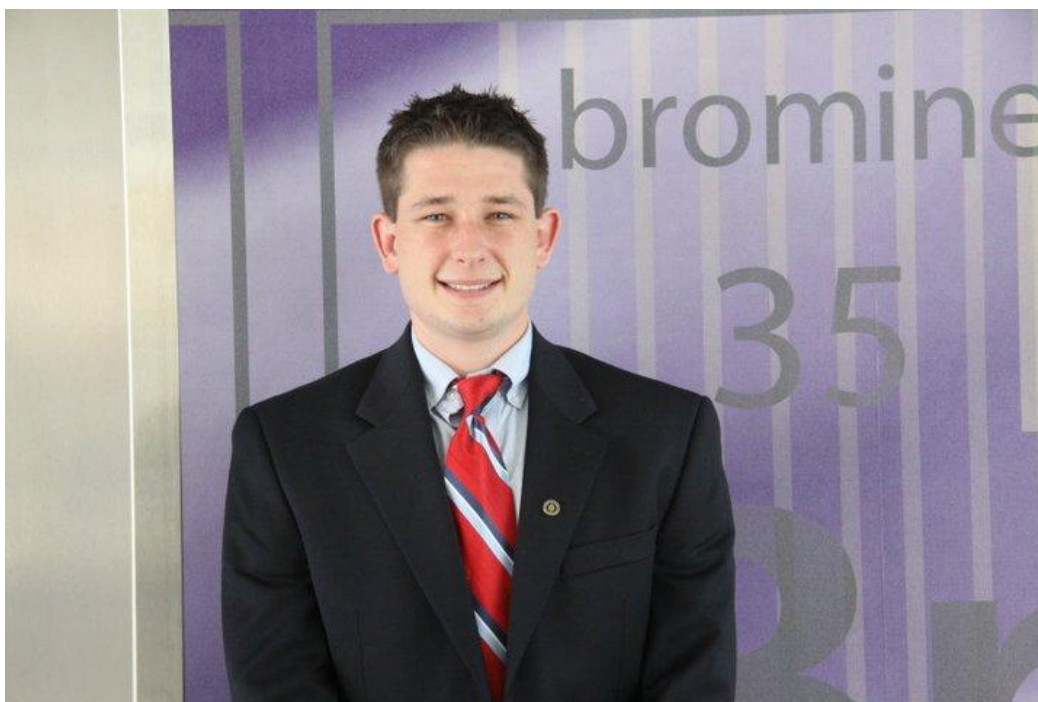


Co-Crystallization and Polymorphism of Naturally Occurring Peptide Derivatives

--Kevin Crowley--



Kevin Crowley is currently a senior at Coastal Carolina University majoring in biochemistry. He is scheduled to graduate in May of 2012. He has always been strong in the fields of biology and chemistry, and he hopes to pursue a career in the field of biochemistry and bioinformatics. Kevin's ultimate goal is to earn a medical degree and practice medicine and conduct research. Since childhood, he has been fascinated with science. He is driven to investigate the numerous unknown or poorly understood biological processes of the human body. Some people say coffee gets them up in the morning, but Kevin is awoken each day by the unanswered questions in science.

ABSTRACT

Carnosine is a dipeptide compound that is found in many dietary supplements and food products. Carnosine has many functions in the body, such as alleviating oxidative stress on tissues by acting as an antioxidant compound. Carnosine, therefore, has important anti-aging properties. Carnosine is also capable of forming protective sequestration structures around heavy metal ions; this process of chelating metals ions in solutions is very beneficial for maintaining the well-being of cells in the body. Thus, carnosine could be useful in pharmaceutical products for creating anti-aging drugs that would reduce tissue stress and promote a healthy cellular environment. I attempted to co-crystallize carnosine with four polycarboxylated aromatic acids

and two Krebs cycle metabolites to generate various supramolecular structures based on the placement of carboxyl groups on the co-crystallants. If a co-crystallization method is created for carnosine, pharmaceutical products can utilize the same method in producing carnosine-based drugs. Furthermore, carnosine chelation of various metal ions was conducted to determine if carnosine would chelate in a variety of solution environments. Co-crystallization of carnosine with the four polycarboxylated aromatic acids and two Krebs cycle metabolites was not fully achieved, possibly due to environmental and stability conditions of solutions. Carnosine demonstrated metal-ion chelation properties with copper ions, whereas iron and zinc and iron ion solutions did not reveal carnosine chelation properties. In conclusion, more experiments with carnosine should be conducted to find optimal co-crystallization conditions for the production of pharmaceutical products.

1.0 Introduction

Carnosine has many functions in the body, including alleviating oxidative stress and chelating metals ions in solutions. Thus, carnosine could be useful in pharmaceutical products. I attempted to co-crystallize carnosine with four polycarboxylated aromatic acids and two Krebs cycle metabolites to generate various supramolecular structures based on the different placement of carboxyl groups on the co-crystallants. Co-crystallization of carnosine with the four polycarboxylated aromatic acids and two Krebs cycle metabolites was not fully achieved, possibly due to environmental and stability conditions of solutions. Carnosine demonstrated metal-ion chelation properties with copper ions, whereas iron and zinc and iron ion solutions did not reveal carnosine chelation properties.

Carnosine is a dipeptide molecule that is synthesized from histidine and alanine (Reeve et al., 1993). Also known as β -alanylhistidine, carnosine is a hydrophilic compound that prevents oxidative stress in a variety of important biomolecules, tissues, and organisms (Boldyrev et al. 2004). Carnosine occurs naturally in high concentrations in active and excitable tissues such as heart, brain, and skeletal muscle (Boldyrev et al. 2004). Many experiments have been conducted to observe the effects of carnosine on vertebrates (Janessen et al. 2005; Brownrigg et al. 2010; Ma et al. 2010).

