Analysis of melanin-like pigment synthesized from homogentisic acid, with or without tyrosine, and its implications in Alkaptonuria.

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Abstract

Alkaptonuria is an iconic disease used by Archibald Garrod to demonstrate the theory of "inborn errors of metabolism". AKU knowledge has advanced in recent years; development of an *in vitro* model, discovery of murine models and advances in understanding bone and cartilage phenotypes and arthropathy in AKU. These discoveries have aided in a new clinical trial into nitisinone. However, there are still knowledge gaps surrounding the pigment in AKU and the pigmentation process. We demonstrate an advance in the understanding in the kinetics and chemistry of the polymerization of homogentisic acid (HGA) into its pigment using size exclusion chromatography and IR spectroscopy. We compared the properties of HGA-based pigments that were freshly prepared to those stored in solution for two years. Our results demonstrate the importance of pH in the polymerisation process and that colour change seen in solution (analogous to AKU patient urine) is not initially due to presence of ochronotic pigment but the quinone intermediary. In addition, we observed pigment formation from HGA can occur in the presence of tyrosine, without the inclusion of this tyrosine into the pigment. These observations have positive implications for patients with alkaptonuria; an increased understanding of the pigment polymer chemistry, the presence of an intermediary and their kinetics present more therapeutic opportunities for treating the condition, including preventing the pigment forming, binding or reversing established pigmentation. AKU patients treated with nitisinone show elevated tyrosine levels causing side effects such as corneal opacities, our data demonstrates that elevated tyrosine levels should not contribute or add to the ochronotic pigment burden in these patients.

"Take-home" message

Our data shows initial colour change in samples of HGA solution are due to the formation of intermediaries which are not polymerised pigment. C-C and C-O bonds are involved in linking aromatic units in the formation of ochronotic pigment. Tyrosine is not incorporated into ochronotic pigment meaning that patients with elevated plasma tyrosine will not have elevated plasma tyrosine adding or contributing to the pigment burden.

Keywords

Alkaptonuria; Homogentisic acid; Tyrosine; Pigment; Size exclusion chromatography

Introduction

Alkaptonuria (AKU) is a rare autosomal recessive condition which results from a single enzyme deficiency on the tyrosine metabolic pathway.(Mistry and Bukhari et al 2013) This enzyme, homogentisate 1,2 dioxygenase (HGD), is responsible for cleaving the benzene ring of homogentisic acid (HGA). However, in AKU patients the deficiency of this enzyme results in systemic elevation of HGA. This systemic elevation occurs even though the kidneys excrete gram quantities of HGA per day in urine. The presence of HGA in gram quantities results in darkening of urine over time or upon addition of alkali substances – this is the first clinic symptom in the triad that are typical of AKU. Regardless of the excretion of gram quantities, the systemic elevation of HGA leads to polymerisation of the HGA monomer in collagenous tissues. This polymerisation leads to darkening, termed ochronosis – the second of the clinical triad. Overtime the ochronosis leads to ochronotic osteoarthropathy, the final clinical symptom, leading to the need for joint replacement, often multiple joints replacements in AKU patients. The darkening of urine is present from birth, ochronosis is believed to begin in the 3rd decade of life with ochronotic osteoarthropathy presenting late in the 4th and 5th decade of life.(Taylor and Boyde et al 2011)

AKU holds a unique place in medical history as one of the diseases that Garrod used to describe "inborn errors of metabolism." In more recent years the defective gene has been mapped and cloned.(Pollak and Chou et al 1993; Zatkova 2011) An *in vitro* and mouse model are also available to aid in understanding the progression of the disease and screening of therapeutic agents.(Preston and Keenan et al 2014; Taylor and Preston et al 2012; Tinti and Taylor et al 2011) Although advances have been made in understanding AKU there is one area that is still somewhat lacking; the analysis and understanding of what occurs to HGA and the chemical changes that occur as it polymerises that may enable or determine how the pigment interacts with the cells and extracellular matrices of AKU patients' tissues.

