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CAPILLARY ELECTROPHORESIS METHOD DEVELOPMENT FOR QUANTIFYING CAROTENOID CONTENT IN THE CRUSTACEAN *MYSIS DILUVIANA*

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CAPILLARY ELECTROPHORESIS METHOD DEVELOPMENT FOR QUANTIFYING CAROTENOID CONTENT IN THE CRUSTACEAN MYSIS DILUVIANA

by

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ABSTRACT

Mysis diluviana is a shrimp-like crustacean that lives in many Canadian lakes and is an important food for fish. Species of *Mysis* contain carotenoids which are a category of pigmented organic compounds produced mainly by bacteria, algae, and plants. *Mysis* obtain carotenoids through the food chain, which results in some carotenoids ending up in the tissues of predatory fish, such as trout and salmon, which feed on *Mysis*. Carotenoids are also responsible for the orange-red coloration in the muscle tissue of trout and salmon. Carotenoids also play a significant role in animals such as antioxidants, precursors in the production of vitamin A, and photo-inhibitory molecules. This research aimed to develop a novel capillary electrophoresis (CE) method using an aqueous buffer to detect and quantify astaxanthin, β-carotene, canthaxanthin, and lutein in *M. diluviana* samples. CE was the chosen analytical method for the identification and quantification of carotenoids due to its high resolution, small sample volumes, and small solvent consumption. A capillary electrophoresis method called micellar electrokinetic chromatography (MEKC) was chosen given its extended functionality with neutral analytes. To develop this novel method, the pH, concentration of sodium dodecyl sulfate (SDS), and concentration of borate were optimized in the aqueous buffer. The optimal conditions were determined to be pH 9.0, 20 mM SDS, and 60 mM borate. The wavelength of analysis was also optimized and determined to be 254nm. Also, 30% acetonitrile was able to separate the carotenoids into three peaks. The optimized method was then applied to *M. diluviana* samples where the solvent and extraction time was optimized for the carotenoid extraction procedure. Further optimization is required for the extraction process. It was determined that the sample extracts contained 67.25 ppm astaxanthin, 24.37 ppm β-carotene, and 13.50 ppm. Lutein was not detected in the *Mysis* samples.

Thesis supervisor: Dr. Louis Gosselin

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1. INTRODUCTION

Mysis diluviana is a pelagic shrimp-like crustacean found in freshwater lakes of northern North America and was introduced into Okanagan and Kalamalka lakes in the mid-1960s (Whall and Lasenby, 2009). It has become a highly abundant and invasive species in those lakes. It has been suggested *M. diluviana* may have been responsible for a sharp decrease in the Kokanee salmon population of Okanagan Lake (Ahsley et. al., 1997). This decrease in Kokanee salmon would have been caused by competition between the two species for food (zooplankton, and particularly cladocerans).

As part of a plan developed by the BC government in the 1990s to help the Okanagan Lake Kokanee population recover, the BC government authorized the harvesting of the invasive *M. diluviana* from Okanagan Lake (Devlin et al., 2017). The harvested *M. diluviana* is primarily being used as fish food in the aquarium industry; however, reports suggest *M. diluviana* from Okanagan Lake might also constitute a superior source of nutrition for captive fish reared in hatcheries relative to other commercially available fish foods (Stafford et., al 2020).

Carotenoids are one of the most common types of natural pigments and can influence the colors of living organisms (Maoka, 2020). More than 750 kinds of carotenoids have been identified and have been separated into two major types: carotene, which only contains hydrocarbons, and xanthophyll which contains oxygen. Many carotenoids are known to improve the antioxidative stress and immune system of an organism, resulting in improved disease resistance, growth performance, and survival (Simat, 2022). Carotenoids are also responsible for the orange-red coloration in the muscle tissues of trout and salmon.

Species in the genus *Mysis* are known to feed on phytoplankton and zooplankton. In Lake Michigan, *M. diluviana* were found to commonly ingest diatoms, chrysophytes, and calanoid copepods (Maoka, 2011). A study that analyzed zooplankton and phytoplankton in *Mysis* of Okanagan Lake supported the importance of diatoms and cladocerans in the diet of *Mysis*. A study of carotenoids in marine animals suggested that the principal carotenoid in crustaceans such as *Mysis* is astaxanthin (Simat, 2022). Many crustaceans can synthesize astaxanthin from β-carotene ingested from dietary algae. Furthermore, reports suggest that carotenoids found in phytoplankton such as diatoms are metabolites of fucoxanthin and alloxanthin (Maoka, 2020).

Previously, separating and analyzing carotenoids were done mostly by high performance liquid chromatography (HPLC) and high-resolution gas chromatography with flame ionization detector (GC-FID) (Rodriguez et al., 2020). Some disadvantages of the HPLC method are the requirement of costly materials and low-resolution. The main disadvantage of GF-FID is that the FID detector does not respond well to organic compounds containing oxygen, which would make the detection of certain carotenoids such as astaxanthin difficult.

CE is an ideal method for carotenoid analysis due to its high resolution and efficiency, small sample volumes, low risk of contamination, and reproducibility. We intend to develop a method that will be accurate and sensitive for detecting low concentrations of carotenoids in *M. diluviana*. Our method will examine common carotenoids that affect tissue coloration in salmonids including astaxanthin, β-carotene, canthaxanthin, and lutein. These carotenoids (Fig. 1) share a similar structure and therefore a similar migration time. These standards were also used for method development because of their prominence within algae and crustacean populations (Maoka, 2020).

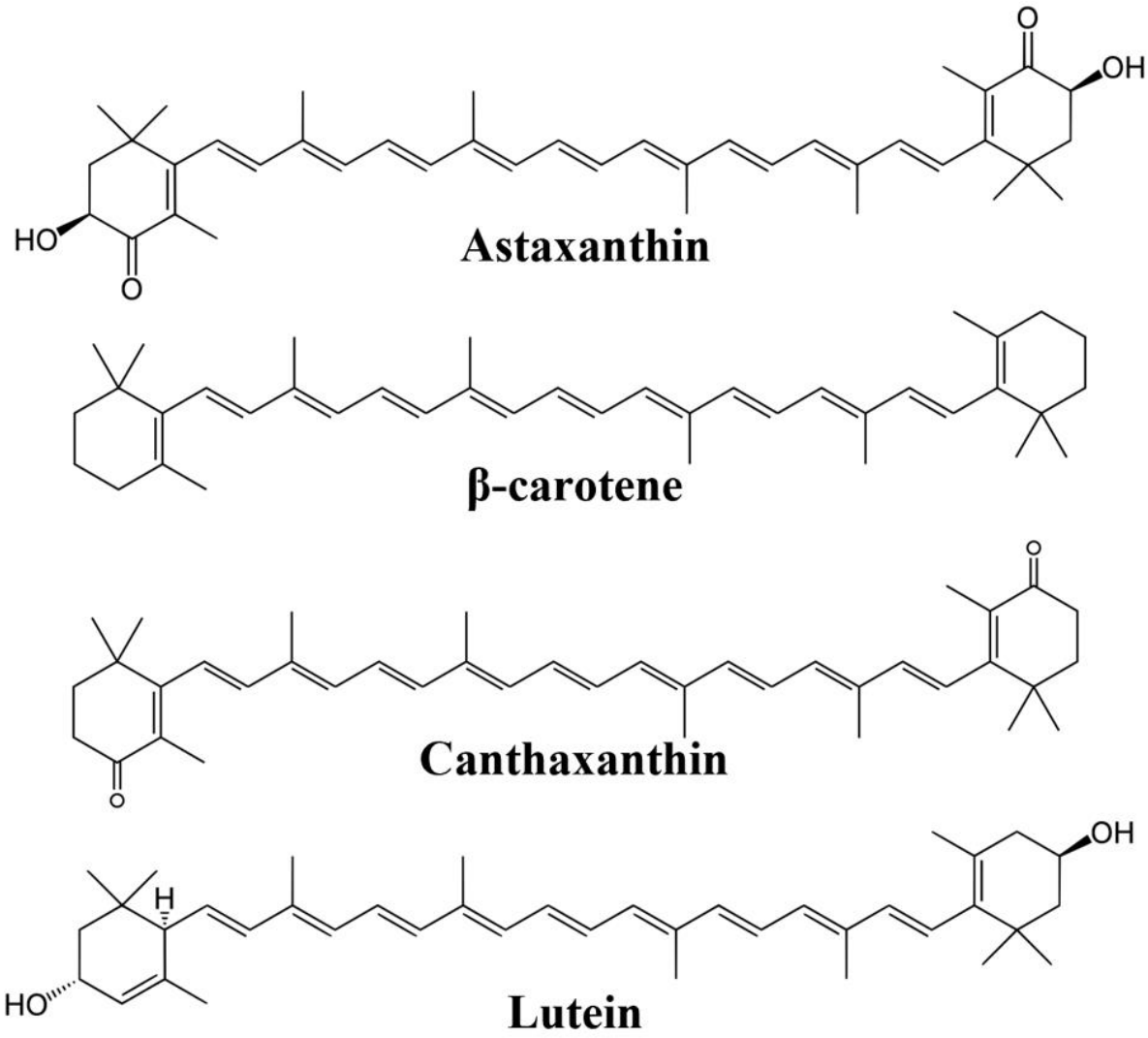

Figure 1. Structures of the four carotenoids.