Carnosine inactivates harmful compounds related to oxidative stress and activation of excitatory compounds; hydrolysis of carnosine into β -alanine and histidine leads to the production of histamine, which is a common neurotransmitter (Suer et al. 2011). Furthermore, carnosine inhibits activity of serotonin-derived melanoid, a compound that decreases neurological activity and may advance Alzheimer's disease (Brownrigg et al. 2010). Moreover, carnosine prevents diabetic neuropathy, a common condition among diabetics usually resulting in kidney failure and even death, by reducing glycosylation and glucose levels in the extracellular matrices of cells (Janssen et al. 2005). When carnosine levels are too high in certain tissues, carnosinase enzymes convert carnosine into β -alanine and histidine; both of these amino acids can be used for protein synthesis or metabolism (Bertinaria et al. 2011). Production of carnosinase from the CNDP1 gene decreases carnosine levels, which can lead to accelerated development of diabetic neuropathy (Riedl et al. 2007). When concentrations of heavy metals, such as copper, cobalt, zinc, and iron, are elevated under normal physiological conditions, carnosine creates chelation

complexes to isolate and sequester harmful heavy metal ions and prevents them from interacting with important biomolecules (Lanza et al. 2011). Carnosine performs a wide array of protective and anti-aging functions that are essential to biological processes throughout the body. Practical applications of carnosine have been studied in the meat-processing industry by including carnosine in the diet of livestock to preserve meat and prevent spoilage (Ma et al. 2010). Thus, it would be beneficial to use carnosine for medicinal needs and pharmaceutical products based on its physical and chemical properties.

Crystallization is a very common method for precipitating compounds out of solution and tightly packing molecules into a stable, solid state. However, not all molecules crystallize easily. Therefore, compounds called co-crystallants are used to form crystals in solution; this process is used frequently by pharmaceutical companies to condense compounds into pill form. Co-crystallants are specifically engineered based on the chemical properties and functional groups of the molecule that needs to be crystallized. For example, molecules that have many carbonyl, hydroxyl, or even nitrogen-bearing functional groups readily form dimers with co-crystallants capable of hydrogen bonding (Mahapatra et al. 2010). Co-crystallants must be fairly stable as to not undergo chemical reactions with other compounds in solution; aromatic compounds can be used to increase stability of crystallization bonds as well as increase molecular interactions with pi-pi stacking of aromatic rings (Qiao et al. 2011). Aromatic compounds capable of hydrogen bonding are likely to co-crystallize with carnosine because of the nitrogen-based and oxygen-based functional groups present in the molecular structure. Compounds that function as important metabolites can also be used successfully for co-crystallization and possible drug development due to their harmless nature. For example, compounds such as citrate and α -ketoglutarate can be used for co-crystallization due to the presence of multiple carboxylic acid groups on those compounds. Citrate and α -ketoglutarate are important metabolites in the Krebs cycle and contain multiple carboxylic acid groups; thus co-crystallization could be established by utilizing these metabolites (Karki et al. 2007). Using Krebs cycle metabolites and aromatic polycarboxylated acids as co-crystallizing agents would be suitable for drug development.

Carboxylic acids and other carbonyl-based molecules readily form hydrogen bonds and dimers in aqueous solutions. By using stable molecules with multiple carboxylic acid groups, such as aromatic polycarboxylated acids and Krebs cycle metabolites, co-crystallization of carnosine may be achieved through hydrogen bonding, dimer formation, and even pi-pi stacking (Santra and Biradha 2009). Various supramolecular structures can be generated by using co-crystallants with different placements of key functional groups needed for co-crystallization; polymorphism as well as co-crystallization can be achieved (Goswami et al. 2008). For instance, when performing co-crystallization with phthalic acid, crystal structures have been documented to resemble long, repeating helices with pi-pi stacking of aromatic rings (Bán et al. 2009). Crystal structures can vary greatly among co-crystallants leading to the formation of numerous types of crystals. For example, co-crystallization with compounds containing *ortho*-carboxylic groups, such as those on phthalic acid, can generate several different supramolecular structures based on the complexity of molecular interactions during crystallization (Baca et al. 2006).

After crystal formation, crystals must be extracted and purified by using simple laboratory techniques based on molecular properties. Three-dimensional crystal structures are determined via x-ray diffraction, IR spectroscopy, and/or Nuclear Magnetic Resonance (NMR) spectroscopy

(Wang et al. 2009). The purpose of this research project is to successfully co-crystallize carnosine with aromatic polycarboxylated acids and examine polymorphism of crystal structures. We hypothesized that co-crystallization of carnosine can be achieved by using various aromatic polycarboxylated acids and Krebs cycle metabolites that produce different crystal structures based on the placement of functional groups on the co-crystallants. We will test four aromatic polycarboxylated compounds: isophthalic acid, phthalic acid, terephthalic acid, and trimesic acid. Each of these compounds contains two or more carboxylic acid groups bound to a phenyl ring, but each compound differs based on the placement of the carboxylic acid groups. Furthermore, two important Krebs cycle metabolites, citrate and α -ketoglutarate, will be used to generate additional crystal structures based on their incorporation in numerous metabolic pathways that can be utilized for possible drug development. To ensure accuracy of results, several trials will be conducted for each co-crystallant with carnosine.

Previous experiments that have been conducted with these polycarboxylated molecules have demonstrated a variety of binding properties unique to each compound. For example, trimesic acid has been shown to create large complicated supramolecular complexes when metal ions are introduced in solution (Chatterjee et al. 2000). Trimesic acid contains three carboxylic acid groups, which is more than the number found in other polycarboxylated compounds such as terephthalic acid. It has been observed that trimesic acid exhibits weak intermolecular bonding due to steric hindrance (Fleischman et al. 2003). Bonding properties of each of the co-crystallants will be unique and therefore produce different crystal structures.

Practical applications of this experiment are possible based on the ion-chelating properties of carnosine. Under conditions where harmful, free iron ions were present, carnosine could prevent iron ions from causing oxidative stress and disrupting cellular activity. Red blood cell disorders that cause denatured hemoglobin to release iron ions into the bloodstream are problematic due to the oxidative properties of free iron ions. Excess amounts of iron can be removed from the body with iron-chelation therapy (Porter 2009). Carnosine has shown chelating properties when exposed to oxidizing copper ions (Decker et al. 2000). However, carnosine has not yet been observed to form iron-chelation complexes (Morrissey et al. 1998). Therefore, one objective for this experiment is to achieve iron chelation of carnosine under a variety of different conditions. By coupling the conditions at which iron chelation of carnosine occurs and crystal structures generated from carnosine co-crystallization, it may eventually be possible to develop a carnosine-based drug to chelate excess iron ions in the bloodstream.

