizes could be used in the analysis. UPLC would be an attractive option when a pump with higher pressure is needed because the maximum operating pressure could be up to 15000 psi. The flow rate of a mobile phase typically ranges from 0.5 to 2.0 mL/min. Degasser is used to remove dissolved gas in HPLC solvents to improve the performance and reliability of the pump and the detector. Stable baselines with reduced drift and pressure fluctuations and reduced detector baseline noise contribute to more reliable quantitation. Dissolved oxygen or air bubbles may affect the response of pumps, columns, and detectors (Harris and Lucy, 2020). Autosampler and manual injection are two modes of the injection system. The system draws samples from vials and injects them into the solvent flow delivered by the pump. Sometimes, HPLC is equipped with a fraction collector to collect purified eluted components and maintain sample integrity.

HPLC column contains chromatographic packing material, which is also called the stationary phase. Most columns are made of stainless steel to resist high pressure. Overall separation power can be determined by mechanical separation power and chemical separation power. Mechanical separation power, also called efficiency, is related to column length, particle size, and packed-bed uniformity. Chemical separation power, also called selectivity, is related to the physicochemical competition for compounds between the stationary and mobile phases. The internal diameter of the column is normally standardized in a range from 1.8 to 4.6 mm. Filtration and using HPLC-grade solvents can prevent the degradation of columns caused by impurities and minimize detector background signals from contaminants. HPLC analysis (separation) might be affected by many

interfering compounds; therefore, careful sample preparation, pre-purification, and suitable chromatographic conditions should be optimized, which might solve the separation problem (San José Rodriguez et al., 2012).

Detectors for HPLC are chosen based on the optical properties of the analytes, such as absorption, fluorescence, and refractive index. UV-Vis detector focuses on ultraviolet and visible regions of the spectrum in the wavelength range between 190-700 nanometers (nm). Detection is selective for compounds with an absorbing chromophore at wavelengths above 210 nm (Harris and Lucy, 2020). The photodiode array (PDA), also called the diode array detector (DAD), can measure the entire wavelength range in real-time. Evaporative Light Scattering Detector (ELSD) measures the light scattered by particle aggregates of analytes that persist after evaporation of the mobile phase matrix. Refractive Index Detector (RID) measures the refractive index when light passes through a cell containing analytes compared to a reference cell containing solvent. Fluorescence Detector (RF) has the highest sensitivity with stable detection in controlled temperature conditions. For example, thiochrome, the oxidized derivative of thiamine, is fluorescent in an alkaline medium, suitable for the HPLC-fluorometric method after pre-column or post-column derivatization (Lynch and Young, 2000). A conductivity detector (CDD) is designed for ion chromatography or organic acid analyses. In summary, it is essential to consider analytes' physio-chemical properties, detectors' sensitivity and detection limits, maintenance features, and temperature control before selecting a suitable detector.

1.3.2 HPLC separation modes

1.3.2.1 Separation based on polarity

Compounds can be separated well by the most suitable mobile phase (Harris and Lucy, 2020). Polarity is one of the primary characteristics of chemical compounds for creating HPLC

separations. Molecules with similar chromatographic polarities tend to attract each other, while those with different polarities exhibit weak attraction. In samples, if compounds are similar in polarity to the stationary phase, they would move slowly. On the contrary, if compounds whose polarity is similar to that of the mobile phase, they would move faster. Water serves as a common polar solvent of the mobile phase, while acetonitrile and hexane act as non-polar mobile phases. In the stationary phase, silica surface can be modified selectively by chemically bonding to more minor polar functional groups, such as n-octylsilyl (C8) and n-octadecylsilyl (C18) moieties on silica. Creating a separation based on polarity involves knowledge of various compounds, kinds of analytes, and retention modes.

### 1.3.2.2 Normal-phase chromatography

In normal-phase chromatography (NP-HPLC), a polar stationary phase and a less polar mobile phase are used. A more polar solvent has a higher eluent strength, which means solutes will quickly be eluted from a normal-phase column. 100% organic non-polar solvent is typically used as the mobile phase for NP-HPLC on silica. Because the stationary phase is polar, the polar chemicals retain, and the non-polar chemicals elute faster (Harris and Lucy, 2020). In NP-HPLC, polar compounds that dissolve only in organic solvents can be separated (Harris and Lucy, 2020).

### 1.3.2.3 Reverse-phase chromatography

In reversed-phase chromatography (RP-HPLC), the stationary phase is non-polar or weakly polar, while the mobile phase is polar. A more robust mobile phase is a less polar solvent, and it elutes polar solutes more rapidly from the column. Due to the non-polar stationary phase, the polar chemicals can be eluted faster, and the non-polar chemicals are firmly retained. RP-HPLC is the most common mode of HPLC, and it is used for approximately 75% of all HPLC methods. Aqueous combinations of water with a miscible, polar organic solvent, such as methanol,

acetonitrile, phosphoric acid, and formic acid, are used as mobile phases. Sometimes, using buffer solutions to adjust pH in the solvent can get more optimal conditions for separation. C18-bonded silica, non-polar and hydrophobic, is the most famous stationary phase in RP-HPLC. Reverse-phase chromatography is usually adequate to separate mixtures of low-molecular-mass neutral or charged organic compounds (Harris and Lucy, 2020).

### 1.3.3 The application of HPLC in food analysis

When using HPLC, appropriate selections of a detector, stationary phase, mobile phase, and program of separation are required (Nikolin et al., 2004) since they are important for the development of cheap, cost-effective, rapid, and reliable analytical methods to determine vitamins, and other nutrients in food products and any laboratory (San José Rodriguez et al., 2012). In summary, HPLC is currently widely used for quantitative and qualitative analyses of pharmaceuticals, drugs, vitamins, and many other chemicals in light of its rapid separation, accurate quantification, and high selectivity.

## 1.4 Introduction of minerals

Minerals possess many functionalities and potentials in the metabolism and homeostasis of the body's systems, including building strong bones, generating different hormones, transmitting nerve impulses, and being structural parts in different enzymes (Wang et al., 2021). Many physiological and dietary variables are capable of influencing the bioavailability of minerals. The only source of mineral intake for human beings is from food. After consuming the minerals, some of them cannot be absorbed and used by body cells because minerals strongly interact with foodstuffs and other components when passing through the digestive system. Deficiency of

micronutrients frequently occurs impacts the health of people all around the world. For example, iron (Fe) deficiency seriously affects women, children, and infants due to insufficient intake or poor iron absorption. Enhancers or inhibitors can strongly influence mineral bioavailability too. For example, the primary inhibitor, phytate, can chelate iron and form insoluble complexes. However, ascorbic acid can reduce iron from the diet to ferrous iron, which can be absorbed by the intestinal cells (Bechoff and Dhuique-Mayer, 2016).

Minerals are extensively divided into significant minerals (macro-minerals) and trace minerals (micro-minerals). The former includes calcium (Ca), magnesium (Mg), potassium (K), sodium (Na), chloride (Cl), phosphorous (P), and sulfur (S), while the latter contains iodine (I), zinc (Zn), selenium (Se), iron (Fe), manganese (Mn), copper (Cu), cobalt (Co), molybdenum (Mo), fluoride (F), chromium (Cr) and boron (B) (Gharibzahedi and Jafari, 2017). Copper (Cu) can be found in legumes, whole grains, and seafood, and its primary functions include repairing injured tissues, promoting Fe and protein metabolism, and neutralizing free radicals caused by intense cell damage. In comparison, iron (Fe) can be found in nuts, chicken livers, dark leafy greens, and cocoa powders, and its functions include stimulating the formation of hemoglobin in red blood cells, energy metabolism, and being a transport medium for electrons within cells (Gharibzahedi and Jafari, 2017).

1.4.1 The required intake of minerals

**Table 1.7** shows the dietary reference intakes (DRIs) for various minerals and elements essential for healthy adults (National academies, 2019; Ross et al., 2011). Macro-minerals, such as calcium, phosphorous, sodium, chlorine, magnesium, and potassium, normally require more than 100 mg in daily diets, while trace-minerals and micro-minerals are required less than 100 mg in daily diets. Copper, fluoride, iron, and zinc are listed in the group of trace minerals, and iodine,

molybdenum, selenium, chromium, and manganese are listed in micro-minerals (see **Table 1.7**) (National academies, 2019; Ross et al., 2011; Balamurugan et al., 2017).

1.4.2 Copper

Copper is one of the essential trace minerals, while excess amounts of copper cause oxidative tissue damage and neurodegenerative disorders. With the extensive use of copper pipes, copper levels in drinking water are found to be highly associated with total dietary copper intake (Pettersson and Rasmussen, 1999; Wu et al., 2022). In the United States, the median copper concentrations for the first-draw $90^{th}$ percentile exceedances in 7307 samples from 1991 to 1999 were slightly more significant than 2 mg/L, which exceeded the regulatory action level of 1.3 mg/ L (Donohue et al., 2005). Therefore, the absorption and toxicity of copper from drinking water cannot be ignored (Wu et al., 2022). There is a more considerable difference in the copper absorption rate in foods and water. In water, copper is mainly derived from the dissolution of copper pipes and water storage facilities, and exists in free ions and soluble inorganic complexes. In foods, proteins, dietary fibers, and other substances would form complex matrices with copper, making more challenges to release copper from foods during digestion (Wu et al., 2022). In addition, the same study showed that during the *in vitro* digestion system, the largest proportion of the copper complexes existed in the range of molecular sizes between 100 Da – 1000 Da, accounting for 60 4%. Smaller sizes of copper complexes were taken from water, and larger sizes were obtained from food during the digestion in the intestines. Generally speaking, the copper from water may have a higher absorption rate and higher toxicity than the copper from foods (Wu et al., 2022).

1.4.3 Iron

It is challenging to directly add iron salts to foodstuffs due to their low solubility, bioaccessibility, and bioavailability at physiological pH. Iron salts might accelerate the formation of reactive oxygen species. The majority of the bioavailable iron compounds have higher reactivity, which can induce changes in flavor, color, appearance alterations, precipitation, and lipid peroxidation and lower the chemical stability of foodstuffs (Kazemi-Taskooh and Varidi, 2021). Protein can enhance the iron bioavailability and reduce its pro-oxidant activity, and lipids can improve iron transport and permeability in the gastrointestinal tract (Kazemi-Taskooh and Varidi, 2021). The absorption of ferrous iron ($Fe^{2+}$) is generally assumed to be better than ferric iron ($Fe^{3+}$). Ferric salts can precipitate when pH rises from the stomach to the duodenal area, and the complexation of iron can prevent this precipitation with compounds that form absorbable chelates that remain soluble at increasing pH. Furthermore, dietary compounds generally increase bioavailability by reducing iron from ferric to ferrous (Blanco-Rojo and Vaquero, 2019).

## 1.5 Research objectives

The general objective of this research is to investigate the effects of different metal ions (i.e., $CuCl_2$, $CuCl$, $FeCl_2$, and $FeCl_3$) at different concentrations under three storage temperatures (i.e., 25°C, 40°C, and 55°C) on the stability of thiamine (vitamin $B_1$) in the solution prepared from thiamine hydrochloride (THCl), which will be measured by HPLC. In detail, Chapter 2 will describe the effects of different types and concentrations of metal ions on the stability of thiamine at 25°C, while Chapter 3 will discuss the effects of different temperatures of storage conditions and different types of metal ions on thiamine stability. The degradation kinetics are also determined in both chapters.
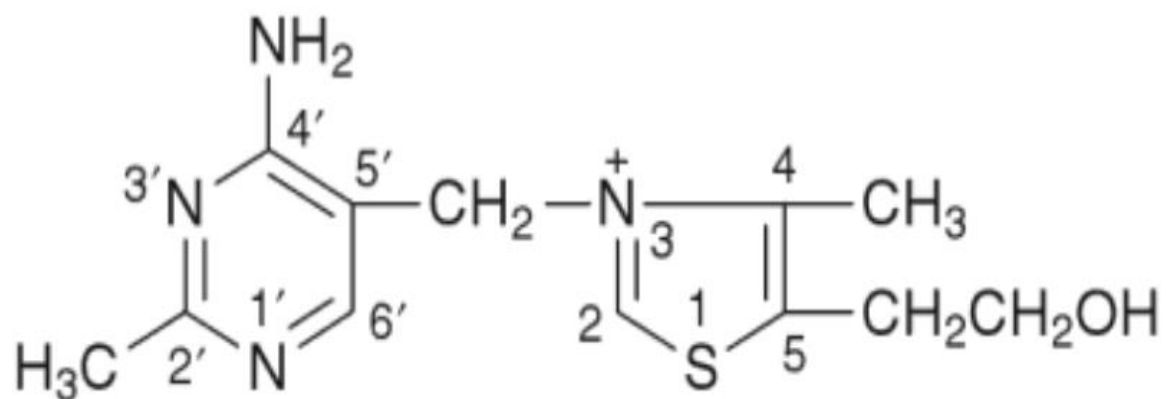
**Figure 1.1** Structures of thiamine (Ball, 2004).
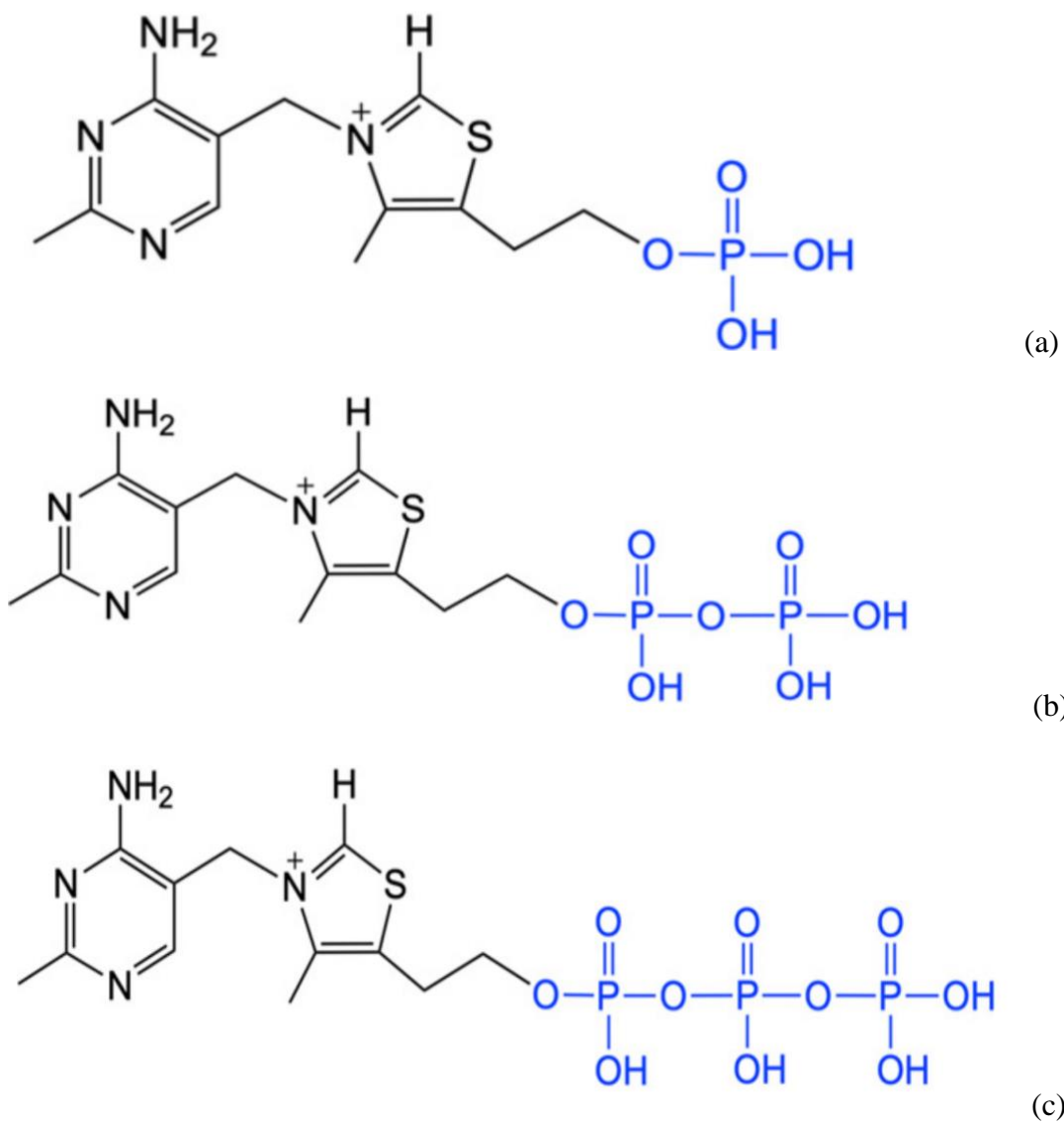
**Figure 1.2** Structures of thiamine in phosphorylated forms: (a) TMP (thiamine monophosphate) (b) TDP (thiamine diphosphate) (c) TTP (thiamine triphosphate) (Bettendorff and Wins, 2009).
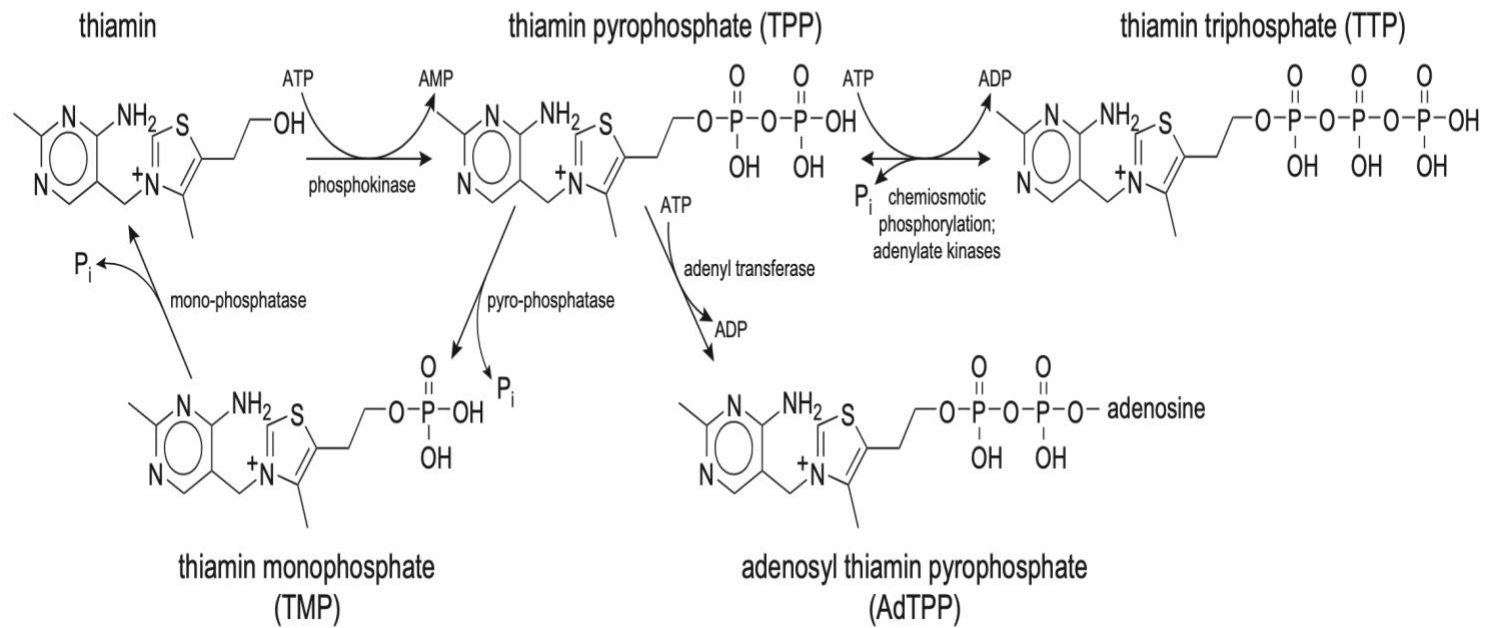
**Figure 1.3** Schematic representation of the metabolic activation of thiamine in peripheral tissues (Combs and McClung, 2017).
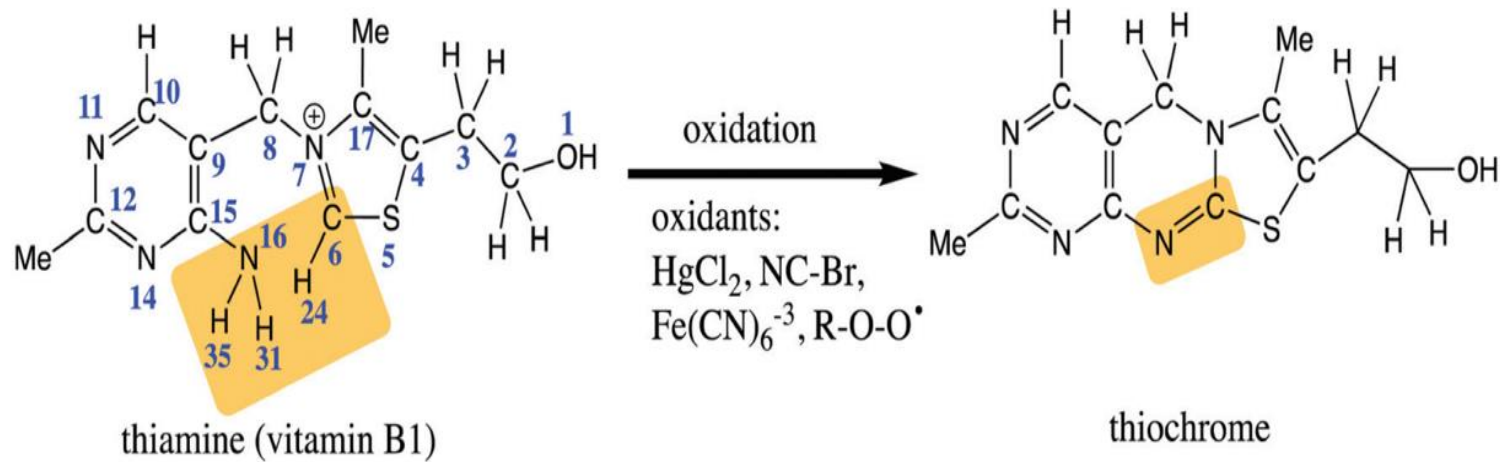
**Figure 1.4** The scheme of the thiamine to thiochrome oxidation. (The orange color showed the reaction center, and the blue-color atom numbering is for the present calculations) (Yamabe, Tsuchida and Yamazaki, 2021).
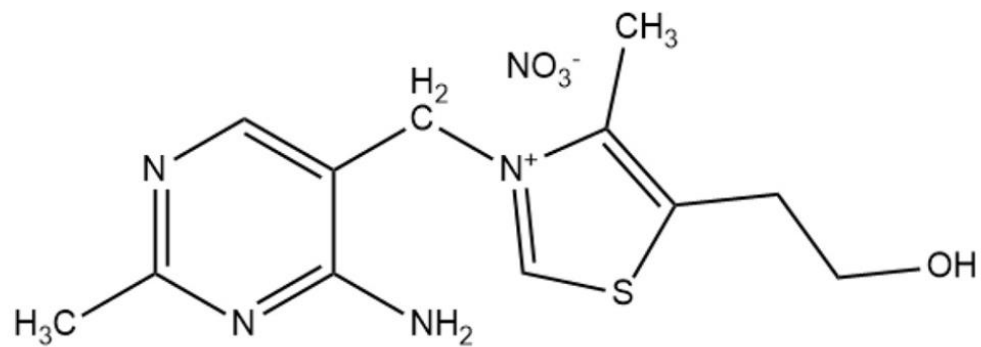
(a)
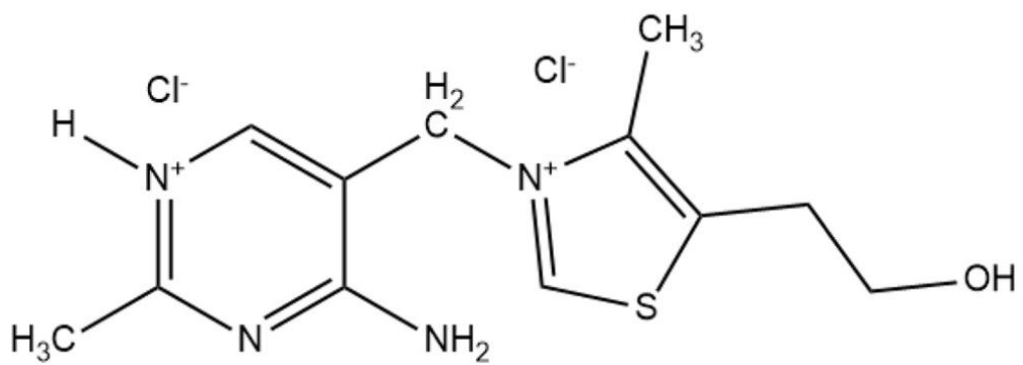


(b)

**Figure 1.5** The chemical structures of thiamine salts: (a) thiamine mononitrate (TMN) and (b) thiamine hydrochloride (THCl) (Voelker et al., 2018).
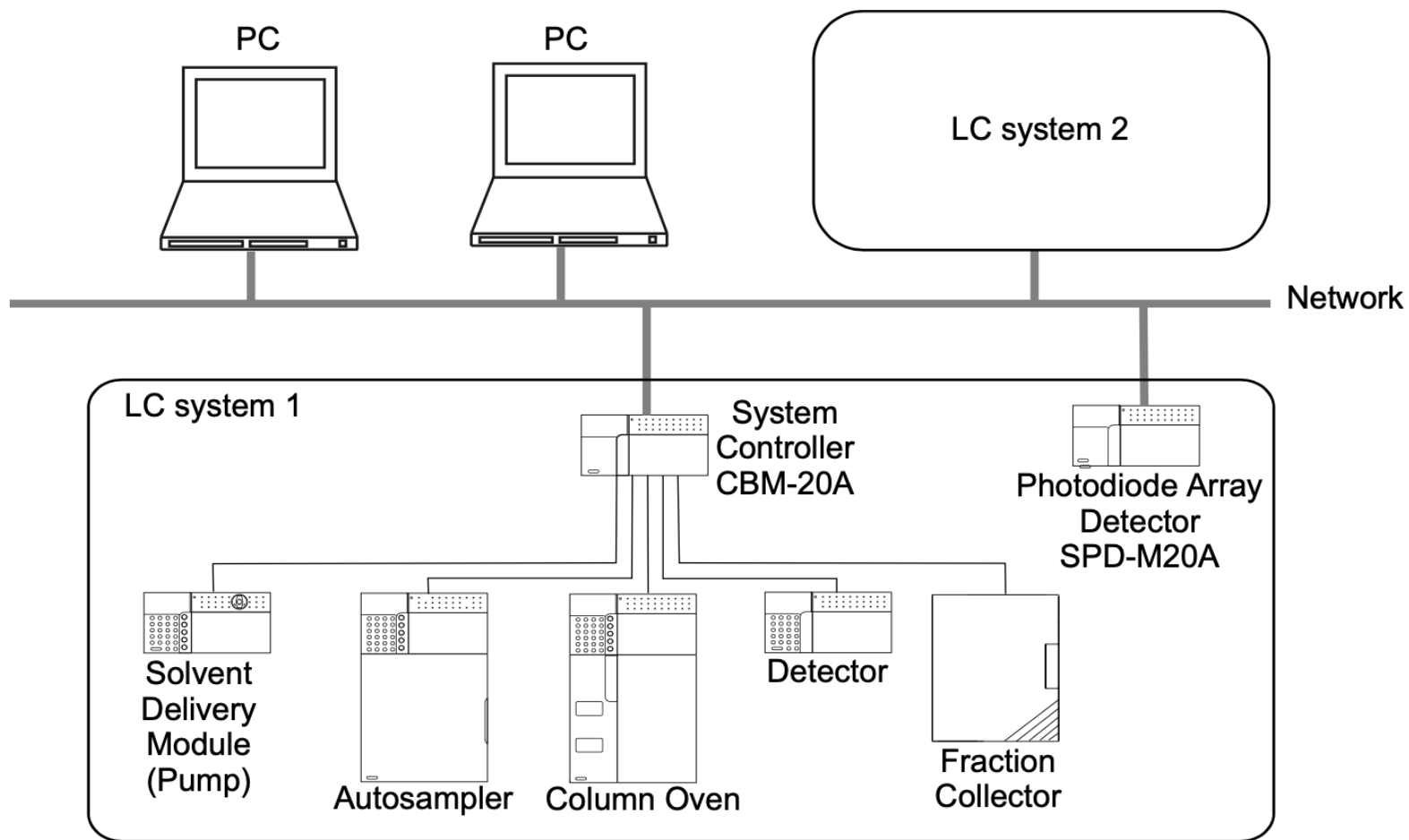
**Figure 1.6** The system configuration of HPLC (Shimadzu Instruments, 2017).
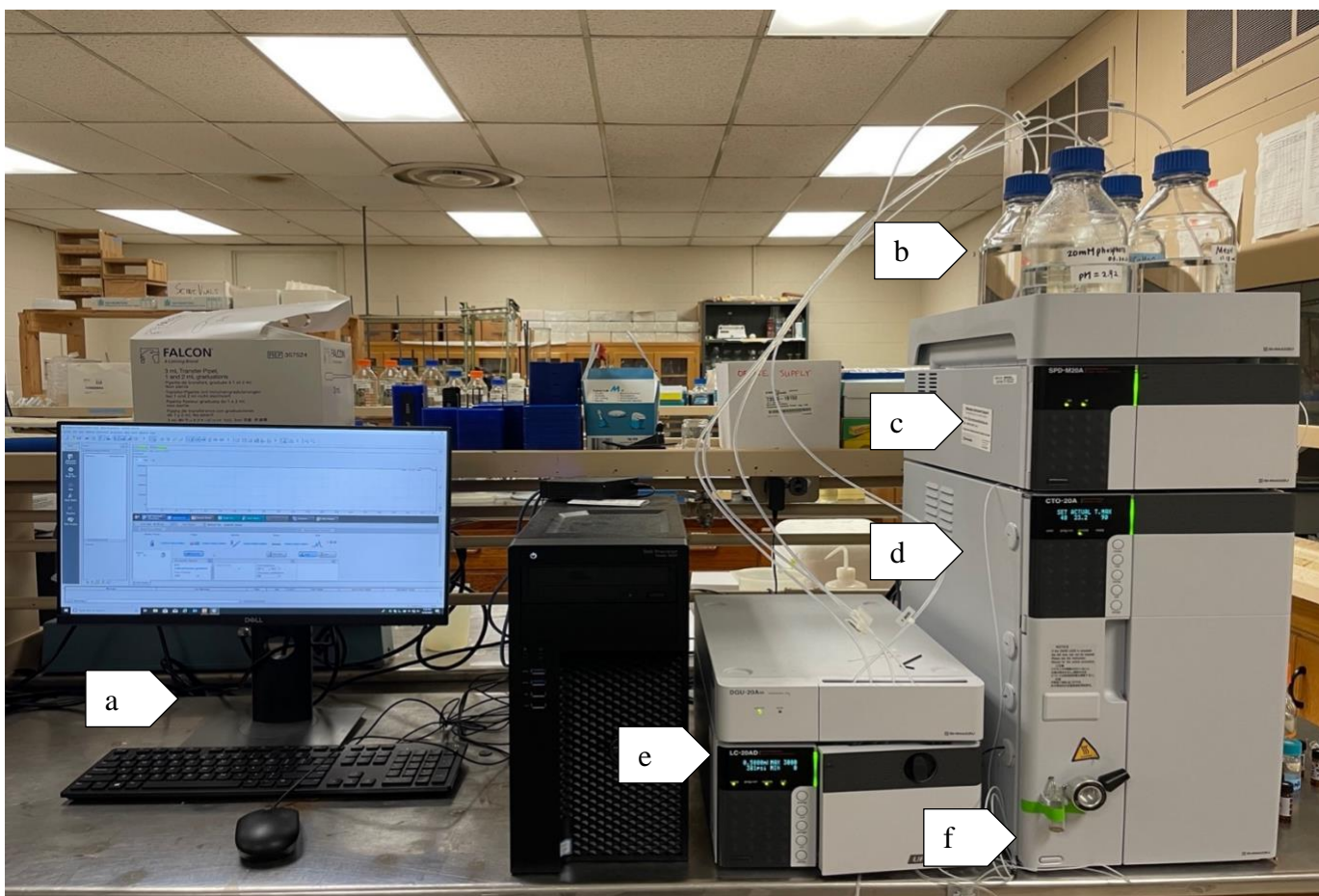
**Figure 1.7** A Shimadzu HPLC system that is located in Dr. Feng Chen's lab, and it is used for this research: (a) software (b) solvents (mobile phase) (c) multi-wavelength detector (d) column oven (e) pump (f) sampling injector.