CE is an analytical method used for the separation of analytes in solution (Voeten et al., 2018). The separation is achieved by passing a solution through a silica capillary in the presence of an electric field. The ions will differ in their mass and charge, meaning they will migrate at different rates through the capillary. In CE, the solution is passed from the anode to the cathode. In general, small cations are eluted first, followed by large cations, neutral analytes, large anions, and finally small anions. The ions will be detected by UV spectrophotometry, in which light is

passed through the sample and the absorbance at UV wavelengths is recorded. The detector response is recorded on an electropherogram where analytes can be identified based on migration time.

A CE method called micellar electrokinetic chromatography (MEKC) was chosen for the analysis. MEKC is based on the addition to the buffer solution of a micellar pseudostationary phase, which interacts with the analytes according to partitioning mechanisms, just like in a chromatographic method (Hancu et al., 2013). The pseudostationary phase is composed of a surfactant added to the buffer solution in a concentration above its critical micellar concentration (CMC). In this system, electroosmotic flow (EOF), which is describes the movement of the buffer solution in response to the applied electric field, acts like a chromatographic mobile phase. From a chromatographic point of view, the EOF's plug-like flow profile is almost ideal as it minimizes band broadening, which can occur during the separation process. The most commonly used surfactant is SDS, an anionic surfactant. The anionic SDS micelles are electrostatically attracted towards the anode. The EOF transports the bulk solution towards the negative electrode due to the negative charge on the internal surface of the silica capillaries. But the EOF is usually stronger than the electrophoretic migration of the micelles and therefore the micelles will migrate also toward the negative electrode with a retarded velocity.

This research aims to develop an accurate and sensitive method to determine the total amount of selected carotenoids present as well as the forms of carotenoids present in *M. diluviana* using CE. The project specifically aims to: (1) optimize the use of an aqueous buffer for the CE analysis of carotenoids; (2) optimize the separation of carotenoid peaks; and (3) determine the total amounts of carotenoids present in *M. diluviana* samples.

2. MATERIALS AND METHODS

2.1. Chemicals

This study sought to develop a methodology, using an aqueous buffer, for the analysis of the carotenoids astaxanthin, β-carotene, canthaxanthin, and lutein standards, obtained from Millipore Sigma, Oakville, ON, Canada. Two chemicals used for the preparation of the aqueous buffer solution, sodium tetraborate decahydrate and SDS (ACS reagent, purity ≥99.5%), were also obtained from Millipore Sigma, Oakville, ON, Canada. Methanol was purchased from Caledon (Georgetown, ON, Canada). All other reagents were of analytical grade, and 18 MΩ water was used to prepare the solutions.

2.2. Aqueous buffer and standard solutions

The effectiveness of the carotenoid analytical procedure being developed was determined by testing standard solutions of each carotenoid. This was done by CE separations performed with an aqueous buffer solution of 60 mM borate and 20 mM SDS, which was prepared by dissolving an appropriate amount of reagent in 18 MΩ water. The pH of the buffer was adjusted to 9.0, using 1.0 M NaOH. The buffer solution was filtered through a 0.45 μm Nylon filter and stored in a plastic bottle at room temperature.

Stock standard solutions of 100 ppm for astaxanthin, β-carotene, canthaxanthin, and lutein were prepared by dissolving each carotenoid in methanol, filtering through a 0.45 μm Nylon filter into an amber glass bottle, and then storing at 4°C until analysis. Seven standard solutions of each carotenoid were then prepared: 5 ppm, 10 ppm, 15 ppm, 20 ppm, 25 ppm, 50 ppm and 100 ppm.

2.3. Instrumentation and analytical procedure

The carotenoids were analyzed using a Beckman Coulter P/ACE^{TM} MDQ system CE unit (Beckman Coulter Inc., Fullerton, CA) equipped with an ultraviolet (UV) detector. Separations were carried out in an uncoated, 60 cm (effective length of 50 cm) fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with an inner diameter of 50 μm. The capillary was held at a temperature of 25°C by a liquid fluorocarbon coolant system. A separation voltage of +20kV was applied for 10 min, and the carotenoids were detected at wavelengths of 200, 214, 254, and 280 nm using a direct absorbance; data acquisition was carried out with 32Karat software.

New capillaries were first rinsed with methanol (20 psi, 5 min) to remove any particulates. They were then rinsed with 1.0 M NaOH (20 psi, 30 min), 0.1 M NaOH (20 psi, 20 min), and 18 MΩ water (20 psi, 10 min) and finally the capillaries were flushed with a buffer of 60 mM borate and 20 mM SDS at pH 9.5 (20 psi, 15 min). Prior to each sequence run, the capillary was pre-conditioned by flushing it with 1.0 M NaOH (20 psi, 15min) and 60 mM borate and 20 mM SDS buffer at pH 9.5 (20 psi, 15 min).

For individual runs, the capillary was rinsed with 1.0 M NaOH (20 psi, 8 min), 0.1 M NaOH (20 psi, 4 min), 18 MΩ water (20 psi, 4 min) and 60 mM borate and 20 mM SDS buffer at pH 9.5 (20 psi, 5 min) before injecting the sample using an injection pressure of 15 psi and injection time of 1 min. When the capillary was not in use, it was filled with water, and the ends were immersed in vials of water to prevent the formation of precipitate that could block the capillary.

2.4. Optimization of buffer pH, sensor wavelength, SDS concentration, and borate concentration

The pH of the buffer, wavelength of the UV detector, the concentration of borate, and the concentration of SDS in the aqueous buffer were optimized to obtain high resolution peaks for

each carotenoid. To identify the optimal pH for carotenoid analysis, aqueous buffers with different pH were obtained by adjusting the pH using 1.0 M NaOH. Buffer solutions were analyzed for each carotenoid at pH 8.5, 9.0, 9.5, and 10.0. The optimal wavelength for carotenoid analysis was determined by testing with UV filters for 200 nm, 214 nm, 254 nm, and 280 nm on the Beckman Coulter P/ACE^{TM} MDQ CE instrument. The optimal wavelength was also confirmed by a Cary UV-Visible spectrophotometer to determine the wavelength of maximum absorption for each carotenoid. The optimal borate buffer concentration was determined for each carotenoid by testing concentrations of 50 mM, 60 mM, and 70 mM. The optimal concentration of SDS in the aqueous buffer for each carotenoid was determined by testing concentrations of, 10 mM, 20 mM, and 30 mM.

2.5. Separation of carotenoids

Astaxanthin, β-carotene, canthaxanthin, and lutein share similar chemical structures and migration times. As a result, a solution containing a mixture of these four carotenoids resulted in the detection of a single peak corresponding to the concentrations of the sum of each individual carotenoid. To detect each individual carotenoid in a mixture solution, organic modifiers were added to the buffer solution. Organic modifiers can increase the window of migration time which would allow for enough separation in the detection of each carotenoid. Acetonitrile and tetrahydrofuran (THF) were analyzed in the range of 10-30% in order to avoid micelle distortion which is observed at higher organic solvent amounts.

2.6. Sample preparation

M. diluviana samples were homogenized by grinding with a mortar and pestle to maximize extraction efficiency. To identify the best extraction solvent for sample analysis, homogenized samples were dissolved in chloroform, acetone, methanol, or water, and each solution was

analyzed using the optimized CE method to determine the maximum sensitivity. The best extraction time was determined by vortexing homogenized sample in methanol for 5 min, 10 min, 15 min, 20 min, 25 min, and 30 min and for each time period, the solution was analyzed by capillary electrophoresis to determine the maximum sensitivity.

2.7. Sample analysis

M. diluviana samples were analyzed using the optimized conditions developed in the steps of the project described above. *M. diluviana* samples were diluted 3-fold in methanol to obtain concentrations within the range of the calibration concentrations used for each carotenoid standard. The presence of each carotenoid was determined by migration time and spiking of the sample with individual carotenoid stock solutions. The carotenoid content of *M. diluviana* samples was quantified using the method of external standard calibration. A calibration curve was generated for each standard and the equation of the line of least squares was used to determine the amount of each carotenoid present in the *M. diluviana* samples.