The histological reaction of HGA and its polymeric derivative with Schmorls' reagent shows identical staining properties to tyrosine and melanin.(Tinti et al. 2011) The conversion of L-tyrosine to its L-3,4-dihydroxyphenylalanine (L-DOPA) intermediary and melanin are catalysed by tyrosinase and it has been proposed that this could also be involved in the conversion of HGA to its quinone intermediary and subsequent ochronotic pigment.(Taylor and Kammath et al 2016) L-Tyrosine is converted into L-DOPA, which is then converted into an *o*-quinone, L-DOPA-quinone. Both reactions are catalysed by tyrosinase and the oxidations of L-tyrosine to L-DOPA and to melanin pigment follow each other consecutively.(Goldfeder and Egozy et al 2013) The *o*-quinone form of L-DOPA, via a series of steps, self-polymerises (non-enzymatically) to form melanin (Faccio, 2012 & Sonmez, 2011). The oxidation pathway of HGA into ochronotic pigment is presumed to be similar to the L-tyrosine_L-DOPA_melanin pathway.(Roberts and Curtis et al 2015) HGA is somewhat similar to L-DOPA as L-DOPA is an *o*-diphenolic compound and HGA is a *p*-diphenolic compound (Rosa, 2011). HGA can oxidize into the *p*-quinone molecule, benzoquinone acetic acid (BQA), before going on to polymerise as the ochronotic pigment.(Roberts et al. 2015) (see supplementary figure) This study set out to examine the polymerization of HGA to melanin-like pigment and to try to understand its chemical composition. A further objective was to examine if in the polymerization of HGA pigment, L-tyrosine could be incorporated into the pigment molecule.

Materials and Methods

Homogentisic acid (HGA), tyrosine sodium chloride and molecular grade water were all purchased from Sigma Aldrich, UK. All other chemicals used were of analytical grade.

The following solutions were prepared:

Solution 1: 2mL with HGA at 50mg/mL and NaOH at 0.25M.

Solution 2: 2.5mL with HGA at 40mg/mL, tyrosine at 0.2M and NaOH at 0.2M.

Solution 3: 0.55mL with HGA at 91 mg/mL.

Solution 4: 0.55mL with HGA at 91 mg/mL and NaOH at 0.1M.

Solution 5: 0.55mL with HGA at 91mg/mL solution of HGA and NaOH at 1M.

Solutions 1 and 2 were placed in sealed vials and left on a laboratory bench for 2 years before being subjected to analysis. At the time of analysis 200μL aliquots of each solution was removed and subjected to the following. The aliquots were diluted with 20mL distilled water and dialyzed using Spectrum Spectra/Por RC dialysis membranes with molecular-weight-cut-off of 3.5kDa obtained from Fisher Scientific (Suwanee, GA, USA) against water (up to 3.5L) for three days with up to four changes of water each day. Following dialysis a 400μL aliquot was diluted with 600μL chromatography solvent, centrifuged and analyzed by size-exclusion chromatography (SEC). The dialyzed mixtures were kept at -20°C for 6 hours and dried for three days using a Labconco FreeZone Plus 4.5L benchtop freeze-dry system obtained from Fisher Scientific (Suwanee, GA, USA). This yielded pigment samples #1 and #2. Solutions 3 through 5 were kept on the lab bench at room temperature. Over the course of 10 days, 5μL aliquots were diluted thousand-fold with solvent for size exclusion chromatography, centrifuged and analysed by size exclusion chromatography. After ten days

standing at room temperature, solution 5 was dialyzed and dried as outlined for solutions 1 and 2, yielding pigment sample #5.

Size Exclusion Chromatography (SEC)

SEC analyses were performed on a Breeze 2 HPLC system equipped with two 1500 series HPLC pumps and a model 2998 Photodiode array detector from Waters, Co (Milford, MA, USA). Analyses were performed using an Ultrahydrogel 500 column (300 X 7.8 mm) obtained from Waters, Co (Milford, MA, USA) in isocratic fashion using a mixture of 25mM Na acetate:methanol:acetic acid (90:10:0.05% v/v) as solvent. Analyses were performed at room temperature and 20μL of sample was injected.

FT-IR spectral analysis

FT-IR spectroscopic scans were made using the NicoletiS10 instrument equipped with the SmartiTR Basic accessory from ThermoScientific (Waltham, MA). Scans were taken with a resolution of 4 cm^{-1} between 650 and 4,000 cm^{-1} at room temperature using a KBr beam splitter and DTGS KBr detector. Each spectrum represents the accumulation of 32 scans.