**Table 1.1** RDAs and AI for water-soluble vitamins in varied age groups (*: AI) (IOM, 1998; IOM, 2000).

| Water-Soluble Vitamin | | 0-0.6 y | 0.6-1 y | 1-3 y | 4-8 y | 9-13 y | >13 y males | >13 y females | Pregnancy | Lactation |
|---|---|---|---|---|---|---|---|---|---|---|
| Name | Unit | | | | | | | | | |
| Vitamin C | | 40* | 50* | 15 | 25 | 45 | 75-90 | 65-75 | 80-85 | 115-120 |
| Thiamine | | 0.2* | 0.3* | 0.5 | 0.6 | 0.9 | 1.2 | 1.0-1.1 | 1.4 | 1.4 |
| Riboflavin | | 0.3* | 0.4* | 0.5 | 0.6 | 0.9 | 1.3 | 1.0-1.1 | 1.4 | 1.6 |
| Niacin | mg/d | 2* | 4* | 6 | 8 | 12 | 16 | 14 | 18 | 17 |
| Pantothenic acid | | 1.7* | 1.8* | 2* | 3* | 4* | 5* | 5* | 6* | 7* |
| Vitamin $B_6$ | | 0.1* | 0.3* | 0.5 | 0.6 | 1.0 | 1.3-1.7 | 1.2-1.5 | 1.9 | 2.0 |
| Biotin | µg/d | 5* | 6* | 8* | 12* | 20* | 25-30* | 25-30* | 30* | 35* |
| Folate | | 65* | 80* | 150 | 200 | 300 | 400 | 400 | 600 | 500 |
| Vitamin $B_{12}$ | | 0.4* | 0.5* | 0.9 | 1.2 | 1.8 | 2.4 | 2.4 | 2.6 | 2.8 |

**Table 1.2** RDI and ULs for water-soluble vitamins in varied age groups (IOM., 1998).

| Water-Soluble Vitamins | RDI | Upper levels in varied age groups | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0-0.6 y | 0.6-1 y | 1-3 y | 4-8 y | 9-13 y | >13 y males | >13 y females | Pregnancy | Lactation |
| Vitamin C[1] | 75 | ND | ND | 400 | 650 | 1200 | 1800-2000 | 1800-2000 | 1800-2000 | 1800-2000 |
| Thiamine[1] | 1.4 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Riboflavin[1] | 1.6 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Niacin[1] | 18 | ND | ND | 10 | 15 | 20 | 30-35 | 30-35 | 30-35 | 30-35 |
| Pantothenic acid[1] | 6 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Vitamin $B_6$[1] | 2 | ND | ND | 30 | 40 | 60 | 80-100 | 80-100 | 80-100 | 80-100 |
| Biotin[2] | 30 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Folate[2] | 400 | ND | ND | 300 | 400 | 600 | 800-1000 | 800-1000 | 800-1000 | 800-1000 |
| Vitamin $B_{12}$[2] | 6 | ND | ND | ND | ND | ND | ND | ND | ND | ND |

[1]: the unit is mg/day. [2]: the unit is µg/d. *ND: Not determinable due to lack of data on adverse effects in this age group and concern with regard to lack of ability to handle excess amounts

**Table 1.3** The properties of TMN and THCl in solid states (Voelker et al., 2018; Hiatt et al., 2008).

| | Thiamine Mononitrate (TMN) | Thiamine Hydrochloride (THCl) |
|---|---|---|
| Molecular Weight (g/mol) | 327.36 | 337.26 |
| Melting Point (°C) | 196-200 | 248 |
| Deliquescence Point ($RH_0$) | 98.5% RH | 88% RH |
| Aqueous solubility (mg/mL) | 30 | 570 |
| Activation Energy (kcal/ mol) | 26.3 | 22.4 |

**Table 1.4** General signs of thiamine deficiency (Combs and McClung, 2017).

| Organ system | Signs |
|---|---|
| General appetite | Severe decrease |
| Growth | Decrease |
| Dermatologic | Edema |
| Muscular | Cardiomyopathy, bradycardia, heart failure, weakness |
| Gastrointestinal | Inflammation, ulcer |
| Vital organs | Hepatic steatosis |
| Nervous | Peripheral neuropathy, opisthotonos |

**Table 1.5** Dosage for thiamine supplementation (DiNicolantonio et al., 2013).

| Cases | Dosage of Supplementation | Route of Administration | Time |
|---|---|---|---|
| RDA | 1.1-1.2 mg | Orally | Daily |
| Risk of thiamine deficiency | 100 mg | | 3 times daily until thiamine levels normalize |
| Proven thiamine deficiency | 200 mg | | 3 times daily until thiamine levels normalize |
| Alcoholics without encephalopathy | 50 mg | Orally | Daily |
| Patients on a refined grain diet | 5-15 mg | Orally | Daily |
| (Dry beriberi) Mild neuropathy | 10-20 mg | Orally | 2 weeks |
| (Dry beriberi) Severe neuropathy | 20-30 mg | Orally | Several weeks |
| Wet beriberi | 100 mg | Intravenously | Several days |
| Prophylactic dose in patients with heart failure (HF) | 10-20 mg | Orally | Daily |

**Table 1.6** The conversion of thiamine during post-absorptive metabolism (Ball, 2004).

| | Chemical Reaction | Involved Enzymes | Locations |
|---|---|---|---|
| (a) | thiamine + ATP → TDP + AMP | Thiamine diphosphokinase | Liver and other tissue |
| (b) | TTP → TDP + PPi | Thiamine triphosphatase | Nervous tissue |
| (c) | TDP + ATP → TTP + ADP | Thiamine pyrophosphate-ATP phosphoryl transferase | Brain and other tissue |
| (d) | TDP → TMP + PPi | Thiamine diphosphatase | Nervous tissue |
| (e) | TMP → thiamine + PPi | Thiamine monophosphatase | Nervous tissue |

**Table 1.7** Dietary Reference Intakes (DRIs) for minerals and elements that are essential for healthy adults (National academies, 2019; Ross et al., 2011).

| Macro-minerals (> 100 mg/day) | | | Trace-minerals (1 to 100 mg/day) | | | Micro-minerals (< 1 mg/day) | | |
|---|---|---|---|---|---|---|---|---|
| Calcium | 1000 | mg/day | Copper | 900 | µg/day | Iodine | 150 | µg/day |
| Phosphorous | 700 | | Fluoride | Males: 3 | mg/day | Molybdenum | 45 | |
| Sodium | 1500 | | | Females:4 | | Selenium | 55 | |
| Chlorine | Males: 550 | | Iron | Males: 8 | | Chromium | Males: 35 | |
| | Females: 425 | | | Females:18 | | | Females: 25 | |
| Magnesium | Males: 420 | | Zinc | Males: 11 | | Manganese | Males: 2.3 | mg/day |
| | Females: 320 | | | Females: 8 | | | Females: 1.8 | |
| Potassium | Males: 3400 | | | | | | | |
| | Females: 2600 | | | | | | | |

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# CHAPTER TWO

EFFECTS OF METAL IONS ON STABILITY OF THIAMINE HYDROCHLORIDE

## Abstract

The effects of four metal ions (i.e., $Cu^+$, $Cu^{2+}$, $Fe^{2+}$, and $Fe^{3+}$) on thiamine hydrochloride (THCl) stability in aqueous solutions were determined. The samples were stored at room temperature (25°C) for seven days. Each sample contained 250 mg $L^{-1}$ of THCl, and copper and iron ions at their final concentrations of 5, 10, 25, and 50 mg $L^{-1}$ in their respective solutions. pH values of the samples were also monitored during the storage. The remaining amount of thiamine was determined by high-performance liquid chromatography equipped with an Eclipse XDB C18 column maintained at 30°C under a gradient elution program, which consisted of solvents A (0.1 M ammonium acetate adjusted to pH of 5.8 by 0.1% acetic acid) and solvent B (acetonitrile) at the flow rate of 1 mL/min. The UV detector was set at 254 nm and the total analysis time lasted 5 minutes. The degradation kinetics of thiamine were plotted and found to obey the first-order reaction model, which was illustrated by the kinetic rate. After seven days of storage, the highest loss of THCl was found in 50 mg $L^{-1}$ copper chloride (I) solution, with a remaining amount of 64.00 %, while the lowest loss of THCl was found in 50 mg $L^{-1}$ iron chloride (III) solution, with a remaining amount of 78.34%.

## 2.1 Introduction

Thiamine, also known as vitamin $B_1$, is relatively unstable, so its retention is a big challenge for food industry. The degradation kinetics of thiamine in three different spaceflight foods (i.e., brown rice, split pea soup, and BBQ beef brisket) for a long-term storage at three temperatures

(4°C, 20°C, and 37°C) was investigated (Goulette et al., 2020), which found the kinetics of thiamine degradation obeyed the first-order kinetic model shown below:

$$-\frac{d[C]}{dt} = k[C]^n \qquad [1]$$

where [C] is the concentration (conc.) of vitamin at time t, k represents the degradation rate constant (day$^{-1}$), and n is the reaction order (dimensionless). After the integration of Eq. (1) for the kinetics reaction, Eq. (2) can be written as follows:

$$\ln\frac{[C_t]}{[C_0]} = -kt \qquad [2]$$

where $\frac{C\,(t)}{C\,(0)}$ is the concentration ratio of vitamin, and $0 < \frac{C\,(t)}{C\,(0)} \leq 1$ (Goulette et al., 2020), k is the reaction rate constant (day$^{-1}$), $C_0$ is the initial concentration, and $C_t$ represents the concentration on time t (day).

Thiamine can be partially degraded to the thiazole and pyrimidine moieties (Dwivedi, Arnold, and Libbey, 1972). Heating the solution of thiamine at pH 6.7 for 15 minutes in a boiling water bath resulted in several thermal degradation products of thiamine, including hydrogen sulfide, 2-methyl furan, 2-methyl thiophene, and 4,5-dihydro-2-methiophene (Arnold, Libbey, and Lindsay, 1969). Moreover, under a thermal processing at 121°C for 40 minutes, free sulfur from the thiazole ring in thiamine hydrochloride was detected both at pH 5.9 (slightly acid) and pH 7.4 (slightly basic) (Morfee and Liska., 1972).

HPLC with UV detector is mainly used to analyze thiamine content in fortified foods because of its straightforward sample preparation, which can avoid inaccurate results caused by the naturally occurring polyphenol compounds (Hilker and Clifford, 1982). Thiamine can be easily detected at 245 nm, 254 nm, and 265 nm when using HPLC with ultraviolet detection.

The aim of this study was to observe the effects of copper and iron ions at varied concentrations on the thiamine stability in the pure thiamine hydrochloride solution under room temperature (at around 25°C) within a short period (i.e., seven days), and determine the degradation kinetics to predict the rates of thiamine loss. HPLC was used for the thiamine analysis with UV detection at the wavelength of 254 nm.

## 2.2 Materials and methods

2.2.1 Chemicals and reagents

Thiamine hydrochloride ($C_{12}H_{17}ClN_4OS \cdot HCl$) (purity: 99%) was obtained from Thermo Fisher Scientific (Ward Hill, Massachusetts, USA). Cupric (II) chloride ($CuCl_2 \cdot H_2O$) (purity: 99.0%) and ferric chloride ($FeCl_3$) (purity: 98 %) were purchased from Sigma Chemical (Louis, Missouri, USA). Copper (I) chloride ($CuCl$) (purity: 99.99%) and iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) (purity: 99%) were purchased from Acros Organics (Morris Plains, New Jersey, USA). Ammonium acetate (crystalline) (purity: $\geq$ 97%) and glacial acetic acid (purity: $\geq$ 99.7%) were purchased from Fisher Chemical (Fair Lawn, New Jersey, USA). All reagents were of analytical grade, except the HPLC grade acetonitrile (purity: 99.9%), which was also purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Water was deionized and purified by using a Millipore Synergy UV system (Millipore Billerica, MA, USA). When analyzing the pH values of the samples, pH electrode storage solution, pH 4.01, pH 7.00, and pH 10.01 buffer were used, and they were purchased from Thermo Fisher Scientific (Chelmsford, MA, USA).

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2.2.2 Instrumentation

The HPLC system was the Shimadzu LC-20A series system (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) with a solvent delivery model (LC-20AD), a column oven (CTO-20A), and a photodiode array detector (SPD-M20A). The LabSolutions software controlled the LC system.

The chromatographic experiments were conducted by a 5 $\mu$m Agilent Eclipse XDB column composed of C18 reversed-phase (RP) in size of 4.6 X 150 mm (P/N: 993967-902). The column temperature was set at 30°C. In the elution condition, the flow rate was 1 mL/min, and the injection volume was 10 $\mu$L. The mobile phase was composed of acetonitrile (solvent B) and 0.1 M ammonium acetate (solvent A) at pH 5.8, which was adjusted by 1% acetic acid. The chemical separation was under a gradient program, which started at an ammonium acetate/ acetonitrile ratio of 92:8, changed to the ratio of 82:18 at 1.6 minutes, was back to the ratio of 92:8 at 3.91 minutes, and finally lasted until 5 minutes. The chromatographic parameters used in the study are tabulated in **Table 2.1**. The wavelength was 254 nm.

In this study, the pH value of the samples was measured by the Denver Instrument UltraBasic pH Benchtop Meters (Arvada, CO, USA).

2.2.3 Preparation of standards and metal chlorides in aqueous solutions and sample storage

The standards of thiamine hydrochloride were prepared by accurately weighing 250 mg of the powder and transferring it to a 500 mL volumetric standard flask. Distilled water was added to form a stock solution of 500 mg $L^{-1}$. The stock solution was stored in the refrigerator at 4.0°C when not in use. The blank control that did not contain any salt solution was prepared by mixing

one volume of 500 mg $L^{-1}$ of the thiamine solution and one volume of distilled water, resulting in its final concentration at 250 mg $L^{-1}$.

Aqueous solutions of metal chloride (i.e., CuCl, $CuCl_2$, $FeCl_2$, and $FeCl_3$) were prepared by weighing 10 mg of respective powders and transferring them in 100 mL volumetric flasks. Distilled water was added to fill the flasks to the calibration mark to form the solutions at 100 mg $L^{-1}$. Linearity levels were prepared by subsequent dilution of the 100 mg $L^{-1}$ salt solutions using the distilled water as a diluent, and the aforementioned respective series of salt solutions contained four concentrations: 10, 20, 50, and 100 mg $L^{-1}$. A vortex mixer was used when required. Before the sample storage, each test sample was prepared with 0.7 mL of 500 mg $L^{-1}$ of thiamine solution and 0.7 mL of a solution with respective metal ions. Therefore, the final concentrations of the mixtures should be half of their original values, which contained 250 mg $L^{-1}$ of thiamine, and 5, 10, 25, and 50 mg $L^{-1}$ of metal chloride, respectively. Each test sample was prepared and analyzed in triplicate.

All the samples were stored in a Fisher Scientific isotemp water jacked $CO_2$ incubator (Revco Technologies, Asheville, NC) at 25 °C (room temperature) for up to 7 days to monitor the thiamine stability.

2.2.4 Determination of remaining amounts of thiamine

After the thiamine solution was mixed with the different metal chlorides at the aforementioned concentrations, the remaining amount of thiamine in each sample was measured at the time intervals on day 0, 1, 2, 3, 4, 5, and 7, respectively. All the samples were determined in triplicate under the same HPLC chromatographic condition. The percentage of remaining thiamine was calculated by the following equation:

$$\frac{C_t}{C_0} \times 100\% = \% \; of \; remaining \; thiamine \qquad [3]$$

where $C_t$ is the average of the detected peak area detected at a certain time (t = day), and $C_0$ is the initial peak area detected on day 0.

2.2.5 First-order reaction of the thiamine degradation

The collected data were applied in a first-order reaction kinetic model, and the degradation kinetics were investigated during a period of 7 days for four metal chlorides. The natural logarithm of the relative percentages of thiamine was plotted against time for each degradation set to estimate the kinetic parameters, such as kinetic reactant value ($k_x$) and coefficient of determination ($R^2$). A general expression of the reaction rate for the degradation kinetics was based on previous reports (Nisha Rekha, Singhal, and Pandit, 2004; Voelker et al., 2018; Goulette et al., 2020):

$$-\frac{d[C]}{dt} = k_x[C]^n \qquad [4]$$

where [C] is the quantitative value of the degradation product under consideration, $k_x$ is the reaction rate constant, and n is the reaction order, which is 1 in this study.

After the integration, the solution of Eq. (4) of the first-order kinetics for thiamine degradation can be described by:

$$ln\frac{[C_0]}{[C_t]} = k_x t \qquad [5]$$

where $C_t$ is the remaining amount of thiamine at the reaction time t (days), $C_0$ is the initial amount of thiamine at time 0, $k_x$ is the reaction rate constant (day$^{-1}$), and t represents the time (days).

2.2.6 Statistical analysis

Each experiment was tested in triplicate. Microsoft Excel 2019 was used for calculating kinetic data and making the figures of degradation curves and regression analysis. The method of

least squares was used to draw the best straight lines (calibration curve) through experimental data points. Excel built-in function LINEST was performed in this study. The slope and intercept and their standard uncertainties were returned in a table, and values of Student's t were as references (Harris and Lucy, 2020). Significant differences ($p < 0.05$) of degradation rates ($k_x$) (slope) were obtained among different groups of metal ions system.

## 2.3 Results and Discussion

My study investigated the thiamine degradation in the aqueous solutions after exposure to different types and concentrations of metal chlorides at 25°C. **Table 2.2** shows the remaining percentages of thiamine in the salt solutions for 7 days, and the degradation profiles are depicted in **Figure 2.1** to **Figure 2.4**. All those thiamine degradations fitted in the first-order kinetics models are profiled in **Figure 2.5**, **Figure 2.6**, **Figure 2.7**, and **Figure 2.8**. **Table 2.3** lists the reaction rate constants (k) (day$^{-1}$) and coefficient of determination ($R^2$) in all samples. The pH values of the samples that were consecutively monitored during 7 days are in **Table 2.5** and **Figure 2.9**. Lastly, the theoretical kinetic models of the thiamine degradation in systems of the four metal chlorides are built and reported in **Figure 2.10**.

2.3.1 Effect of copper chlorides (I) and (II) on thiamine stability in THCl solution at 25°C

**Table 2.2, Figure 2.1**, and **Figure 2.2** show the remaining percentages of thiamine in the copper chloride systems. In the control group, the thiamine content was 89.15% on day 7. In comparison, 64.00% and 75.47 % of thiamine were detected in the CuCl and CuCl$_2$ solutions, respectively, at 50 mg L$^{-1}$ at the same day. The most loss of thiamine was found in the CuCl system among the results. Moreover, the trending lines showed that the degradation in both copper

chlorides solutions proceeded slowly during the entire storage time. As shown in **Table 2.3**, firstly, in the CuCl system, its $k_x$ increased from $4.68 \times 10^{-2}$ to $6.63 \times 10^{-2}$ along with the increased concentrations from 5 to 50 mg L$^{-1}$. In other words, the $k_x$ value became higher with increased concentrations, and the degradation rate under the higher concentration was faster than under the lower concentration in the salt (i.e., Cu$^+$) solution. Secondly, in the CuCl$_2$ system, its $k_x$ value also increased from $2.53 \times 10^{-2}$ at 5 mg L$^{-1}$ to $4.06 \times 10^{-2}$ at 50 mg L$^{-1}$. In **Figure 2.12**, the $k_x$ value in the CuCl solution at 50 mg L$^{-1}$ was significantly higher ($p < 0.05$) than that in the CuCl$_2$ solution at the same concentration, meaning a more considerable impact of CuCl on the thiamine degradation. Compared to the control group ($k_x = 1.92 \times 10^{-2}$), the degradation rates were significantly faster ($p < 0.05$) in both copper chloride solutions during the entire storage time.

Both copper chloride (I) and (II) solutions contain moderate concentrations of hydrogen ions, resulting in acidic solutions. The pH values of the CuCl solutions from 5 to 50 mg L$^{-1}$ were measured in a range from 5.57 to 5.89, and the pH values of the CuCl$_2$ solutions were from 5.90 to 6.14 (see **Table 2.4**). Besides, slightly higher pH was found in the CuCl$_2$ solution at the same concentration. Also, the pH of the mixture made from thiamine and copper chloride solutions was monitored (see **Table 2.5** and **Figure 2.9**). Higher pH was detected at higher concentrations in the CuCl solutions. In summary, the pH values among these three systems at 25°C on day 7 were in the following order: CuCl (pH = 4.47 at 50 mg L$^{-1}$) > CuCl$_2$ (pH = 3.83 at 50 mg L$^{-1}$) > the control group (pH = 3.78).

According to previous research results, thiamine was stable in an acid environment. With addition of copper chlorides in the thiamine solution, the pH values of the solutions slightly increased even though those solutions were still in acidic condition. Thus, the accelerated thiamine degradation in the copper chlorides solutions were ascribed to their higher pH values.

2.3.2 Effect of iron chlorides (II) and (III) on thiamine stability in THCl solution at 25°C

As shown in **Table 2.2**, **Figure 2.3**, and **Figure 2.4**, on the day 7, the remaining thiamine in the $FeCl_2$ solutions was from 69.86% at 5 mg $L^{-1}$ to 76.34% at 50 mg $L^{-1}$, while 78.34% to 81.96% of thiamine remained in the studied concentrations of $FeCl_3$ solutions. The trends also indicated that thiamine degradation in iron chlorides solutions slowly proceeded during the entire storage time. In **Table 2.3**, **Figure 2.7**, and **Figure 2.8**, the $k_x$ value of 5 mg $L^{-1}$ and 50 mg $L^{-1}$ of the $FeCl_2$ solutions were 4.27 x $10^{-2}$ and 6.07 x $10^{-2}$, respectively. The degradation rate at higher concentration was more rapid than at the lower concentration. In comparison, in the $FeCl_3$ system, the $k_x$ value at 5 mg $L^{-1}$ was 2.89 x $10^{-2}$, and its value at 50 mg $L^{-1}$ was 4.33 x $10^{-2}$. **Figure 2.12** shows that the degradation rates in $FeCl_2$ were not significantly higher than ($p > 0.05$) in $FeCl_3$.

$Fe^{2+}$ ion was found to be more soluble in water ($Ksp = [Fe^{2+}] [OH^-] = 8$ x $10^{-16}$) than $Fe^{3+}$ ion ($Ksp = [Fe^{3+}] [OH^-] = 1$ x $10^{-36}$) at $25°C$ (Stumm and Lee., 1961). Besides, $FeCl_3$ in an aqueous solution undergoes hydrolysis and tends to be precipitated as ferric hydroxide. As a result, a solution with a higher amount of $FeCl_3$ will result into more ferric hydroxide precipitation and release more $H^+$ ion in the solution, leading to a more acidic solution (Giri et al., 2022). In **Table 2.5** and **Figure 2.9**, the pH values of the thiamine solutions with $FeCl_3$ were lower than those in thiamine solutions with $FeCl_2$. On the other hand, 78.34% of thiamine was detected to be in the 50 mg $L^{-1}$ of $FeCl_3$ with pH 3.03, and 69.86% of thiamine was remained in the 50 mg $L^{-1}$ of $FeCl_2$ with pH 3.36 on day 7, which indicated that the $FeCl_3$, compared to its counterpart $FeCl_2$, had less impact on the thiamine degradation. This result is consistent with the theory that thiamine is more stable in acidic solution.

2.3.3 Comparison of thiamine degradation in the four metal chlorides solutions

The remaining percentages of thiamine at the highest concentration (50 mg $L^{-1}$) of the salt solutions at the end of the storage time (i.e., day 7) were in the following order: $FeCl_3 > CuCl_2 > FeCl_2 > CuCl$ with 78.34%, 75.47%, 69.86%, and 64.00%, respectively (see **Table 2.2**). As expected, the thiamine content (89.15 %) in the control group was the highest compared to the salt solutions.

The kinetic constants also reflect the degradation rates of the catalyzed reactions. Firstly, the degradation rates in aqueous salt solutions were compared (see **Figure 2.12**). Thought the $k_x$ value at 50 mg $L^{-1}$ of CuCl ($6.63 \times 10^{-2}$) was not significantly larger ($p > 0.05$) than its counterpart of $FeCl_2$ ($6.07 \times 10^{-2}$), the $k_x$ values in all metal chlorides systems were significantly higher ($p < 0.05$) the that of the control group, which means the thiamine stability was significantly influenced in the aqueous salt solutions. Secondly, before making a comparison of the degradation rates caused by metal ions, the concentrations of metal chlorides should be set at same molarity. In **Figure 2.11**, the $k_x$ values at the certain concentration (around 0.3 mmole $L^{-1}$) were determined and ranked in the following order: $Fe^{2+} > Cu^{+} > Fe^{3+} > Cu^{2+}$. At this point, $Fe^{2+}$ might form a metal complex with thiamine and cause apparent catalytic effects for the degradation even though iron ions could provide a more acidic environment for stabilizing thiamine. However, more research need to be conducted in the future. Nevertheless, the chemical properties of the metal chloride have been considered to be a main factor of the degradation of vitamins (Farrer., 1947; Dwivedi, Arnold, and Libbey., 1972). Although the concentrations in each metal chlorides solutions are different at the same molarity (i.e., 0.3 mmole $L^{-1}$) due to their different molar mass, which are 38.03 mg $L^{-1}$, 40.34 mg $L^{-1}$, 29.70 mg $L^{-1}$, and 48.66 mg $L^{-1}$ for $FeCl_2$, $CuCl_2$, CuCl, and $FeCl_3$, their effects on thiamine stability were significant.

Thiamine stability affected by pH has been well-documented, and it is known that higher retention of thiamine can be achieved in an acidic environment. My research results showed the pH values of the metal solutions at 50 mg $L^{-1}$ on day 7 were in the following order: $CuCl > CuCl_2 > FeCl_2 > FeCl_3$ for 4.47, 3.83, 3.36, and 3.03, respectively (see **Table 2.5**), which led the loss of thiamine by 64.00%, 75.47%, 69.86%, and 78.34%, respectively. It is obvious that thiamine is more stable in more acidic solution (low pH values). In other word, CuCl was the most significant catalytic agent, while $FeCl_3$ was the least significant catalyst.

In **Figure 2.10**, the theoretical kinetic models reflect the relationships between the remaining percentages of thiamine and the kinetic constants. For example, the highest thiamine loss was found in the 50 mg $L^{-1}$ of CuCl solution (64.00%) on day 7, resulting from its highest $k_x$ value among the systems, which was $6.63 \times 10^{-2}$. Among the models, the distributed data points (remaining thiamine contents) under various concentrations in the $CuCl_2$ system were much close to their trending lines, which means its theoretical model is fitful or close to the real degradation of thiamine in the $CuCl_2$ system.

Nevertheless, this study only discussed the effects of metal chlorides on the stability of thiamine. Other factors, such as air (oxygen, nitrogen, and carbon dioxide), moisture (water activity), the reactions with metal ions, and exposure to light, were not included in discussion. Those factors and the potentially degraded products were not investigated in this research due to the time limit. It is worth of conducting more research on thiamine stability in more studies.

## 2.4 Conclusion

The effect of the studied metal chlorides at 50 mg $L^{-1}$ on the thiamine stability at 25°C on day 7 was ranked in the following order: $CuCl > FeCl_2 > CuCl_2 > FeCl_3$. 78.34% was the maximum

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amount of thiamine remained in the $FeCl_3$ solution, while 64.00% was the minimum amount of thiamine in the CuCl solution. According to the kinetic constants, the degradation rates in 50 mg $L^{-1}$ of four metal chlorides are significantly higher ($p < 0.05$) than that of the control group. In copper system, the degradation rate of thiamine in CuCl was significantly faster ($p < 0.05$) than in $CuCl_2$, while the rate in $FeCl_2$ was insignificantly faster ($p > 0.05$) than in $FeCl_3$. Also, the $k_x$ values caused by metal ions at the same molarity of metal chlorides were in the following order: $Fe^{2+} > Cu^+ > Fe^{3+} > Cu^{2+}$. $Fe^{2+}$ might be prone to combine with thiamine to form a metal complex and increase the oxidation-reduction system in thiamine degradation. The pH values of thiamine in different metal chlorides solutions were in the following order: $CuCl >$ control group $> CuCl_2 > FeCl_2 > FeCl_3$. Acidic conditions were favorable for the thiamine stability, which was confirmed in our study that CuCl could accelerate the degradation due to its highest pH value among the 4 test models, while the $FeCl_3$ solutions had the least impact on the thiamine degradation. The current research is expected to be able to improve our understanding of the thiamine stability, and be useful for the development of fortified products with thiamine. In summary, the issues of thiamine degradation caused by the metal ions (i.e., $Cu^+$, $Cu^{2+}$, $Fe^{2+}$, and $Fe^{3+}$) cannot be ignored.

## 2.5 Figures and Tables
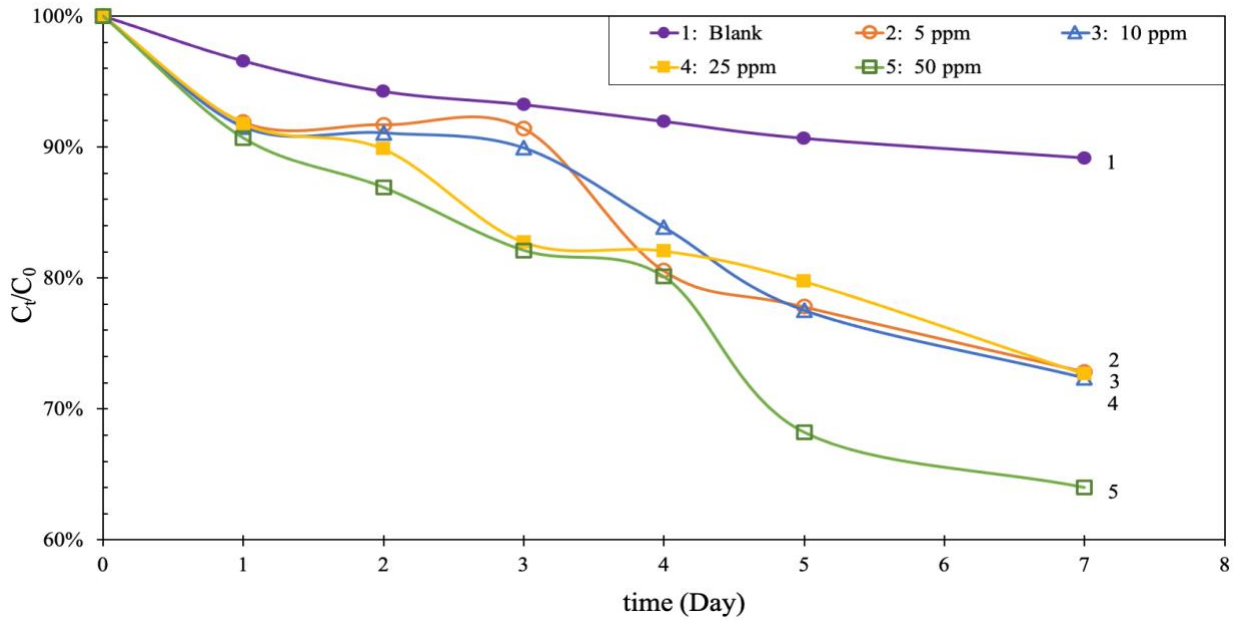


**Figure 2.1** Effects of varied CuCl concentrations on remaining percentage of thiamine vs. time at 25 °C for 7 days. * $C_t$: Concentration of thiamine at time t, $C_0$: Concentration of thiamine at time 0, t: Storage time in days.
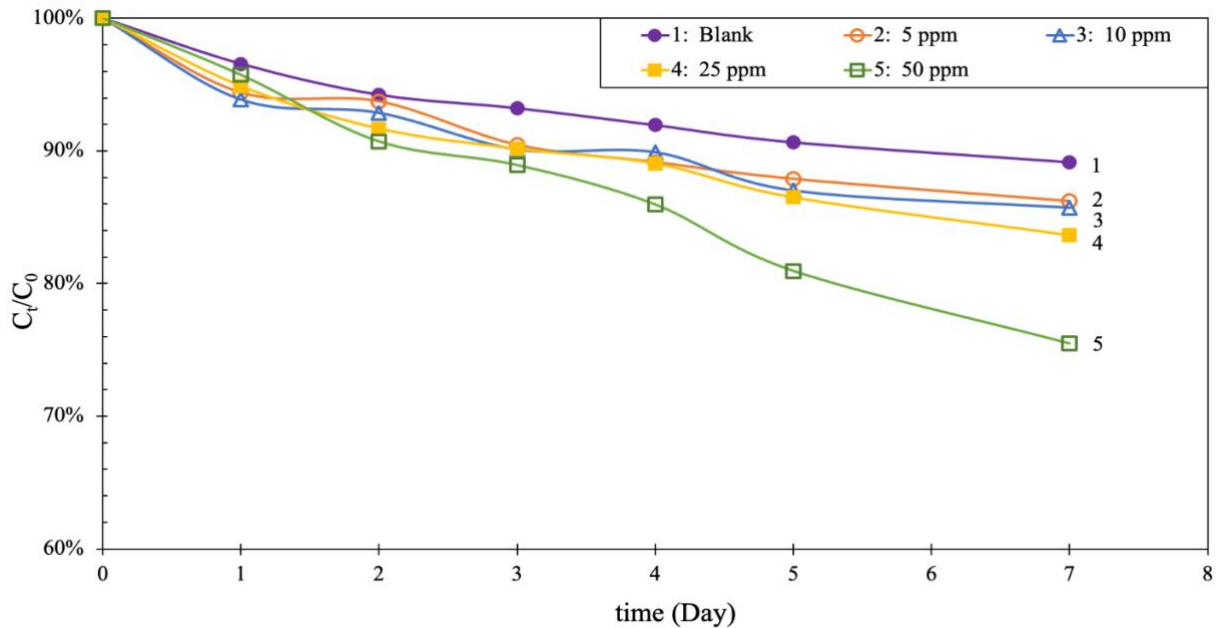


**Figure 2.2** Effects of varied $CuCl_2$ concentrations on remaining percentage of thiamine vs. time at 25 °C for 7 days.