3. RESULTS AND DISCUSSION

3.1. Stock solution preparation

The four carotenoid standards did not dissolve in water but did fully dissolve in chloroform, acetone, and methanol. The CE analysis of samples dissolved in chloroform or in acetone, however, resulted in several interference peaks (Fig. 2a) that prevented accurate determination of the carotenoid. Using methanol as a solvent, on the other hand, did not result in any interference peaks (Fig. 2b); and methanol was therefore selected as a solvent for the carotenoid standards to create the stock solutions.

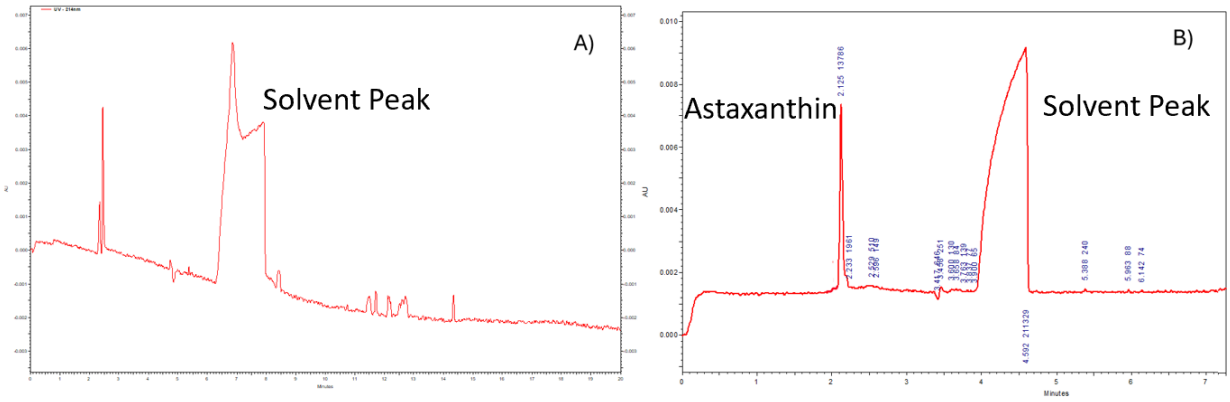

Figure 2. Electropherogram of an astaxanthin solution as a function of time in CE analysis at 214 nm. Astaxanthin standard was dissolved in one of the following two solvents: (A) chloroform (B) methanol. Identity of the analyte peak in chloroform could not be determined with certainty due to the presence of several minor peaks.

3.2. Detection of carotenoids and optimization of wavelength

The first step in selecting a wavelength for carotenoid analysis involved the determination of the absorption spectrum for each carotenoid using a UV-VIS scanning spectrophotometer. For all four carotenoids, this analysis revealed absorption for all wavelengths from 240 nm to 280 nm as well as a maximum at 475 nm (Fig. 3). Given that the CE unit did not have filters for wavelengths above 280 nm, we then ran optimization trials with the CE unit at 200 nm, 214 nm, 254 nm, and 280 nm. Analyses using 200 nm and 214 nm wavelengths were not optimal due to low-moderate sensitivity; analyses using 280 nm wavelength analyses revealed high sensitivity but produced peaks that were asymmetric and thus the 280 nm wavelength was also not optimal. Analyses using the 254 nm wavelength, however, produced high sensitivity and the narrowest and most symmetric peak shape (Fig. 4), and therefore was selected as the optimal wavelength for analysis of these carotenoids.

Studies by Furr (2004) and Gamze et al. (2007) analyzed carotenoids with organic buffers at 410 nm and 445 nm wavelengths respectively. These wavelengths correspond to the absorption maximum at 475 nm which was determined by the UV-Vis absorption spectrum for each

carotenoid. These studies were therefore able to detect carotenoids with much higher sensitivity and at much lower concentrations. This suggests that the optimal wavelength for detection could be between 400 nm and 500 nm and requires further testing for aqueous buffers.

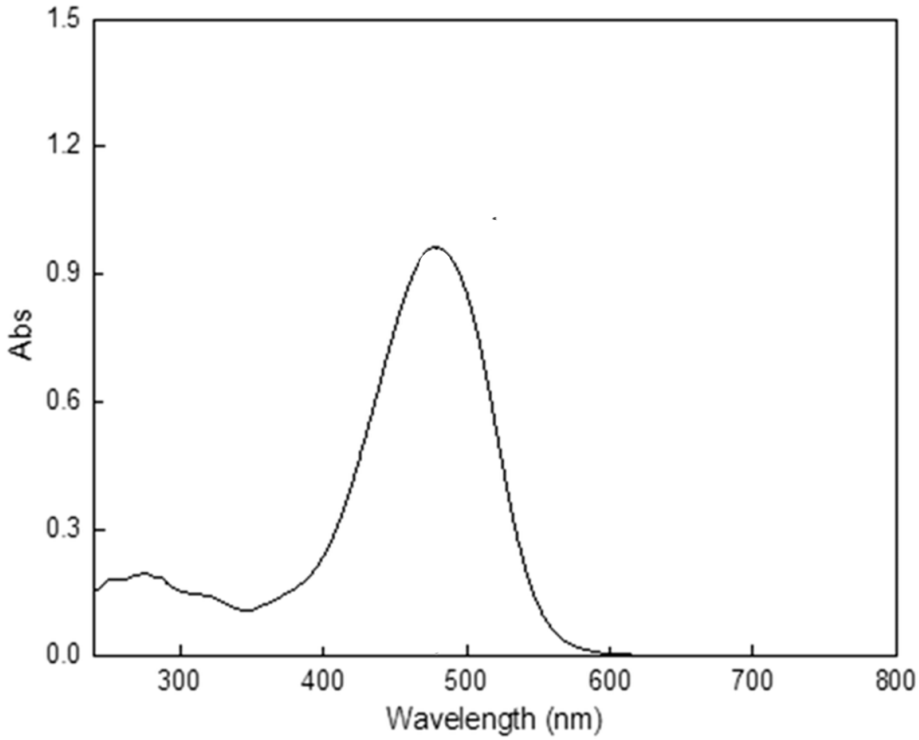

Figure 3. UV-Visible spectrum for astaxanthin showing peak maximum at 475 nm and another peak at 250 nm.

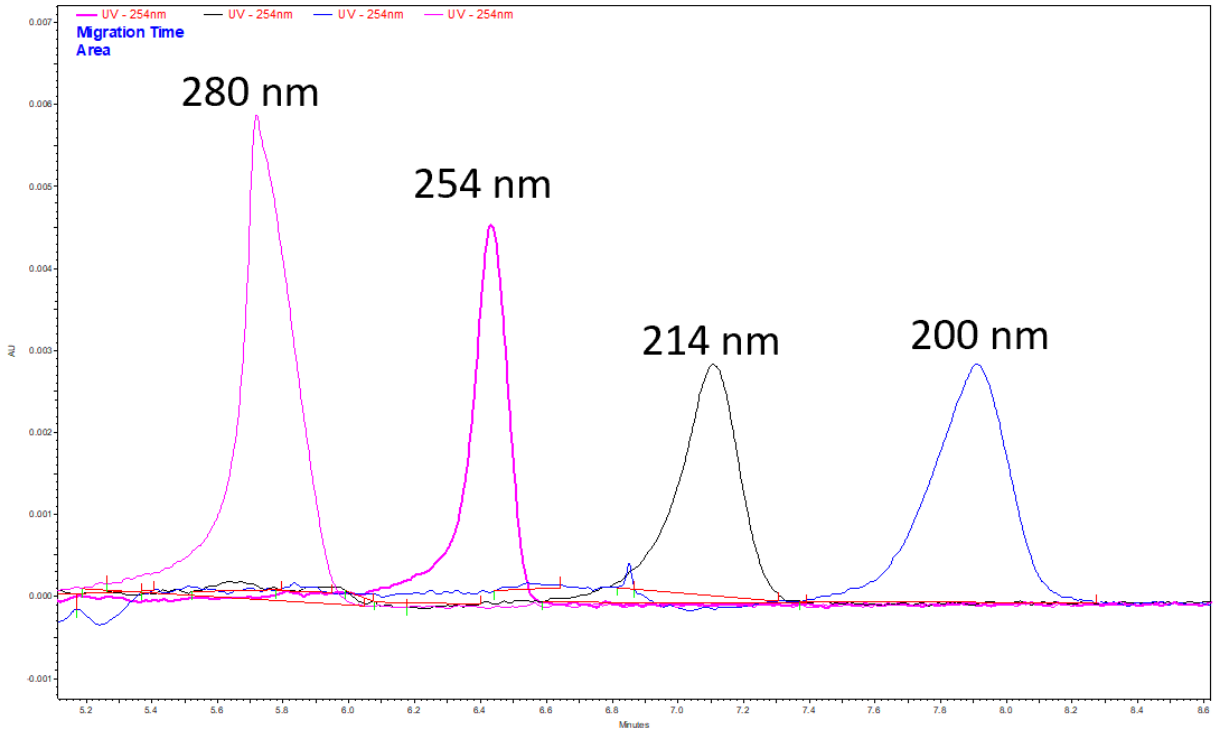

Figure 4. Electropherogram overlay for wavelength optimization by CE showing (from left to right), 280 nm, 254 nm, 214 nm, 200 nm for an astaxanthin standard solution.