Results

Macroscopic examination of the mixtures showed that solutions 1, 2 and 5 were dark black with no light able to pass through the samples, while solution 4 had turned dark orange. Solution 3 had maintained the light yellow colour it had at the start of the experiment. The aliquots from solutions 1, 2 and 5 did not show any precipitations upon dilution for size exclusion chromatography analysis or during the dialysis process; the solutions maintained a dark-brown to black colouration. However, during the freezing process of the dialyzed mixtures a phase separation occurred. The frozen mixtures displayed a heterogeneous colouration. They had a central core of black ice surrounded by clear-coloured ice. Upon freeze drying, flaky to powdery dark-brown material was obtained, but the sides of the plastic tubes that contained the dialyzed mixtures during the freeze drying process were stained with rings of dark-brown material.

Figure 1 illustrates the SEC profile of a diluted aliquot from solution 5 after 48 hours reaction at room temperature. In SEC analyses, molecules are separated on the basis of differences in hydrodynamic volume (often related to the molecular mass of the molecule); the lower the retention time, the higher the hydrodynamic volume of the analyte. In our SEC analyses, the lower limit of exclusion, as determined by the injection of water, was about 15 minutes. Peaks with a retention time lower than 15 minutes are high molecular mass compounds, while peaks with retention times of 15 minutes or higher are low molecular mass compounds. HGA was determined to have a retention time of about 21 minutes in our SEC analyses. Following reaction between HGA and NaOH in solution 5, a new peak emerged with a retention time of about 12.4 minutes and with absorbance in the UV and visible range of the electromagnetic spectrum. This peak was presumed to correspond to the pigment generated from HGA. Kinetic monitoring of the area-under-the-curve (AUC) of the peak corresponding to HGA and the new peak with retention time of about 12.4 minutes obtained through SEC

analyses of diluted aliquots from solution 5 indicated that near all HGA reacted away within ten days and the amount of high molecular mass pigment increased steadily over this period (results not shown). SEC analyses revealed that no pigment was generated in solutions 3 and 4 (though a change in colour was observed) and that the concentration of HGA did not change significantly over the course of ten days (results not shown). The change in colour observed in solution 4 was attributed to pH effects on HGA and possible oxidation to the quinone form of HGA. SEC analyses of diluted aliquots from dialyzed solutions 1 and 2 yielded peaks with retention times around 12.4 minutes and no peaks corresponding to HGA (results not shown). Figure 2 illustrates the UV_Vis profiles of the peak corresponding to the HGA-derived pigment observed in the SEC analyses of purified solutions 1, 2 and 5. All three samples had similar UV_Vis absorbance profiles: strong absorbance in the UV range and modest absorbance in the visible range. Figure 3 illustrates an overlay of the FT-IR spectra obtained of pigment samples 1, 2 and 5. The FT-IR spectra of the three pigment samples are qualitatively identical to each other. This suggests that the pigment left standing for two years did not undergo any significant chemical alterations compared to freshly prepared pigment materials. Figure 4 illustrates an overlay of the FT-IR spectra obtained of the dialyzed and dried pigment from solution 1 and HGA.

The dried pigment samples can be redissolved in water and an attempt was made to take an NMR spectrum of pigment sample 1 by dissolving about 15mg in 0.7mL D_2O. The attempt failed as no signals were obtained in the ^{1}H or ^{13}C scans. It was observed that the pigment material had precipitated during the NMR analysis and this was attributed to the cooler temperature of the NMR core environment. This constituted a second observation that some physical instability occurs when pigment solutions are cooled off below ambient temperatures.

Discussion

The polymerisation of HGA monomer is well defined in the scientific literature, however little is known about how HGA pigment molecules interact with each other or with other biomolecules that are present in solutions or more importantly, biological matrices. Furthermore, little is known about the kinetics of the reaction and how the conversion of HGA to its intermediary and finally the ochronotic pigment seen in AKU occurs. This study is the first to examine aged ochronotic pigment formed from HGA and to examine the kinetics of the polymerisation of HGA to pigment using SEC. Our observations suggest that, when studying the formation of pigment from HGA, merely monitoring change in absorbance may be misleading as changes in colour without formation of pigment can occur. Our SEC analyses confirmed that a high molecular mass pigment was generated in solutions 1, 2 and 5 and that the colour of these samples was not due to a low molecular mass chromophore. SEC analyses of solution 5 at intermediate stages did not show any other peaks than those corresponding to pigment or HGA. If other intermediates were generated in the reaction between HGA and NaOH they may have had a short life-time. Our kinetic studies indicate that no to very little pigment formation occurred when HGA was kept in water or in a 0.1M NaOH environment and that almost 100% of the HGA reacted away within ten days when present in a 1M NaOH environment. This strongly suggests that for HGA to polymerize into its pigment, an alkalinity threshold needs to be surpassed for the HGA molecules to be activated. Any enzyme or other biomolecule responsible for the *in vivo* formation of pigment from HGA probably needs to act as a base catalyst in addition to being an oxidase.