2.0 Materials and Methods

2.1 Stock Solutions

Each of the six co-crystallant solutions and the carnosine solution were created in mass quantities to easily replicate crystallization trials. Optimal crystallization conditions for small molecules occurs around 0.1M of each solution. Stock solutions were composed of 100 mL deionized water with 0.1 moles of solute; 0.1M (10 mmol/100 mL solution) solutions of carnosine, phthalic acid, isophthalic acid, terephthalic acid, trimesic acid, citrate, and α -ketoglutarate were created as stock solutions. Carnosine powder and 0.1M carnosine were stored under refrigeration because carnosine degrades at room temperature. The other co-crystallant solutions and corresponding

powder samples were kept at room temperature. The polycarboxylated aromatic acids (phthalic acid, isophthalic acid, terephthalic acid, and trimesic acid) are partially insoluble in water; therefore additional solvents such as ethanol, acids, and bases were added to the solutions to create homogenous mixtures.

2.2 Co-crystallization at Room Temperature

Glassware used in the experiment consisted primarily of 25 mL, sealed, round-bottom flasks and class-A 10 mL pipets to ensure accuracy and precision of results. Ten mL of 0.1M carnosine solution were mixed with 10 mL of 0.1M phthalic acid in a flask and left at room temperature for slow-evaporation crystallization to occur. This procedure was repeated for isophthalic acid, terephthalic acid, trimesic acid, citrate, and α -ketoglutarate solutions in separate flasks. The procedure was repeated for each co-crystallant in unsealed, open-top flasks, thus allowing faster evaporation at room temperature, which could possibly produce crystals at a faster rate or produce different types of crystals.

2.3 Carnosine Chelation with Metal Ions

Chelation properties of carnosine were tested by using iron, copper, and zinc ions in solution. Optimal chelation conditions were obtained by using 0.1M solutions of both carnosine and metal ion solutions. Stock solutions were created at 0.1M of iron (Fe^{3+} from $\text{Fe}(\text{NO}_3)_3$), copper (Cu^{2+} from $\text{Cu}(\text{NO}_3)_2$), and zinc (Zn^{2+} from $\text{Zn}(\text{NO}_3)_2$). Ten mL of 0.1M carnosine were mixed with 10 mL of 0.1M metal ion solution in 25 mL, sealed, round-bottom flasks and kept at room temperature. The process was repeated for each metal ion solution using 25 mL, open-top flasks.

2.4 Co-Crystallization and Chelation with Metal Ions

Stock solutions were created at 0.1M with iron (Fe^{3+} from $\text{Fe}(\text{NO}_3)_3$), copper (Cu^{2+} from $\text{Cu}(\text{NO}_3)_2$), and zinc (Zn^{2+} from $\text{Zn}(\text{NO}_3)_2$) with the addition of 10 mL of 0.1M α -ketoglutarate to each solution as a co-crystallizing agent. Equal volumes of 0.1M metal ion solution and α -ketoglutarate solution were added to 0.1M carnosine in 25 mL, sealed, round-bottom flasks and kept at room temperature. This process was repeated with citrate; a total of six 0.1M metal ion stock solutions with co-crystallants were made.

2.5 Analysis of Crystal Structures

After crystals-like structures were achieved in solution, crystals would then be observed via x-ray diffraction to determine the three-dimensional crystal lattices created from co-crystallization. The materials needed for x-ray diffraction were not present at Coastal Carolina University; therefore crystals would be sent to Dr. Frank Fronzcek at Louisiana State University Department of Chemistry (an associate of Dr. Evans). NMR spectroscopy was used to further analyze crystal structures in order to corroborate the data from x-ray diffraction. Metal ion solutions were analyzed by using UV-Vis spectroscopy in the Smith Science Center at Coastal Carolina University. Solutions containing pure metal ions were run as blanks in the UV-Vis spectroscopy; differences in absorbance peaks between the blanks and carnosine solutions would be indicative of chelation or alternative-bonding structures, as opposed to two free molecular species in

solution. The results were compiled and analyzed to generate supramolecular bonding models for carnosine.

3. Results

Stock solutions were made successfully for five of seven compounds. Carnosine, citrate, and α -ketoglutarate readily dissolved in solution without the need for additional solvents. Phthalic acid, isophthalic acid, terephthalic acid, and trimesic acid were mostly insoluble in water and did not dissolve initially. Carbonic acid was added to each of the four polycarboxylated aromatic acid solutions in increments of 10 mL to observe changes in solubility. A total of 50 mL of carbonic acid was added to each of the four solutions, which yielded no change in solubility; undissolved solids remained. New solutions were created by adding 0.01 mole of each polycarboxylated aromatic acid to 100 mL of ethanol to create 0.1M solutions. Ethanol-based solutions of trimesic acid and phthalic acid were created successfully, whereas terephthalic acid and isophthalic acid remained largely insoluble. Stronger base solutions were used in an attempt to dissolve isophthalic acid and terephthalic acid; 6M NaOH successfully dissolved isophthalic acid, but not terephthalic acid. Organic solvents were needed to make terephthalic acid miscible in water. Accordingly, 0.01 mole of terephthalic acid successfully dissolved in 100 mL of pyridine to create a stock solution.

Precipitate and crystals formed at different rates in the co-crystallization solutions; therefore crystal solutions were not analyzed until the end of the experiment. In addition, it was unknown whether or not structures generated in solution were crystals until extraction and analysis were performed. Precipitated and/or crystal structures quickly formed in citrate and α -ketoglutarate solutions. It was noted that citrate solutions in both sealed and open-top flasks generated three to five large crystal-like structures, whereas α -ketoglutarate solutions generated ten to twelve small crystal-like structures. Open-top 0.1M trimesic acid in ethanol with 0.1M carnosine generated one massive crystal-like structure stuck to the bottom of the flask with needle-shaped projections; five to seven small floating crystals were also formed in solution. All other solutions did not contain any precipitate or crystals; only a homogenous mixture was present with no signs of solids forming in solution.