3.3. Optimization of the aqueous buffer

The development of an aqueous buffer method required optimizing the pH as well as the concentrations of SDS and borate in the buffer solution.

3.3.1. Optimization of pH

The optimal pH of the aqueous buffer was determined following the analyses of standards for all four carotenoids using pH 8.5, 9.0, 9.5, and 10.0. For each carotenoid, analyses using a pH of 9.0 produced the most symmetric and largest peak area and had the narrowest base (Fig. 5), and therefore pH 9.0 was identified as the optimal pH. For CE methods utilizing aqueous buffers, it is necessary to optimize pH whereas for methods utilizing organic buffers it is not. Organic

solvents do not have a pH since hydronium ion formation is not possible without the presence of water (Deleebeeck 2021).

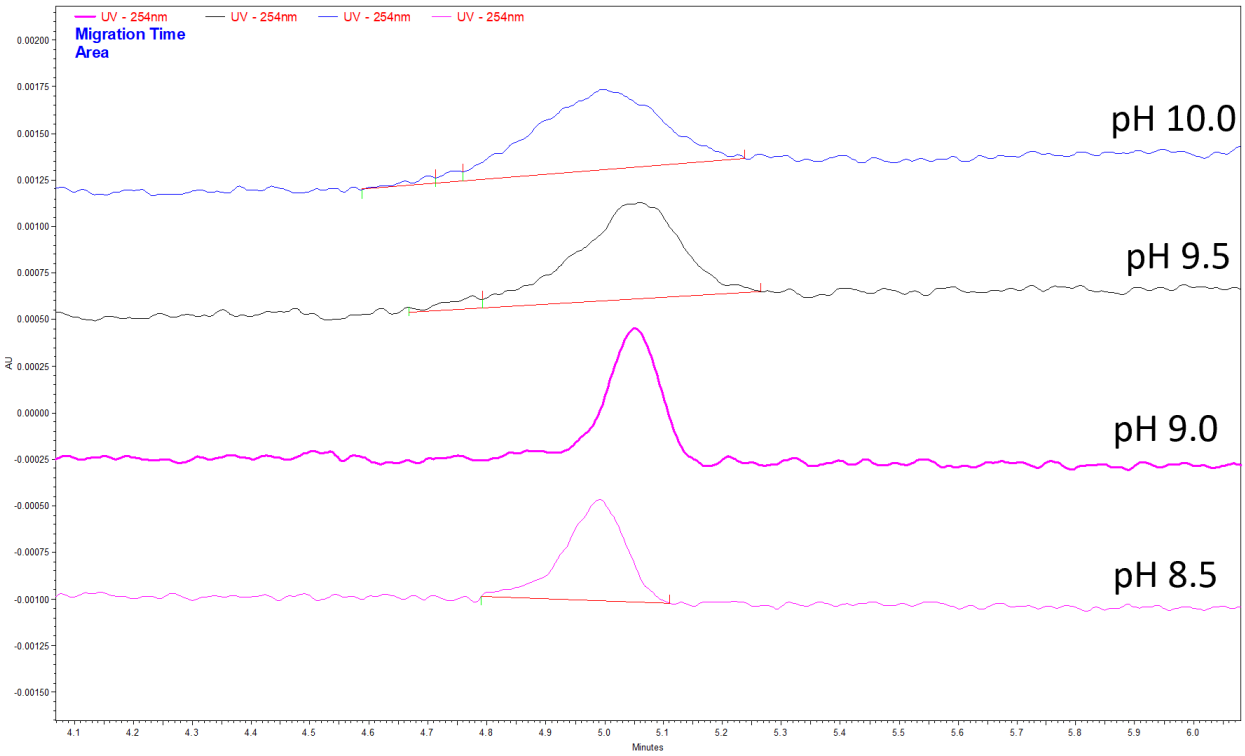

Figure 5. Electropherogram overlay of absorbance of astaxanthin solution as a function of time in CE analysis using aqueous buffer at different pH and analysed using 254 nm wavelength. The optimal pH for astaxanthin was pH 9.0.

3.3.2. Optimization of sodium dodecyl sulfate (SDS) concentration

To maximize the sensitivity of the CE method, peak area and shape were examined following analyses of standards using SDS concentrations of 10 mM, 20 mM, and 30 mM. As suspected, the electropherogram for 5 mM SDS concentration resulted in no detection for all the carotenoids since this was below the critical micelle concentration of 8 mM (Hammouda 2013). SDS concentrations of 10 mM, 20 mM, and 30 mM SDS all showed similar sensitivity and peak

shape (Fig. 6). The 20 mM SDS concentration had a slightly higher sensitivity compared to 10 mM and 30 mM and therefore, 20 mM SDS was determined to be the optimal concentration.

In MEKC, the separation of analytes depends on the partitioning equilibrium of the different analytes between the micellar and the aqueous phase (Hancu 2013). The greater percentage of analyte is distributed into the micelle, the slower it will migrate and therefore, the optimal concentration of the surfactant (often from 10 mM to 50 mM for SDS) will be dependent on the affinity of each molecule for the micelles (Maoka, 2020). There were no CE studies which analysed carotenoids utilizing aqueous buffers; however, a study by Li et al. (1999), developed a CE method to separate cefalexin and its relatives. This study determined that 40 mM was the optimal SDS concentration, however, the optimal SDS concentration will depend on the molecules being separated.

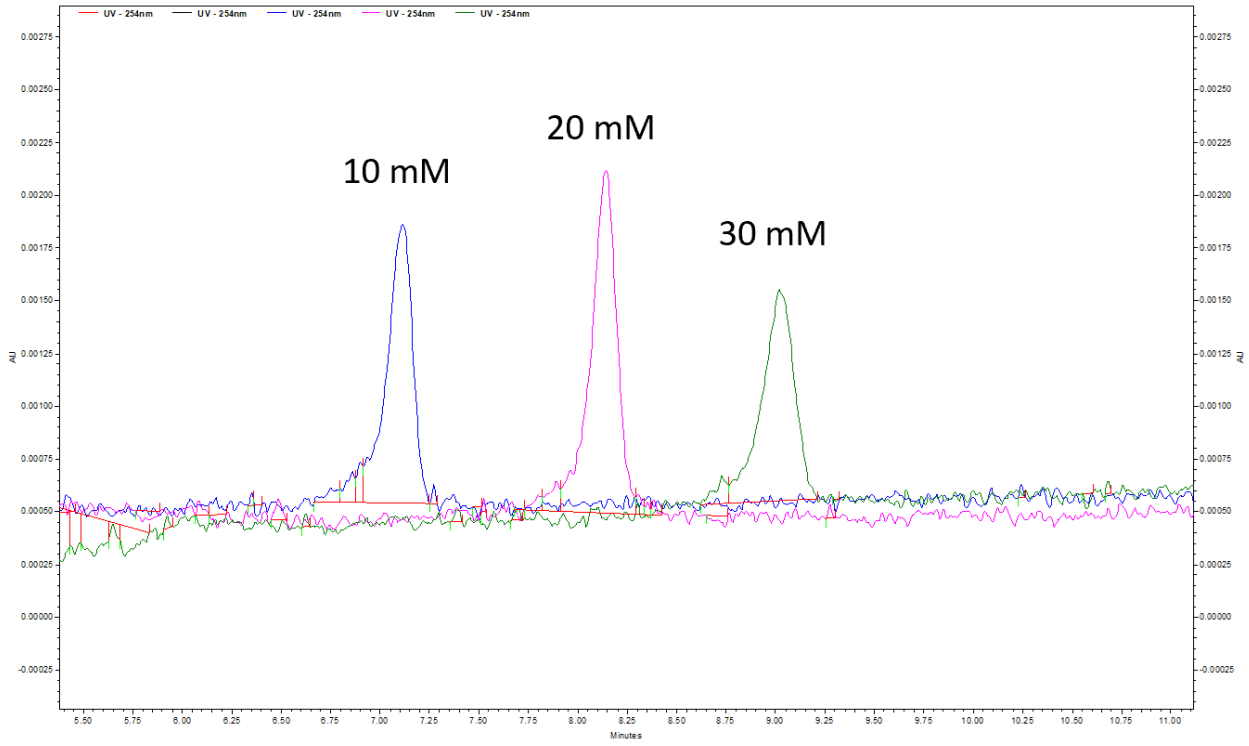

Figure 6. Electropherogram overlay of astaxanthin solution as a function of time in CE analysis using aqueous buffer with SDS concentration from 10 mM, 20 mM, 30 mM (left to right) and analysed using 254 nm wavelength.