In the ochronosis process in AKU the polymer is suggested to be present in urine in solution giving a darkened colour, particularly at alkali pH, although this is not damaging to AKU patients the detrimental effects of ochronosis are seen in later life where the ochronosis is widely seen in joint tissues, bound to collagen fibres.(Taylor and Wilson et al 2010). The UV absorbance of the pigment of solution 5 appeared to be relatively stronger than the UV

absorbance of the pigment in solutions 1 or 2. This difference in UV_Vis spectra may reflect a physical difference between the samples, as FT-IR analyses of the pigments 1, 2 and 5 did not reveal any chemical differences between the samples. As solutions 1 and 2 were left standing for two years, we envision that more aggregation, possibly through π-π stacking interactions between the aromatic units of the pigment molecules, could have occurred. Overall, the UV_Vis spectra are similar to the UV_Vis spectrum of the HGA-based pigment reported before.(Menon and Persad et al 1991)

Whilst the association of pigment with collagen or other collagen associated proteins has been shown, it is still not clear exactly what atoms are bound together between the biological matrices and HGA and/or its polymer.(Chow and Taylor et al 2011) One of the major unanswered questions is as to whether the binding of HGA or pigment to the matrix is by HGA which then subsequently polymerises, or if the polymerisation occurs within the space surrounding collagen fibres and it is the BQA intermediary or the ochronotic polymer which first binds to the collagen fibres. Ochronotic cartilage shows a significant decrease in the amount of glycosaminoglyan (GAG) present in the cartilage matrix and it may be the spaces left from the removal of these GAGs that the polymer is filling. Alternatively, GAGs present in the matrix may act as the nucleation sites for HGA or BQA. (Taylor, 2016) In this context it is worth noting that in a study of the interaction between carbohydrates and proteins, it was observed that carbohydrates favoured interaction with aromatic types of amino acids.(Hudson and Bartlett et al 2015) Alternatively, another such nucleation interaction could occur between the OH group of hydroxyproline and HGA. These hypotheses will be addressed in future research efforts.

The spectrum of sample 2 is qualitatively very similar to the spectra of samples 1 and 5. This suggests that the presence of tyrosine had not altered the pigment formation from HGA in

any way. The region of the spectra between wavenumbers 1000 and 1700 cm^{-1} is very similar to the FT-IR spectrum obtained from a pigment obtained from HGA under similar conditions.(Turick and Tisa et al 2002) The spectra of the pigments exhibit a shoulder at a wavenumber of about 1080 cm^{-1} and broad peaks with wavenumbers of about 1200, 1380 and 1580 cm^{-1}. These absorbances can be attributed to C-H in-plane/out-of-plane deformation, C-O stretching, phenolic OH-bending and aromatic C=C bending vibrations respectively. In addition, a peak at a wavenumber of about 1700 cm^{-1} can be observed and such a peak is associated with the presence of carbonyl stretching.(Turick et al. 2002) Absent in the spectrum of sample 2 are peaks with wavenumbers between 1500 and 1540 cm^{-1}. Such peaks have been assigned to amino functionalities in melanin pigments derived from DOPA.(Apte and Girme et al 2013; Tu and Xie et al 2009) This further suggests that tyrosine was not built into the pigment sample when mixed with HGA. The overlay of FT-IR spectra of pigment sample 1 and HGA shows that all the typical, well-defined peaks associated with HGA are not present in the pigment sample. An exception to this may be the peak at wavenumber of about 1690 cm^{-1}. This peak corresponds to the C=O bond of the carboxylic acid functional group. Noticeably absent in the spectrum of sample 1 is the sharp peak at wavenumber of about 3480 cm^{-1}. This peak represents free, not hydrogen-bonded OH groups and its absence in the spectrum of sample 1 suggests that the phenols of HGA are linked into new chemical bonds or hydrogen-bonded into the new structure. The broad peak at wavenumber of about 3300 cm^{-1} suggests the presence of hydrogen-bonded OH groups. In addition, the sharp peaks at wavenumbers between 700 and 900 cm^{-1} present in the spectrum of HGA are absent in the spectrum of sample 1. Peaks in that region correspond to aromatic C-H bonds and their absence in the spectrum of the dried sample suggests that in the pigment, aromatic units are linked to each other via C-C or C-O bonds.