The five 0.1M carnosine co-crystallization solutions (0.1M citrate open-top and sealed flasks, 0.1M α -ketoglutarate open-top and sealed flasks, and 0.1M trimesic acid with ethanol) that contained precipitate/crystals underwent extraction of solids in solution. It was observed that the solids in solution were not actually crystal structures based on their appearance and their inability to remain solid at room temperature. NMR was not conducted on any of the solutions due to difficulties that arose with the instrument over the course of the semester.

Data collected from UV-Vis spectroscopy revealed the chelation properties of carnosine with different metal ions in solution. There was a shift in the absorbed light spectrum for copper ion solutions with 0.1M carnosine ([Figure 1](#)). The shift in light absorbance is indicative of alternative binding species in solution, such as chelation; therefore the results indicated copper-carnosine chelation in solution for both sealed and open-top solutions. There were no apparent shifts in spectral absorbance in the other eight chelation solutions indicating that no chelation occurred ([Figures 2 – 9](#)).

4. Discussion

Based on the results from the experiment, the hypothesis was not fully supported. Attempted co-crystallization of carnosine with polycarboxylated aromatic acids and Krebs cycle metabolites was mostly unsuccessful. Crystals did not form in any of the solutions, but there were precipitates and crystal-like structures that formed in carnosine co-crystallization solutions containing α -ketoglutarate (sealed and open-top), citrate (sealed and open-top), and trimesic acid in ethanol (open-top). It was observed that the solids in solution were not actually crystal structures based on their appearance and their inability to remain in solid form at room temperature. Crystals are condensed, solid, organized structures; the solids extracted from all the solutions were somewhat gel-like (Harris 2010). Crystallization in early stages can be regarded as a physical gelling process where gel-like structures are generated before actual crystals form (Pogodina & Winter 1998). It is possible that the amorphous solids in solution were actually early stages of crystals, and would require additional time to crystallize. None of the solutions yielded crystals over the course of the semester and there was no apparent co-crystallization that occurred. We planned to use NMR spectroscopy to determine if there was co-crystallization occurring on a small scale in solution, but the NMR instrument at Coastal Carolina University was nonfunctional.

Citrate and α -ketoglutarate solutions formed precipitate quickly, possibly because both of these metabolites and carnosine are hydrophilic and contain many carboxylic acid groups to form amorphous solids. Co-crystallization may have occurred, not on the supramolecular level as hypothesized, but on a microscopic level, which was unable to be determined with the instrumentation at Coastal Carolina University. Furthermore, the inability to co-crystallize carnosine with the co-crystallants could have arisen due to environmental conditions and/or properties of the stock solutions that were created. It was anticipated that the placement of carboxylic acid groups would stabilize polycarboxylated compounds in water and therefore create a sufficient environment for co-crystallization. The polycarboxylated aromatic acids were not fully soluble in water and the addition of strong bases and organic solvents was needed to create stock solutions. Inability to attain water-based solutions could have affected the co-crystallization process due to the fact that carnosine is hydrophilic and does not react well with organic solvents and strong bases. Additionally, crystallization is a slow process and requires stable environmental conditions (Dale et al. 2003). Co-crystallization may have been successful in a refrigerated or climate-controlled environment to assure more stable conditions.

Based on data collected from UV-Vis spectroscopy, only copper-ion solutions exhibited carnosine chelation, whereas the other eight solutions did not. Carnosine has been known to chelate with copper ions and in some scenarios with iron and zinc (Trombley et al. 1999; Porter 2009). The data from the eight solutions that did not experience chelation show slight peak shifts in spectral data that could be misinterpreted as chelation (Figures 2 – 9). Figures 2, 4 and 8 have spectral peak shifts that seem indicative of chelation because they exhibit slight shifts in light absorbance. Moreover, chelation can be detected via NMR and chelation-ion chromatography (Huang et al. 2002). Therefore, using UV-Vis spectroscopy may not have been the best choice for testing chelation properties of carnosine in metal ion solutions; additional instrumentation, such as NMR and chelation-ion chromatography, could be used in future experiments to further test and record chelation results from this experiment.

To improve the methods for future experiments, co-crystallization of carnosine should be conducted under more stable environmental conditions and possibly at colder temperatures to ensure slow crystallization, rather than quick precipitation and formation of amorphous solids. Additionally, optimal solubility conditions should be identified for carnosine and the polycarboxylated aromatic acid co-crystallants; the crystallization process should occur easily if both solutes are stable and not precipitating in solution. Crystallization is a slow process that requires adequate time for slow formation of crystals in solution (Young et al. 1999). Thus, the time span over which the experiment occurred could be lengthened in order to form crystals that are suitable for x-ray diffraction. Finally, chelation properties of carnosine could be tested using NMR spectroscopy to corroborate data collected from UV-Vis spectroscopy.

In conclusion, carnosine co-crystallization with polycarboxylated aromatic acids and Krebs cycle metabolites was not achieved, possibly due to conditions of the environment and of the aqueous solutions. Furthermore, chelation of metal ions with carnosine was not achieved, with the exception of copper. Improvements such as changing environmental conditions, allotting more time, and using more up-to-date instrumentation could lead to future success with this experiment.