3.3.3. Optimization of borate concentration

Three concentrations of borate in the aqueous buffer were tested: 50 mM, 60mM, and 70 mM. The different concentrations of borate did not appear to significantly impact the sensitivity of analysis. A concentration of 60 mM was determined to be the optimal condition due to producing a slightly narrower and more symmetric peak shape than the two other concentrations (Fig. 7).

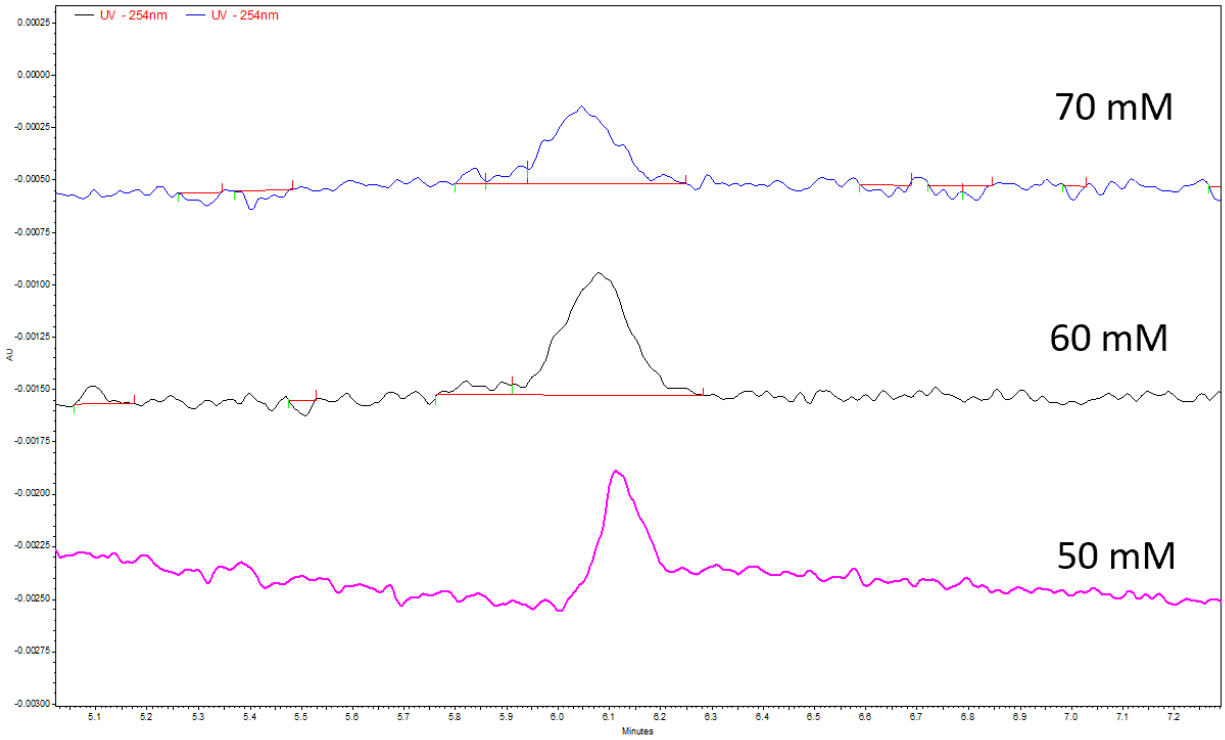

Figure 7. Electropherogram overlay of an astaxanthin solution in CE analysis using aqueous buffer with borate concentrations (from bottom to top) 50 mM, 60 mM and 70 mM, and the results showing optimal borate concentration as 60 mM.

3.4. Separation of carotenoids

Acetonitrile and THF were analyzed in the range of 10-30% (v/v for acetonitrile or THF) to avoid micelle distortion which is observed at higher organic solvent amounts (Maoka, 2020). All concentrations of THF as well as 10-20% acetonitrile resulted in one individual peak for a solution

containing the four carotenoids (Fig. 8a). The 30% acetonitrile buffer solution was able to fully separate three carotenoids into individual peaks (Fig. 8b). The first analyte peak also is not fully symmetric and contains a shoulder indicating that the fourth carotenoid is probably present but was not able to be fully separated.

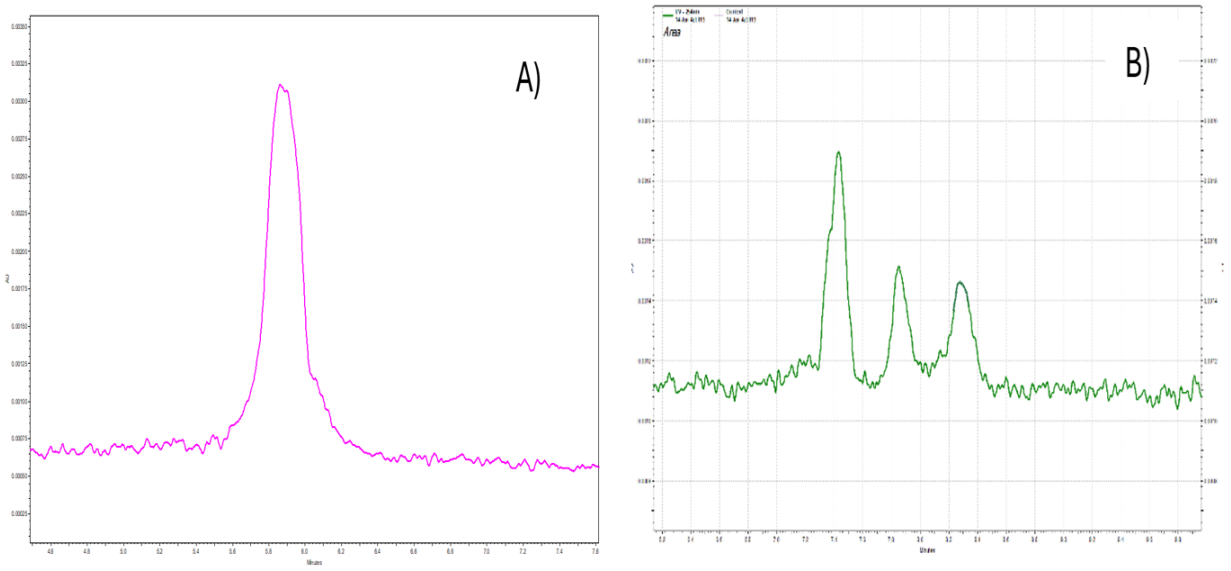

Figure 8. Electropherogram for a solution containing astaxanthin, β-carotene, canthaxanthin, lutein. A) Separation using THF and 10-20% acetonitrile (no separation); B) Separation using 30% acetonitrile and no THF (carotenoids separated into three peaks).

These results suggest that adding acetonitrile as an organic modifier was effective at separating three carotenoids in solution; further investigation is necessary, however, to determine the amount and type of organic modifier which is optimal for separating carotenoids. Organic solvents are used in other CE methods to increase the solubility of analytes but their role in MEKC is to increase the migration times and migration time window of the analytes (Hancu 2013). A study by Gamze 2007 tested acetonitrile, THF, and DMF as organic modifiers in the range of 5%-30% v/v but was unable to separate the carotenoids. It is important to note that the buffer solution

optimized in that study contained SDS and cetyltrimethylammonium (CTAB) whereas our research used a SDS and borate buffer solution and therefore their testing of organic modifiers would have different results.

3.5. Quantification of carotenoids

A calibration curve was developed for astaxanthin, β-carotene, canthaxanthin, and lutein individually. This was done by preparing standards of increasing concentrations and obtaining the peak areas of each signal and plotting on a graph. The concentrations used were 2 ppm, 5ppm, 10 ppm 15 ppm, 20 ppm, 25 ppm, 50 ppm and 100 ppm. The range of concentrations was intentionally wide in an effort to account for the potential variation in the concentration of carotenoids in the *Mysis* samples.

The standard calibration curves for all four carotenoids were highly linear with a high level of variance explained by the peak area: astaxanthin (R^2=0.9754, Fig. 9), β-carotene (R^2=0.9972), canthaxanthin (R^2=0.9992), and lutein (R^2=0.9925). Most of the work in the literature has aimed to detect and separate carotenoids in solution and therefore no other studies produced calibration curves for carotenoids to which we can compare the results of this study.