Our results demonstrate the absence of distinct tyrosine peaks from the 2 year old pigment generated in the presence of tyrosine, which is clinically interesting. A current ongoing trial looking at the suitability of nitisinone in AKU patients is looking to be very promising for reducing HGA. However, the major risk in these patients is an increase in plasma tyrosine, leading to corneal opacities and other more severe complications.(Ranganath and Milan et al 2016) An absence of detectable tyrosine from the spectra in our study suggests that patients who have established ochronosis will likely not have to worry about the polymerisation of tyrosine and it binding to the already established ochronotic pigment in their tissues. The presence of potentially high molecular mass material or nanoparticulate material is also of clinical interest. It has been shown that the pigmented material alters the biomechanical properties of cartilage and is resistant to enzymatic degradation. (Taylor et al. 2011) High molecular mass pigments, as well as being unable to be broken down, is unlikely to be able to be moved from the cells and tissues of the body, thereby adding pressure for any therapeutic agent to be targeted as early as possible in life prior to any pigment formation or deposition. (Taylor et al. 2011) Finally, we show that SEC presents a useful tool for examining the conversion of HGA to intermediaries and pigment polymer and should be used in assessing the effectiveness of anti-oxidant and anti-polymeric agents *in vitro* to gain a greater understanding of HGA pigments and how to inhibit their formation.

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Contribution

Dr Taylor and Vercruysse have been involved in conception, design, analysis and interpretation of data. Both authors drafted the article and revised it critically for important intellectual content.

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Legends to the figures

Figure 1: SEC profile viewed at 290nm (solid line) and 400nm (dashed line) of a diluted aliquot from solution 5 after 48 hours reaction at room temperature.

Figure 2: UV_Vis spectra of the peak with retention time of about 12.4 minutes of the SEC analysis of solution 1 (dashed line), solution 2 (dotted line) and solution 5 (solid line).

Figure 3: FT-IR spectra obtained of pigment sample 1 (dashed line), 2 (dotted line) and 5 (solid line).

Figure 4: FT-IR spectra of dried pigment from solution 1 (black line) and HGA (dashed line).

Supplementary Figure: A diagram showing the potential fate of tyrosine through its various pathways.