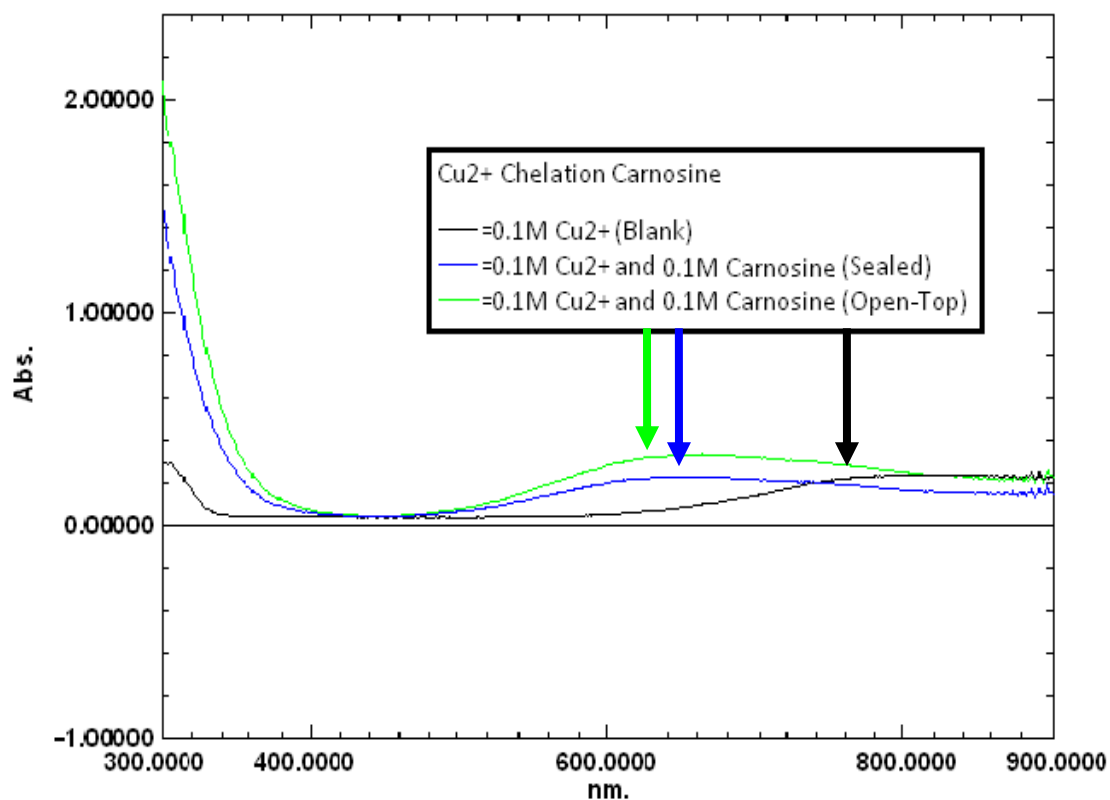
FIGURES

Figure 1--Spectral analysis of copper and carnosine chelation in sealed and open-top solutions. X-axis is wavelength of light in nanometers (nm) and the Y-axis is absorbance of light (arbitrary absorbance units). The arrows pointing to peak shifts in absorbance indicate copper ion chelation with carnosine.

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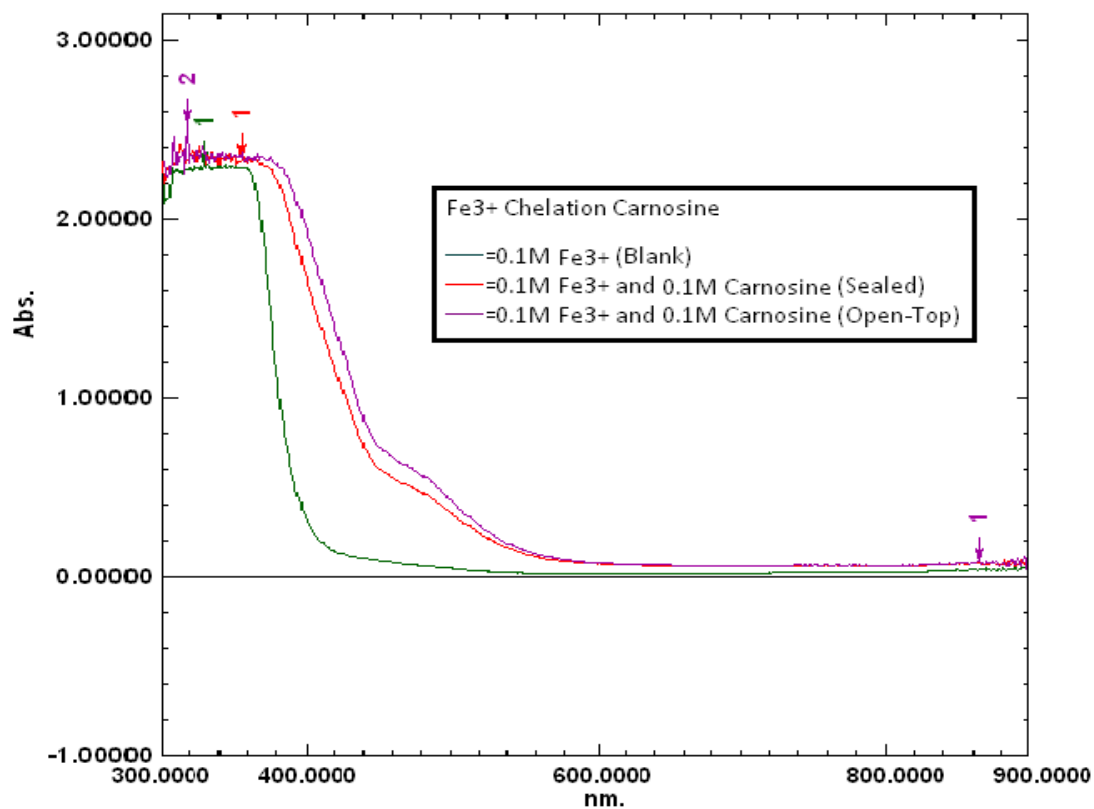


Figure 2--Spectral analysis of iron and carnosine chelation in sealed and open-top solutions. X-axis is wavelength of light in nanometers (nm) and the Y-axis is absorbance of light (arbitrary absorbance units). There are no peak shifts in absorbance values between both solutions, which indicate no iron chelation with carnosine.