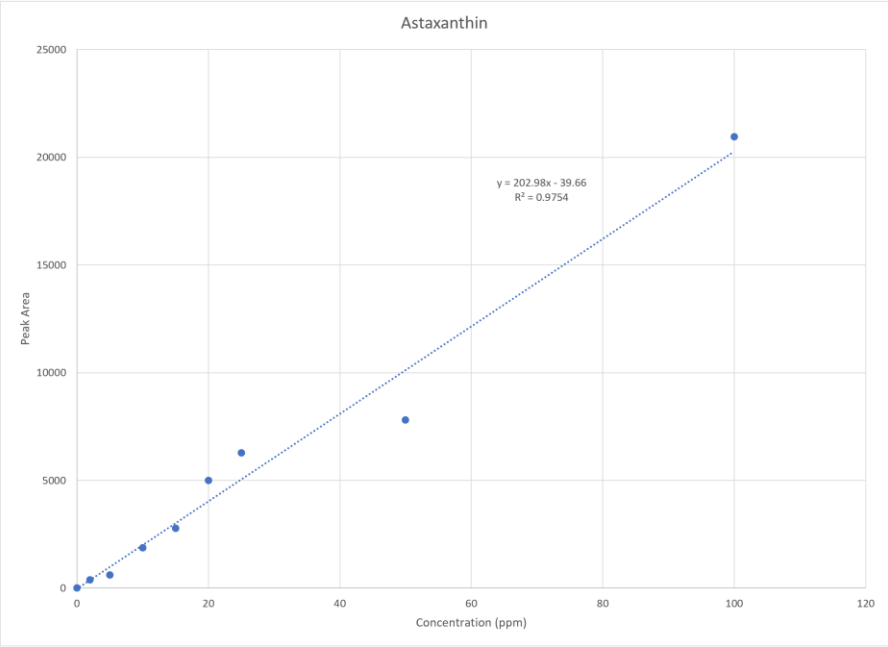

Figure 9. Calibration curve for astaxanthin showing the linearity between peak area and concentration.

3.6. Sample preparation and analysis

The electropherogram using methanol and vortexing for 30 min showed the presence of astaxanthin, β-carotene, and canthaxanthin. Extractions using methanol for less time than 20 min showed no analyte peak for canthaxanthin. Extractions using chloroform and acetone resulted in interference peaks once again and therefore prevented analysis. Therefore, it was determined that the best extraction conditions that were tested were using methanol as a solvent and vortexing for 30 min.

A review by Simat et al. (2022) summarized astaxanthin extraction procedures for different crustacean species. In shrimp and prawn species, solvent extraction using different organic solvents such as acetone: methanol (7:3, v/v) and dichloromethane: methanol (1:3, v/v) yielded the highest astaxanthin concentration with a shorter extraction time. In crab species, supercritical fluid extraction yielded 1.5-fold higher content of astaxanthin compared to other methods. A study by

Hooshmand et al. (2017) optimized carotenoid extraction from blue crab and shrimp for β-carotene, and canthaxanthin. It was determined that acetone and sunflower oil at a solvent ratio of 6:1 (v/w) in a simple organic solvent extraction procedure resulted in the highest amount of carotenoid yield. No studies were found that extracted lutein from crustaceans.

Using the optimized aqueous buffer conditions and extraction procedure, the analysis of *Mysis* extracts revealed three main analyte peaks (Fig. 10). The presence of astaxanthin, β-carotene, and canthaxanthin were confirmed by spiking the sample solution with the individual carotenoid stock solutions. When the *Mysis* extract was spiked with lutein, this produced an additional analyte peak between canthaxanthin and β-carotene, which suggests that lutein is not present in *Mysis* in detectable concentrations (Fig 11).

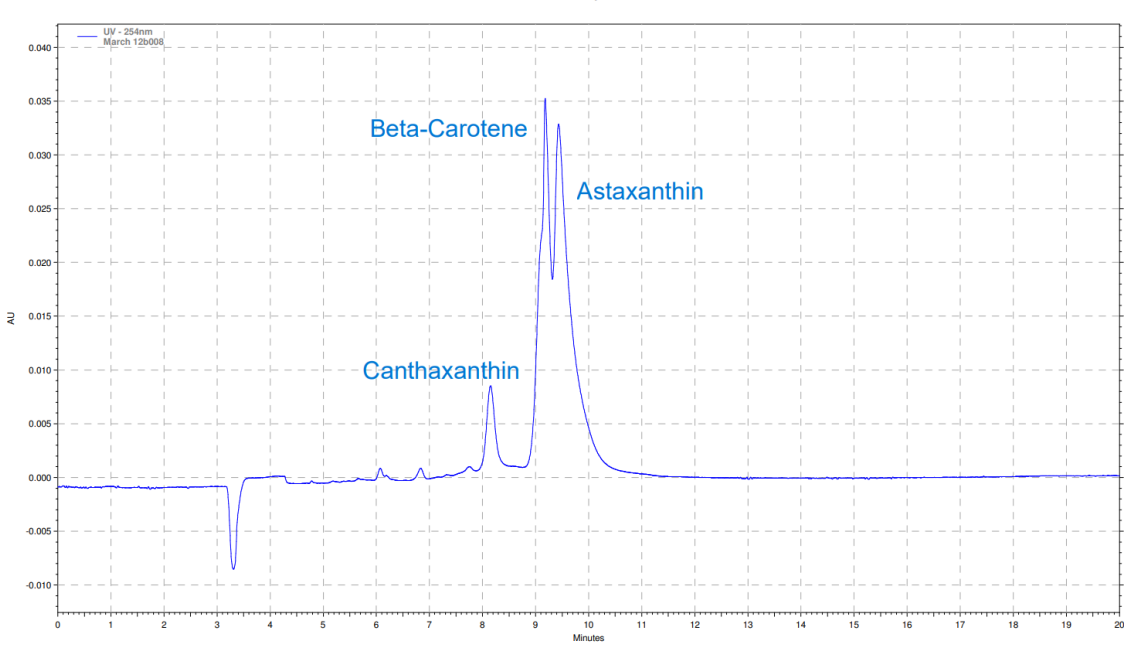

Figure 10. An electropherogram for the *Mysis* sample showing astaxanthin, β-carotene, and canthaxanthin.

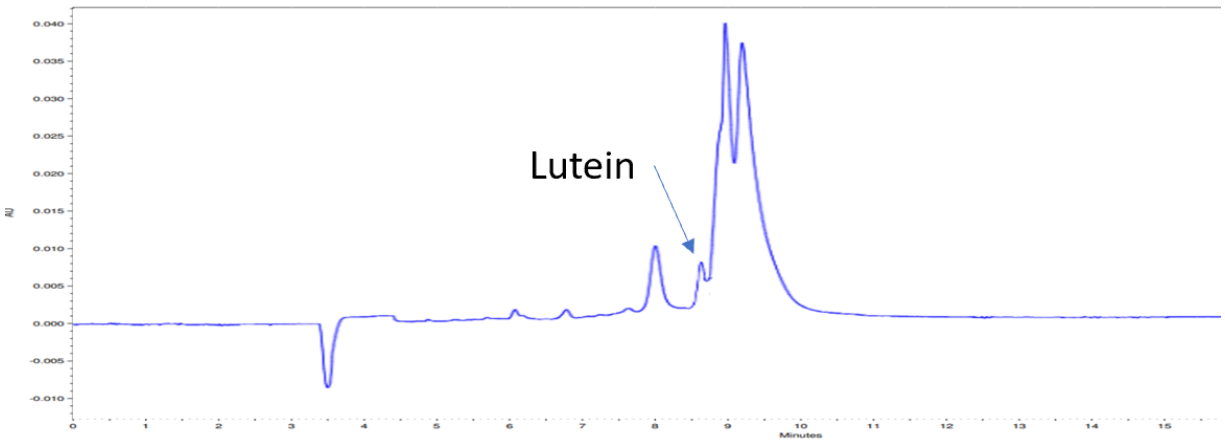

Figure 11. An electropherogram for the *Mysis* sample spiked with lutein.

The concentration of each carotenoid was determined by interpolating from the equation of the line from the standard curve. The concentration of astaxanthin, β-carotene, and canthaxanthin were determined to be 67.25 ppm, 24.37 ppm, and 13.50 ppm respectively. Lutein did not appear to be above the limit of detection in this sample. However, further optimization of the extraction procedure may result in better results for lutein.

These preliminary results are consistent with carotenoid concentrations found in other studies which analysed carotenoid content in crustaceans. One such study by Irna et al. (2018) determined that *Penaeus monodon* (tiger shrimp) contained 58.50 ± 2.62 ppm of astaxanthin. Another study by Sachindra et al. (2005) analysed astaxanthin, β-carotene, and canthaxanthin concentrations in four species of shrimp and it was determined that the concentration of astaxanthin ranged from 50.3 ppm to 72.5 ppm, the concentration of β-carotene ranged from 10.4 ppm to 26.5 ppm, and the concentration of canthaxanthin ranged from 1.7 ppm to 12.2 ppm. In crustaceans, lutein is used to synthesize astaxanthin through metabolic reactions and therefore could explain the low levels of lutein which are not able to be detected. No studies were found that determined the concentration

of lutein in crustacean; however, the results for astaxanthin, β-carotene, and canthaxanthin suggest that this method is relatively accurate in the determination of carotenoid content in *Mysis.*

The migration order of astaxanthin, β-carotene, canthaxanthin, and lutein was determined to first be canthaxanthin, followed by lutein, β-carotene, and finally astaxanthin. This migration order cannot be explained by examining the two main characteristics that determine migration time: molar mass and charge of each carotenoid at pH 9.0. From table 1. It can be shown all four carotenoids remain neutral at pH 9.0 and that the increasing molar mass does not correspond to the increasing migration time. Therefore, some other factors must be considered to understand the migration order.