**Figure 2.3** Effects of varied FeCl$_2$ concentrations on remaining percentage of thiamine vs. time at 25 °C for 7 days. * C$_t$: Concentration of thiamine at time t, C$_0$: Concentration of thiamine at time 0, t: Storage time in days.
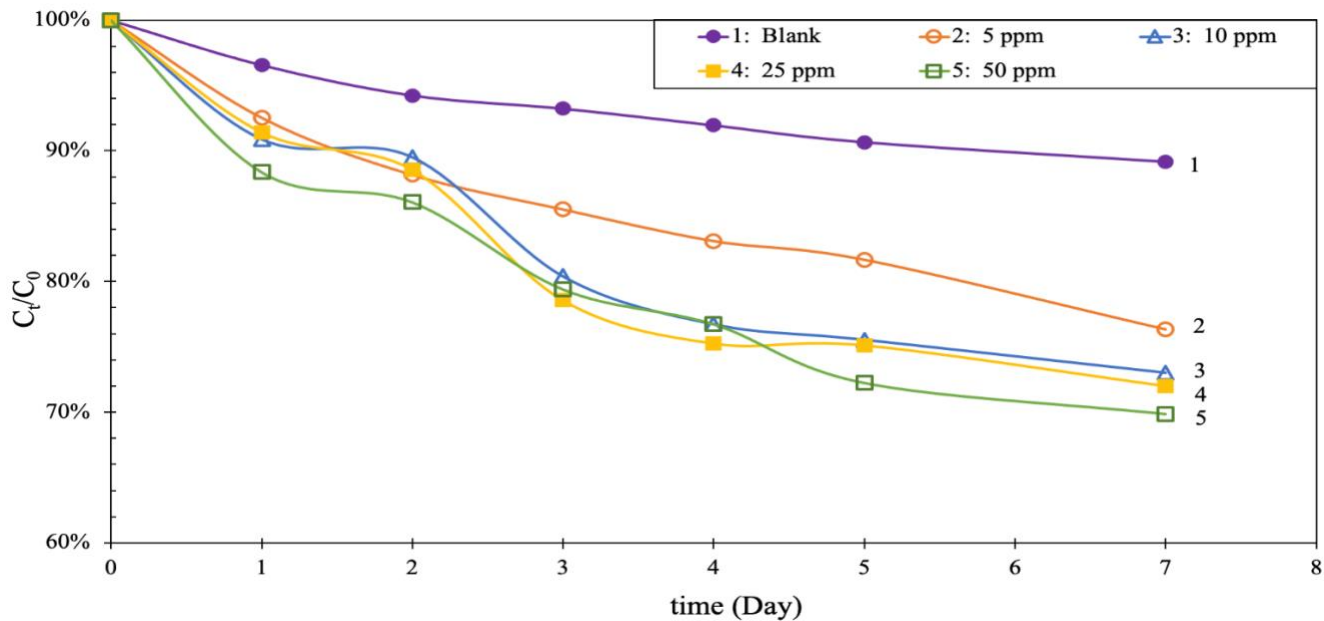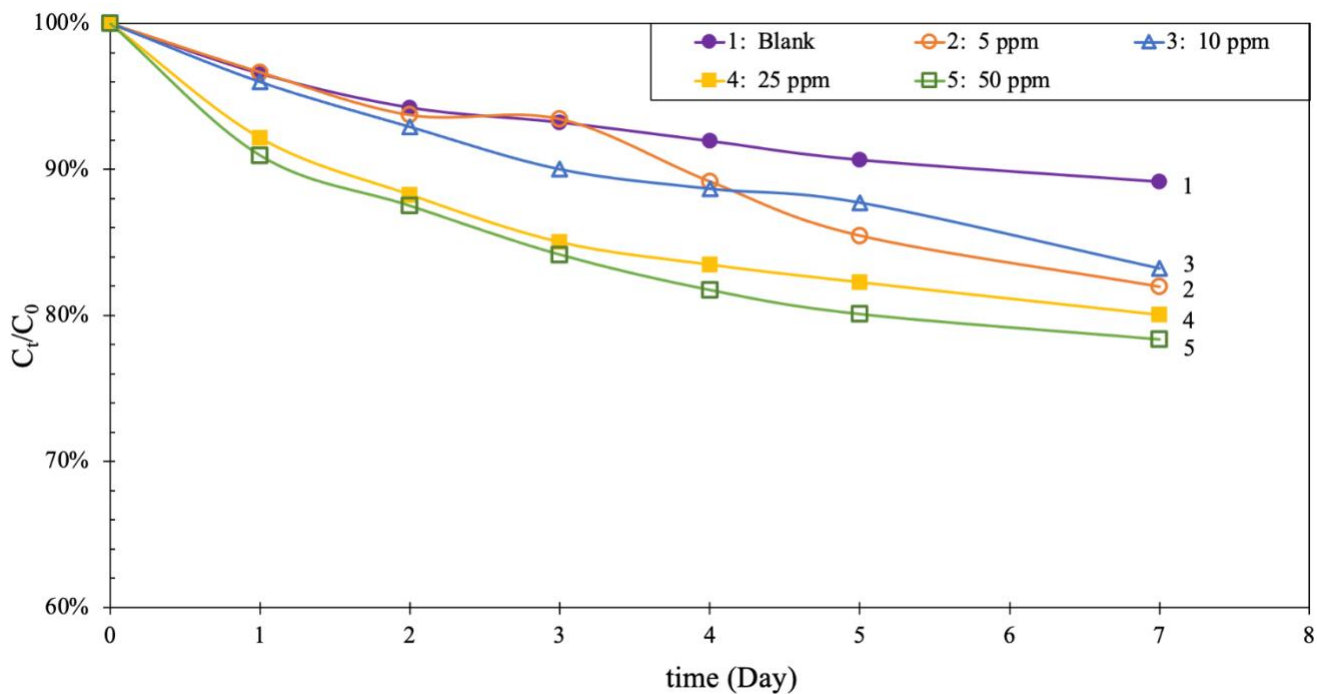


**Figure 2.4** Effects of varied FeCl$_3$ concentrations on remaining percentage of thiamine vs. time at 25 °C for 7 days.
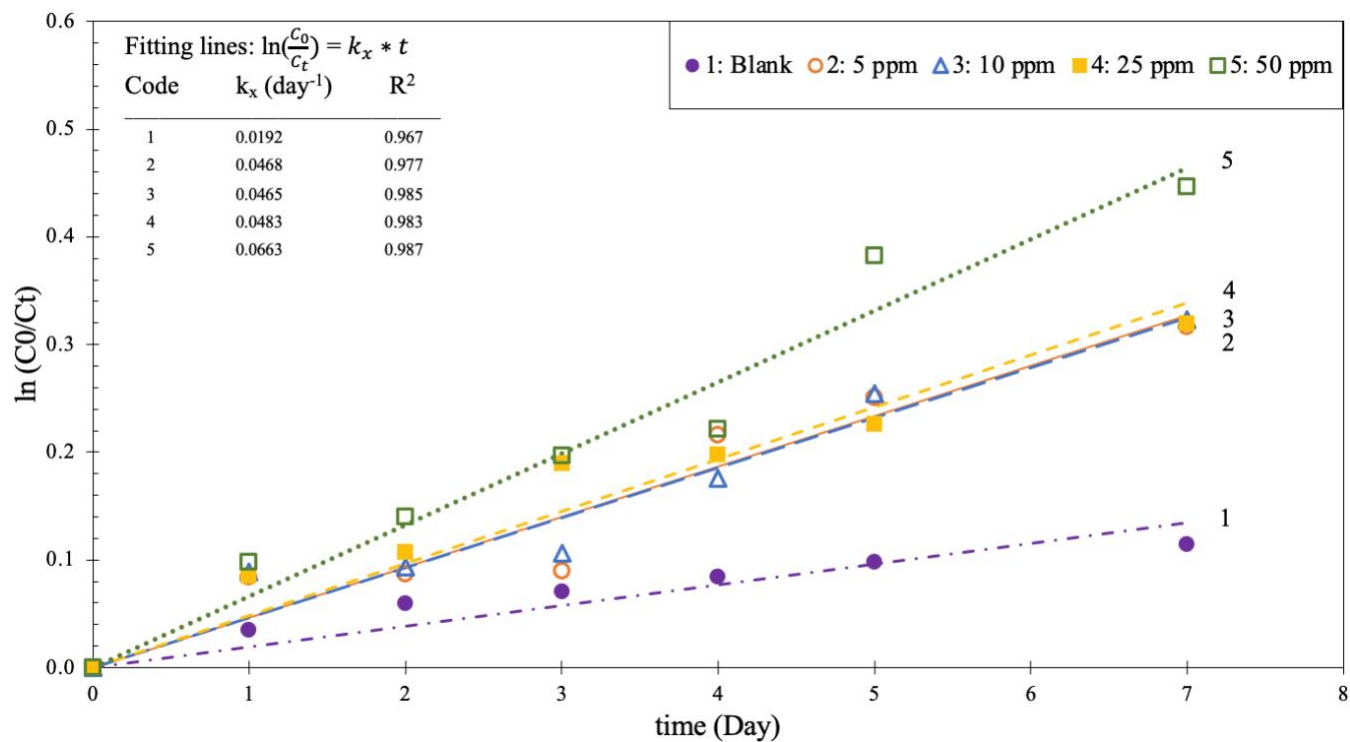
**Figure 2.5** First-order reaction of thiamine degradation with CuCl at 25 °C for 7 days.

The fitting table for Figure 2.5:

| Code | $k_x$ (day$^{-1}$) | $R^2$ |
|---|---|---|
| 1 | 0.0192 | 0.967 |
| 2 | 0.0468 | 0.977 |
| 3 | 0.0465 | 0.985 |
| 4 | 0.0483 | 0.983 |
| 5 | 0.0663 | 0.987 |

Fitting lines: $\ln(\frac{C_0}{C_t}) = k_x * t$
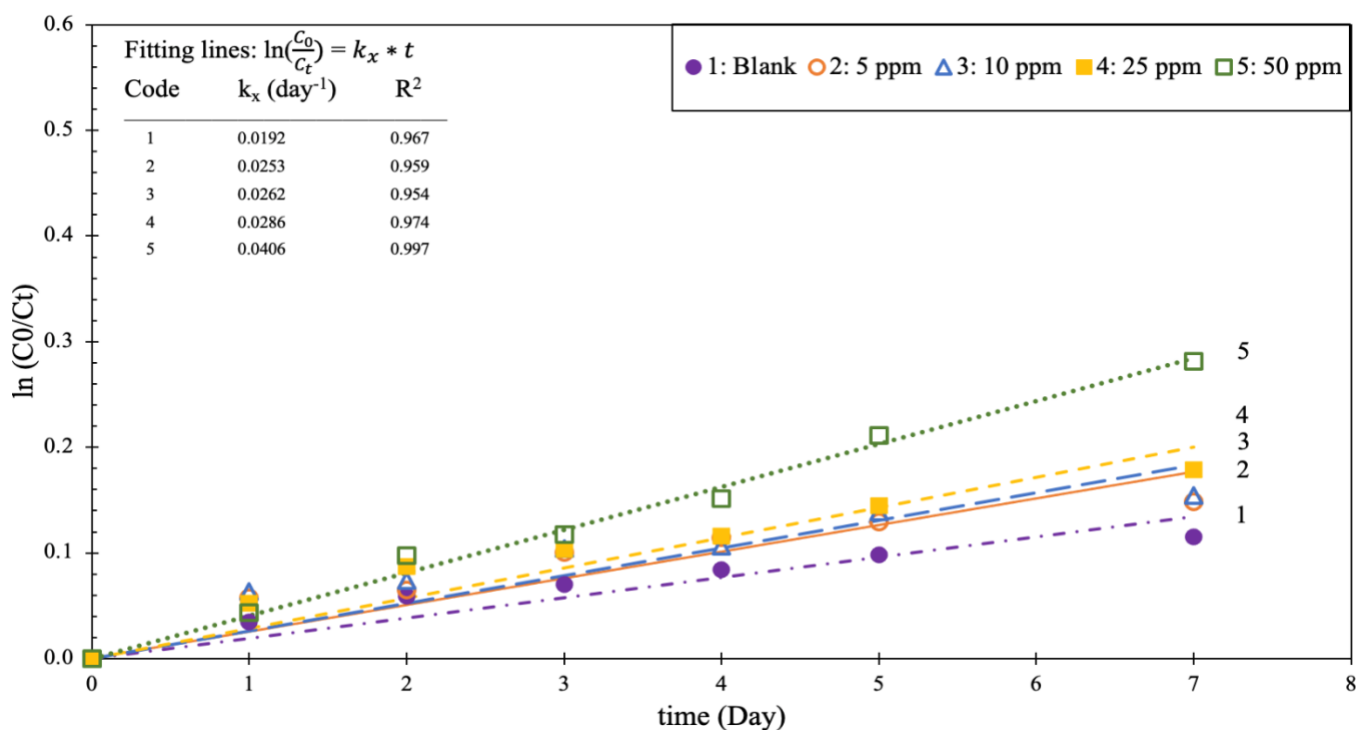


**Figure 2.6** First-order reaction of thiamine degradation with CuCl$_2$ at 25 °C for 7 days.

The fitting table for Figure 2.6:

| Code | $k_x$ (day$^{-1}$) | $R^2$ |
|---|---|---|
| 1 | 0.0192 | 0.967 |
| 2 | 0.0253 | 0.959 |
| 3 | 0.0262 | 0.954 |
| 4 | 0.0286 | 0.974 |
| 5 | 0.0406 | 0.997 |

Fitting lines: $\ln(\frac{C_0}{C_t}) = k_x * t$

**Figure 2.7** First-order reaction of thiamine degradation with $FeCl_2$ at 25 °C for 7 days.
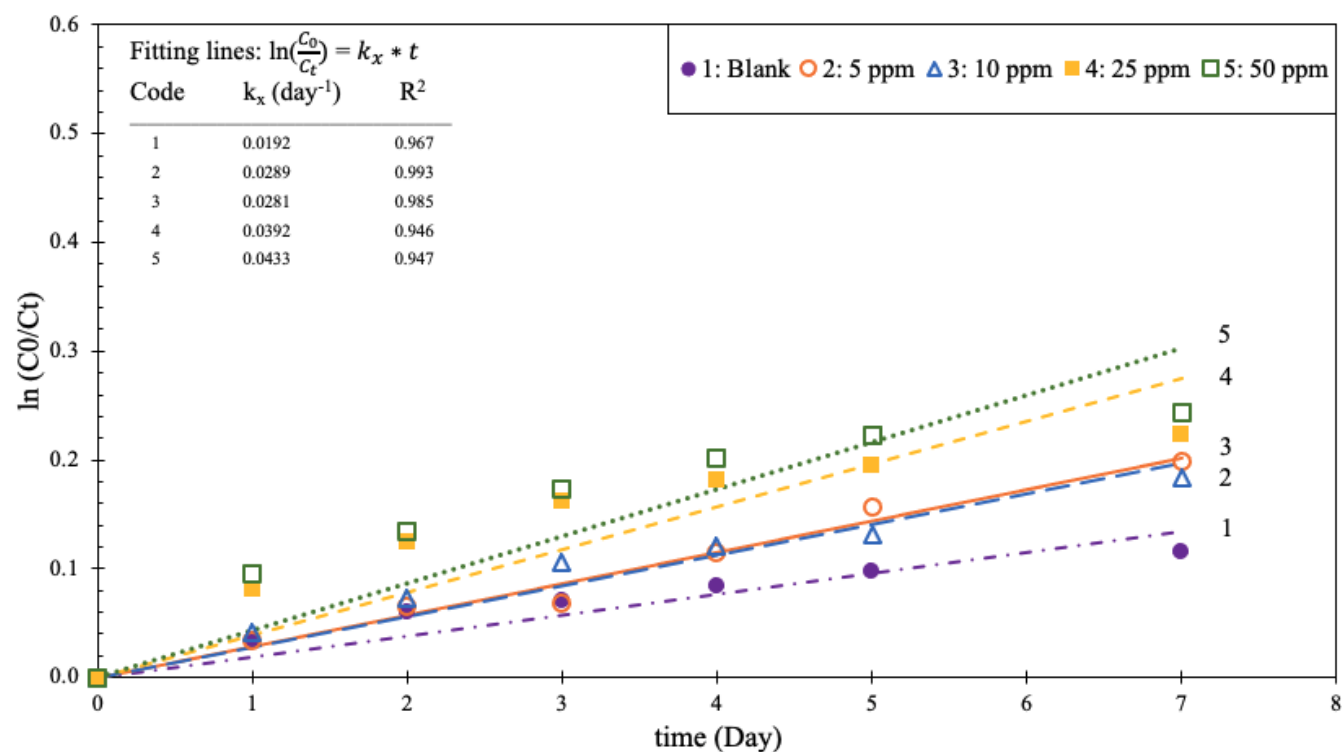


**Figure 2.8** First-order reaction of thiamine degradation with $FeCl_3$ at 25 °C for 7 days.
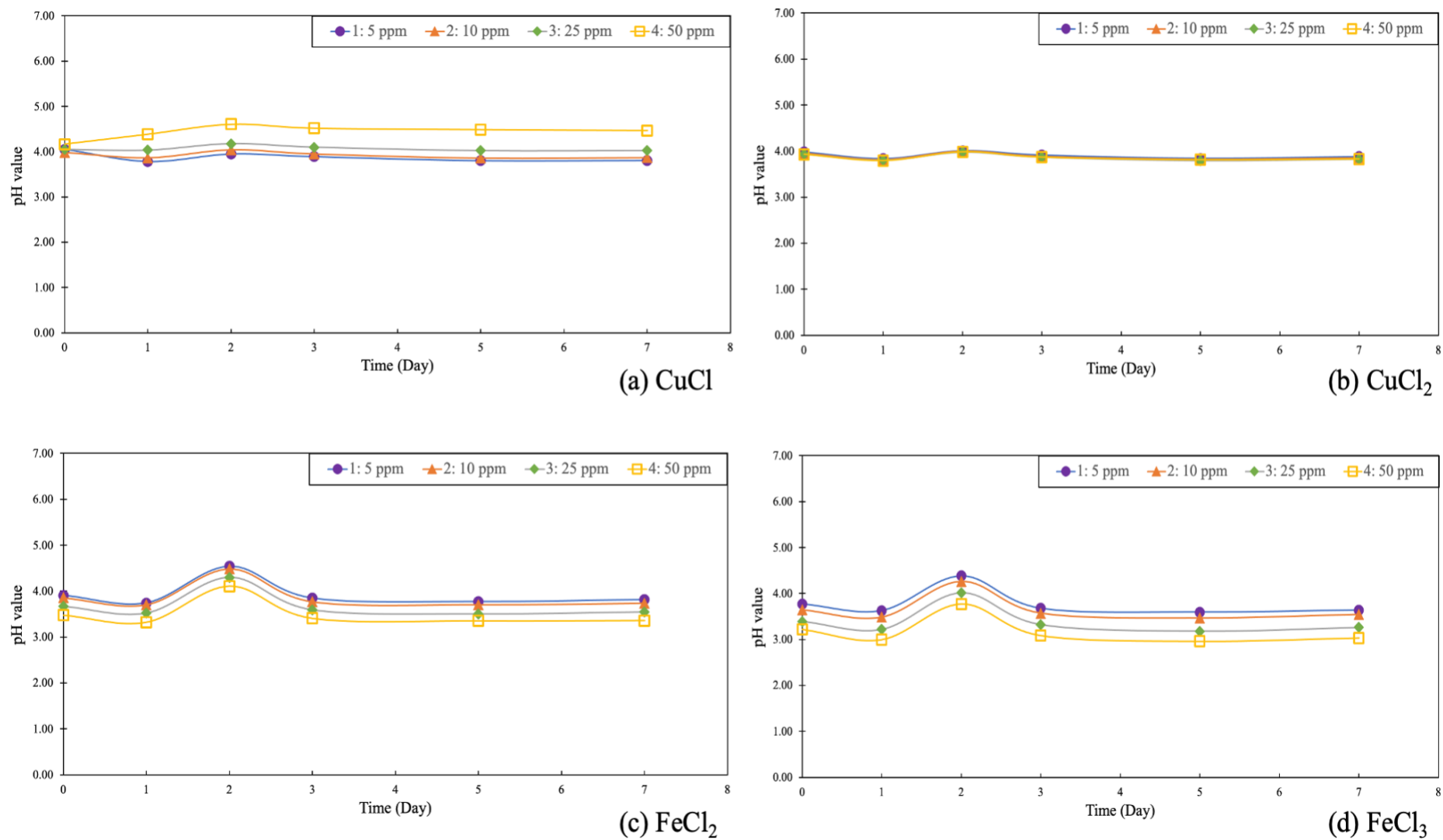
**Figure 2.9** pH changes in thiamine solutions with 4 metal chlorides (i.e., a) CuCl; b) CuCl$_2$; c) FeCl$_2$; d) FeCl$_3$) at studied concentrations (i.e., 5, 10, 25, and 50 ppm) during 7 days at 25 °C. *The pH values are also listed in **Table 2.5.**
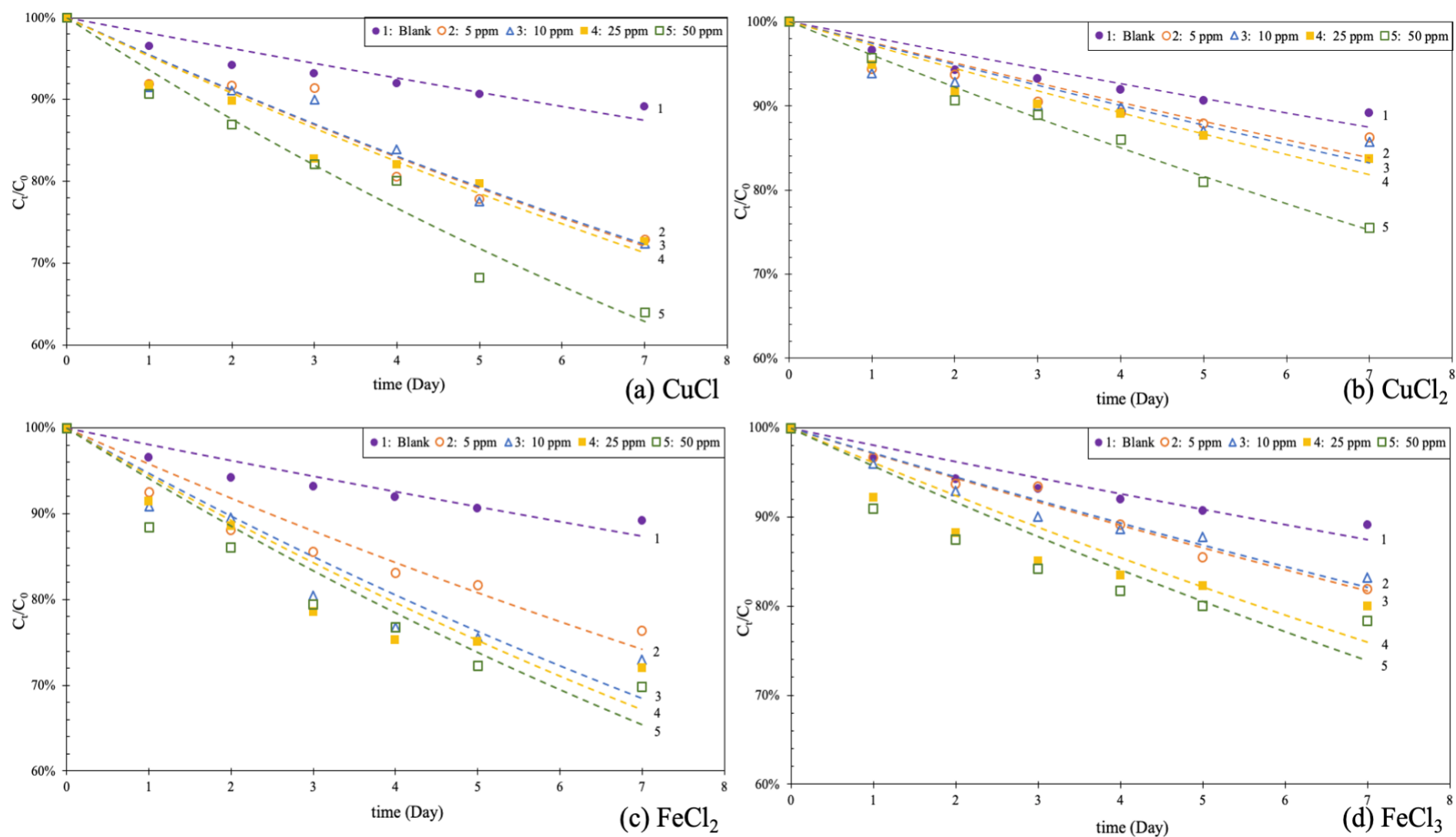
**Figure 2.10** The theoretical kinetic model for the four systems (i.e., a) CuCl; b) CuCl$_2$; c) FeCl$_2$; d) FeCl$_3$): the remaining percentage of thiamine vs. time for the degradation reactions in aqueous solutions at varied concentrations at 25 °C. The fitting line is $C_t/C_0 = \exp(-k_x t)$.
* $C_t$: Concentration of thiamine at time t, $C_0$: Concentration of thiamine at time 0, t: Storage time in days.

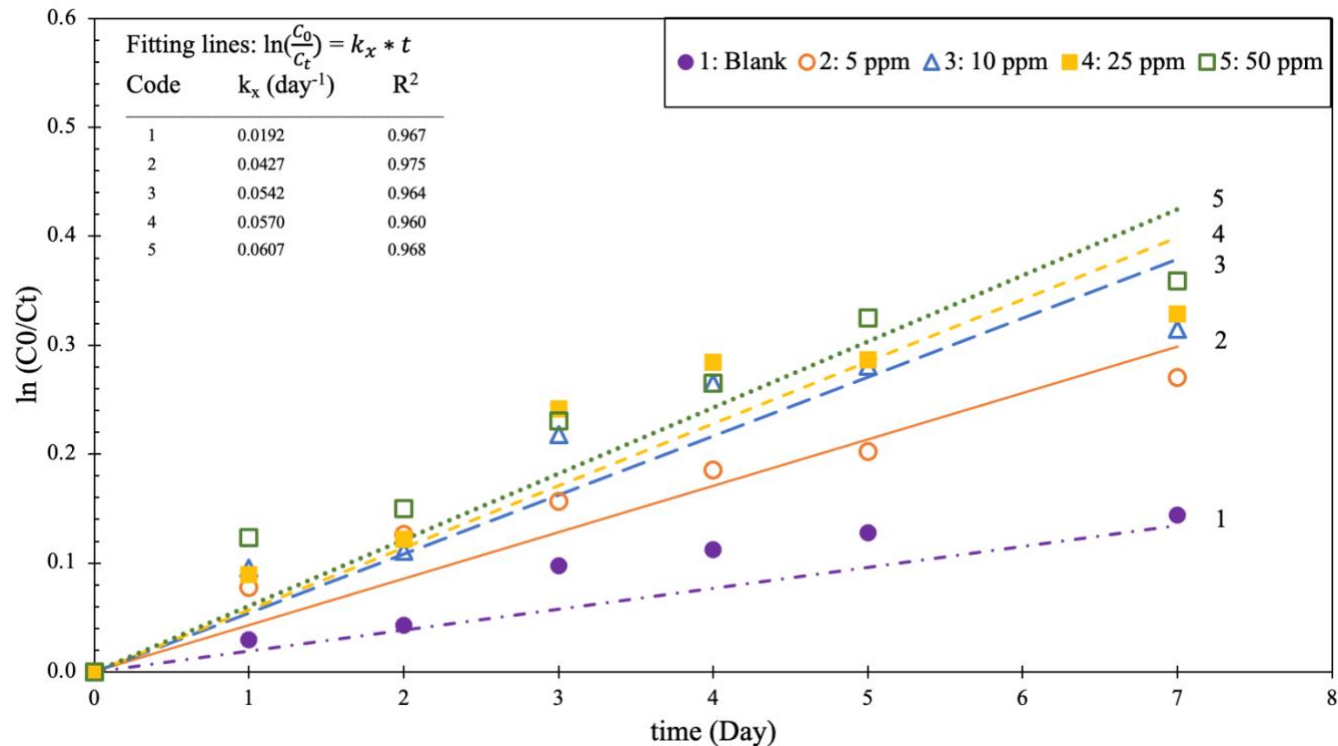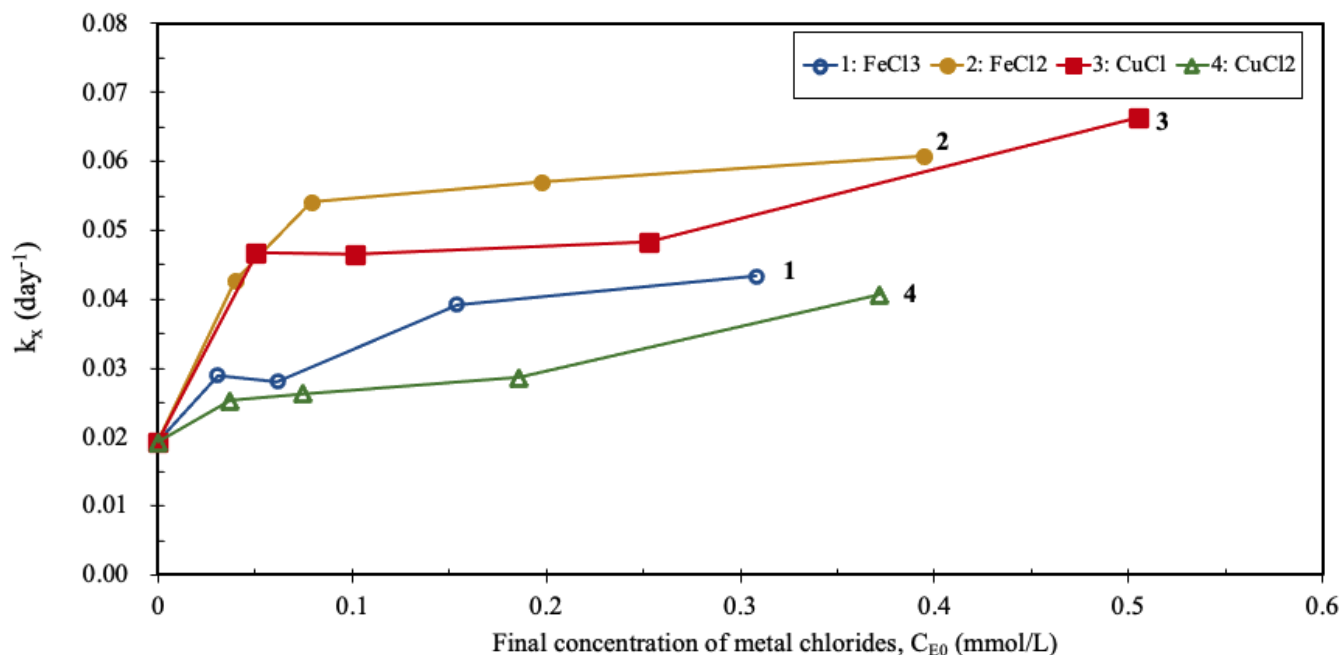**Figure 2.11** The comparison of $k_x$ of four types of metal chloride with different concentrations at 25 °C for thiamine degradation. [1]$C_{E0}$: the final concentration of metal chloride (i.e., 5, 10, 25, 50 ppm). [2]The unit of concentration was converted from ppm to mmol/L. [3]The mmol/L are also listed in **Table 2.3**.
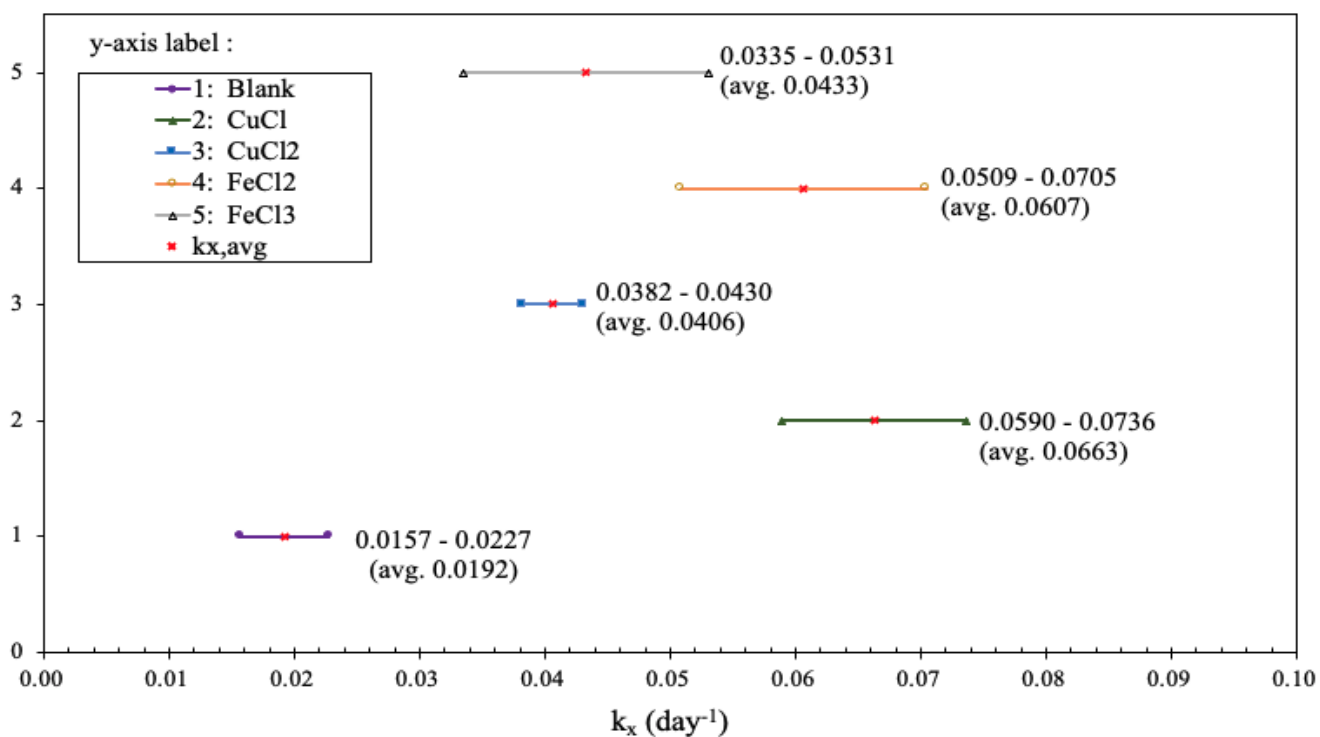


**Figure 2.12** The $k_x$ for thiamine degradation in four metal chlorides solution at 50 mg L^{-1} in 95% confidence interval (CI). *$k_x$ is in the fitting line: ln (Ct/ C0) = $k_x$ t.