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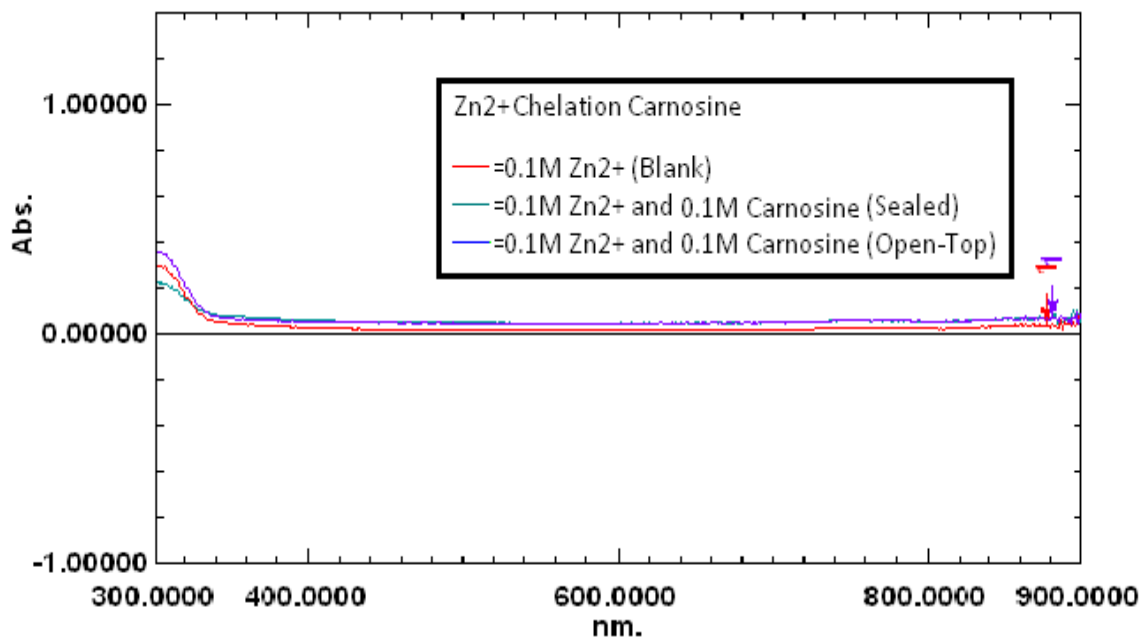


Figure 3 Spectral analysis of zinc and carnosine chelation in sealed and open-top solutions. X-axis is wavelength of light in nanometers (nm) and the Y-axis is absorbance of light (arbitrary absorbance units). There are no peak shifts in absorbance values between both solutions, which indicate no zinc chelation with carnosine.

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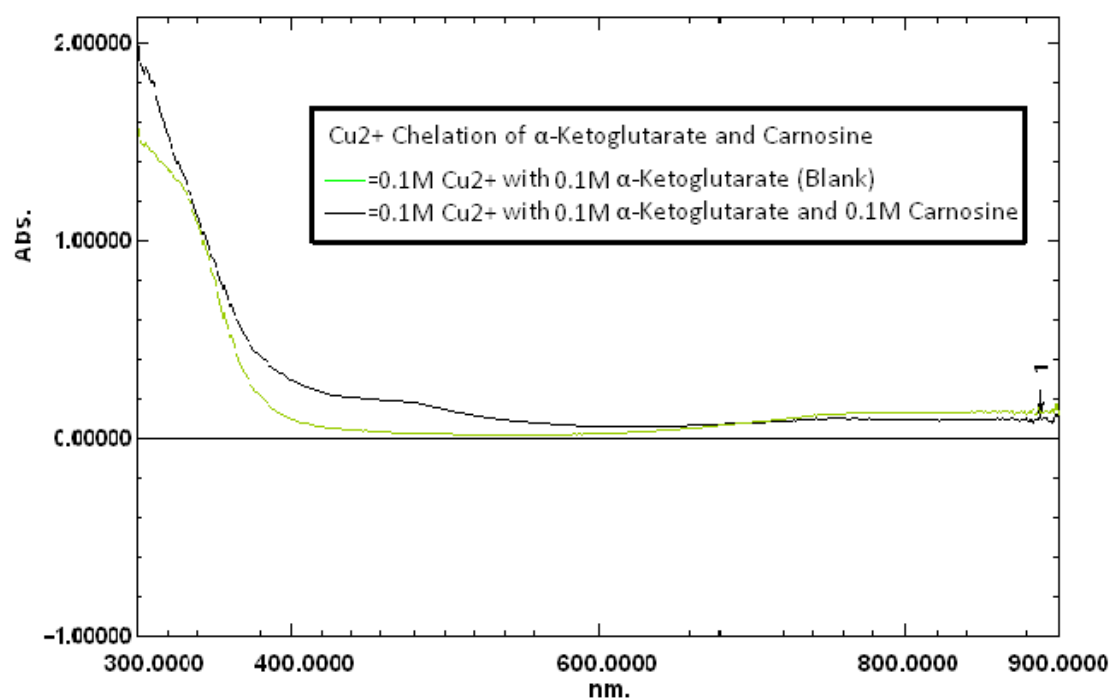


Figure 4--Spectral analysis of copper, α -ketoglutarate, and carnosine chelation in sealed and open-top solutions. X-axis is wavelength of light in nanometers (nm) and the Y-axis is absorbance of light (arbitrary absorbance units). There are no peak shifts in absorbance values between both solutions, which indicate no copper chelation with α -ketoglutarate and or carnosine.

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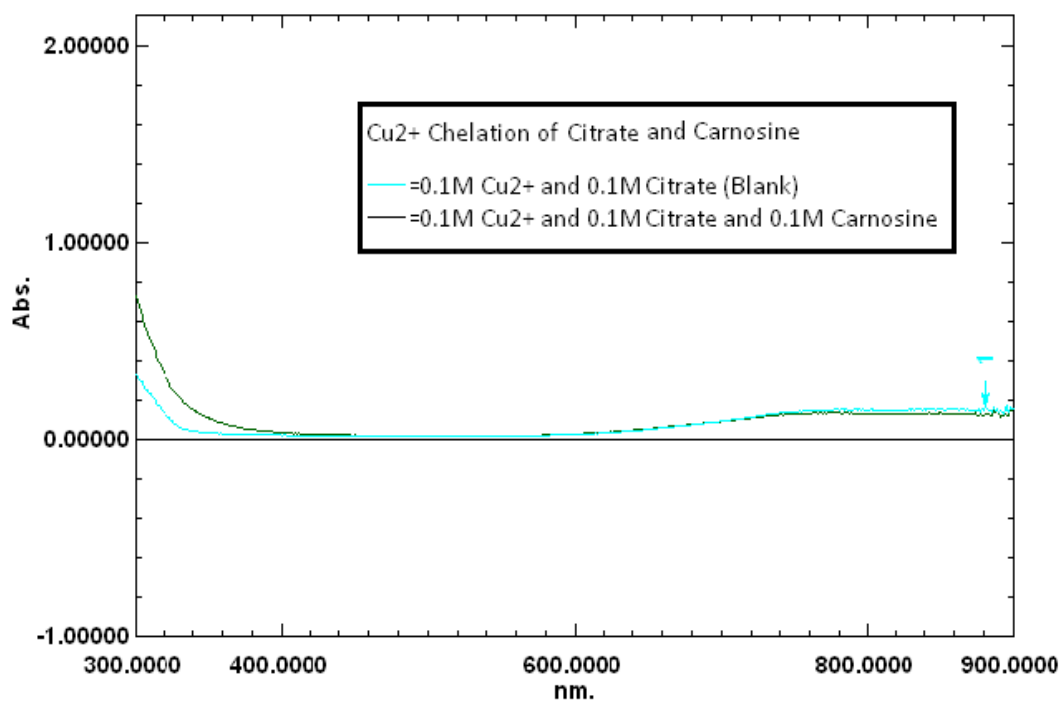