Table 1. Carotenoid molecular information.

	Molecular Formula	**Molar Mass (g/mol)**	**Charge at pH 9.0**
Astaxanthin	$C_{40}H_{52}O_4$	596.8	Neutral
β-carotene	$C_{40}H_{56}$	536.9	Neutral
Canthaxanthin	$C_{40}H_{52}O_2$	564.8	Neutral
Lutein	$C_{40}H_{56}O_2$	568.9	Neutral

4. CONCLUSIONS

In conclusion, a CE method was developed using an aqueous buffer to detect and quantify the carotenoids astaxanthin, β-carotene, canthaxanthin, and lutein. It was determined that *M. diluviana* had the highest concentration of astaxanthin, followed by β carotene, and finally the smallest

concentration of canthaxanthin. There was no signal detected for lutein which indicates that this carotenoid is not present or is present in concentrations too low to detect. The results of this study also suggest that this method could be applied to other crustacean samples. In addition, *M. diluviana* could provide two benefits to fish feeds: a protein source and essential carotenoids which are currently being supplemented. The development of a CE method using an aqueous buffer to detect and quantify carotenoids is significant because of the short analysis time and the aqueous buffer has less environmental consequences by using approximately 35% less volume of organic solvents (approximate number since the volume extracted varies depending on the sample size). The results of this research also suggest that using an aqueous buffer is as accurate and sensitive when compared with organic buffer methods and therefore could be a promising method for further analysis of carotenoids. Further research would be required to determine if organic buffers are able to separate different carotenoids in solution more effectively than aqueous buffers.

5. FUTURE WORKS

Future work for this research would aim to optimize this method to fully separate a mixture of carotenoids. Future work could include adding different organic modifiers to the buffer solution to increase the migration time window in which the carotenoids are detected. Future work should also consider optimizing the extraction procedure of the carotenoids from the *M. diluviana* samples. A simple extraction procedure was used but other methods should be considered such as Soxhlet extraction and saponification (Parise 2018). Finally, future work should include validation of the proposed method by evaluating the precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ).

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7. APPENDICES

7.1 Electropherogram for astaxanthin, β-carotene, canthaxanthin, and lutein.

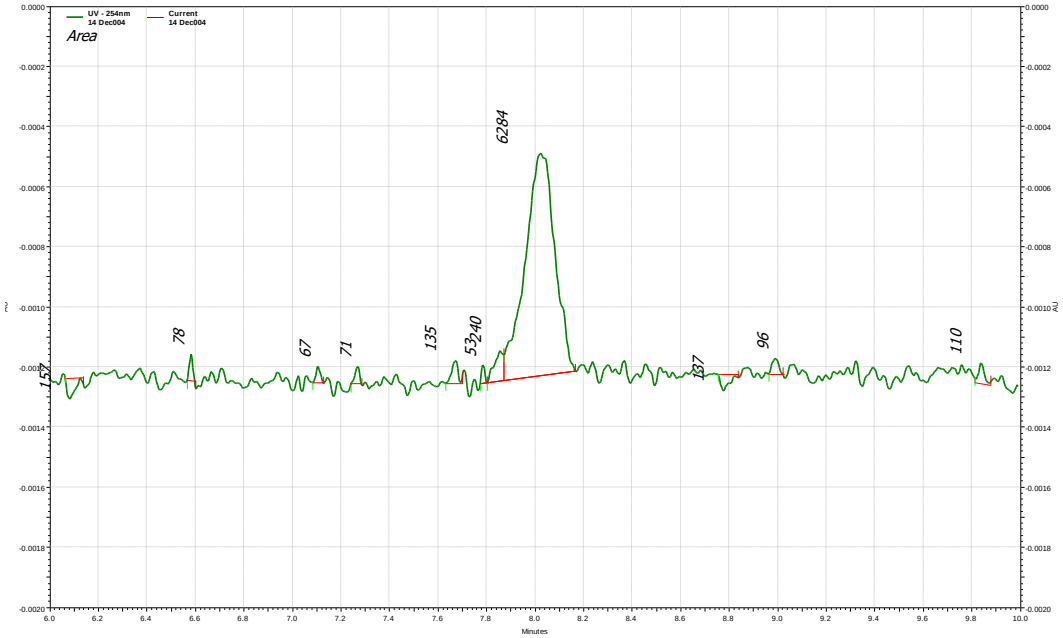

Figure 7.1a. Electropherogram for astaxanthin stock solution using optimized conditions.

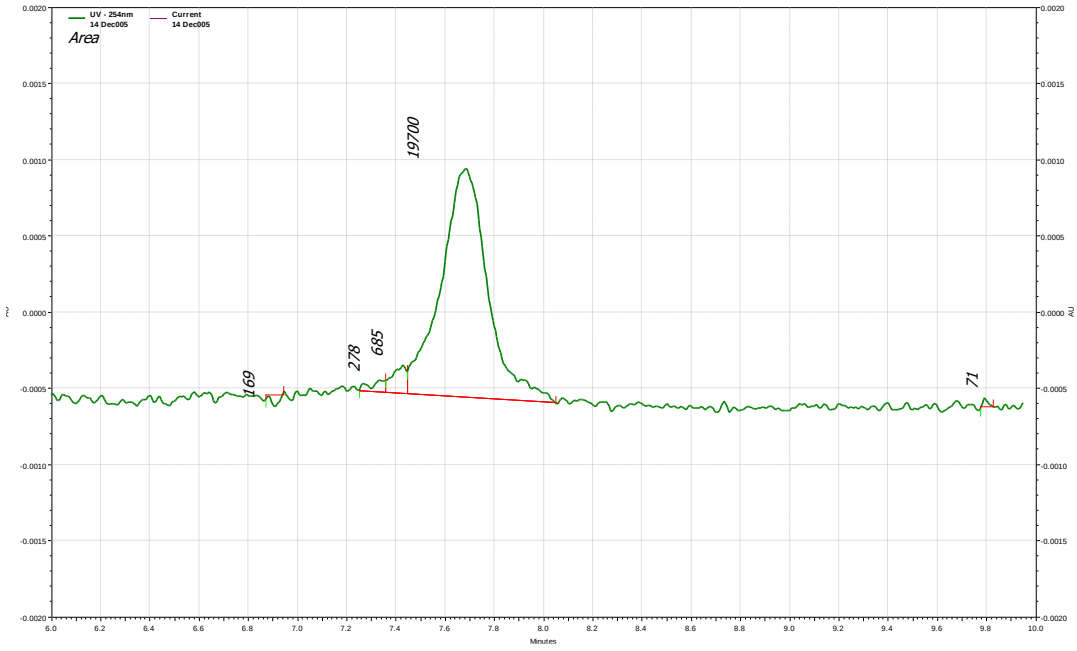

Figure 7.1b. Electropherogram for β-carotene stock solution using optimized conditions.

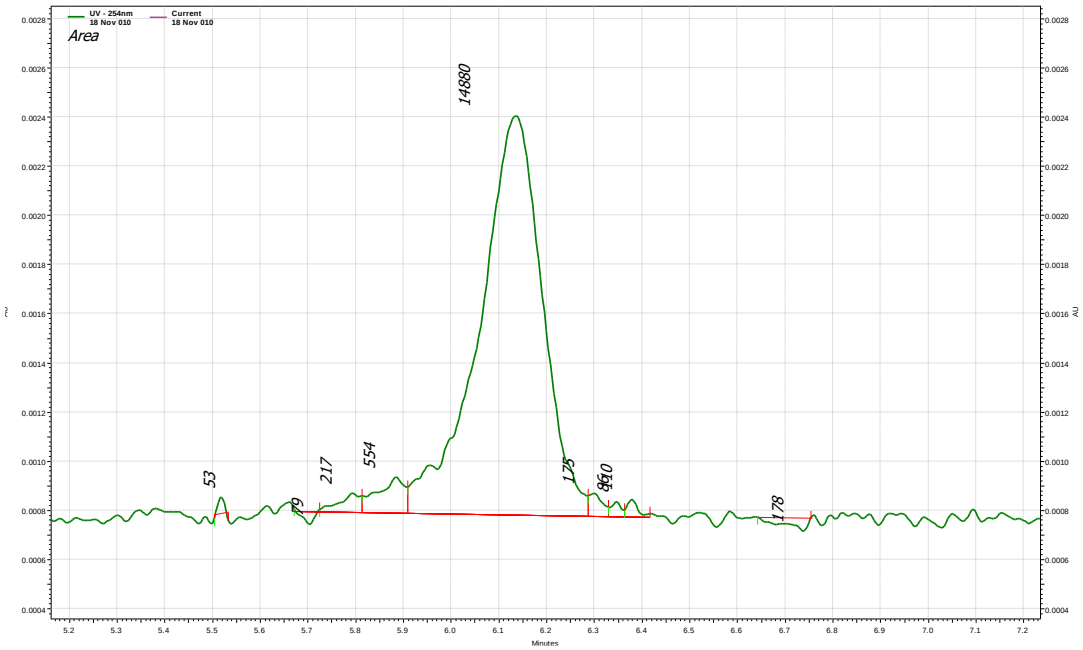

Figure 7.1c. Electropherogram for canthaxanthin stock solution using optimized conditions.