Figure 1

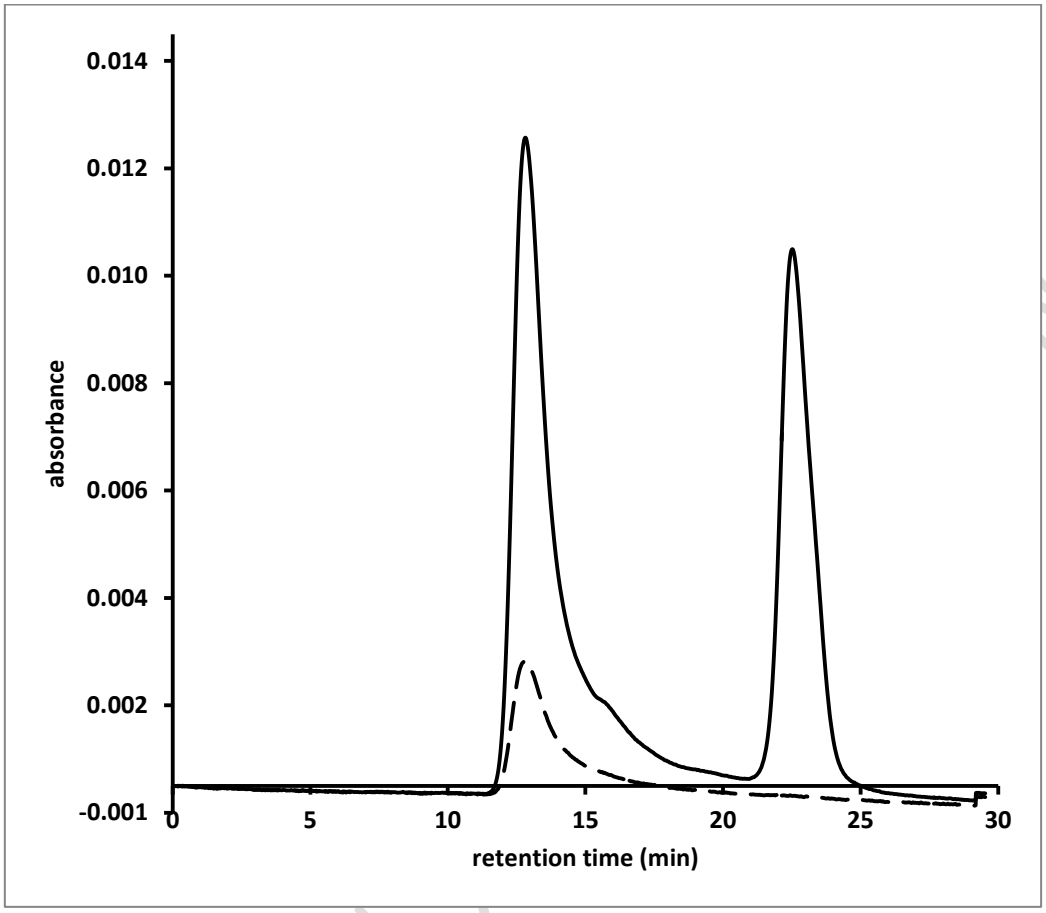

Figure 2