Figure 5: Spectral analysis of copper, citrate, and carnosine chelation in sealed and open-top solutions. X-axis is wavelength of light in nanometers (nm) and the Y-axis is absorbance of light (arbitrary absorbance units). There are no peak shifts in absorbance values between both solutions, which indicate no copper chelation with citrate and/or carnosine.

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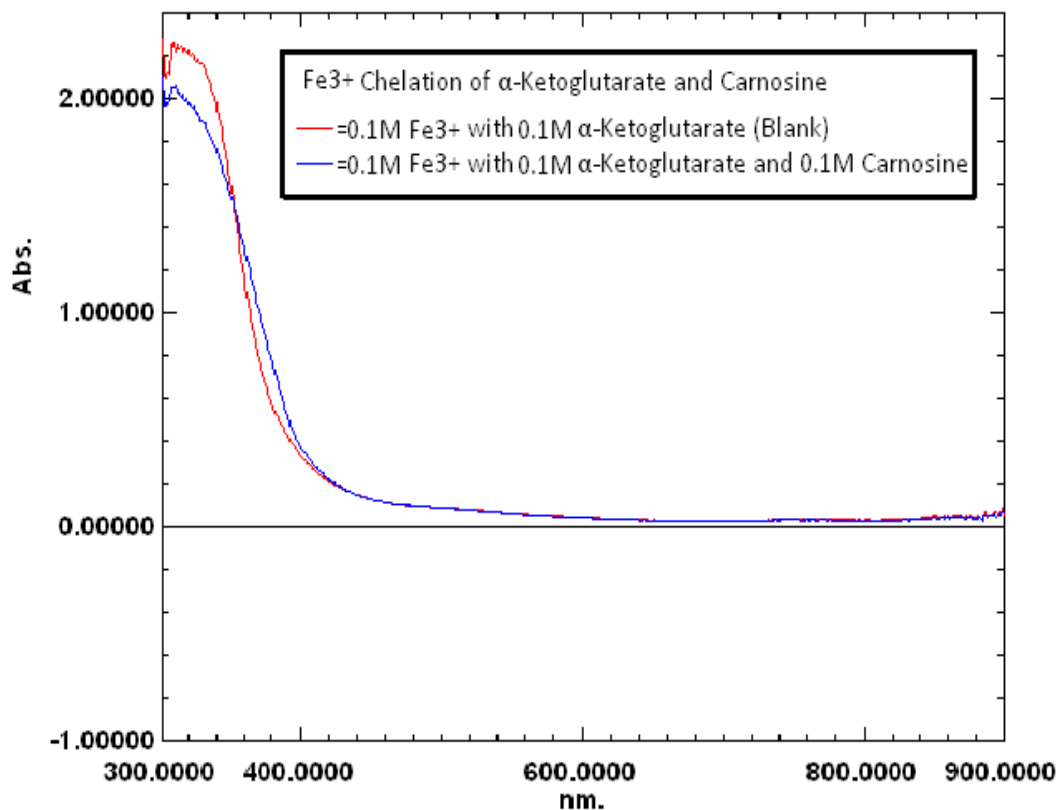


Figure 6: Spectral analysis of iron, α -ketoglutarate, and carnosine chelation in sealed and open-top solutions. X-axis is wavelength of light in nanometers (nm) and the Y-axis is absorbance of light (arbitrary absorbance units). There are no peak shifts in absorbance values between both solutions, which indicate no iron chelation with α -ketoglutarate and/or carnosine.

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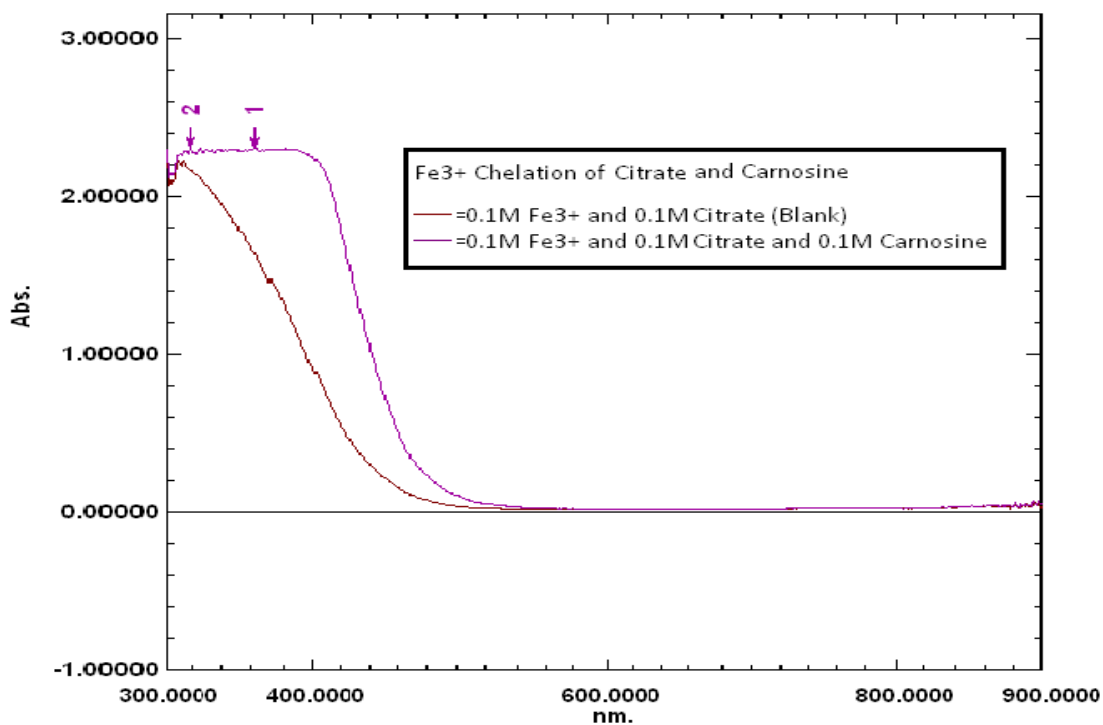