**Table 2.1** Gradient elution program of thiamine quantification by HPLC

| Time (min) | Solvent A (%, v/v) 0.1 M Ammonium Acetate in water | Solvent B (%, v/v) Acetonitrile |
|---|---|---|
| 0 - 1.6 | 92 | 8 |
| 1.6 - 3.9 | 88 | 12 |
| 3.9 – 5.0 | 92 | 8 |

[1] The pH value of the solvent A was adjusted by 1 % acetic acid to 5.80 ± 0.02.

**Table 2.2** Remaining percentages of thiamine with four metal chloride (i.e., CuCl, CuCl$_2$, FeCl$_2$, FeCl$_3$) in aqueous solution at the studied concentrations (i.e., 5, 10, 25, 50 mg L$^{-1}$) during a storage time of 7 days at 25°C.

| Sample | Final Conc. (mg L$^{-1}$) | 0 | 1 | 2 | 3 (day) | 4 | 5 | 7 |
|---|---|---|---|---|---|---|---|---|
| **Thiamine** (Blank) | 250 | 100% | 96.56% | 94.23% | 93.22% | 91.94% | 90.65% | 89.15% |
| **CuCl** | 5 | 100% | 91.89% | 91.71% | 91.42% | 80.55% | 77.81% | 72.85% |
| | 10 | 100% | 91.54% | 91.08% | 89.94% | 83.88% | 77.54% | 72.38% |
| | 25 | 100% | 91.80% | 89.84% | 82.73% | 82.06% | 79.74% | 72.67% |
| | 50 | 100% | 90.69% | 86.92% | 82.11% | 80.10% | 68.22% | 64.00% |
| **CuCl$_2$** | 5 | 100% | 94.40% | 93.73% | 90.46% | 89.16% | 87.90% | 86.22% |
| | 10 | 100% | 93.86% | 92.86% | 90.10% | 89.89% | 87.01% | 85.71% |
| | 25 | 100% | 94.88% | 91.68% | 90.16% | 89.05% | 86.50% | 83.65% |
| | 50 | 100% | 95.72% | 90.70% | 88.92% | 85.95% | 80.95% | 75.47% |

**Table 2.2 (continued).**

| Sample | Final Conc. (mg L$^{-1}$) | 0 | 1 | 2 | 3 (day) | 4 | 5 | 7 |
|---|---|---|---|---|---|---|---|---|
| **FeCl$_2$** | 5 | 100% | 92.54% | 88.16% | 85.52% | 83.10% | 81.67% | 76.34% |
| | 10 | 100% | 90.90% | 89.52% | 80.40% | 76.73% | 75.54% | 73.02% |
| | 25 | 100% | 91.42% | 88.55% | 78.56% | 75.25% | 75.11% | 72.00% |
| | 50 | 100% | 88.37% | 86.07% | 79.40% | 76.73% | 72.24% | 69.86% |
| **FeCl$_3$** | 5 | 100% | 96.65% | 93.72% | 93.44% | 89.17% | 85.46% | 81.96% |
| | 10 | 100% | 95.99% | 92.91% | 90.01% | 88.68% | 87.73% | 83.23% |
| | 25 | 100% | 92.15% | 88.26% | 85.01% | 83.47% | 82.26% | 80.03% |
| | 50 | 100% | 90.95% | 87.50% | 84.15% | 81.73% | 80.08% | 78.34% |

**Table 2.3** Rate constants $k_x$ (day$^{-1}$) and the coefficient of determination ($R^2$) of thiamine in blank sample and different metal chlorides

(i.e., CuCl, CuCl$_2$, FeCl$_2$, FeCl$_3$) at the studied concentrations (i.e., 5, 10, 25, 50 mg L$^{-1}$) for 7 days.

| Sample type | Final concentration | | First-order kinetics parameters | |
|---|---|---|---|---|
| | (mg L$^{-1}$) | (mmol L$^{-1}$) | $k_x$ (day$^{-1}$) | $R^2$ |
| **Thiamine solution** (Blank sample) | 250 | | 0.0192 | 0.9674 |
| **CuCl** | 5 | 0.051 | 0.0468 | 0.9772 |
| | 10 | 0.101 | 0.0465 | 0.9848 |
| | 25 | 0.253 | 0.0483 | 0.9834 |
| | 50 | 0.505 | 0.0663 | 0.9874 |
| **CuCl$_2$** | 5 | 0.037 | 0.0253 | 0.9588 |
| | 10 | 0.074 | 0.0262 | 0.9542 |
| | 25 | 0.186 | 0.0286 | 0.9743 |
| | 50 | 0.372 | 0.0406 | 0.9971 |

**Table 2.3** (continued).

| Sample type | Final concentration | | First-order kinetics parameters | |
|:---:|:---:|:---:|:---:|:---:|
| | (mg L$^{-1}$) | (mmol L$^{-1}$) | $k_x$ (day$^{-1}$) | $R^2$ |
| **FeCl$_2$** | 5 | 0.039 | 0.0427 | 0.975 |
| | 10 | 0.079 | 0.0542 | 0.964 |
| | 25 | 0.197 | 0.0570 | 0.960 |
| | 50 | 0.394 | 0.0607 | 0.968 |
| **FeCl$_3$** | 5 | 0.031 | 0.0289 | 0.9931 |
| | 10 | 0.062 | 0.0281 | 0.9853 |
| | 25 | 0.154 | 0.0392 | 0.9455 |
| | 50 | 0.308 | 0.0433 | 0.9466 |

**Table 2.4** pH values of thiamine (blank sample) and different types of metal chlorides solutions.

| Final Conc. (mg L$^{-1}$) | Sample name | | | |
|---|---|---|---|---|
| | **Thiamine solution** (Blank sample) | | | |
| 250 | 3.94 $\pm$ 0.04 | | | |
| | **CuCl** | **CuCl$_2$** | **FeCl$_2$** | **FeCl$_3$** |
| 5 | 5.89 $\pm$ 0.02 [a] | 6.14 $\pm$ 0.05 [a,b] | 4.37 $\pm$ 0.05 [a] | 3.71 $\pm$ 0.02 [a] |
| 10 | 5.76 $\pm$ 0.04 [b] | 6.03 $\pm$ 0.08 [a,b] | 3.90 $\pm$ 0.01 [b] | 3.48 $\pm$ 0.01 [b] |
| 25 | 5.68 $\pm$ 0.01 [c] | 5.93 $\pm$ 0.02 [b,c] | 3.51 $\pm$ 0.03 [c] | 3.08 $\pm$ 0.02 [c] |
| 50 | 5.57 $\pm$ 0.02 [d] | 5.90 $\pm$ 0.01 [c,d] | 3.26 $\pm$ 0.02 [d] | 2.90 $\pm$ 0.01 [d] |

[1]all the pH values are mean $\pm$ standard deviation (M $\pm$ SD), n = 3. [2]The different letters in the same columns for each form (i.e., CuCl, FeCl$_2$, and FeCl$_3$) indicate that there are statistical differences between concentrations ($p < 0.05$), while the same letters in the column of CuCl$_2$ indicate there are no significant differences ($p > 0.05$).

**Table 2.5** pH value of thiamine samples with four types of metal chlorides solution at 25 °C during the storage time of 7 days.

| Sample name | Final Conc. (mg L$^{-1}$) | Storage time (days) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 5 | 7 |
| **Blank** | 250 | 3.94 ± 0.02 | 3.68 ± 0.01 | 3.90 ± 0.04 | 3.86 ± 0.05 | 3.81 ± 0.04 | 3.78 ± 0.02 |
| **CuCl** | 5 | 4.05 ± 0.03 [b,c] | 3.78 ± 0.01 [d] | 3.95 ± 0.01 [d] | 3.89 ± 0.01 [c,d] | 3.80 ± 0.02 [d] | 3.81 ± 0.01 [d] |
| | 10 | 3.98 ± 0.01[d] | 3.86 ± 0.01 [c] | 4.04 ± 0.01 [c] | 3.95 ± 0.02 [c,d] | 3.86 ± 0.02 [c] | 3.87 ± 0.01 [c] |
| | 25 | 4.05 ± 0.02 [b,c] | 4.03 ± 0.00 [b] | 4.17 ± 0.01 [b] | 4.10 ± 0.01 [b] | 4.02 ± 0.01 [b] | 4.02 ± 0.01 [b] |
| | 50 | 4.16 ± 0.04 [a] | 4.38 ± 0.01 [a] | 4.61 ± 0.02 [a] | 4.52 ± 0.01 [a] | 4.49 ± 0.02 [a] | 4.47 ± 0.01 [a] |
| **CuCl$_2$** | 5 | 3.98 ± 0.03 [a,b,c,d] | 3.83 ± 0.01 [a,b,c,d] | 4.01 ± 0.03 [a,b,c,d] | 3.91 ± 0.02 [a,b] | 3.84 ± 0.03 [a,b,c] | 3.88 ± 0.01 [a] |
| | 10 | 3.95 ± 0.02 [a,b,c,d] | 3.82 ± 0.02 [a,b,c,d] | 4.00 ± 0.01 [a,b,c,d] | 3.89 ± 0.01 [a,b,c,d] | 3.83 ± 0.02 [a,b,c] | 3.85 ± 0.00 [b] |
| | 25 | 3.94 ± 0.02 [a,b,c,d] | 3.80 ± 0.02 [a,b,c,d] | 3.98 ± 0.00 [a,b,c,d] | 3.87 ± 0.01 [b,c,d] | 3.79 ± 0.02 [a,b,c,d] | 3.82 ± 0.01 [c,d] |
| | 50 | 3.93 ± 0.02 [a,b,c,d] | 3.79 ± 0.03 [a,b,c,d] | 3.98 ± 0.01 [a,b,c,d] | 3.87 ± 0.02 [b,c,d] | 3.81 ± 0.02 [a,b,c] | 3.83 ± 0.02 [c,d] |

**Table 2.5** (continued)

| Sample name | Final Conc. (mg L$^{-1}$) | Storage time (days) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 5 | 7 |
| **FeCl$_2$** | 5 | 3.91 ± 0.02 $^a$ | 3.74 ± 0.03 $^{a,b}$ | 4.54 ± 0.01 $^a$ | 3.85 ± 0.03 $^a$ | 3.77 ± 0.02 $^a$ | 3.81 ± 0.03 $^a$ |
| | 10 | 3.85 ± 0.02 $^b$ | 3.70 ± 0.04 $^{a,b}$ | 4.48 ± 0.03 $^b$ | 3.77 ± 0.03 $^b$ | 3.70 ± 0.02 $^b$ | 3.74 ± 0.01 $^b$ |
| | 25 | 3.67 ± 0.03 $^c$ | 3.52 ± 0.02 $^c$ | 4.30 ± 0.03 $^c$ | 3.59 ± 0.02 $^c$ | 3.50 ± 0.01 $^c$ | 3.54 ± 0.02 $^c$ |
| | 50 | 3.48 ± 0.02 $^d$ | 3.32 ± 0.01 $^d$ | 4.11 ± 0.03 $^d$ | 3.41 ± 0.02 $^d$ | 3.35 ± 0.03 $^d$ | 3.36 ± 0.01 $^d$ |
| **FeCl$_3$** | 5 | 3.77 ± 0.02 $^a$ | 3.63 ± 0.03 $^a$ | 4.38 ± 0.03 $^a$ | 3.68 ± 0.01 $^a$ | 3.59 ± 0.04 $^{a,b}$ | 3.64 ± 0.01 $^a$ |
| | 10 | 3.65 ± 0.03 $^b$ | 3.49 ± 0.02 $^b$ | 4.27 ± 0.02 $^b$ | 3.57 ± 0.02 $^b$ | 3.47 ± 0.03 $^{a,b}$ | 3.55 ± 0.03 $^b$ |
| | 25 | 3.39 ± 0.02 $^c$ | 3.22 ± 0.03 $^c$ | 4.01 ± 0.02 $^c$ | 3.32 ± 0.02 $^c$ | 3.18 ± 0.01 $^c$ | 3.26 ± 0.01 $^c$ |
| | 50 | 3.22 ± 0.01 $^d$ | 2.99 ± 0.02 $^d$ | 3.77 ± 0.01 $^d$ | 3.08 ± 0.02 $^d$ | 2.96 ± 0.03 $^d$ | 3.03 ± 0.01 $^d$ |

[1]The concentrations represent the final concentrations in each sample solution. [2]All the pH values are mean ± standard deviation (M±SD), and n = 4. [3]The different letters in the same columns for each form indicate that there are statistical differences between concentrations ($p < 0.05$) (e.g., FeCl$_2$ on day 0 and FeCl$_3$ on day 7), while the same letters in the column of indicate there are no significant differences ($p > 0.05$) (e.g., CuCl$_2$ on day 0).

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CHAPTER THREE

EFFECT OF METAL IONS AND TEMPERATURES ON STABILITY OF THIAMINE

**Abstract**

The effects of four metal ions (i.e., $Cu^+$, $Cu^{2+}$, $Fe^{2+}$, and $Fe^{3+}$) on thiamine stability in aqueous solutions were also studied at different temperatures. The samples were stored in the incubator at 40°C and 55°C and monitored for seven days. A series of 250 mg $L^{-1}$ of THCl solutions containing four concentrations of copper and iron ions (i.e., 5, 10, 25, and 50 mg $L^{-1}$) were prepared, and their pH values were measured during the entire storage time. The thiamine content was analyzed by HPLC-UV/Vis instrument, of which the chromatographic condition was as same as that mentioned in Chapter 2. The experimental results demonstrated the thiamine degradation followed the first-order kinetic reaction under the test condition. Regarding the sample group stored at 40°C, the highest loss of thiamine was measured at 40.24% found in the 50 mg $L^{-1}$ $FeCl_2$ solution after seven days, while the lowest loss of thiamine was at 8.52% in the 50 mg $L^{-1}$ $FeCl_3$ solution. In comparison, for the sample group in a higher temperature at 55°C, the highest loss of thiamine was determined at 38.06% in the 50 mg $L^{-1}$ CuCl solution after seven days, while the lowest loss of thiamine was only 4.02% in 50 mg $L^{-1}$ $FeCl_3$ solution.

**3.1 Introduction**

As mentioned in Chapter 2, thiamine is subject to degradation under some conditions. Thiamine in aqueous solutions was found to follow a first-order reaction rate of degradation when being thermally treated at 110, 120, 130, 140, and 150 °C by two heating techniques (i.e., ampoule and capillary) (Ramaswamy et al., 1990). Moreover, the remaining amounts of thiamine were from 92.2% to 95.0% in orange juice and peas after the thermal treatment at 40°C to 120°C for up to 60

mins, so thiamine was thermostable in acid solutions in the aforementioned range of temperatures (Chuaqui-Offermanns et al., 1989). However, it was reported that pH and electrolyte systems might significantly affect the thiamine stability under high temperatures. When the samples were set at pH 5.4 and thermally treated in boiling water for an hour, there was an observation of 100% of thiamine destruction in the solutions with borates, 57% of thiamine destruction in unbuffered solution, 10% of the destruction in the solution with acetate, and 3% of loss in the solutions with phosphate. When increasing the pH of the samples to 7.0 under a similar thermal condition, the results showed 100% of thiamine destruction in the borate, acetate, and unbuffered solutions, except of 40% of its loss in the phosphate solution. Therefore, salt solutions can affect the thiamine stability (Beadle et al., 1943). An experiment showed that 4.1% of thiamine was lost at pH 3.5 under autoclaving at 121°C for 15 mins, while 32.7% of thiamine was degraded at pH 8.0 under the same thermal condition (Dwivedi and Arnold., 1972). This phenomenon was ascribed to the hypothesized mechanism for thiamine degradation under high pH that could change the chemical properties of thiamine. The protonated form of thiamine (I) is mainly present in an acidic environment, such as at pH 3.5, which was less prone to thermal destruction compared to thiamine in form II. The chemical equilibrium is depicted in **Figure 3.25** and can be described by the following equation:

$$thiamine\ (II) + H^+ \rightleftharpoons thiamine\ (I) \hspace{3cm} [1]$$
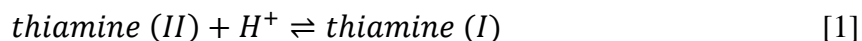
In Eq (1), the C-N bond of the methylene "bridge" between thiazole and pyrimidine moieties of thiamine was broken, leading to the formation of two by-products, 2-methyl-4-amino-5-hydroxymethyl pyrimidine and 4-methyl-5 ($\beta$-hydroxyethyl) thiazole. In acidic conditions, thiamine is present as form (I) or form (II), depending on the pH of the solution. In contrast, thiamine primarily exists as form (II) with small parts of pseudo base (III) and thiol form (IV) in

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neutral and slightly alkaline conditions, such as at pH 7.0 and 8.0. Besides, hydrogen sulfide might be the primary degradation product of the thiazole moiety of thiamine in these conditions. When hydrogen sulfide is released from thiamine, more form (II) of thiamine can be converted to the pseudo base form (III) and thiol (IV) (see **Figure 3.25**) (Dwivedi and Arnold., 1972). In thiamine solution, the pH decreases when being stored for a long-time due to the self-degradation or thiamine hydrolysis. It also results in the cleavage of methylene bridge between thiazole and pyrimidine along with the release of hydrogen ions in the solutions. Thiamine stability was enhanced over time in favorable pH condition (Voelker et al., 2021).

Some fortified foods with thiamine are commonly arranged for transportation nationally or internationally. Those products might be subject to fluctuating temperatures during the transportation and storage. Therefore, the retention of labile vitamins, such as thiamine, after a long-term duration is worth discussing. The objective of this study was to investigate the remaining thiamine contents at 40°C and 55°C during a storage time of 7 days, and establish the reaction kinetics for thiamine degradation.

### 3.2 Materials and methods

3.2.1 Chemicals and reagents

All chemical reagents used in this study were as same as those mentioned in Chapter 2.

3.2.2 Instrumentation

The HPLC system mentioned in Chapter 2 was also used for chemical analysis in this Chapter.

3.2.3 Preparation of standards and metal chlorides in aqueous solutions and sample storage

The preparation of the stock solution and aqueous solutions of metal chlorides (i.e., CuCl, CuCl$_2$, FeCl$_2$, and FeCl$_3$) were as same as those introduced in Chapter 2. The test samples were stored at 40°C and 55°C to monitor the thiamine stability. The samples were stored in a Fisher Scientific isotemp water jacked CO$_2$ incubator (Revco Technologies, Asheville, NC), of which temperatures were controlled in a fluctuation within ± 0.2°C. On day 0, the fresh samples were analyzed at room temperature. After that, all samples were stored in the temperature-controlled environments at 40°C or 55°C for up to 7 days. Each test sample was conducted in triplicate at room temperature. The thiamine degradation was determined by HPLC-UV.

3.2.4 Determination of remaining amounts of thiamine

The remaining amounts of thiamine in each sample were also expressed as relative percentages.

3.2.5 First-order reaction of the thiamine degradation

The kinetic parameters of the thiamine degradation under high temperatures and with four metal chlorides (i.e., CuCl, CuCl$_2$, FeCl$_2$, and FeCl$_3$) were investigated for a period of 7 days. The expression of the kinetic rate for the degradation reaction is as same as that described in Chapter 2.

3.2.6 Statistical analysis

The Microsoft Excel 2019 mentioned in Chapter 2 was also used for the statistical analysis in this chapter.

## 3.3 Results and discussion

3.3.1 Effect of four metal chlorides on thiamine stability in THCl solution at 40°C

The remaining percentages of thiamine are displayed in **Table 3.1**, and the corresponding results are profiled in **Figure 3.1** to **Figure 3.4**. The first-order kinetic models are shown in **Figure 3.5** to **Figure 3.8**. The $k_x$ values and $R^2$ were shown in **Table 3.2**. The pH values in each mixture of thiamine and salt solution are listed in **Table 3.3** and **Figure 3.9**. The theoretical kinetic models are exhibited in **Figure 3.10**.

3.3.1.1 Effect of CuCl and $CuCl_2$ on thiamine stability

In **Table 3.1**, **Figure 3.1**, and **Figure 3.2**, on day 7, compared to the control group (80.18% of thiamine remained), 73.01% of thiamine was detected in the 50 mg $L^{-1}$ of CuCl solutions, and 61.96% of thiamine remained in the 50 mg $L^{-1}$ of $CuCl_2$ solutions. It was out of expectation that thiamine degradation in $CuCl_2$ was more apparent than in CuCl. In **Table 3.2**, **Figure 3.5**, and **Figure 3.6**, firstly, the $k_x$ value increased from 2.93 x $10^{-2}$ at 5 mg $L^{-1}$ to 5.37 x $10^{-2}$ at 50 mg $L^{-1}$ in the CuCl solution. Secondly, the $k_x$ value also increased from 5.89 x $10^{-2}$ at 5 mg $L^{-1}$ to 7.68 x $10^{-2}$ at 50 mg $L^{-1}$ in the $CuCl_2$ solution. In **Figure 3.23**, the degradation rates in the $CuCl_2$ solution were significantly faster (p <0.05) than in 50 mg $L^{-1}$ of the CuCl solution. Although thiamine in the acidic solutions is more stable, the trend of increasing pH values in the copper solutions during the storage might be expected to speed up the thiamine degradation. For example, in **Table 3.3** and **Figure 3.9**, the pH value of the 50 mg $L^{-1}$ of CuCl solution increased from 3.91 on day 0 to 4.23 on day 7, and the pH in 50 mg $L^{-1}$ of $CuCl_2$ solution also increased from 3.73 on day 0 to 4.85 on day 7. Besides, thiamine complexes with copper ions might be formed in the aqueous solution, which might also affect the thiamine stability.

3.3.1.2 Effect of $FeCl_2$ and $FeCl_3$ on thiamine stability

In **Table 3.1**, **Figure 3.3**, and **Figure 3.4**, the remaining thiamine in the $FeCl_2$ solution at 40 °C was determined to be around 59.76 % to 68.17% from 5 mg $L^{-1}$ to 50 mg $L^{-1}$ on day 7. In comparison, around 82.40% to 91.48% of thiamine at the studied concentrations was remained in $FeCl_3$ solutions. In **Table 3.2**, **Figure 3.7**, **Figure 3.8**, and **Figure 3.23**, the $k_x$ value was 7.52 x $10^{-2}$ at 50 mg $L^{-1}$ of the $FeCl_2$ solution, which was higher than 1.58 x $10^{-2}$ at 50 mg $L^{-1}$ of the $FeCl_3$ solution, which was consistent with the observation that the speed of thiamine degradation in 50 mg $L^{-1}$ of $FeCl_2$ solution was significantly faster ($p < 0.05$) than the counterpart of $FeCl_3$. The phenomenon was ascribed to the acidity of the solutions. For example, both $FeCl_2$ and $FeCl_3$ solutions at 50 mg $L^{-1}$ were measured at pH 3.57 and pH 3.08 on day 7 (see **Table 3.3** and **Figure 3.9**). As a result, 91.48% thiamine was detected in the 50 mg $L^{-1}$ $FeCl_3$ solution on day 7, which was the lowest loss of thiamine among all the samples due to the low pH value in the $FeCl_3$ solution. Besides the factor of pH value, the formation of a $Fe^{2+}$ complex with thiamine that results in an observable thiamine degradation was also hypothesized though the solutions were quite acidic.

3.3.2 Effect of four metal chlorides on thiamine stability in THCl solution at 55°C

At 55°C, results of the remaining thiamine in the salt solutions are listed in **Table 3.4**, and the degradation trends are depicted in **Figure 3.11** to **Figure 3.14**. The first-order kinetic models and their diagrams with $k_x$ values and $R^2$ are shown in **Table 3.5** and **Figure 3.15** to **Figure 3.18**. The pH values of each sample were also tested (see **Table 3.6** and **Figure 3.19**). Lastly, the models of thiamine degradation in a theoretical condition are also released in **Figure 3.20**.

3.3.2.1 Effect of CuCl and $CuCl_2$ on thiamine stability

In **Table 3.4**, **Figure 3.11**, and **Figure 3.12**, on day 7, only 61.94% and 85.81% of thiamine were detected in the CuCl and CuCl$_2$ solutions at 50 mg L$^{-1}$, compared to 92.08% of thiamine in the control group. As shown in **Table 3.5**, the $k_x$ value increased from 2.84 x 10$^{-2}$ at 5 mg L$^{-1}$ to 7.60 x 10$^{-2}$ at 50 mg L$^{-1}$ of the CuCl solution, and the $k_x$ value also increased from 9.7 x 10$^{-3}$ at 5 mg L$^{-1}$ to 2.54 x 10$^{-2}$ at 50 mg L$^{-1}$ of the CuCl$_2$ solution. The degradation rate in CuCl solution was significantly lower ($p < 0.05$) than that in the CuCl$_2$ solution (see **Figure 3.24**). In **Table 3.6** and **Figure 3.19**, on day 7, the pH value of the CuCl solution at concentration of 50 mg L$^{-1}$ was measured at 3.84, compared to the pH value of 3.67 for the 50 mg L$^{-1}$ CuCl$_2$ solution, and pH of 3.73 of the control group. As a result, more substantial thiamine degradation occurred in the CuCl solution due to its pH value compared to that of the control group and CuCl$_2$ solution,

3.3.2.2 Effect of FeCl$_2$ and FeCl$_3$ on thiamine stability

As shown in **Table 3.4**, **Figure 3.13**, and **Figure 3.14**, on day 7, the remaining thiamine in the FeCl$_2$ solution within concentrations from 5 to 50 mg L$^{-1}$ was from 82.73% to 87.20%. However, in the FeCl$_3$ solution, it was unexpectedly that 89.12% to 95.98% of thiamine still remained in the solutions at the studied concentrations. In **Table 3.5**, **Figure 3.17**, and **Figure 3.18**, the $k_x$ value increased from 2.09 x 10$^{-2}$ at 5 mg L$^{-1}$ to 3.12 x 10$^{-2}$ at 50 mg L$^{-1}$ of the FeCl$_2$ solution. In contrast, the $k_x$ value decreased from 1.81 x 10$^{-2}$ at 5 mg L$^{-1}$ to 5.4 x 10$^{-3}$ at 50 mg L$^{-1}$ in the FeCl$_3$ solution. As shown in **Table 3.6** and **Figure 3.19**, on day 7, the pH 3.19 was measured at 50 mg L$^{-1}$ of the FeCl$_2$ solution, and pH 2.97 was at 50 mg L$^{-1}$ of the FeCl$_3$ solution. Thus, it was speculated that the thiamine was stable even at relatively high temperatures such as 55°C due to more acidic condition in the FeCl$_3$ solution. However, the degradation rate in FeCl$_2$ was significantly faster ($p < 0.05$) than those in FeCl$_3$ and control group, which means FeCl$_2$ has more

influence on the thiamine stability even though this salt still provides an acidic environment, which was considered in favor of stabilizing thiamine.

3.3.3 Comparison of thiamine stability in different salt solutions at 40 and 55°C

As shown in **Figure 3.23** and **Figure 3.24**, at 40°C, the $k_x$ values of the thiamine degradation in salt solutions were determined in the following order: $FeCl_2 > CuCl_2 > CuCl > FeCl_3$. At 55°C, the $k_x$ values were as follows: $CuCl > FeCl_2 > CuCl_2 > FeCl_3$. The highest degradation rate was found in the $FeCl_2$ solution at 40°C, while the highest rate was in the CuCl solution at 55°C. Higher temperature (from 40 to 55°C) speed up the thiamine degradation in the CuCl solutions. Nevertheless, the lowest degradation rates at 40°C and 55°C were both in the $FeCl_3$ solutions. The degradation rates in $FeCl_3$ were significantly lower ($p < 0.05$) than the other systems at both temperatures. The degradation rate was not so obvious ($p > 0.05$) between the $CuCl_2$ and $FeCl_2$ solutions at both 40 and 55°C.

When discussing the $k_x$ values of metal ions, the unit of concentrations in salt solutions should be converted from ppm to molarity. At 40°C, the $k_x$ values at around 0.3 mmol $L^{-1}$ in the solution were determined in the following order: $Fe^{2+} = Cu^{2+} > Cu^+ > Fe^{3+}$ (see **Figure 3.21**). At 55°C, the $k_x$ values of the salt solutions at around 0.3 mmol $L^{-1}$ were in the following order: $Cu^+ > Fe^{2+} > Cu^{2+} > Fe^{3+}$ (see **Figure 3.22**). The degradation rate caused by $Cu^+$ ion apparently increased with the increased concentration of the ion at both 40 and 55°C. Moreover, $Fe^{2+}$ might form a metal complex to increase the oxidation-reduction system in thiamine degradation, which might overshadow the influence of its acidity on thiamine stability.

For the CuCl system, its pH value increased along with the addition of CuCl in the solution, resulting in the highest losses of thiamine, which were observed with the remaining amounts of thiamine at 64.00% in pH 4.47 at 25°C and 61.94% in pH 3.84 at 55°C, although 73.01% of

thiamine remained in the 50 mg $L^{-1}$ of the same solution at pH 4.23 on day 7. These unexpected results might be caused by some unknown factors leading to the experimental errors, such as the contamination of HPLC column that caused poor elution during the analysis.

For the $FeCl_3$ system, the lowest thiamine losses were observed in the solution with 91.48% at 40°C and 95.98% at 55°C, which was in agreement with my observation of stronger acidity (or lower pH value) in the solutions with the addition of $FeCl_3$, which benefits the thiamine stability.

Theoretical kinetic models for the thiamine degradation in each metal chloride system at 40°C and 55°C were formed to explain theoretical trends of the degradation along with time (see **Figure 3.10** and **Figure 3.20**). At 40°C, some data deviated from the theoretical line, such as those in the control group and in CuCl solutions at 25 and 50 mg $L^{-1}$, which means other factors might also be involved in the degradation, or there are experimental errors in the collected data (see **Figure 3.10**). Compared to the results at 40°C, most data at 55°C were close to their theoretical lines, which means the models have been more accurately illustrated for the thiamine degradation during the storage time at 55°C (see **Figure 3.20**).

Due to the opposing effect of pH on thiamine degradation, higher temperature (at 40 and 55°C) might not significantly influence thiamine stability. However, it might be a mistake that the metal solutions were not kept in the buffer solutions when determining the thiamine stability at higher temperatures. In summary, it is worthy of mentioning that my research results were only based on the experiments on these four metal chlorides at the studied concentrations for short storage time without considering other factors.

### 3.4 Conclusion

In this chapter, at 40°C, the metal chlorides at the 50 mg $L^{-1}$ on day 7 showed their impacts on thiamine stability in the following order: $FeCl_2 > CuCl_2 > CuCl > FeCl_3$, with the remaining content of thiamine in 59.76%, 61.96%, 73.01%, and 91.48%, respectively. At 55°C, the effect of the metal ions at the 50 mg $L^{-1}$ on day 7 on thiamine stability changed to the following order: $CuCl > FeCl_2 > CuCl_2 > FeCl_3$, with the remaining content of 61.94%, 82.73%, 85.81%, and 95.98%. The highest loss of thiamine that was observed in the CuCl solution at 55°C on day 7, which was attributed to its highest pH value in the solution, leading to a significant thiamine degradation. In contrast, the lowest loss of thiamine was found in the $FeCl_3$ solution at 55°C on day 7. Besides, the chemical properties of the metal chlorides (i.e., $FeCl_2$ and $CuCl_2$) might be also involved in the thiamine degradation because they might be prone to form a metal complex for causing a self-catalytic effect on thiamine stability. The thiamine degradation followed the first-order kinetic model, and the speed of degradation could be determined. The CuCl solutions at 55°C had the fastest rate of thiamine degradation, while the $FeCl_3$ solution gave the slowest degradation rate at 55°C.