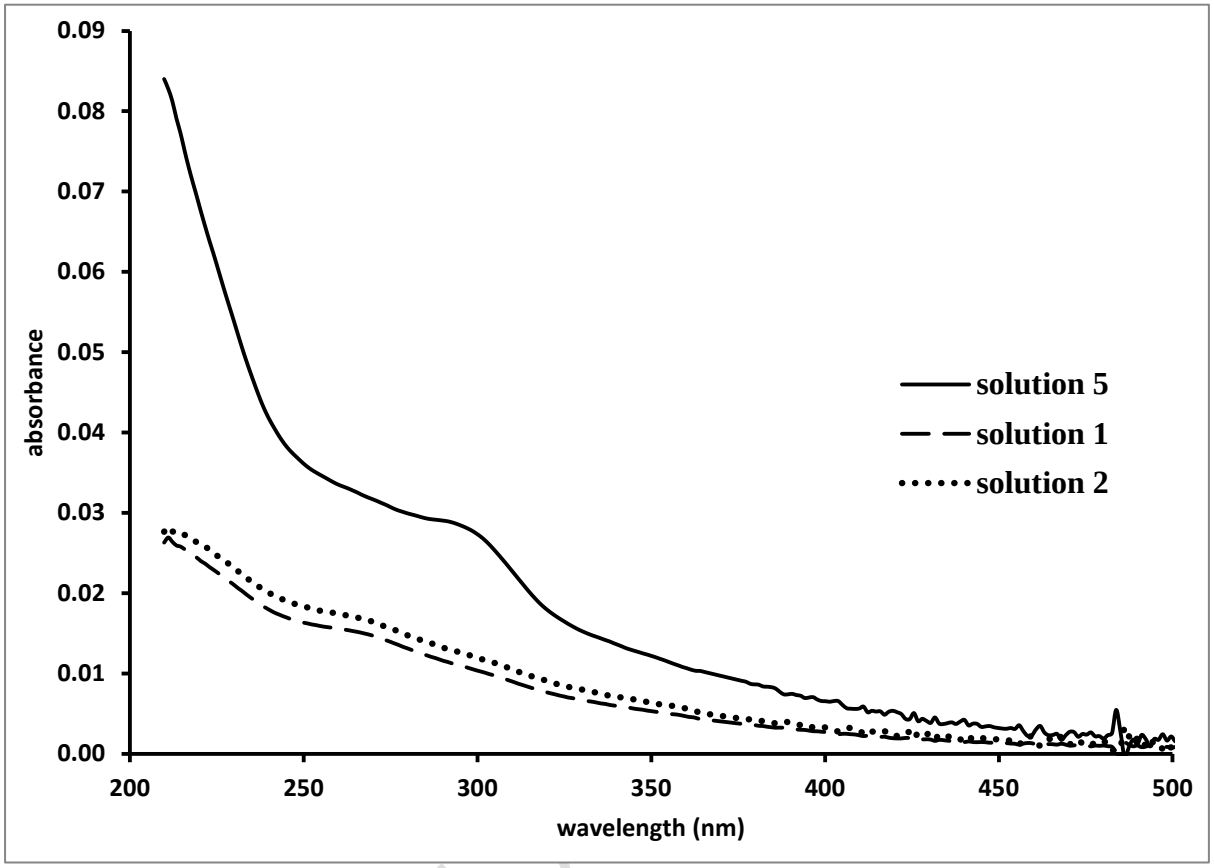

Figure 3

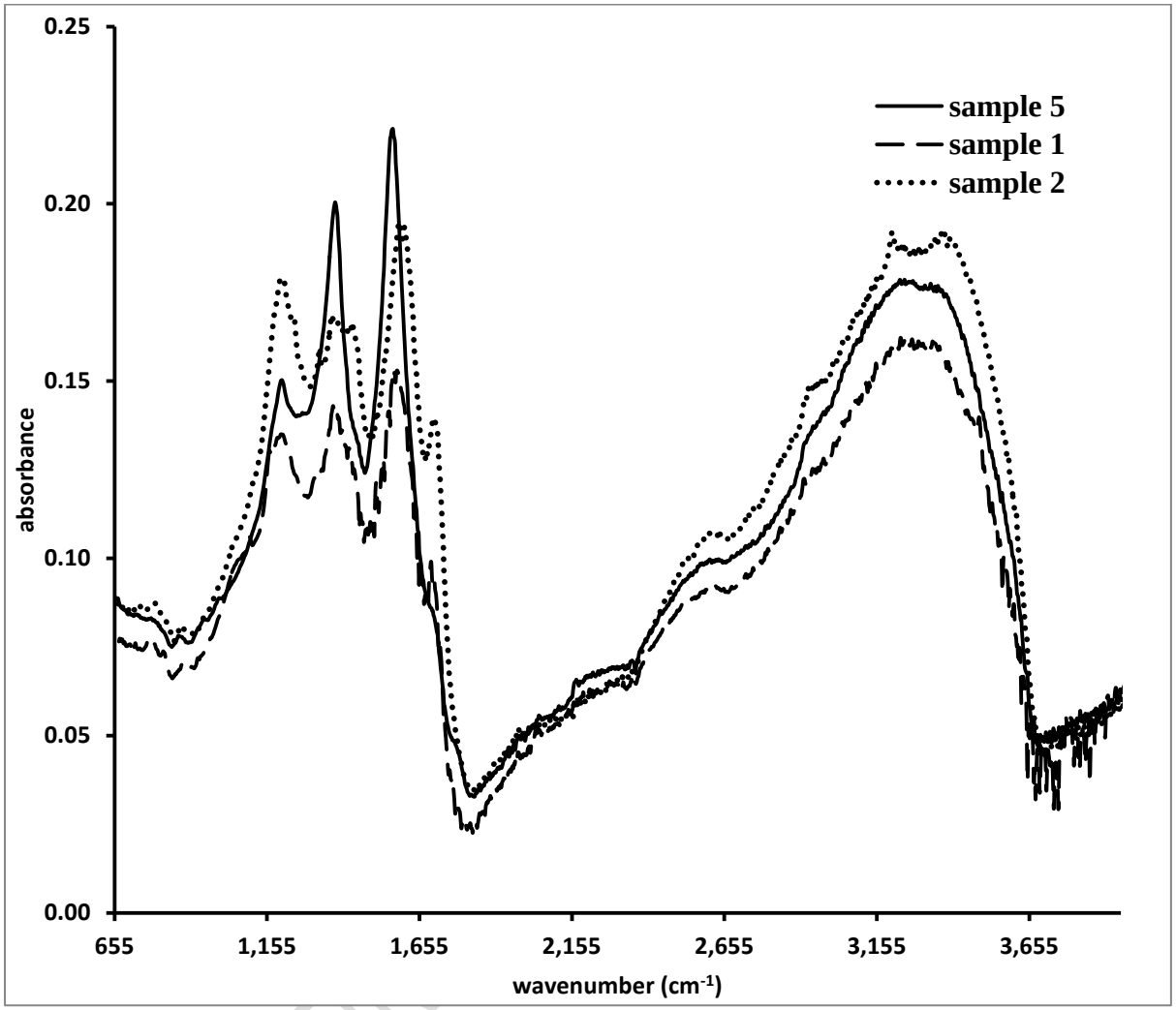

Figure 4