Figure 7: Spectral analysis of iron, citrate, and carnosine chelation in sealed and open-top solutions. X-axis is wavelength of light in nanometers (nm) and the Y-axis is absorbance of light (arbitrary absorbance units). There are no peak shifts in absorbance values between both solutions, which indicate no iron chelation with citrate and/or carnosine.

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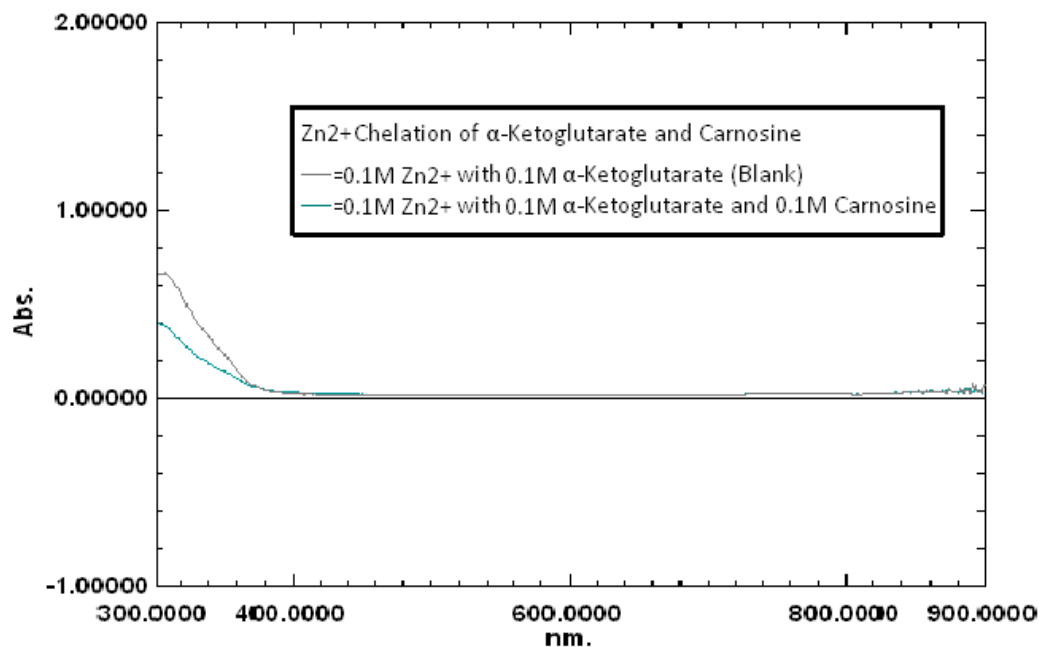


Figure 8: Spectral analysis of zinc, α -ketoglutarate, and carnosine chelation in sealed and open-top solutions. X-axis is wavelength of light in nanometers (nm) and the Y-axis is absorbance of light (arbitrary absorbance units). There are no peak shifts in absorbance values between both solutions, which indicate no zinc chelation with α -ketoglutarate and/or carnosine.

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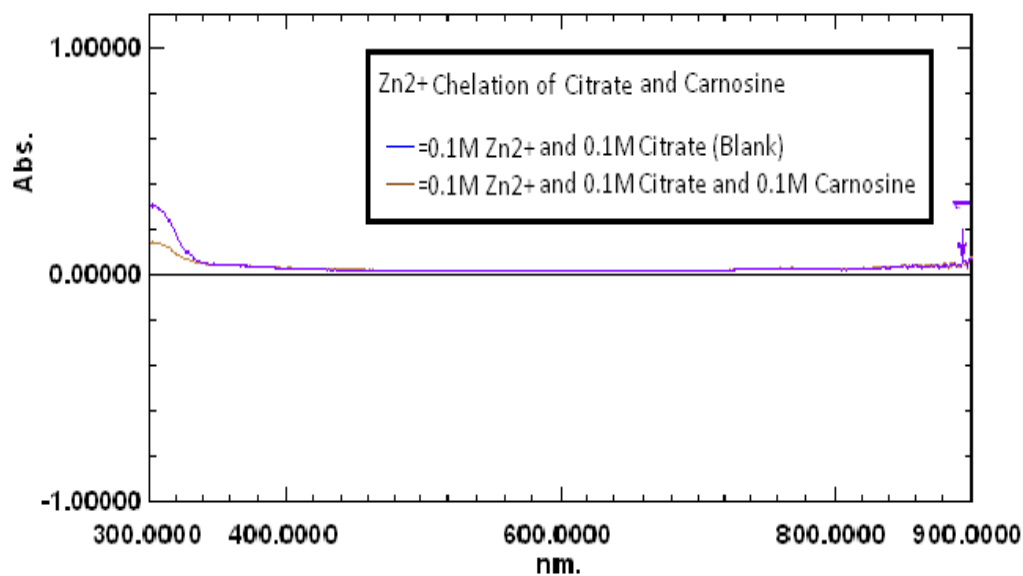


Figure 9: Spectral analysis of zinc, citrate, and carnosine chelation in sealed and open-top solutions. X-axis is wavelength of light in nanometers (nm) and the Y-axis is absorbance of light (arbitrary absorbance units). There are no peak shifts in absorbance values between both solutions, which indicate no zinc chelation with citrate and/or carnosine.

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