In conclusion, thiamine stability could be influenced by temperatures, pH values, and types of metal chlorides. Based on my research results, it was believed that $Cu^+$ ions have more impact on the thiamine stability, while $Fe^{3+}$ ions have the least influence on the thiamine stability. I hope my research results will provide more insights on the study of thiamine stability, and be useful for the quality control of products fortified with thiamine.
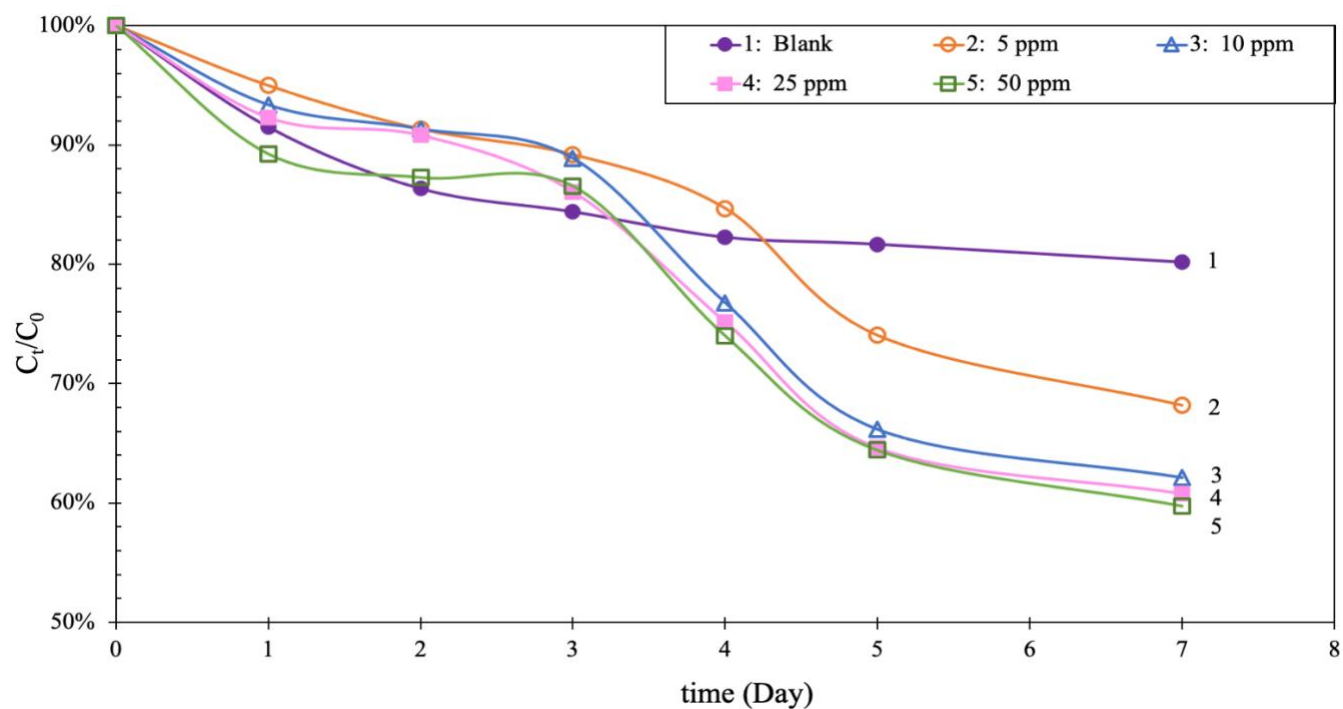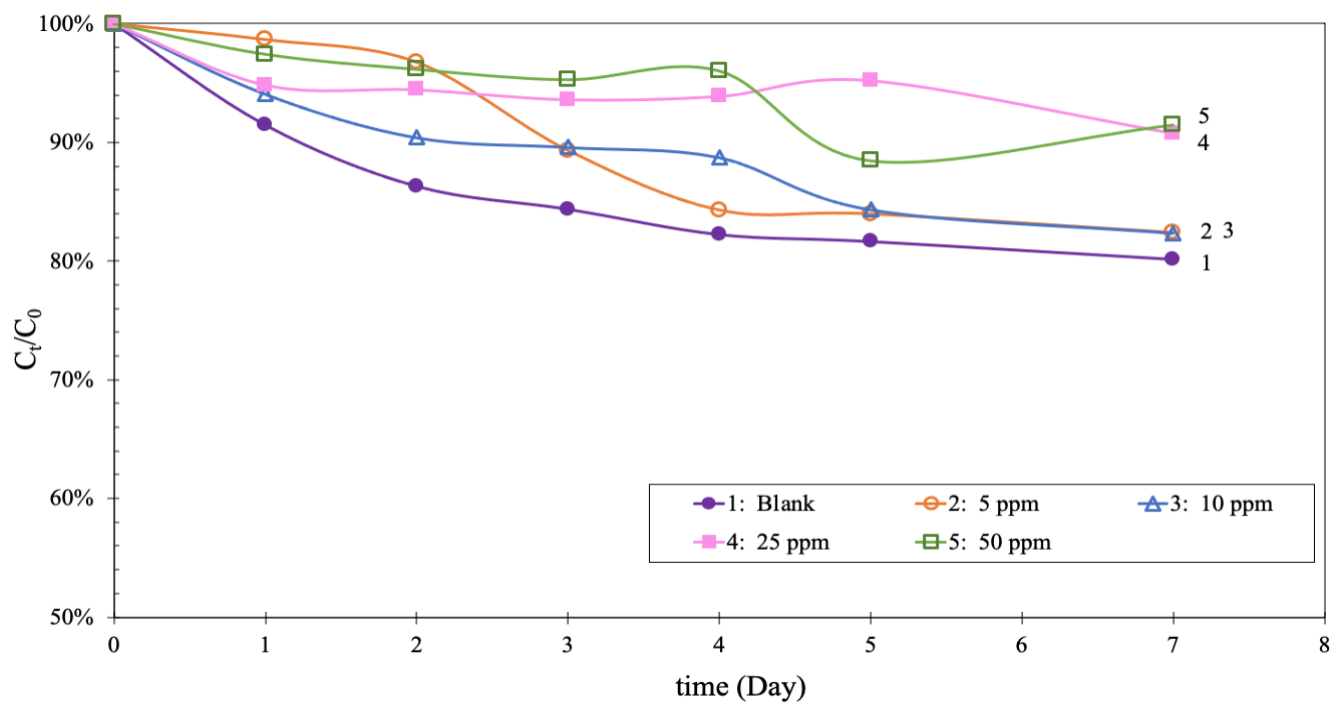
## 3.5 Figures and Tables
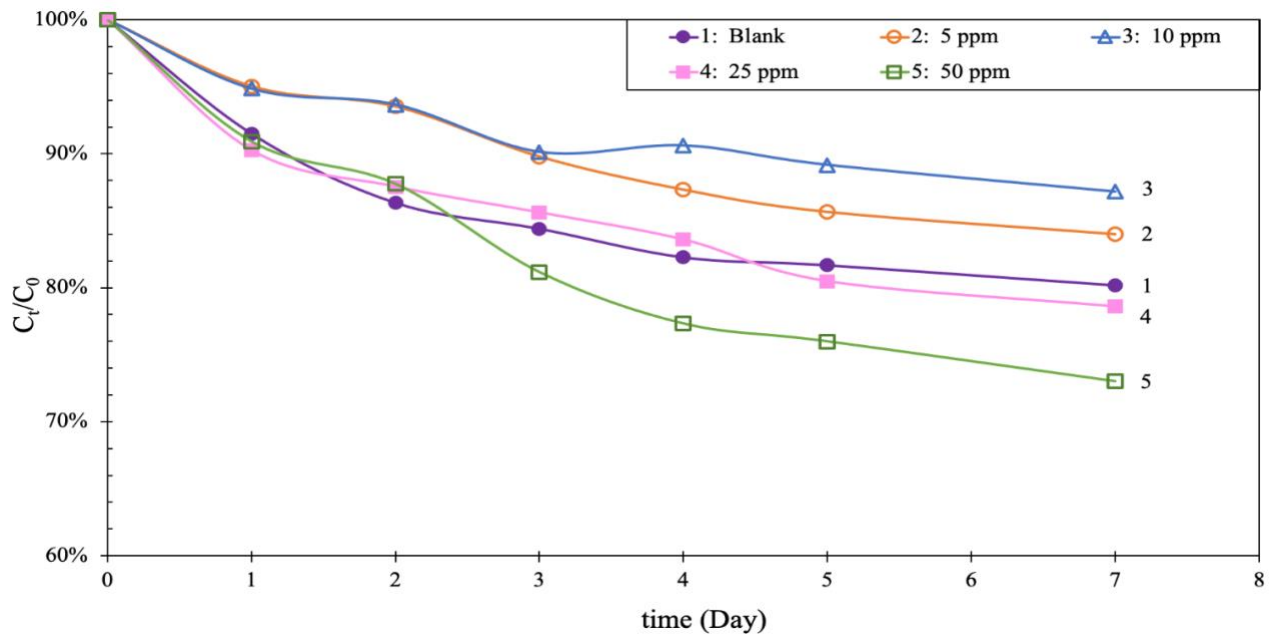


**Figure 3.1** 40°C group: effects of varied CuCl concentrations on remaining percentage of thiamine vs. time for 7 days. * $C_t$: Concentration of thiamine at time t, $C_0$: Concentration of thiamine at time 0, t: Storage time in days.
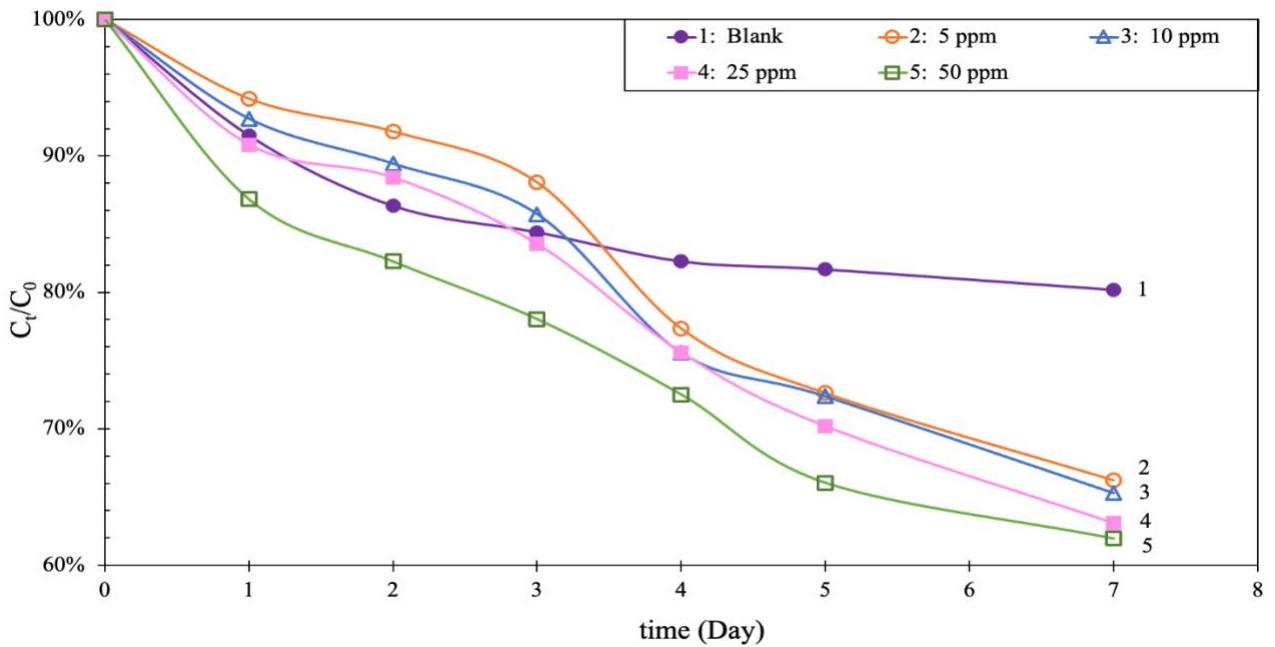


**Figure 3.2** 40°C group: effects of varied $CuCl_2$ concentrations on remaining percentage of thiamine vs. time for 7 days.

**Figure 3.3** 40°C group: effects of varied $FeCl_2$ concentrations on remaining percentage of thiamine vs. time for 7 days. * $C_t$: Concentration of thiamine at time t, $C_0$: Concentration of thiamine at time 0, t: Storage time in days.
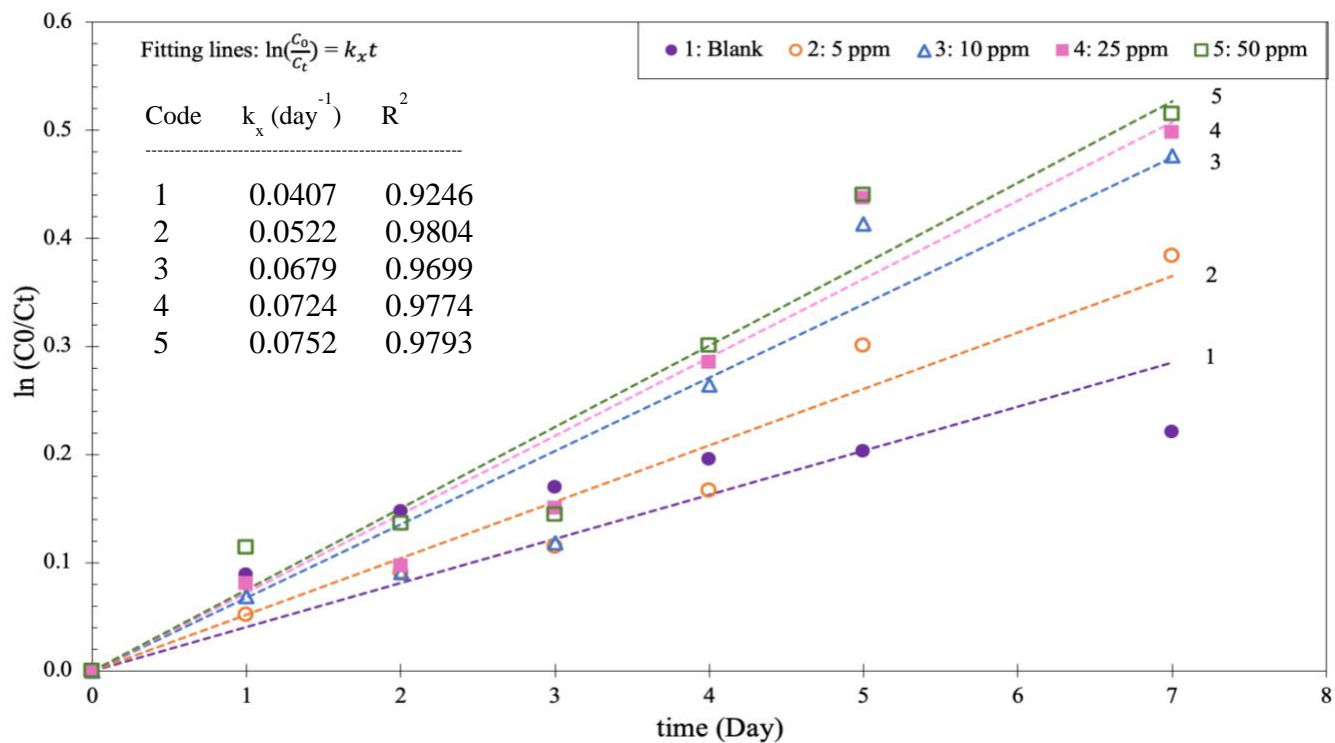


**Figure 3.4** 40°C group: effects of varied $FeCl_3$ concentrations on remaining percentage of thiamine vs. time for 7 days.
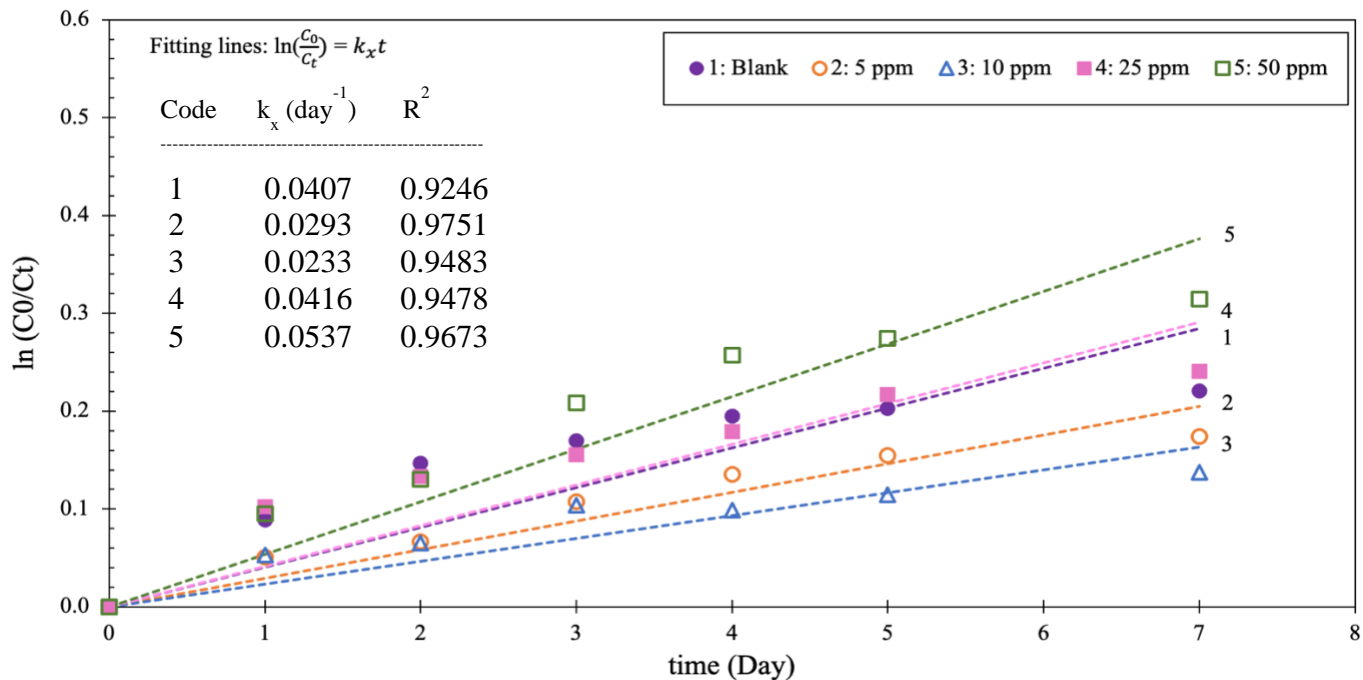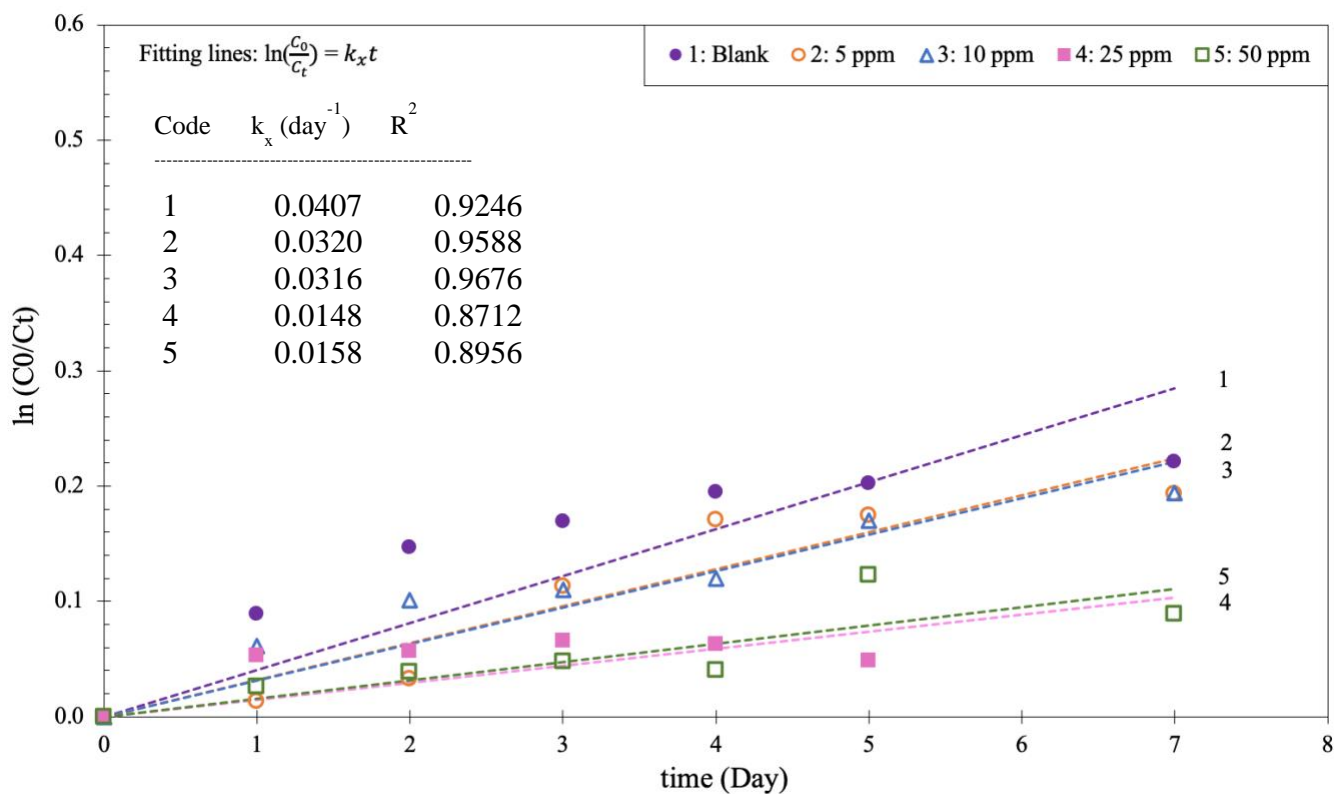
**Figure 3.5** First-order reaction of thiamine degradation with CuCl at 40 °C for 7 days.
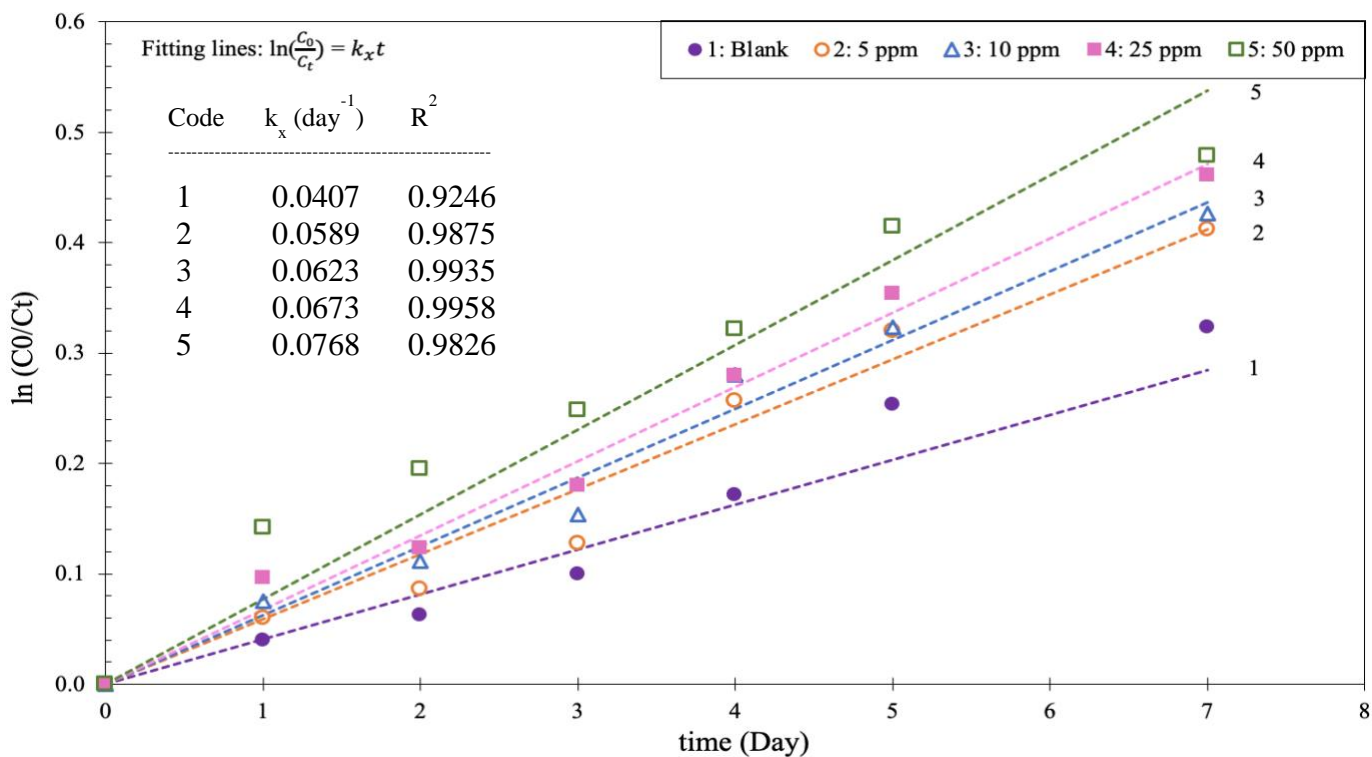


**Figure 3.6** First-order reaction of thiamine degradation with CuCl$_2$ at 40 °C for 7 days.
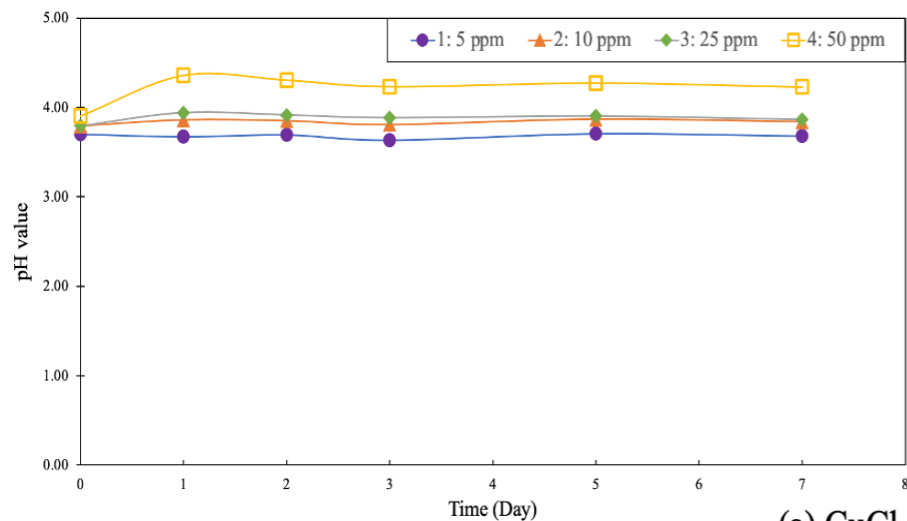
**Figure 3.7** First-order reaction of thiamine degradation with $FeCl_2$ at 40 °C for 7 days.



**Figure 3.8** First-order reaction of thiamine degradation with $FeCl_3$ at 40 °C for 7 days.
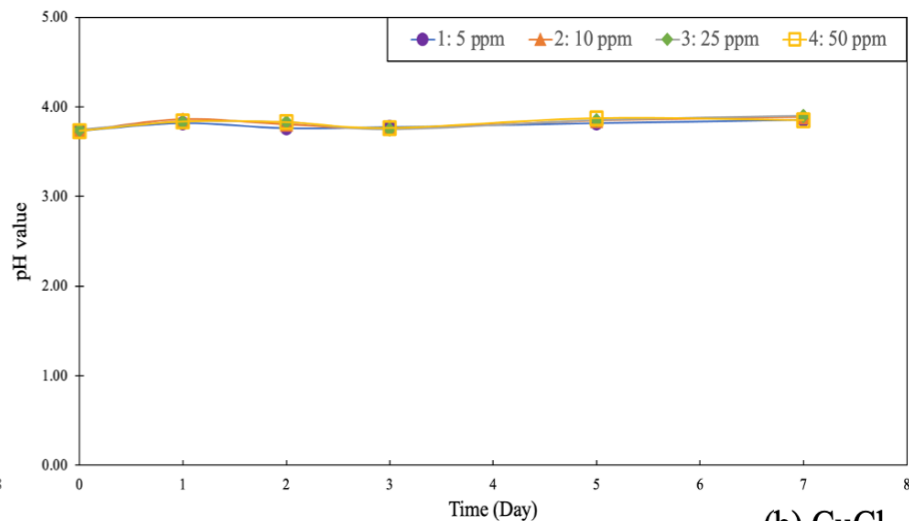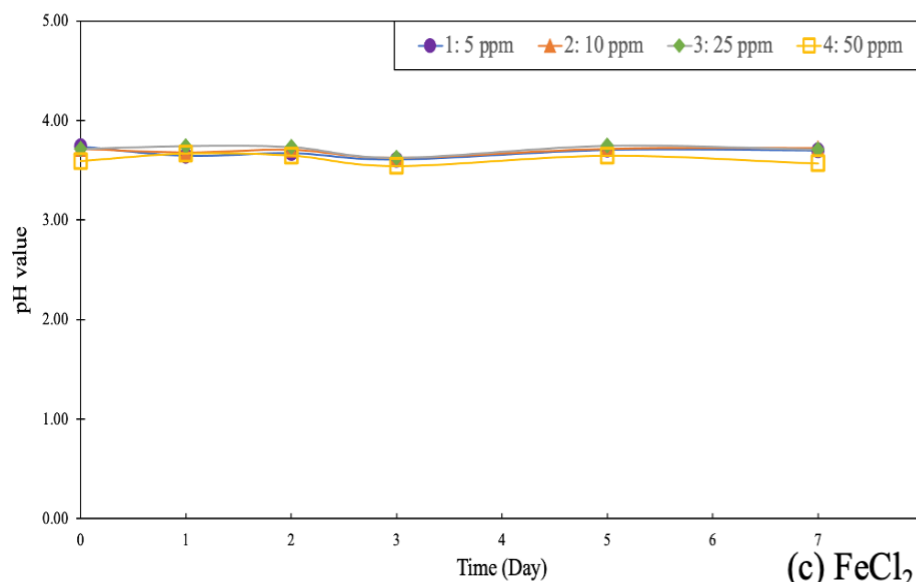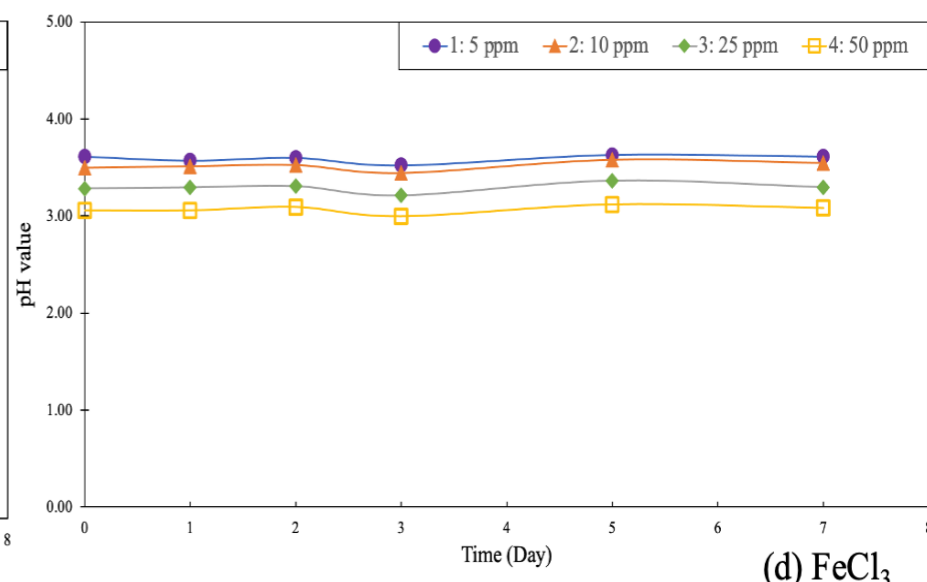
**Figure 3.9** At 40 °C, pH variations of thiamine samples with 4 metal chlorides (i.e., a) CuCl; b) CuCl$_2$; c) FeCl$_2$; d) FeCl$_3$) at studied concentrations (i.e., 5, 10, 25, and 50 ppm) during 7 days.
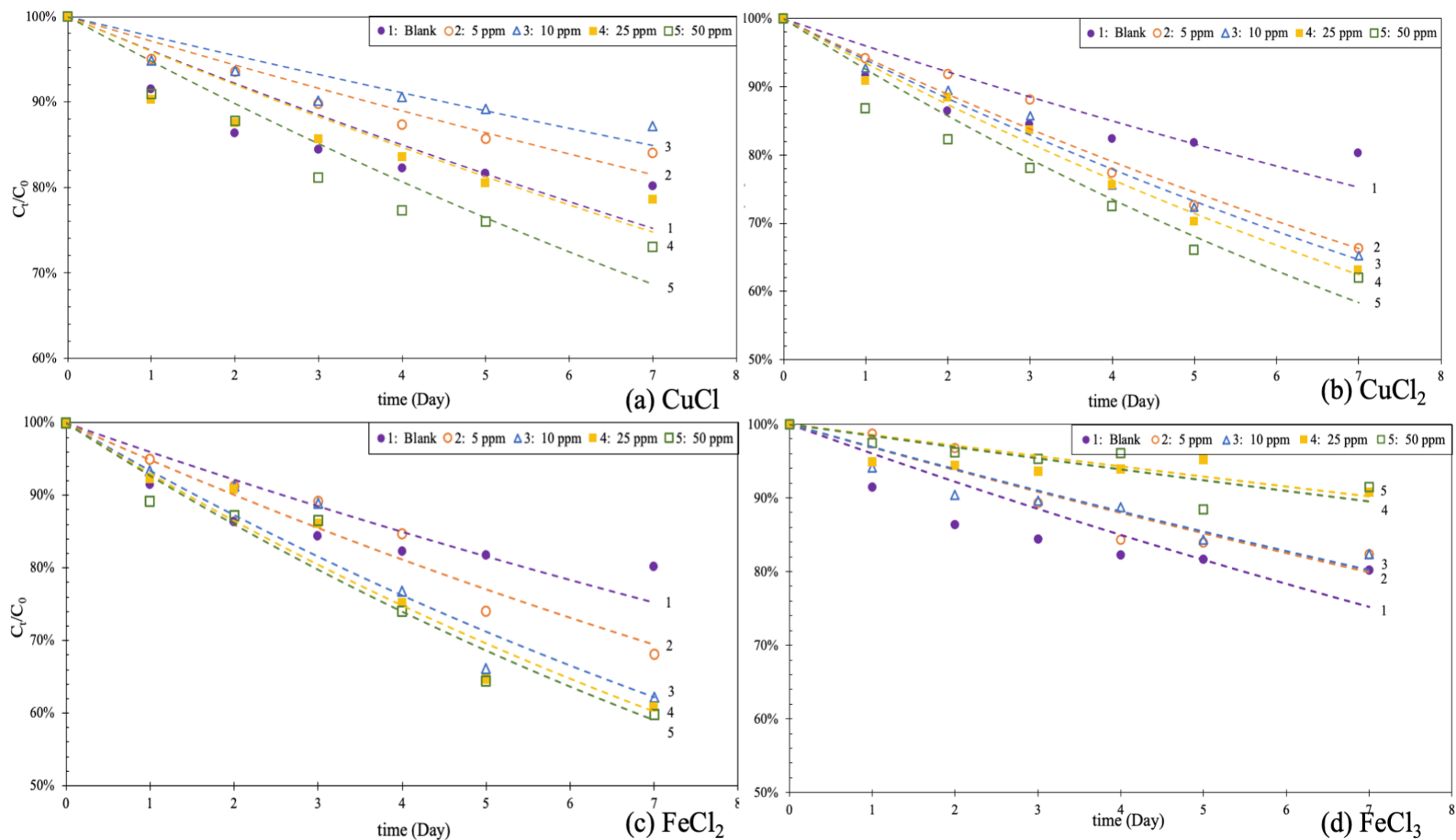
**Figure 3.10** At 40°C, the theoretical kinetic model in the four metal chlorides system (i.e., a) CuCl; b) CuCl$_2$; c) FeCl$_2$; d) FeCl$_3$): the remaining percentage of thiamine vs. time for the degradation reactions in aqueous solutions at varied concentrations. The fitting line is $C_t/C_0 = \exp(-k_x t)$. * $C_t$: thiamine content at time t (t: day); $C_0$: initial thiamine content at t (0).
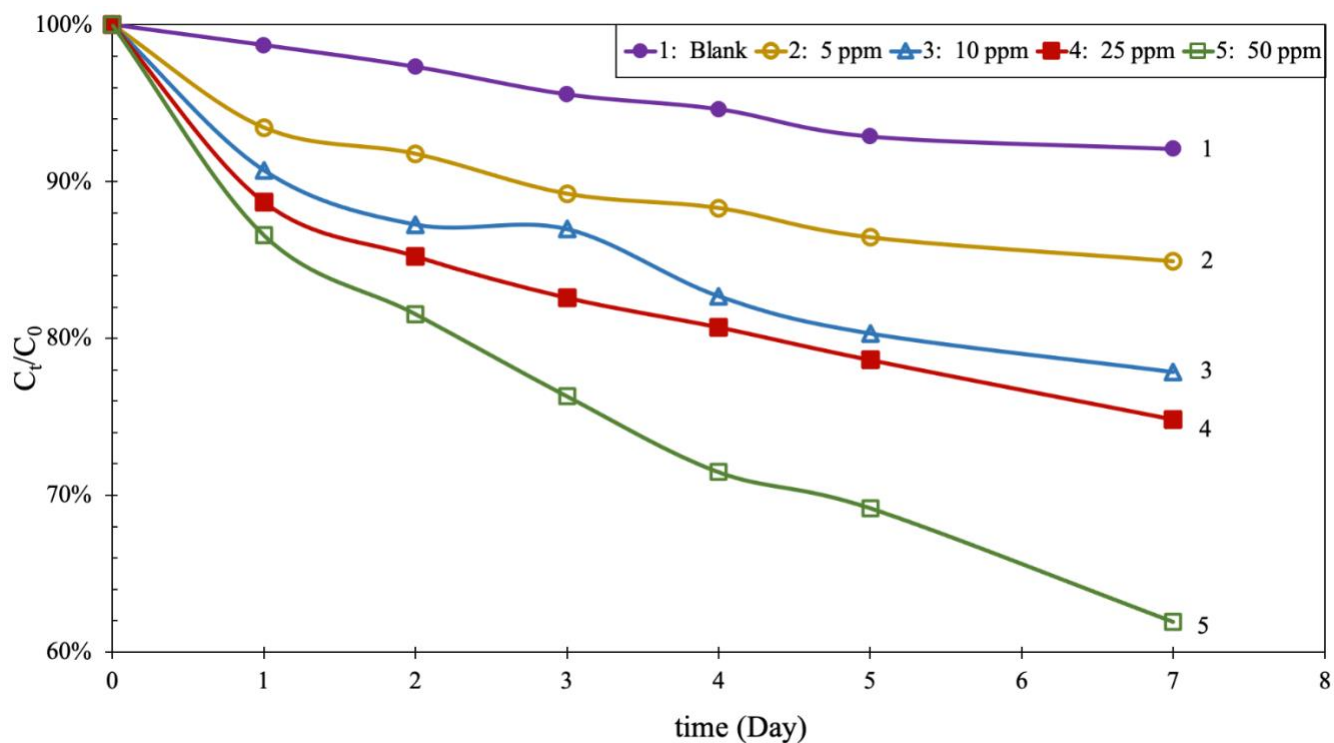
**Figure 3.11** 55°C group: effects of varied CuCl concentrations on remaining percentage of thiamine vs. time for 7 days. * $C_t$: Concentration of thiamine at time t, $C_0$: Concentration of thiamine at time 0, t: Storage time in days.
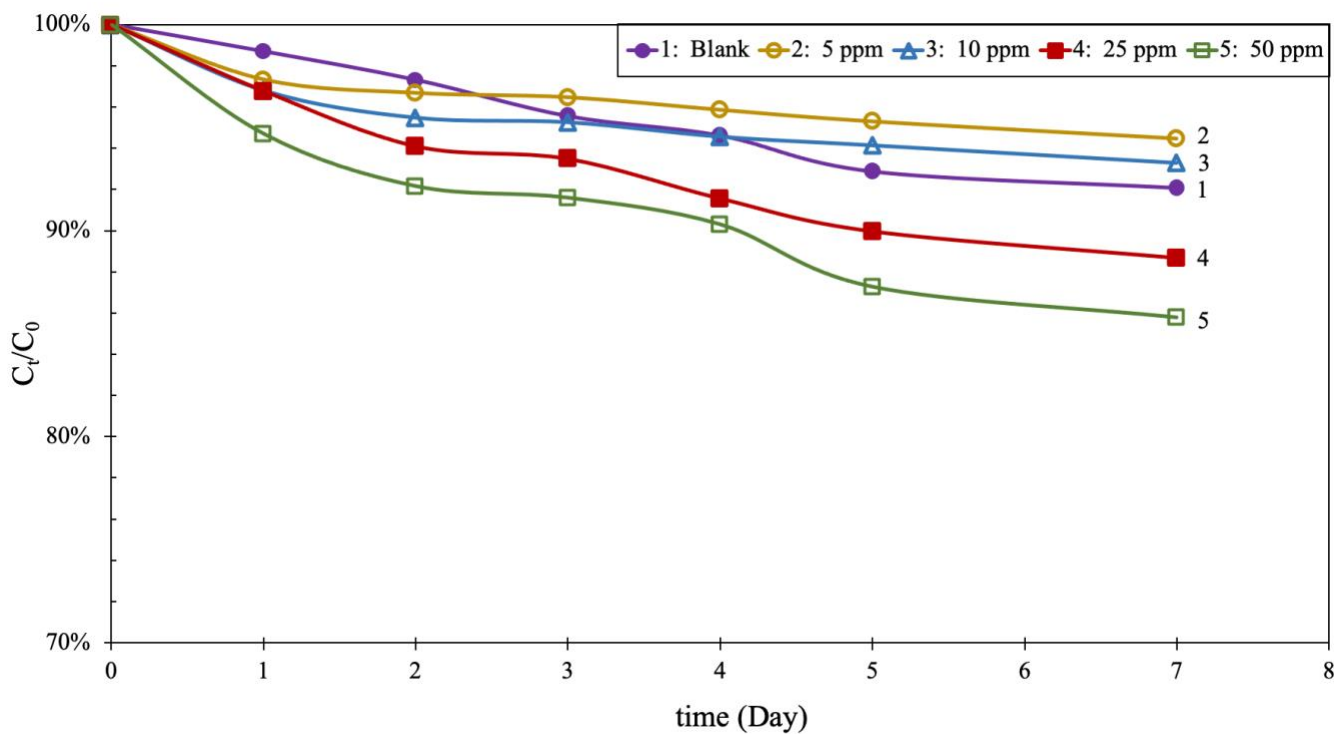


**Figure 3.12** 55°C group: effects of varied $CuCl_2$ concentrations on remaining percentage of thiamine vs. time for 7 days.
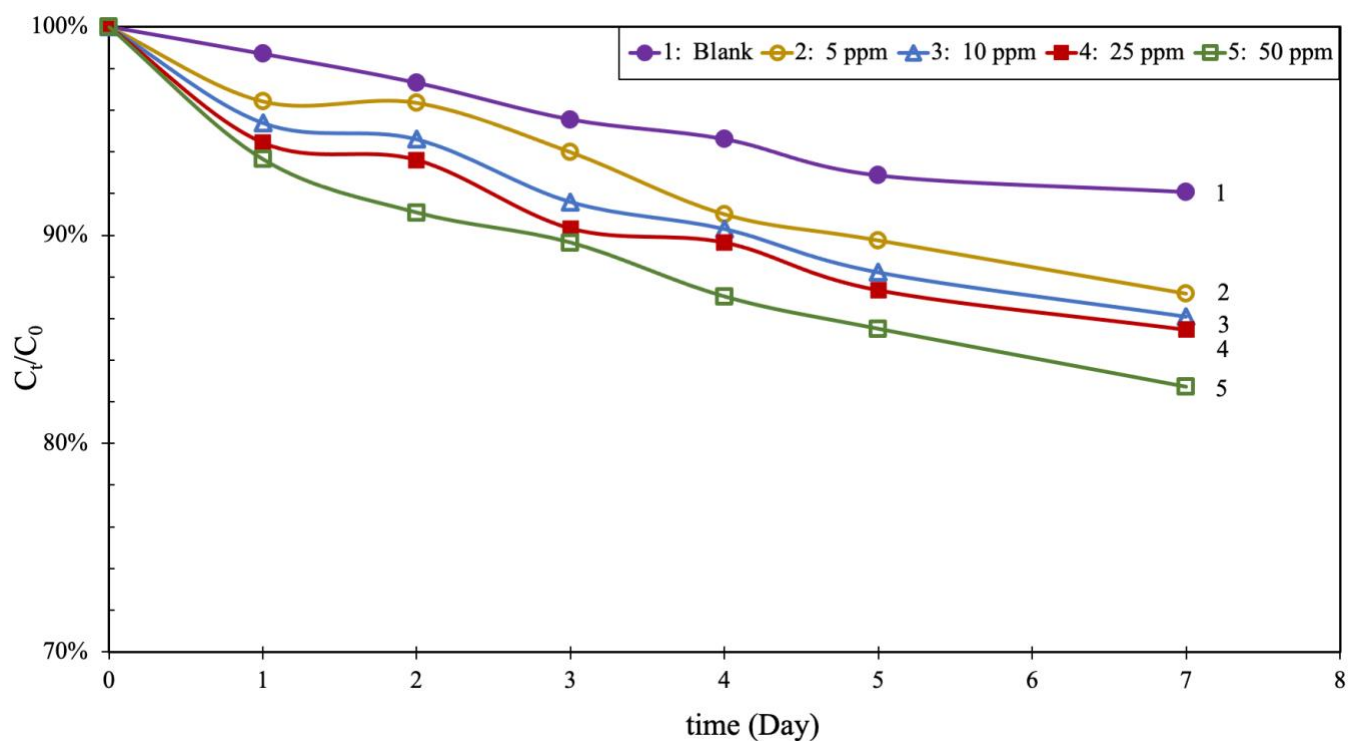
**Figure 3.13** 55°C group: effects of varied $FeCl_2$ concentrations on remaining percentage of thiamine vs. time for 7 days. * $C_t$: Concentration of thiamine at time t, $C_0$: Concentration of thiamine at time 0, t: Storage time in days.



**Figure 3.14** 55°C group: effects of varied $FeCl_3$ concentrations on remaining percentage of thiamine vs. time for 7 days.

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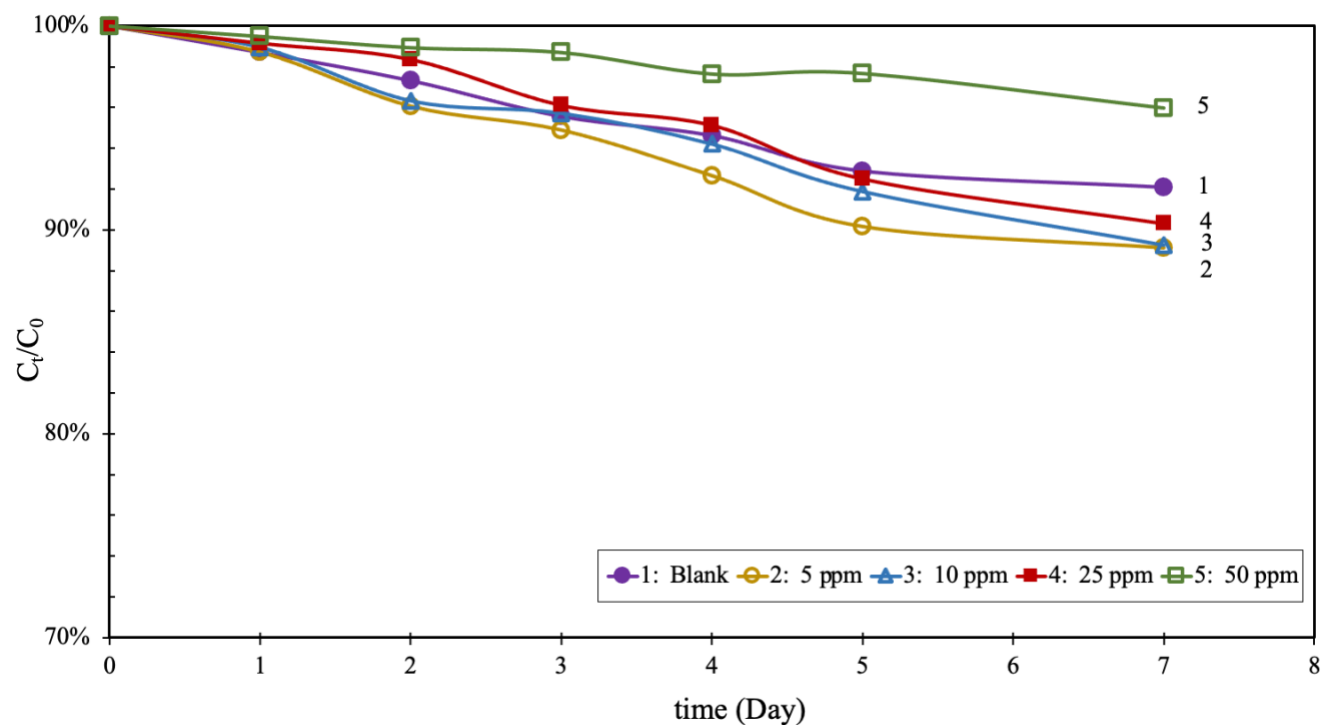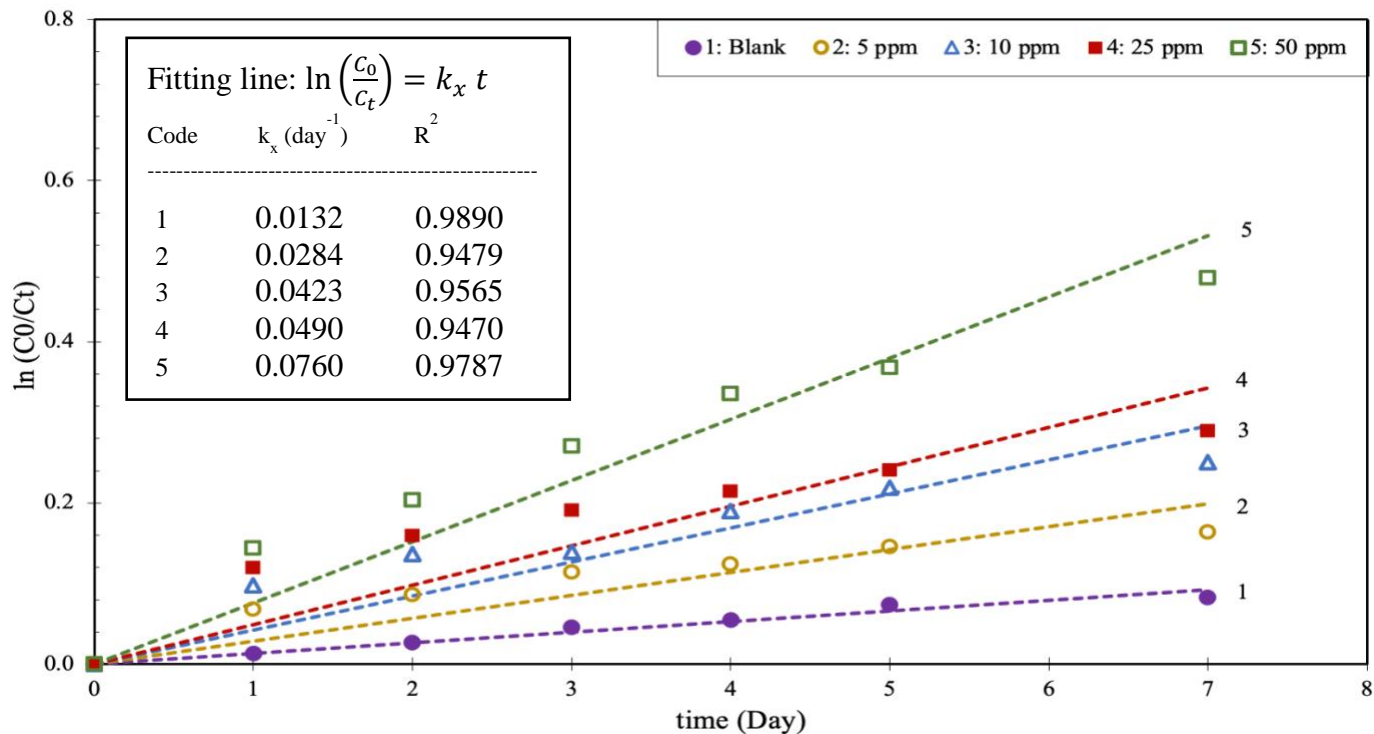**Figure 3.15** First-order reaction of thiamine degradation with CuCl at 55 °C for 7 days.
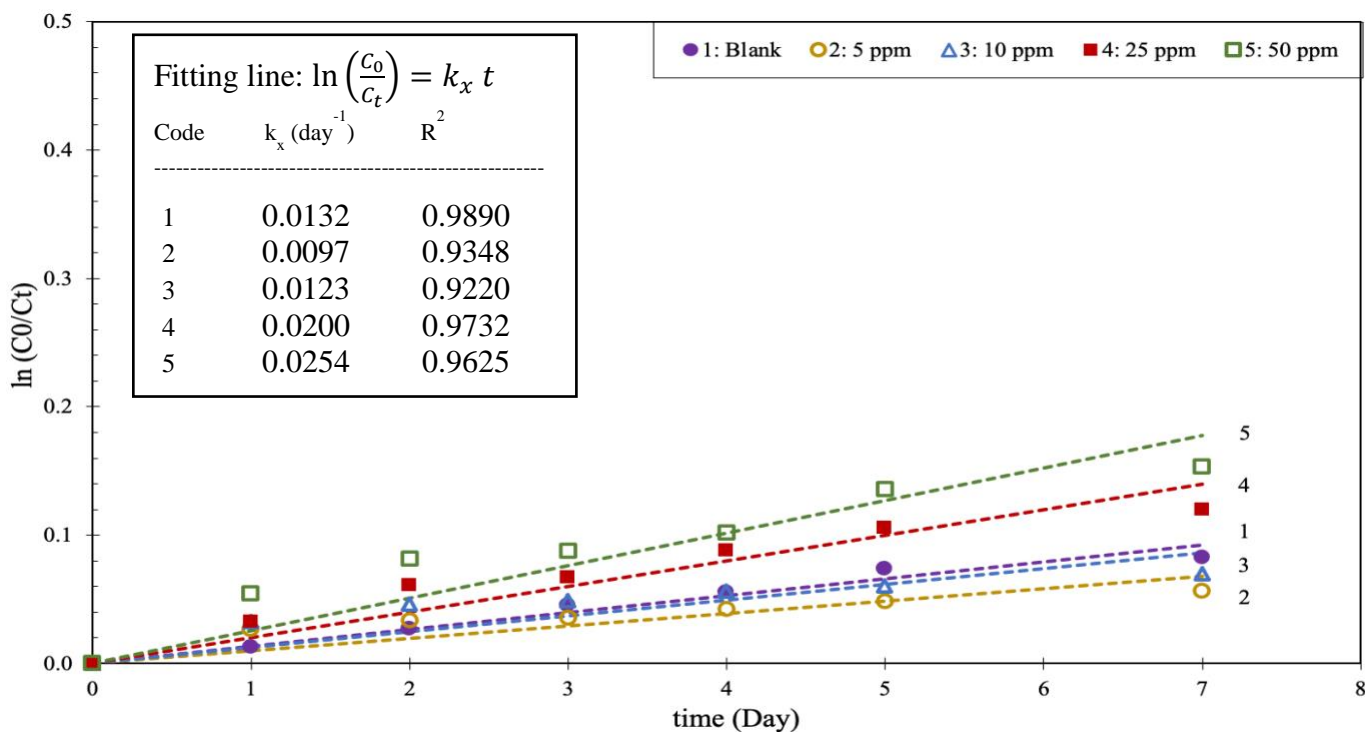


**Figure 3.16** First-order reaction of thiamine degradation with CuCl₂ at 55 °C for 7 days.
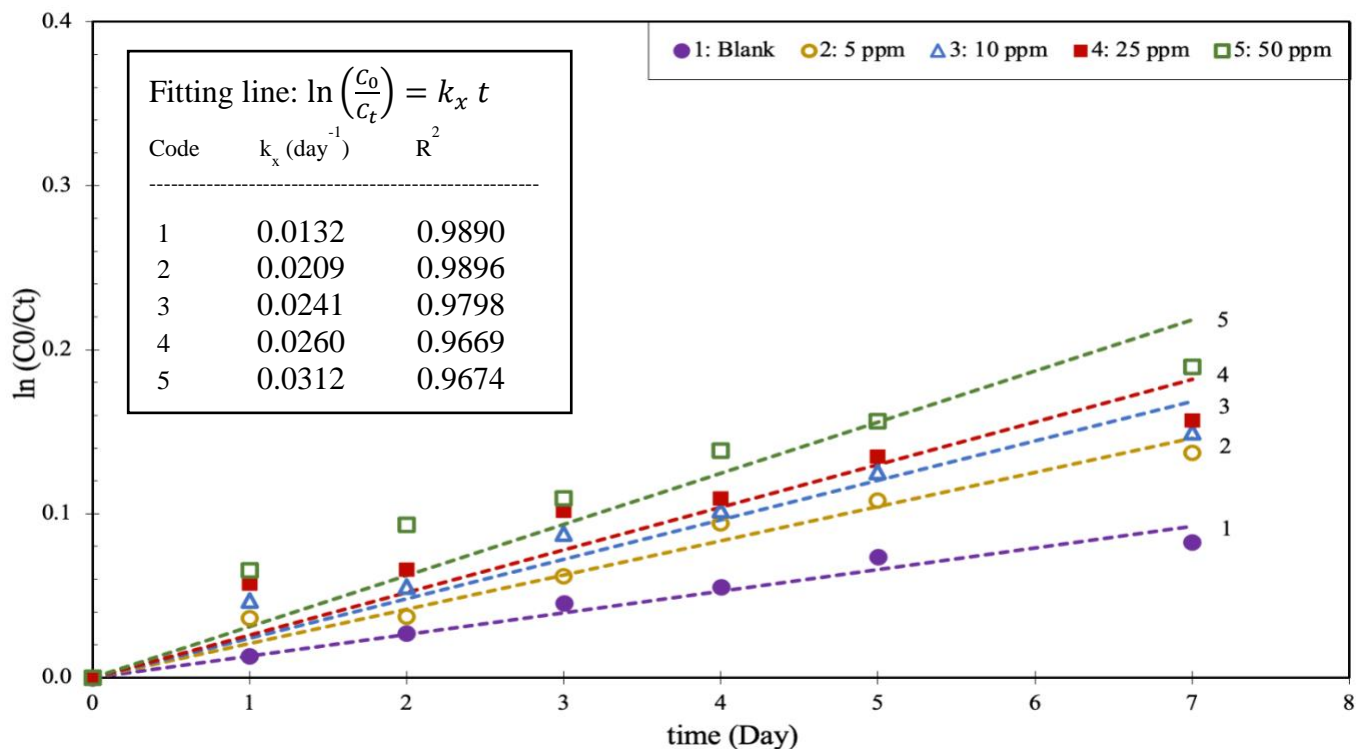
**Figure 3.17** First-order reaction of thiamine degradation with $FeCl_2$ at 55 °C for 7 days.

Fitting line: $\ln\left(\dfrac{C_0}{C_t}\right) = k_x\, t$

| Code | $k_x$ (day$^{-1}$) | $R^2$ |
|------|------|------|
| 1 | 0.0132 | 0.9890 |
| 2 | 0.0209 | 0.9896 |
| 3 | 0.0241 | 0.9798 |
| 4 | 0.0260 | 0.9669 |
| 5 | 0.0312 | 0.9674 |
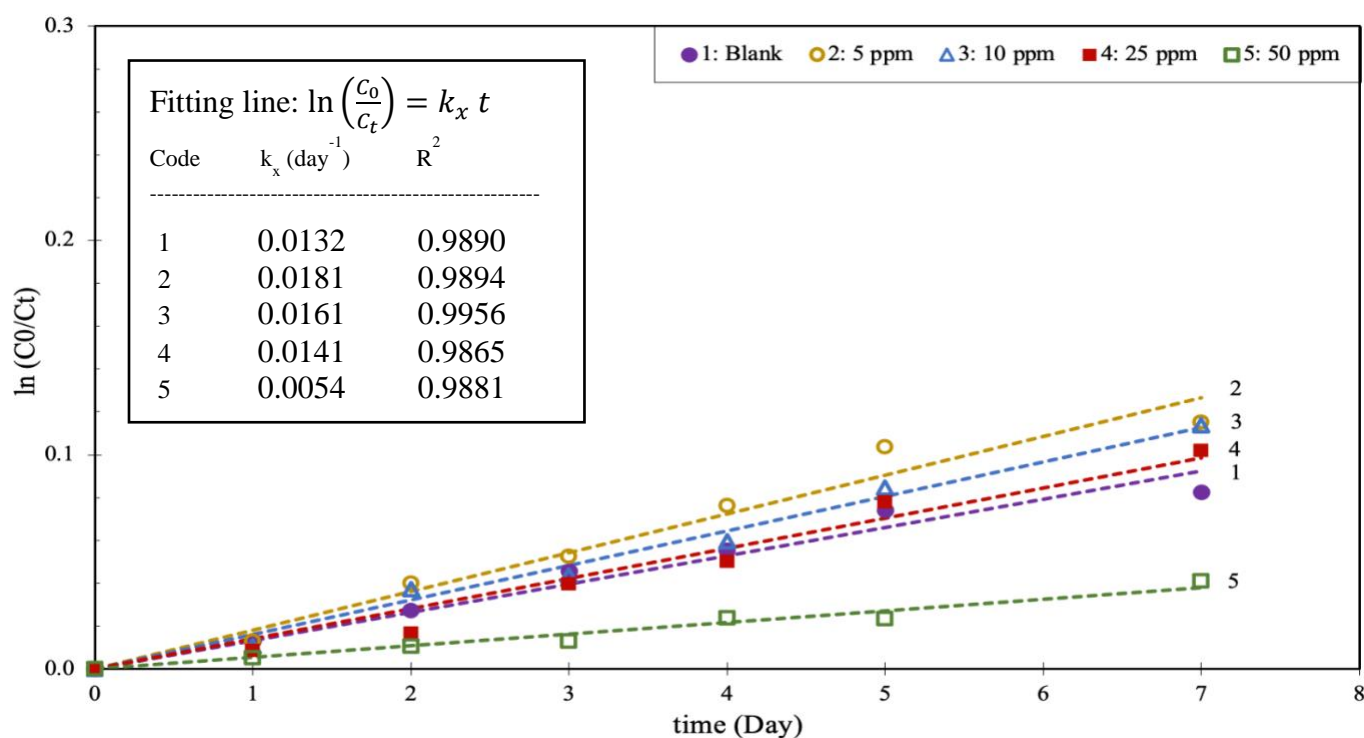


**Figure 3.18** First-order reaction of thiamine degradation with $FeCl_3$ at 55 °C for 7 days.

Fitting line: $\ln\left(\dfrac{C_0}{C_t}\right) = k_x\, t$

| Code | $k_x$ (day$^{-1}$) | $R^2$ |
|------|------|------|
| 1 | 0.0132 | 0.9890 |
| 2 | 0.0181 | 0.9894 |
| 3 | 0.0161 | 0.9956 |
| 4 | 0.0141 | 0.9865 |
| 5 | 0.0054 | 0.9881 |

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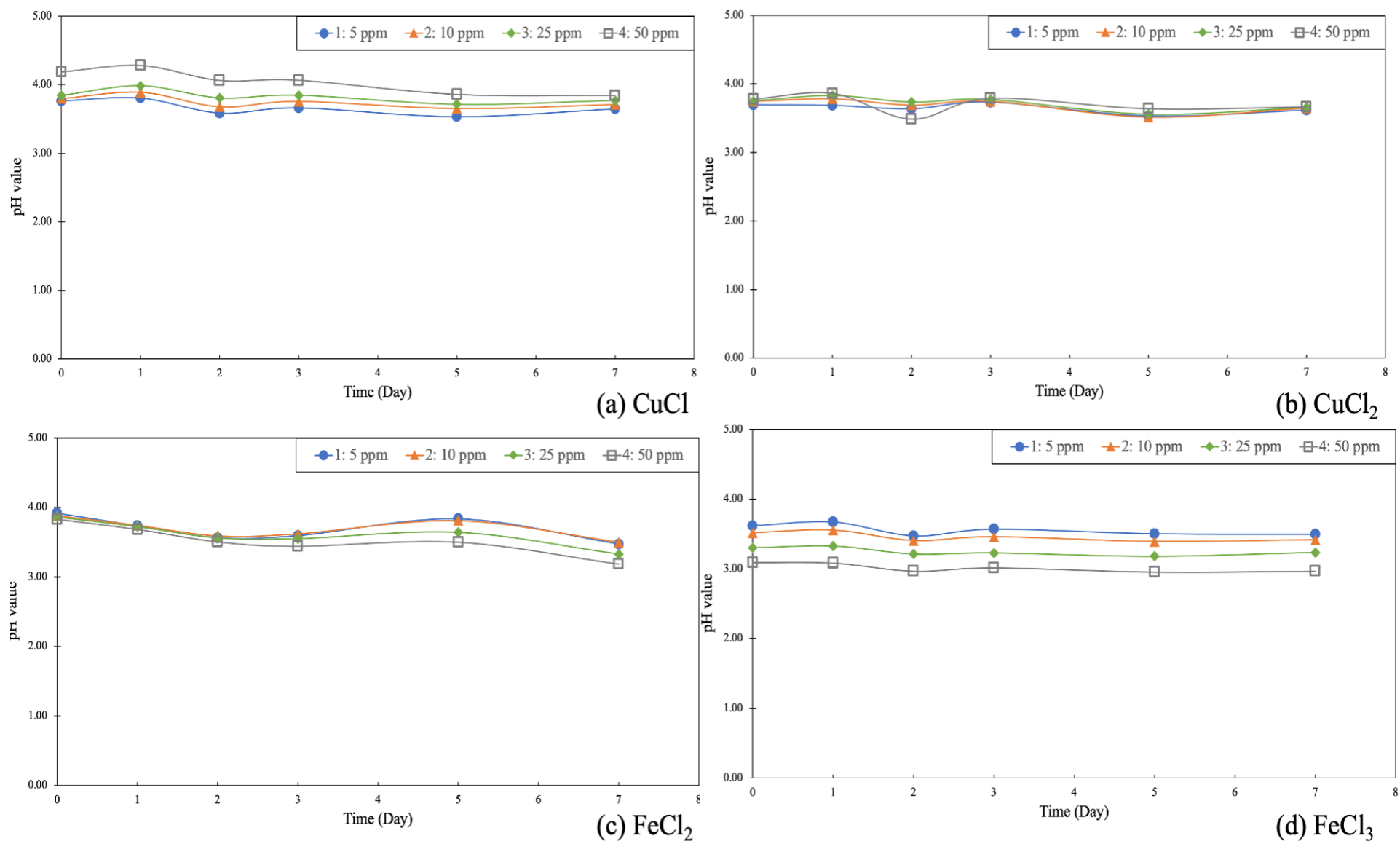
**Figure 3.19** At 55 °C, pH variations of thiamine samples with 4 metal chlorides (i.e., a) CuCl; b) $CuCl_2$; c) $FeCl_2$; d) $FeCl_3$) at studied concentrations (i.e., 5, 10, 25, and 50 ppm) during 7 days.
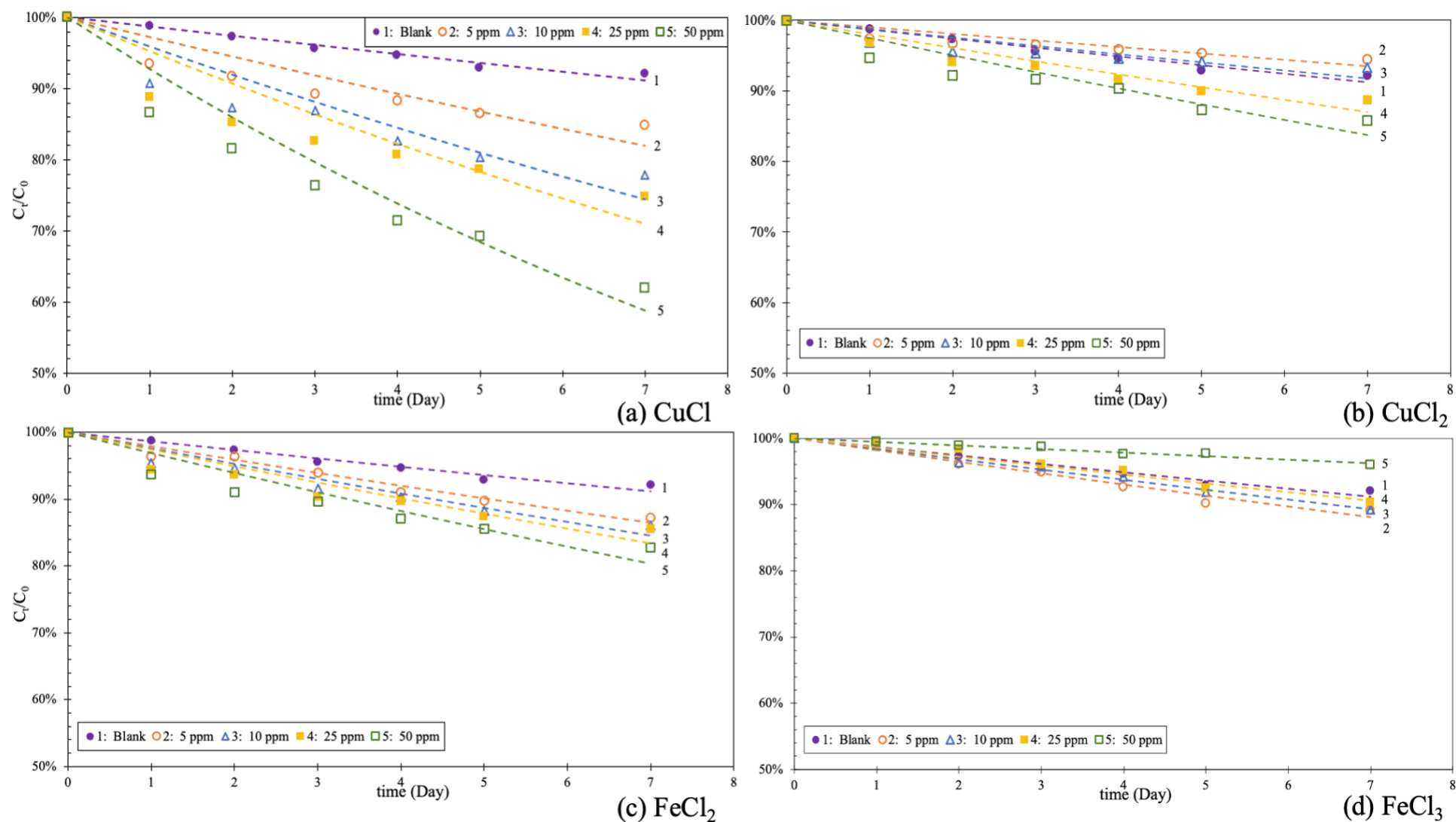
**Figure 3.20** At 55°C, the theoretical kinetic model in the four metal chlorides system (i.e., a) CuCl; b) CuCl$_2$; c) FeCl$_2$; d) FeCl$_3$): the remaining percentage of thiamine vs. time for the degradation reactions in aqueous solutions at varied concentrations. The fitting line is $C_t/C_0 = \exp(-k_x t)$. *$C_t$: thiamine content at time t, $C_0$: initial thiamine content (t = 0), t: days.
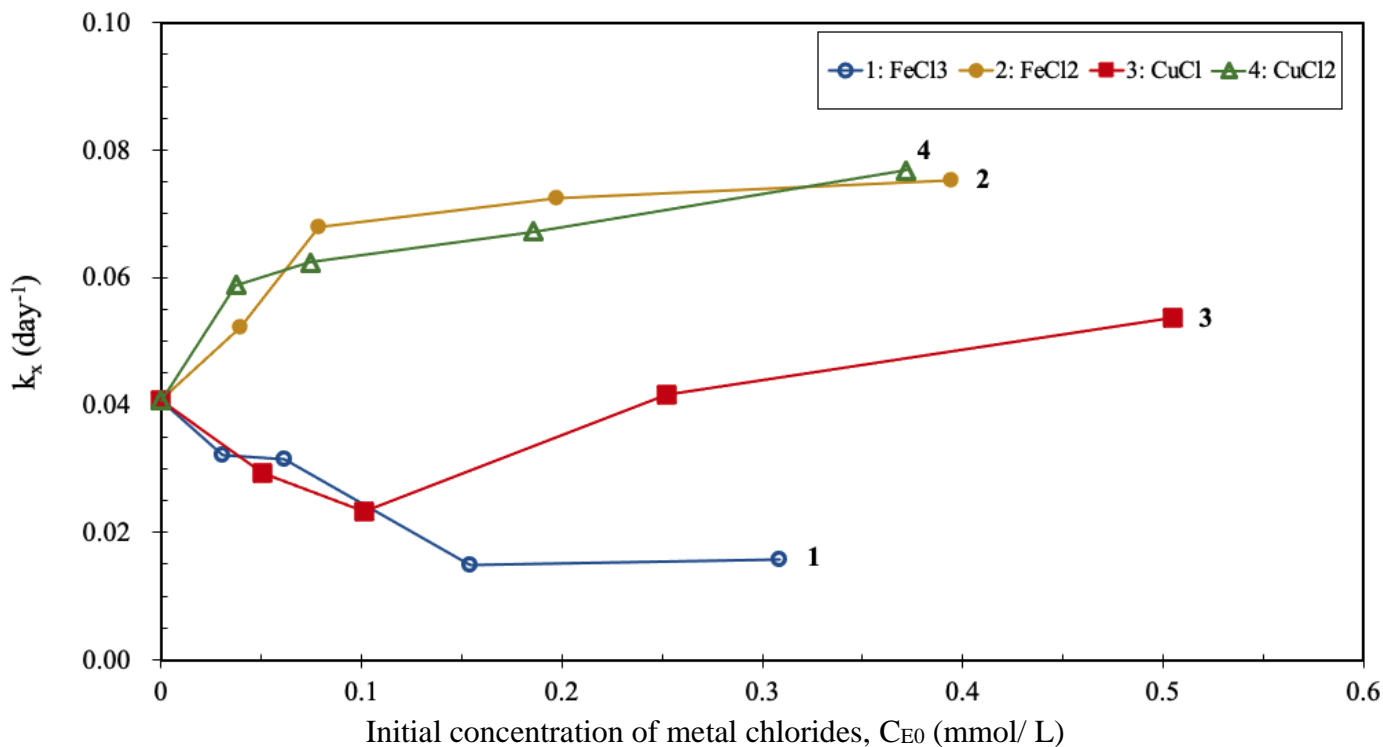
**Figure 3.21** At 40°C, the comparison of $k_x$ of four types of metal chloride with different concentrations for thiamine degradation. [1]$C_{E0}$: the initial concentration of metal chloride (i.e., 5, 10, 25, 50 ppm). [2]The unit of concentration was converted from ppm to mmol/L. [3]The mmol/L was also listed in **Table 3.2**.
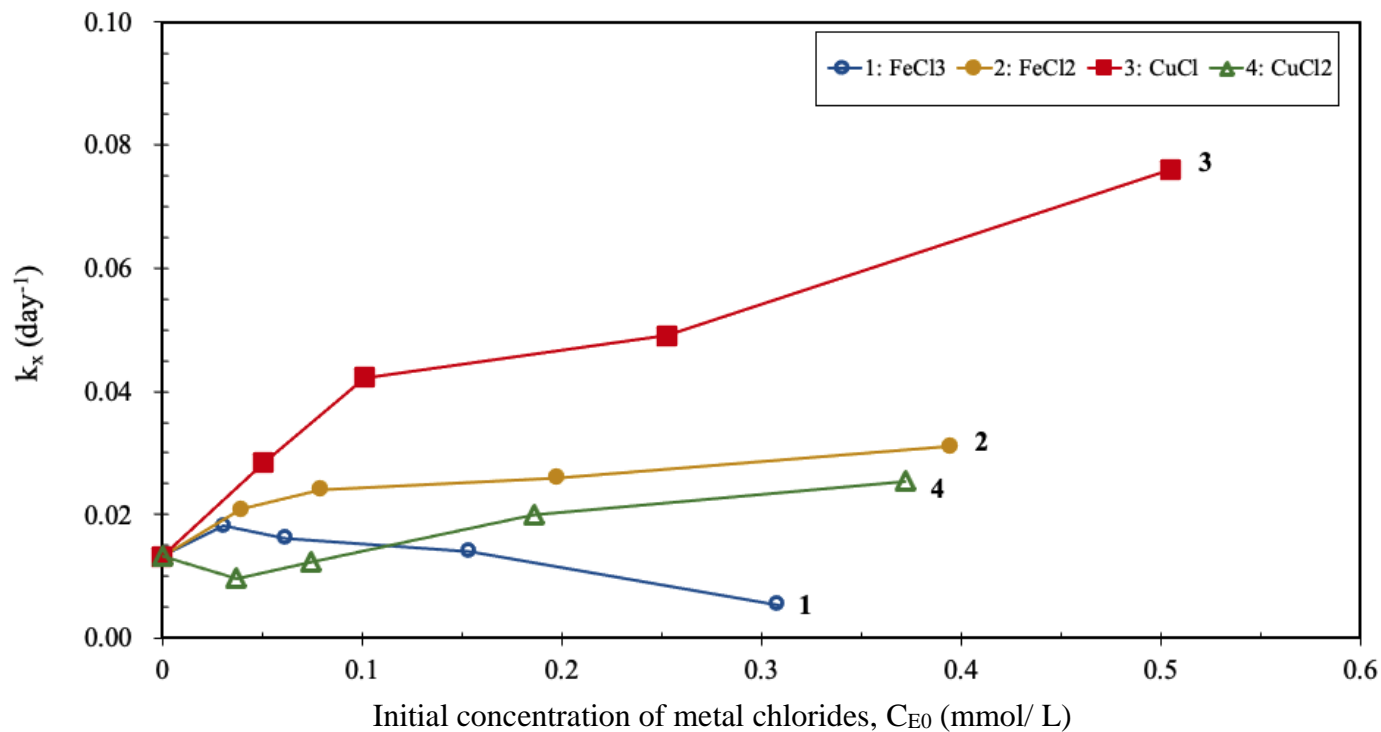


**Figure 3.22** At 55°C, the comparison of $k_x$ of four types of metal chloride with different concentrations for thiamine degradation. [1]The mmol/L were also listed in **Table 3.5**.
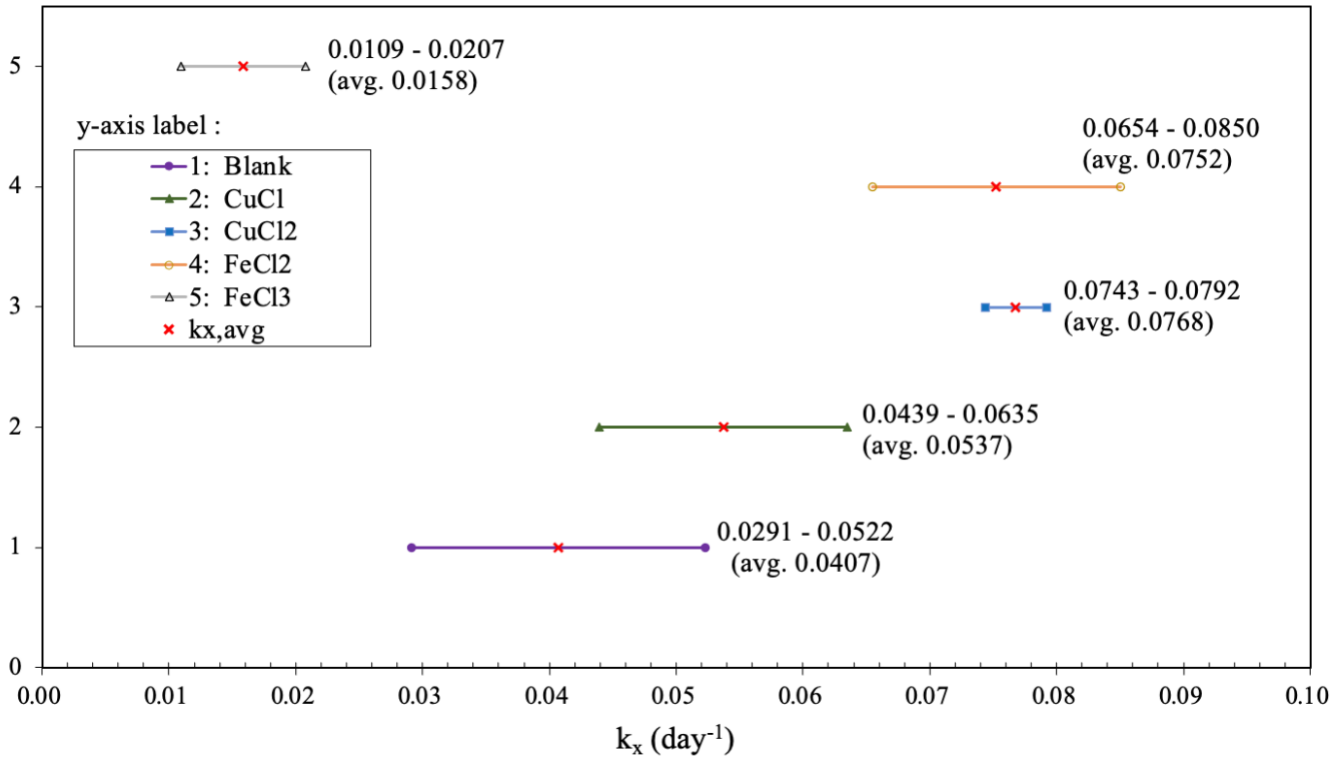
**Figure 3.23** At 40°C, the $k_x$ for thiamine degradation in four metal chlorides solution at 50 mg L$^{-1}$ in 95% confidence interval (CI). *$k_x$ is in the fitting line: ln (Ct/ C$_0$) = $k_x$ t.
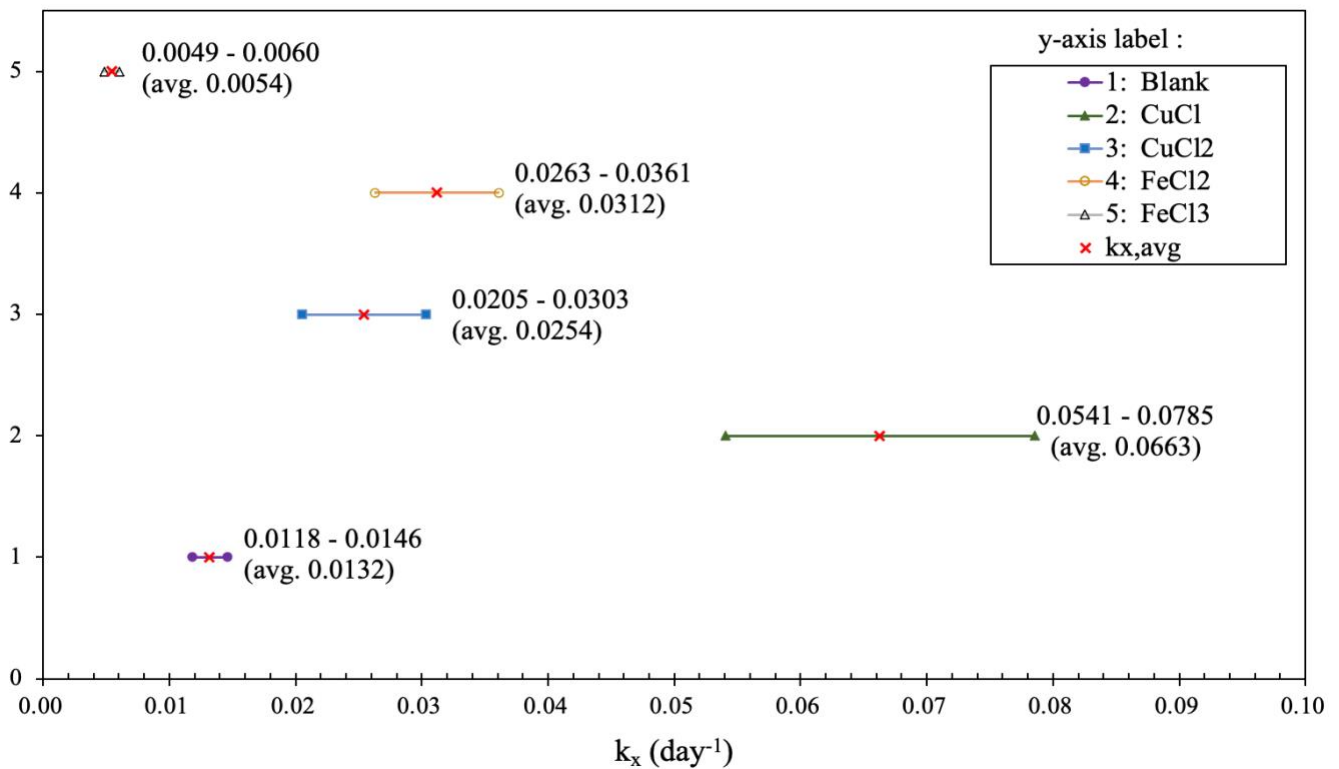


**Figure 3.24** At 55°C, the $k_x$ for thiamine degradation in four metal chlorides solution at 50 mg L$^{-1}$ in 95% confidence interval (CI). *$k_x$ is in the fitting line: ln (Ct/ C$_0$) = $k_x$ t.
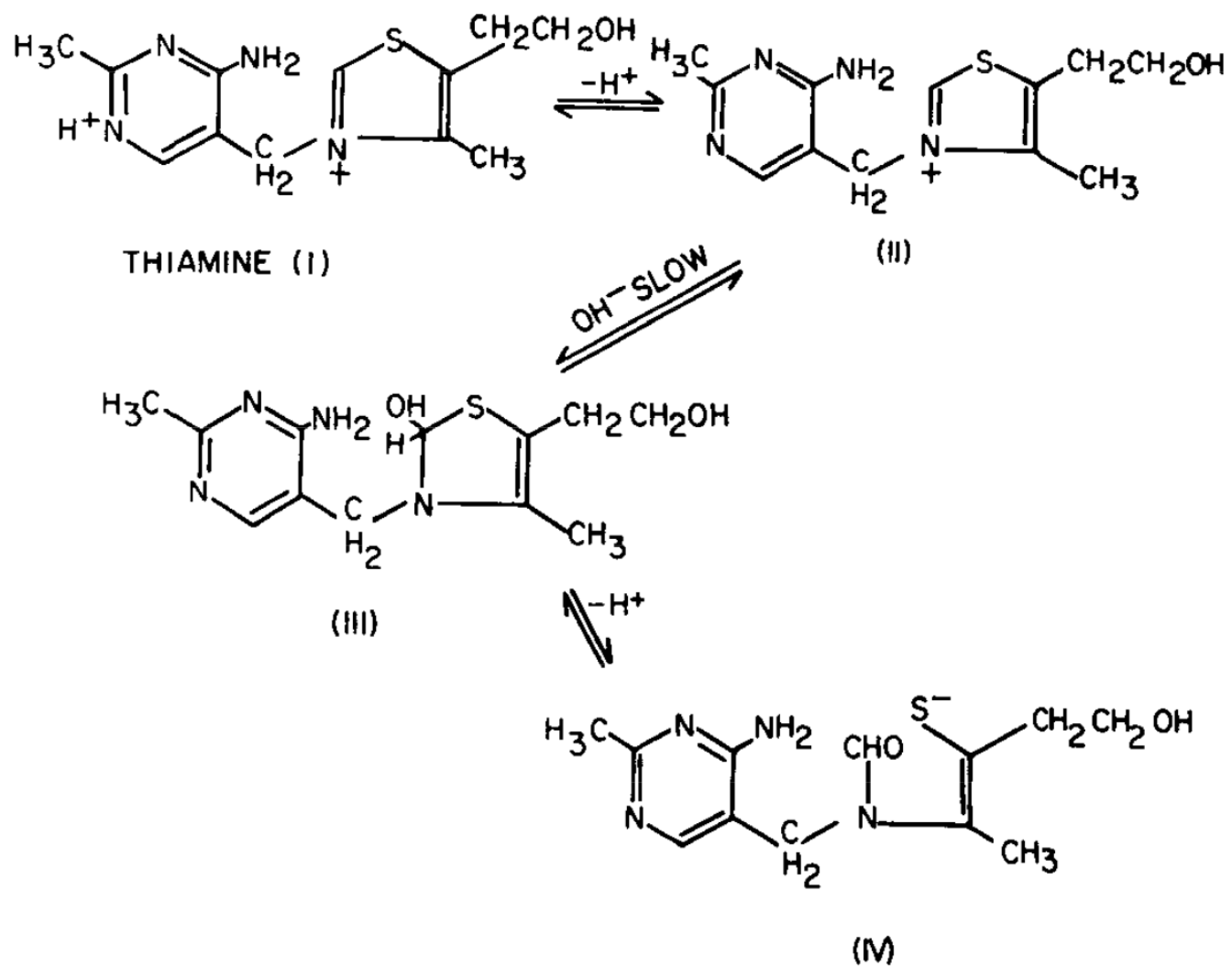
**Figure 3.25** Acid-base equilibria of thiamine (Dwivedi and Arnold., 1972).

**Table 3.1** At 40°C, remaining percentages of thiamine with different metal chlorides (i.e., CuCl, CuCl$_2$, FeCl$_2$, FeCl$_3$) at the studied concentrations (i.e., 5, 10, 25, 50 mg L$^{-1}$) during a storage time of 7 days in different salt solutions.

| Sample | Final Conc. (mg L$^{-1}$) | 0 | 1 | 2 | 3 (Time: day) | 4 | 5 | 7 |
|---|---|---|---|---|---|---|---|---|
| **Thiamine** | 250 | 100% | 91.49% | 86.33% | 84.40% | 82.27% | 81.67% | 80.18% |
| **CuCl** | 5 | 100% | 95.04% | 93.53% | 89.79% | 87.33% | 85.67% | 84.01% |
|  | 10 | 100% | 94.84% | 93.66% | 90.12% | 90.61% | 89.61% | 87.16% |
|  | 25 | 100% | 90.26% | 87.54% | 85.61% | 83.59% | 80.48% | 78.59% |
|  | 50 | 100% | 90.91% | 87.75% | 81.16% | 77.33% | 75.99% | 73.01% |
| **CuCl$_2$** | 5 | 100% | 94.19% | 91.79% | 88.06% | 77.36% | 72.62% | 66.24% |
|  | 10 | 100% | 92.73% | 89.43% | 85.73% | 75.56% | 72.37% | 65.29% |
|  | 25 | 100% | 90.83% | 88.42% | 83.55% | 75.60% | 70.19% | 63.08% |
|  | 50 | 100% | 86.82% | 82.26% | 78.04% | 72.51% | 66.05% | 61.96% |

**Table 3.1 (continued).**

| Sample | Final Conc. (mg L$^{-1}$) | 0 | 1 | 2 | 3 | 4 | 5 | 7 |
|--------|------|------|------|------|------|------|------|------|
| | | | | | (Time: day) | | | |
| **FeCl$_2$** | 5 | 100% | 94.96% | 91.31% | 89.19% | 84.68% | 74.03% | 68.17% |
| | 10 | 100% | 93.35% | 91.34% | 88.88% | 76.78% | 66.16% | 62.12% |
| | 25 | 100% | 92.28% | 90.80% | 86.08% | 75.18% | 64.62% | 60.79% |
| | 50 | 100% | 89.24% | 87.27% | 86.55% | 74.02% | 64.43% | 59.76% |
| **FeCl$_3$** | 5 | 100% | 98.67% | 96.77% | 89.33% | 84.29% | 83.96% | 82.40% |
| | 10 | 100% | 94.07% | 90.40% | 89.59% | 88.73% | 84.34% | 82.36% |
| | 25 | 100% | 94.85% | 94.45% | 93.62% | 93.89% | 95.22% | 90.80% |
| | 50 | 100% | 97.43% | 96.17% | 95.29% | 96.03% | 88.46% | 91.48% |

**Table 3.2** 40°C group: rate constants $k_x$ (day$^{-1}$) and the coefficient of determination ($R^2$) of thiamine in blank sample and different metal chlorides (i.e., CuCl, CuCl$_2$, FeCl$_2$, FeCl$_3$) at the studied concentrations (i.e., 5, 10, 25, 50 mg L$^{-1}$) for 7 days.

| Sample type | Final concentration | | First-order kinetics parameters | | | Final concentration | | First-order kinetics parameters | |
|---|---|---|---|---|---|---|---|---|---|
| | (mg L$^{-1}$) | (mmol L$^{-1}$) | $k_x$ (day$^{-1}$) | $R^2$ | | (mg L$^{-1}$) | (mmol L$^{-1}$) | $k_x$ (day$^{-1}$) | $R^2$ |
| **Thiamine solution (Blank sample)** | 250 | | 0.0407 | 0.9246 | | | | | |
| **CuCl** | 5 | 0.051 | 0.0293 | 0.9751 | **FeCl$_2$** | 5 | 0.039 | 0.0522 | 0.9804 |
| | 10 | 0.101 | 0.0233 | 0.9483 | | 10 | 0.079 | 0.0679 | 0.9699 |
| | 25 | 0.253 | 0.0416 | 0.9478 | | 25 | 0.197 | 0.0724 | 0.9774 |
| | 50 | 0.505 | 0.0537 | 0.9673 | | 50 | 0.394 | 0.0752 | 0.9793 |
| **CuCl$_2$** | 5 | 0.037 | 0.0589 | 0.9875 | **FeCl$_3$** | 5 | 0.031 | 0.0320 | 0.9588 |
| | 10 | 0.074 | 0.0623 | 0.9935 | | 10 | 0.062 | 0.0316 | 0.9676 |
| | 25 | 0.186 | 0.0673 | 0.9958 | | 25 | 0.154 | 0.0148 | 0.8712 |
| | 50 | 0.372 | 0.0768 | 0.9826 | | 50 | 0.308 | 0.0158 | 0.8956 |

**Table 3.3** 40°C group: pH value of the mixtures of thiamine and four metal chlorides solution at different concentrations for 7 days.

| Sample name | Final Conc. (mg L$^{-1}$) | Storage time (days) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 5 | 7 |
| **Blank** | 250 | $3.71 \pm 0.01$ | $3.84 \pm 0.02$ | $3.78 \pm 0.01$ | $3.86 \pm 0.01$ | $3.78 \pm 0.03$ | $3.89 \pm 0.01$ |
| **CuCl** | 5 | $3.70 \pm 0.01$ [d] | $3.67 \pm 0.01$ [d] | $3.69 \pm 0.01$ [d] | $3.64 \pm 0.01$ [d] | $3.71 \pm 0.01$ [d] | $3.68 \pm 0.00$ [d] |
| | 10 | $3.79 \pm 0.00$ [b,c] | $3.86 \pm 0.01$ [c] | $3.85 \pm 0.01$ [c] | $3.81 \pm 0.01$ [c] | $3.87 \pm 0.01$ [c] | $3.84 \pm 0.01$ [b,c] |
| | 25 | $3.80 \pm 0.01$ [b,c] | $3.94 \pm 0.01$ [b] | $3.92 \pm 0.01$ [b] | $3.89 \pm 0.02$ [b] | $3.90 \pm 0.01$ [b] | $3.87 \pm 0.01$ [b,c] |
| | 50 | $3.91 \pm 0.03$ [a] | $4.36 \pm 0.06$ [a] | $4.31 \pm 0.02$ [a] | $4.23 \pm 0.02$ [a] | $4.27 \pm 0.01$ [a] | $4.23 \pm 0.02$ [a] |
| **CuCl$_2$** | 5 | $3.73 \pm 0.02$ [a,b,c,d] | $3.82 \pm 0.01$ [a,b,c,d] | $3.76 \pm 0.01$ [d] | $3.78 \pm 0.04$ [a,b,c,d] | $3.82 \pm 0.01$ [d] | $3.86 \pm 0.00$ [c,d] |
| | 10 | $3.74 \pm 0.01$ [a,b,c,d] | $3.86 \pm 0.01$ [a,b,c,d] | $3.81 \pm 0.01$ [a,b,c] | $3.76 \pm 0.04$ [a,b,c,d] | $3.85 \pm 0.01$ [a,b,c] | $3.89 \pm 0.01$ [a,b] |
| | 25 | $3.75 \pm 0.01$ [a,b,c,d] | $3.83 \pm 0.02$ [a,b,c,d] | $3.83 \pm 0.01$ [a,b,c] | $3.75 \pm 0.02$ [a,b,c,d] | $3.85 \pm 0.01$ [a,b,c] | $3.90 \pm 0.03$ [a,b] |
| | 50 | $3.73 \pm 0.01$ [a,b,c,d] | $3.84 \pm 0.01$ [a,b,c,d] | $3.83 \pm 0.02$ [a,b,c] | $3.76 \pm 0.01$ [a,b,c,d] | $3.87 \pm 0.01$ [a,b,c] | $3.85 \pm 0.01$ [c,d] |

**Table 3.3** (continued)

| Sample name | Final Conc. (mg L$^{-1}$) | Storage time (days) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 5 | 7 |
| **FeCl$_2$** | 5 | $3.74 \pm 0.02$ [a,b,c] | $3.65 \pm 0.02$ [b,c,d] | $3.67 \pm 0.02$ [a,b,c,d] | $3.61 \pm 0.02$ [a,b,c] | $3.71 \pm 0.03$ [a,b,c] | $3.70 \pm 0.01$ [a,b,c] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 10 | 3.72 ± 0.02 [a,b,c] | 3.68 ± 0.01 [b,c,d] | 3.71 ± 0.03 [a,b,c,d] | 3.62 ± 0.02 [a,b,c] | 3.72 ± 0.02 [a,b,c] | 3.72 ± 0.02 [a,b,c] |
| | 25 | 3.72 ± 0.01 [a,b,c] | 3.75 ± 0.01 [a] | 3.74 ± 0.02 [a,b,c,d] | 3.63 ± 0.03 [a,b,c] | 3.75 ± 0.01 [a,b,c] | 3.71 ± 0.01 [a,b,c] |
| | 50 | 3.60 ± 0.01 [d] | 3.67 ± 0.01 [b,c,d] | 3.65 ± 0.01 [a,b,c,d] | 3.55 ± 0.03 [d] | 3.65 ± 0.01 [d] | 3.57 ± 0.01 [d] |
| FeCl$_3$ | 5 | 3.61 ± 0.02 [a] | 3.57 ± 0.01 [a] | 3.60 ± 0.02 [a] | 3.52 ± 0.01 [a] | 3.63 ± 0.01 [a] | 3.61 ± 0.01 [a] |
| | 10 | 3.50 ± 0.02 [b] | 3.51 ± 0.01 [b] | 3.53 ± 0.01 [b] | 3.44 ± 0.01 [b] | 3.58 ± 0.01 [b] | 3.55 ± 0.01 [b] |
| | 25 | 3.28 ± 0.02 [c] | 3.29 ± 0.00 [c] | 3.31 ± 0.01 [c] | 3.21 ± 0.01 [c] | 3.36 ± 0.02 [c] | 3.30 ± 0.01 [c] |
| | 50 | 3.06 ± 0.01 [d] | 3.06 ± 0.01 [d] | 3.09 ± 0.01 [d] | 3.00 ± 0.00 [d] | 3.12 ± 0.00 [d] | 3.08 ± 0.01 [d] |

[1]The pH values were measured at room temperature, and the samples were stored in the incubator at $40 \pm 0.2$ °C when not in analysis. [2]All the pH values are mean ± standard deviation (M± SD), and n = 4. [3]The different letters in the same columns for each form indicate that there are statistical differences between concentrations ($p < 0.05$) (e.g., CuCl on day 1 and FeCl$_3$ on day 0), while the same letters in the column of indicate there are no significant differences ($p > 0.05$) (e.g., CuCl$_2$ on day 0).

**Table 3.4** At 55°C, remaining percentages of thiamine with different metal chlorides (i.e., CuCl, CuCl$_2$, FeCl$_2$, FeCl$_3$) at the studied concentrations (i.e., 5, 10, 25, 50 mg L$^{-1}$) during a storage time of 7 days in different salt solutions.

| Sample | Final Conc. (mg L$^{-1}$) | 0 | 1 | 2 | 3 (Time: day) | 4 | 5 | 7 |
|---|---|---|---|---|---|---|---|---|
| **Thiamine** | 250 | 100% | 98.70% | 97.32% | 95.56% | 94.62% | 92.88% | 92.08% |
| **CuCl** | 5 | 100% | 93.44% | 91.77% | 89.22% | 88.31% | 86.44% | 84.91% |
| | 10 | 100% | 90.72% | 87.26% | 86.97% | 82.70% | 80.32% | 77.86% |
| | 25 | 100% | 88.70% | 85.24% | 82.60% | 80.72% | 78.64% | 74.85% |
| | 50 | 100% | 86.58% | 81.55% | 76.31% | 71.48% | 69.18% | 61.94% |
| | 5 | 100% | 97.34% | 96.69% | 96.48% | 95.87% | 95.31% | 94.48% |
| **CuCl$_2$** | 10 | 100% | 96.80% | 95.48% | 95.26% | 94.56% | 94.14% | 93.29% |
| | 25 | 100% | 96.78% | 94.10% | 93.40% | 91.57% | 89.98% | 88.69% |
| | 50 | 100% | 94.71% | 92.17% | 91.61% | 90.32% | 87.30% | 85.81% |

**Table 3.4 (continued).**

| Sample | Final Conc. (mg L$^{-1}$) | 0 | 1 | 2 | 3 | 4 | 5 | 7 |
|---|---|---|---|---|---|---|---|---|
| | | | | | (Time: day) | | | |
| **FeCl$_2$** | 5 | 100% | 96.42% | 96.35% | 93.98% | 91.01% | 89.75% | 87.20% |
| | 10 | 100% | 95.39% | 94.60% | 91.60% | 90.31% | 88.23% | 86.10% |
| | 25 | 100% | 94.44% | 93.62% | 90.33% | 89.66% | 87.37% | 85.48% |
| | 50 | 100% | 93.65 % | 91.09% | 89.65% | 87.06% | 85.51% | 82.73% |
| **FeCl$_3$** | 5 | 100% | 98.73% | 96.07% | 94.90% | 92.66% | 90.17% | 89.12% |
| | 10 | 100% | 98.95% | 96.33% | 95.72% | 94.21% | 91.88% | 89.25% |
| | 25 | 100% | 99.16% | 98.36% | 96.11% | 95.13% | 92.50% | 90.30% |
| | 50 | 100% | 99.49% | 98.94% | 98.70% | 97.65% | 97.68% | 95.98% |

**Table 3.5** 55°C group: rate constants $k_x$ (day$^{-1}$) and the coefficient of determination ($R^2$) of thiamine in blank sample and different

metal chlorides (i.e., CuCl, CuCl$_2$, FeCl$_2$, FeCl$_3$) at the studied concentrations (i.e., 5, 10, 25, 50 mg L$^{-1}$) for 7 days.

| Sample type | Final concentration | | First-order kinetics parameters | | | Final concentration | | First-order kinetics parameters | |
|---|---|---|---|---|---|---|---|---|---|
| | (mg L$^{-1}$) | (mmol L$^{-1}$) | $k_x$ (day$^{-1}$) | $R^2$ | | (mg L$^{-1}$) | (mmol L$^{-1}$) | $k_x$ (day$^{-1}$) | $R^2$ |
| **Thiamine solution (Blank sample)** | 250 | | 0.0132 | 0.9890 | | | | | |
| **CuCl** | 5 | 0.051 | 0.0284 | 0.9479 | **FeCl$_2$** | 5 | 0.039 | 0.0209 | 0.9896 |
| | 10 | 0.101 | 0.0423 | 0.9565 | | 10 | 0.079 | 0.0241 | 0.9798 |
| | 25 | 0.253 | 0.0490 | 0.9470 | | 25 | 0.197 | 0.0260 | 0.9669 |
| | 50 | 0.505 | 0.0760 | 0.9787 | | 50 | 0.394 | 0.0312 | 0.9674 |
| **CuCl$_2$** | 5 | 0.037 | 0.0097 | 0.9348 | **FeCl$_3$** | 5 | 0.031 | 0.0181 | 0.9894 |
| | 10 | 0.074 | 0.0123 | 0.9220 | | 10 | 0.062 | 0.0161 | 0.9956 |
| | 25 | 0.186 | 0.0200 | 0.9732 | | 25 | 0.154 | 0.0141 | 0.9865 |
| | 50 | 0.372 | 0.0254 | 0.9625 | | 50 | 0.308 | 0.0054 | 0.9881 |

**Table 3.6** 55°C group: pH value of the mixtures of thiamine and four metal chlorides solution at different concentrations for 7 days.

| Sample name | Final Conc. (mg L$^{-1}$) | Storage time (days) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 5 | 7 |
| **Blank** | 250 | 3.53 ± 0.03 | 3.70 ± 0.01 | 3.67 ± 0.01 | 3.84 ± 0.03 | 3.66 ± 0.03 | 3.73 ± 0.01 |
| **CuCl** | 5 | 3.77 ± 0.01 [d] | 3.81 ± 0.02 [d] | 3.59 ± 0.01 [d] | 3.66 ± 0.02 [d] | 3.54 ± 0.01 [d] | 3.65 ± 0.02 [d] |
| | 10 | 3.80 ± 0.00 [c] | 3.89 ± 0.00 [c] | 3.68 ± 0.00 [c] | 3.76 ± 0.02 [c] | 3.66 ± 0.02 [c] | 3.71 ± 0.01 [c] |
| | 25 | 3.85 ± 0.01 [b] | 3.99 ± 0.01 [b] | 3.81 ± 0.01 [b] | 3.85 ± 0.01 [b] | 3.72 ± 0.01 [b] | 3.77 ± 0.00 [b] |
| | 50 | 4.19 ± 0.04 [a] | 4.28 ± 0.01 [a] | 4.06 ± 0.01 [a] | 4.07 ± 0.01 [a] | 3.86 ± 0.00 [a] | 3.84 ± 0.01 [a] |
| **CuCl$_2$** | 5 | 3.69 ± 0.02 [a,b,c,d] | 3.69 ± 0.01 [d] | 3.64 ± 0.02 [c] | 3.73 ± 0.02 [c,d] | 3.53 ± 0.01 [b,c,d] | 3.62 ± 0.01 [a,b,c,d] |
| | 10 | 3.75 ± 0.04 [a,b,c,d] | 3.78 ± 0.01 [c] | 3.69 ± 0.01 [b] | 3.75 ± 0.01 [c,d] | 3.52 ± 0.02 [b,c,d] | 3.65 ± 0.01 [a,b,c,d] |
| | 25 | 3.75 ± 0.01 [a,b,c,d] | 3.83 ± 0.00 [b] | 3.74 ± 0.01 [a] | 3.77 ± 0.02 [a,b] | 3.56 ± 0.02 [b,c,d] | 3.66 ± 0.01 [a,b,c,d] |
| | 50 | 3.78 ± 0.03 [a,b,c,d] | 3.86 ± 0.01 [a] | 3.49 ± 0.03 [d] | 3.79 ± 0.01 [a,b] | 3.64 ± 0.01 [a] | 3.67 ± 0.03 [a,b,c,d] |

**Table 3.6** (continued)

| Sample name | Final Conc. (mg L$^{-1}$) | Storage time (days) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 5 | 7 |
| **FeCl$_2$** | 5 | 3.92 ± 0.04 [a,b,c,d] | 3.74 ± 0.00 [a,b,c] | 3.56 ± 0.01 [b,c] | 3.59 ± 0.01 [a,b] | 3.84 ± 0.02 [a,b] | 3.47 ± 0.02 [a,b] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 10 | $3.88 \pm 0.02$ [a,b,c,d] | $3.74 \pm 0.00$ [a,b,c] | $3.59 \pm 0.01$ [a] | $3.62 \pm 0.01$ [a,b] | $3.81 \pm 0.02$ [a,b] | $3.50 \pm 0.01$ [a,b] |
| | 25 | $3.86 \pm 0.03$ [a,b,c,d] | $3.73 \pm 0.00$ [a,b,c] | $3.56 \pm 0.00$ [b,c] | $3.55 \pm 0.01$ [c] | $3.64 \pm 0.02$ [c] | $3.33 \pm 0.01$ [c] |
| | 50 | $3.83 \pm 0.04$ [a,b,c,d] | $3.69 \pm 0.01$ [d] | $3.51 \pm 0.01$ [d] | $3.44 \pm 0.01$ [d] | $3.50 \pm 0.01$ [d] | $3.19 \pm 0.01$ [d] |
| $FeCl_3$ | 5 | $3.62 \pm 0.02$ [a] | $3.67 \pm 0.01$ [a] | $3.48 \pm 0.01$ [a] | $3.57 \pm 0.01$ [a] | $3.51 \pm 0.01$ [a] | $3.50 \pm 0.01$ [a] |
| | 10 | $3.52 \pm 0.03$ [b] | $3.56 \pm 0.01$ [b] | $3.41 \pm 0.01$ [b] | $3.46 \pm 0.01$ [b] | $3.39 \pm 0.00$ [b] | $3.42 \pm 0.02$ [b] |
| | 25 | $3.30 \pm 0.03$ [c] | $3.33 \pm 0.01$ [c] | $3.21 \pm 0.01$ [c] | $3.23 \pm 0.01$ [c] | $3.18 \pm 0.01$ [c] | $3.24 \pm 0.02$ [c] |
| | 50 | $3.09 \pm 0.01$ [d] | $3.08 \pm 0.00$ [d] | $2.97 \pm 0.02$ [d] | $3.02 \pm 0.01$ [d] | $2.96 \pm 0.01$ [d] | $2.97 \pm 0.01$ [d] |

[1]The pH values were measured at room temperature, and the samples were stored in the incubator at $55 \pm 0.2$ °C when not in analysis.

[2]All the pH values are mean $\pm$ standard deviation (M$\pm$ SD), and n = 4. [3]The different letters in the same columns for each form indicate that there are statistical differences between concentrations ($p < 0.05$) (e.g., CuCl on day 0 and $FeCl_3$ on day 7), while the same letters in the column of indicate there are no significant differences ($p > 0.05$) (e.g., $CuCl_2$ on day 0).

## 3.6 References

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CHAPTER FOUR

GENERAL CONCLUSIONS

Thiamine was investigated for its stability in aqueous salt solutions at four different concentrations (i.e., 5, 10, 25, and 50 mg $L^{-1}$) of four types of metal chlorides (i.e., CuCl, $CuCl_2$, $FeCl_2$, and $FeCl_3$). In addition, effect of temperature on thiamine stability under three temperatures (i.e., 25, 40, and 55°C) was monitored for seven days. At 25 and 55°C, the effects of metal chlorides at 50 mg $L^{-1}$ on thiamine stability are in the following order: CuCl> $FeCl_2$ > $CuCl_2$> $FeCl_3$. At 40°C, the effects of the metal chlorides at 50 mg $L^{-1}$ on thiamine stability are ranked as follows: $FeCl_2$ > $CuCl_2$ > CuCl > $FeCl_3$.

Thiamine is relatively stable in more acidic solutions. As expected, the most significant thiamine degradation occurred in the CuCl solution due to the least acidity of its solution at three temperatures. For example, the highest losses of thiamine were observed in 50 mg $L^{-1}$ of CuCl at 25 and 55°C, which accounted for 64.00% (pH 4.47) and 61.94% (pH 3.84) of remaining amounts of thiamine on day 7, respectively.

In contrast, $FeCl_3$ solutions provide the most acidic environment which is favorable for stabilizing thiamine, which was confirmed by the lowest thiamine losses in 50 mg $L^{-1}$ of $FeCl_3$ at 25, 40, and 55°C, which showed 78.34%, 91.48%, and 95.98% of the remaining amounts of thiamine on day 7, respectively. Compared to the pH values in the $FeCl_3$ solutions at 25, 40 and 55°C, the latter had a lower pH value, which might be the reason for the thiamine stability at the high temperature.

The degradation rates caused by $Fe^{2+}$ ions were fast even though the salt solutions ($FeCl_2$) were acidic (around pH 3.5 at three temperatures). Similarly, $Cu^{2+}$ also exerted the most considerable degradation at 40°C. It was speculated that metal ions could form a metal complex

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with thiamine to increase the rate of thiamine degradation, which means some metal ions (i.e., iron and copper ions) are prone to become metal complexes and cause self-catalytic effects on thiamine stability.

The pH value, temperature, and chemical states of metal ions were observed to influence the thiamine stability. For example, a more acidic environment can ease off thiamine degradation, while higher temperatures may threaten thiamine stability. However, not all factors were able to be tested or be accurately controlled. For instance, there was a drawback of the experimental design, in which buffer solution was not integrated for maintaining the pH values of the test solutions when evaluating the effects of variables of temperature and metal ions. Moreover, the formation of metal complexes might also cause a self-catalytic effect on thiamine stability. In this context, more research ought to be conducted in the future.

In conclusion, thiamine degradation was indeed affected by four metal chlorides (i.e., CuCl, CuCl$_2$, FeCl$_2$, and FeCl$_3$) in the studied concentrations at three temperatures (i.e., 25, 40, and 55°C), though those tests only lasted for 7 days.

APPENDICES

## Appendix A

## PROPERTIES OF METAL CHLORIDES

**Table A.1** Properties of thiamine and metal chlorides.

| Compound name | CAS number | Purity | Molecular formula | Molar mass (g mole$^{-1}$) | Physical properties |
|---|---|---|---|---|---|
| Thiamine hydrochloride | 67-03-8 | 99 % | $C_{12}H_{17}CIN_4OS \bullet HCl$ | 337.28 | - White crystalline powder.<br>- Melting point: 250°C.<br>- Soluble in water (1 g mL$^{-1}$), glycerol (1g 18 mL$^{-1}$), 95% alcohol (1 g 100 mL$^{-1}$), abs. alcohol (1g 315 mL$^{-1}$), and propylene glycol.<br>- Insoluble in hexane, chloroform, benzene, and diethyl ether. |
| Copper (I) chloride | 7758-89-6 | 99.99% | CuCl | 99 | - Gray to green crystalline powder.<br>- Boiling point: 1490.0°C; melting point: 430.0°C.<br>- Soluble in water (0.06 g L$^{-1}$ at 25°C); conc. HCl and $NH_4OH$ (forming complexes).<br>- Insoluble in alcohol and acetone. |
| Copper (II) chloride dihydrate | 10125-13-0 | 99.0 % | $CuCl_2 \bullet 2H_2O$ | 170.48 | - Blue to blue-green and odorless powder.<br>- Melting point: 100°C (decomposition).<br>- Solubility: clear blue solution at 100 mg mL$^{-1}$ in water; soluble in methanol, ethanol, and acetone. |
| Iron (II) chloride tetrahydrate | 13478-10-9 | 99+ % | $FeCl_2 \bullet 4H_2O$ | 198.81 | - Yellow to green crystalline powder, crystals, and chunks.<br>- Melting point: 105°C. |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | - Solubility in water: 64.4g 100 mL$^{-1}$ at 10°C, 68.5g 100 mL$^{-1}$ at 20°C, and 105.7 g 100 mL$^{-1}$ at 100°C; soluble in tetrahydrofuran (THF). |
| Iron (III) chloride anhydrous | 7705-08-0 | - | FeCl$_3$ | 162.2 | - Dark green to black powder.<br>- Boiling point: 316°C; melting point: 307.6°C.<br>- Solubility in water or 1 M HCl (100 mg mL$^{-1}$) (yielding a turbid solution with a color mixture of yellow, brown, and orange).<br>- Highly soluble in water, acetone, methanol, ethanol, and diethyl ether.<br>- Sensitive to light and moisture. |

**\*References come from the product labels and the descriptions on Fisher scientific and Millipore Sigma websites.

## Appendix B

## DETERMINATION OF THIAMINE BY HPLC

**Table B.1** Determination of thiamine by the varied HPLC methods.

| Sample matrix | Preparation/ Extraction | Chromatographic condition |
|---|---|---|
| Vitamin B1 in milk matrix (Sierra et al., 1996; Sierra and Vidal-Valverde., 2001) | Milk (10 mL) was hydrolyzed with 0.3 M HCl at 121°C for 20 mins. Adjust to pH 5.5. Add Taka-Diastase and incubate the sample at 45°C for 3 hours. Filter and dilute. | - µBondapak C$_{18}$ (300 x 3.9 mm, 10µm).<br>- Mobile phase: methanol/water /acetic acid (31:68.5:0.5) with 5 mM sodium hexanesulfonate.<br>- Flow rate: 1 ml min$^{-1}$; column temperature set at 35°C<br>- Post-column derivatization to form thiochrome, and set fluorescence detector at $\lambda_{Ex} = 360\ nm$ and $\lambda_{Em} = 435\ nm$. (Wimalasiri and Wills., 1985) |

| | | |
|---|---|---|
| Thiamine mononitrate (TMN) for enriching bread dough (Voelker et al., 2021). | TMN was added to the diluted dough at 1 mg mL$^{-1}$ and 20 mg mL$^{-1}$. Before the analysis, dilute the sample at 0.5 mg mL$^{-1}$ by using 0.1% TFA. | - Waters Xterra RP-C$_{18}$ column (100 x 3.9 mm, 3.5μm).<br>- Mobile phase: 0.1% TFA in water/ acetonitrile in a gradient program, which is 100/0 at 0 min, 97/3 at 4 min, 90/10 at 6 min, 100/0 at 10 min, and 100/0 at 15 min.<br>- Flow rate: 1 ml min$^{-1}$<br>- UV at 254 nm. |
| Thiamine in malted and unmalted cereal samples (Hucker et al., 2012). | Mill the samples into powders. Add 5 ml of 0.5 M trichloroacetic acid (TCA) to a 0.5 g powdered sample. Centrifuge and take 2 ml of the supernatant. Add 1 ml 10 mM phosphate buffer and adjust to pH 6.5-6.6 with 2M KOH. Dilute and filter. | - Varian pursuit C$_{18}$ (250 x 4.6 mm, 5μm).<br>- Mobile phase: 10 mM phosphate buffer (KH$_2$PO$_4$/ K$_2$HPO$_4$) at pH 6.5/ methanol in a gradient program, which is 95/5 at 0 min, 65/35 at 0.5 min, 95/5 at 15 min, and run until 25 min.<br>- Flow rate: 1 ml min$^{-1}$; column temperature set at 30°C<br>- Covert the thiamine to thiochrome esters and set the fluorescence detector at $\lambda_{Ex} = 360\ nm$ and $\lambda_{Em} = 425\ nm$. |
| Thiamine in meat and fresh liver samples (Tang et al., 2006). | Heat the mixture of 5 g of meat and fresh liver samples, 0.5 g of lyophilized BCR certified liver powder, and 60 mL 0.1M HCl at 100°C for 15 mins. Homogenize for 1 min, and heat again for 45 mins. After cooling, adjust pH to 4.3-4.7 and incubator at 37°C for 18 hours. Add 2 ml 50% trichloroacetic acid and heat for 10 mins. Dilute and filter. | - Inertsil ODS-2 column (200 x 3.0 mm, 5μm).<br>- Mobile phase: methanol/ water (80:20).<br>- Flow rate: 0.3 ml min$^{-1}$.<br>- Convert to thiochrome for the fluorescence detector at $\lambda_{Ex} = 366\ nm$ and $\lambda_{Em} = 434\ nm$. |
| Vitamin B1 in complex cereal food products (Rodriguez et al., 2012). | Mix cereal sample (15 g) with 0.1 N H$_2$SO$_4$ (90 ml). Adjust pH 4.5 with 2.5 M Na acetate, and add papain, diastase, and alpha-amylase. Incubate the samples at 37°C overnight. Dilute and filter. | - Purospher® STAR RP-18e (250 x 4.0 mm, 5μm).<br>- Mobile phase: 12.5 mM sodium acetate in a mixture of methanol/ water (25/75) + 2.5 mM sodium heptanesulphonate.<br>- Flow rate: 0.9 ml min$^{-1}$.<br>- UV at 268 nm. |

HPLC-UV method was commonly used to determine thiamine in pharmaceutical products and foods because it is simple and fast. Reversed-phase HPLC with C18 column and the solvents of methanol/water as the eluent were mostly applied. Careful hydrolysis and purification procedures could improve the chromatographic conditions when analyzing food products by using HPLC-method because food samples contain several interfering compounds which might absorb light in the UV range. The presence of buffers is often useful to adjust pH in the solvent and get more reproducible conditions (Rodriguez et al., 2012). Besides, the option of the columns affects the separation of water-soluble vitamins because of the difference in their chemical structures (Heudi et al., 2005). The silica-based column is advantageous in separating polar vitamins, such as vitamin B1 and vitamin C. The polar nature of those vitamins can lead to hydrophilic interaction with the stationary phase (Patle et al., 2022).

In one study, Varian LC 5060 (Palo Alto, CA, USA) with a Vatuchrom-5 UV detector was used. It was reported that thiamine had a characteristic UV spectrum with two absorption maxima, 245 and 260 nm, respectively. This study also showed the UV spectra of the various forms of thiamine, such as the maximum absorption of three phosphate esters (thiamine monophosphate (TMP), thiamine diphosphate (TDP), and thiamine triphosphate (TTP)) was at 248 nm and maximum absorption of thiamine disulfide (TDS) was at 245 nm (see **Figure B.1**) (Hilker et al.,1982). In another study, an Agilent 1100 LC system (Agilent Technology Inc., Urdorf, Switzerland) with a diode array spectrophotometric detector was used, and the UV spectra of thiamine were also presented in **Figure B.1** (Heudi et al., 2005).
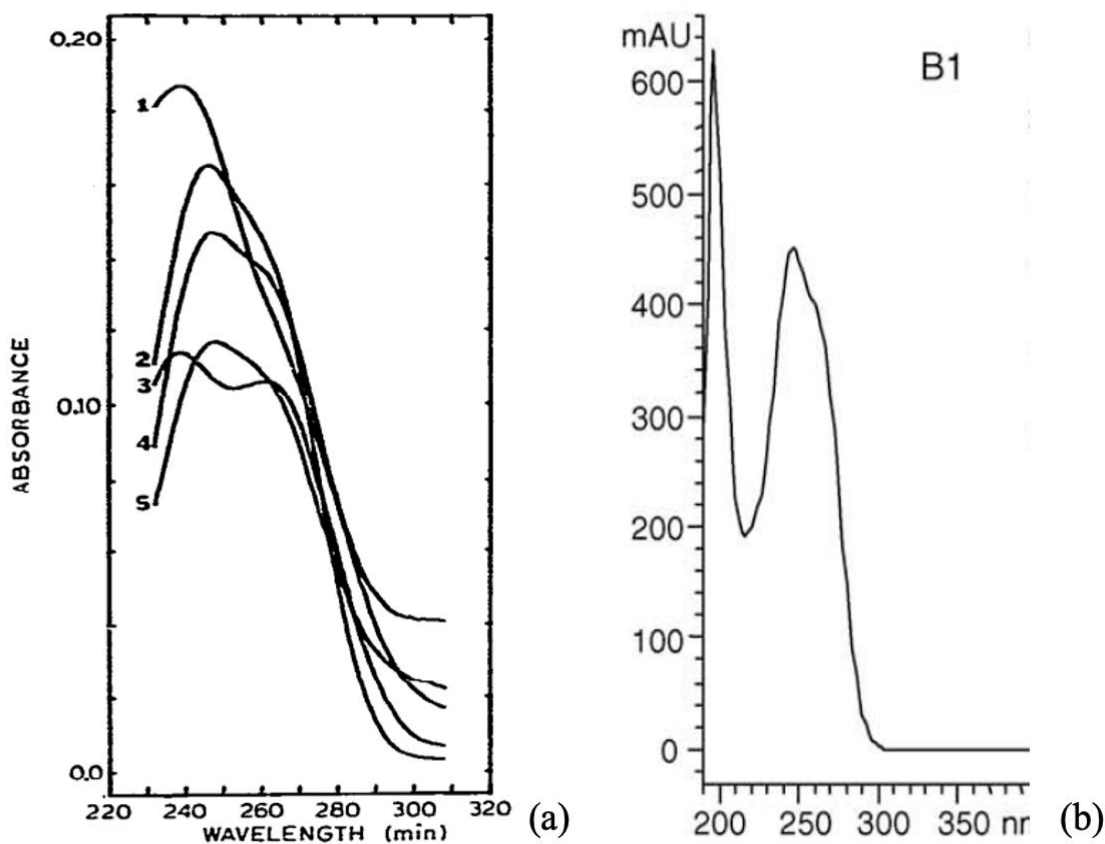
**Figure B.1** UV absorption spectra of the thiamine determined by HPLC. a) thiamine in the various forms. The absorbance values are relative. *Curves: 1= thiamine disulfide (TDS); 2= thiamine monophosphate (TMP); 3=thiamine; 4= thiamine diphosphate (TDP); 5= thiamine triphosphate (TTP) (Hilker et al., 1982); b) thiamine mononitrate ($V_{B1}$). *The unit for abscissa was nm (wavelengths) (Heudi et al., 2005).

In this study, the HPLC-UV chromatogram of the standard thiamine solution and the mixture of thiamine with each metal chloride solution was described in **Figure B.2** and **Figure B.3**. The HPLC model was listed in Chapter 2 and Chapter 3. The flow rate was 1 ml min$^{-1}$, the injection volume was 10 μL, the column was maintained at 30°C, and the wavelength was set at 254 nm. Thiamine was separated to the baseline and eluted as a sharp peak within 5 mins, and the retention time of the thiamine peak was around 2.7 mins. The peak area and height were reproducible in each metal ions system.
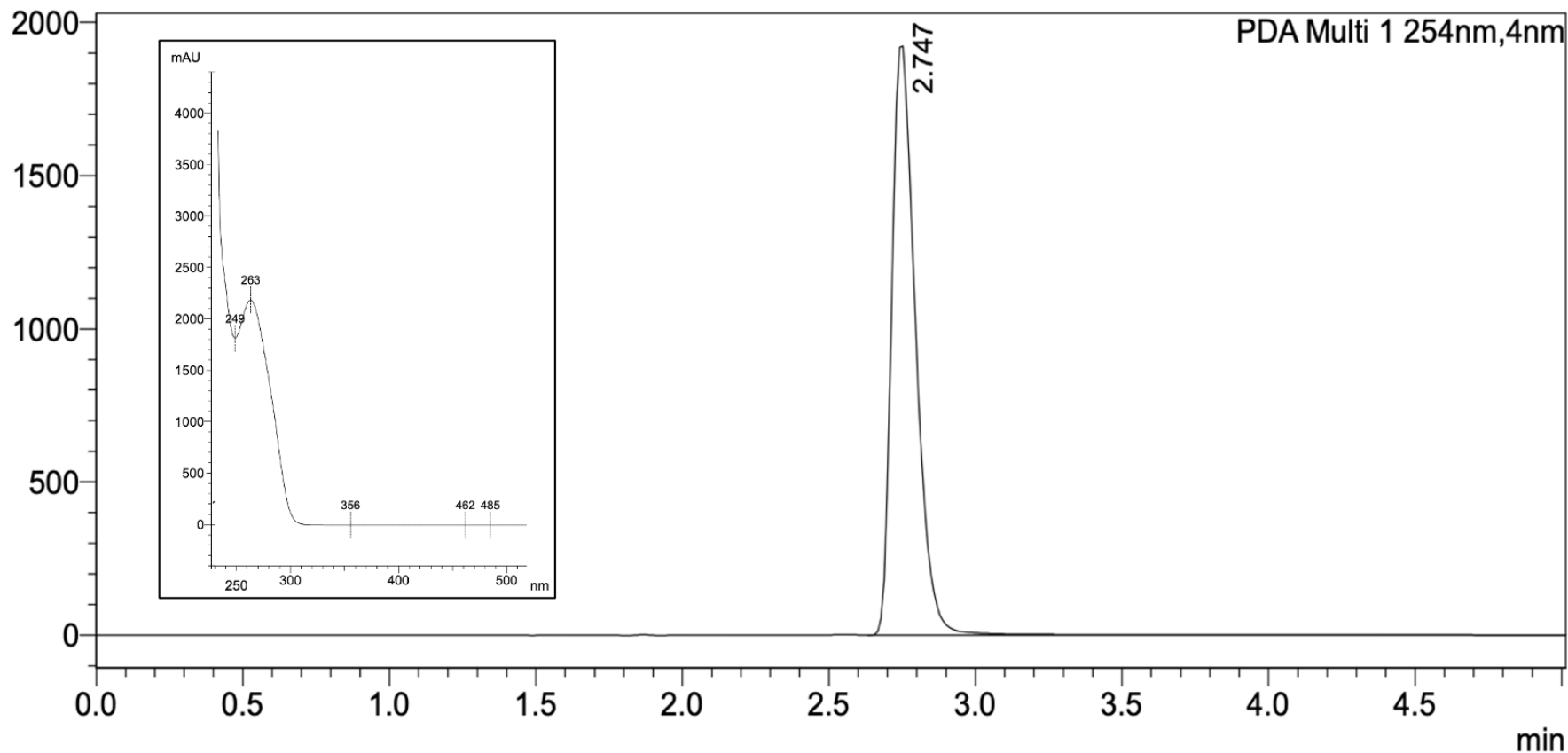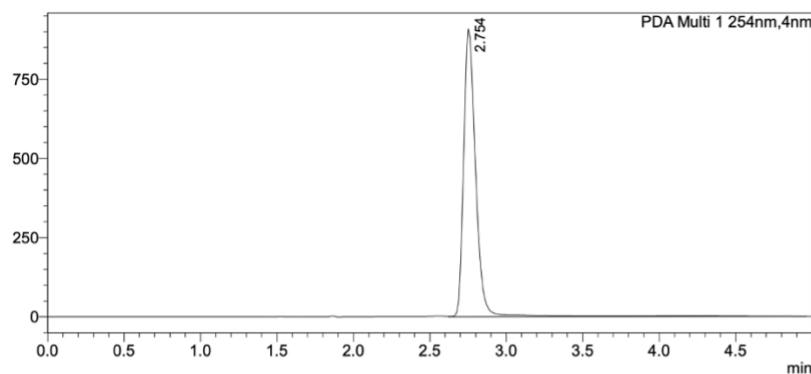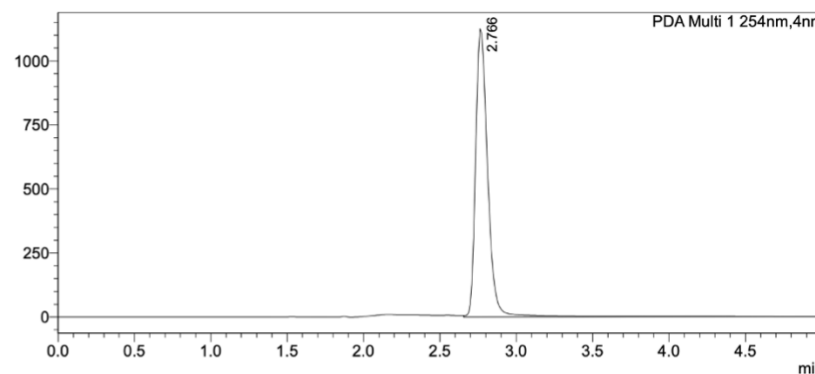
**Figure B.2** An example of a chromatogram of 500 mg L$^{-1}$ of standard thiamine solution at 25°C on day 0 by HPLC-UV and the UV spectrum used in this study. *254 nm was one of the wavelengths with higher absorptions of UV light, and 2.747 min was the retention time of the thiamine sample.
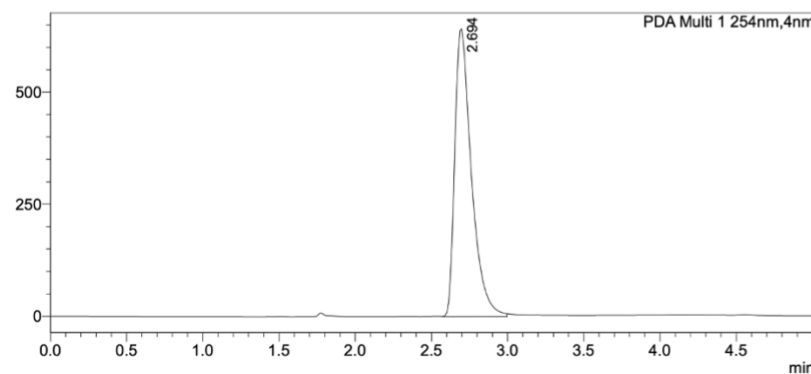
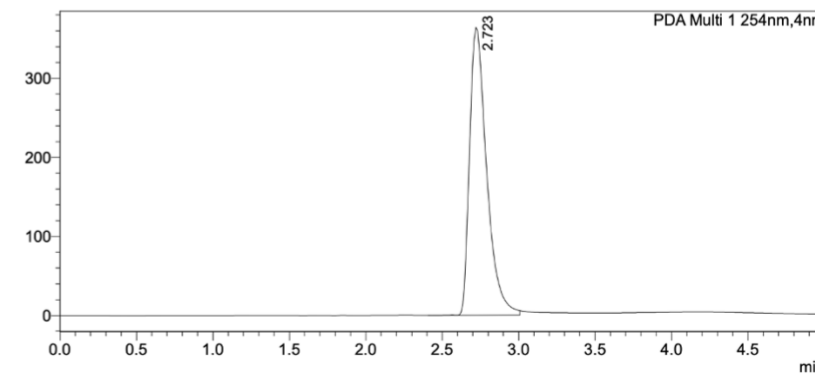**Figure B.3** Examples of the chromatogram of thiamine solution in the aqueous solutions with 100 mg L$^{-1}$ of varied metal chlorides (i.e., a) CuCl; b) CuCl$_2$; c) FeCl$_2$; d) FeCl$_3$) at 25°C on day 1 by HPLC-UV. [1]The peaks showed around 2.7 mins (retention time of thiamine) in each analysis. [2]After mixing with 500 mg L$^{-1}$ of thiamine solution, the final concentration of the metal chloride was 50 mg L$^{-1}$ and thiamine was 250 mg L$^{-1}$.

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