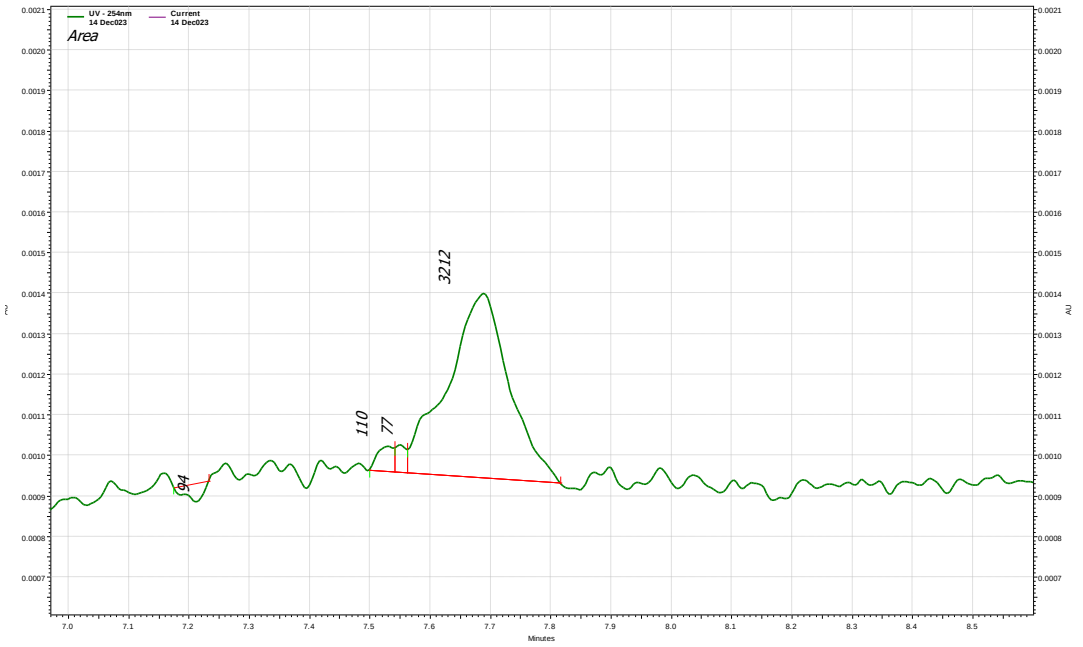

Figure 7.1d. Electropherogram for lutein stock solution using optimized conditions.

7.2. Calibration curves for β-carotene, canthaxanthin, and lutein.

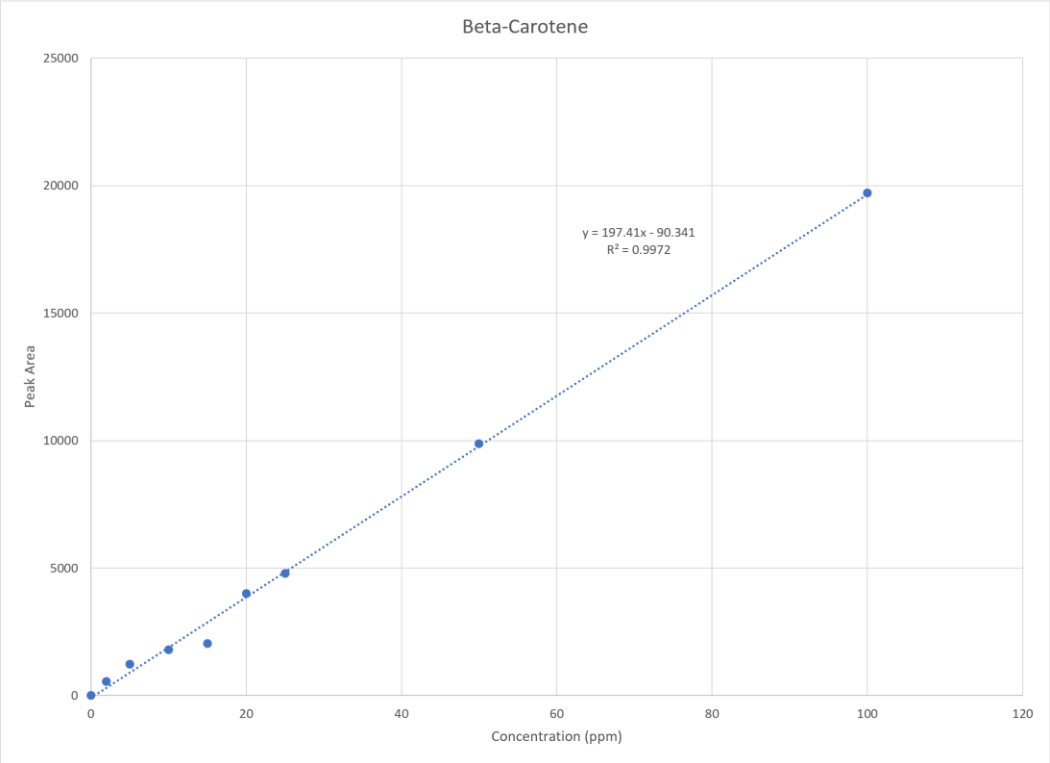

Figure 7.2a. Calibration curve generated from β-carotene stock solution using optimized conditions.

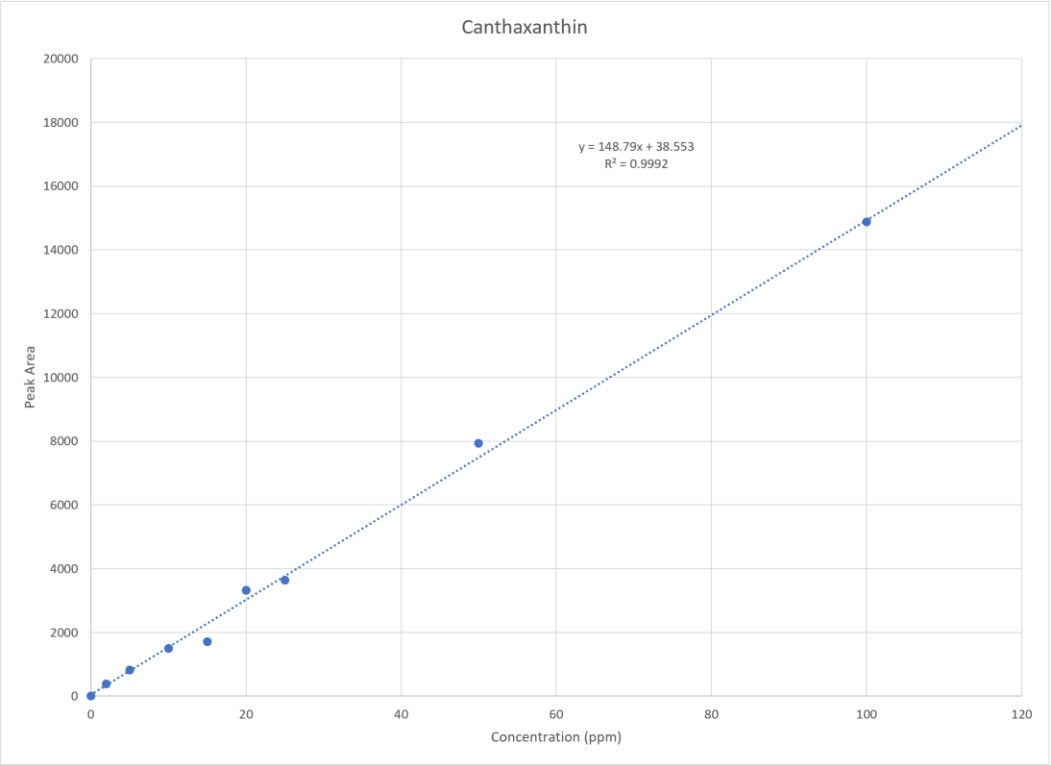

Figure 7.2b. Calibration curve generated from canthaxanthin stock solution using optimized conditions.

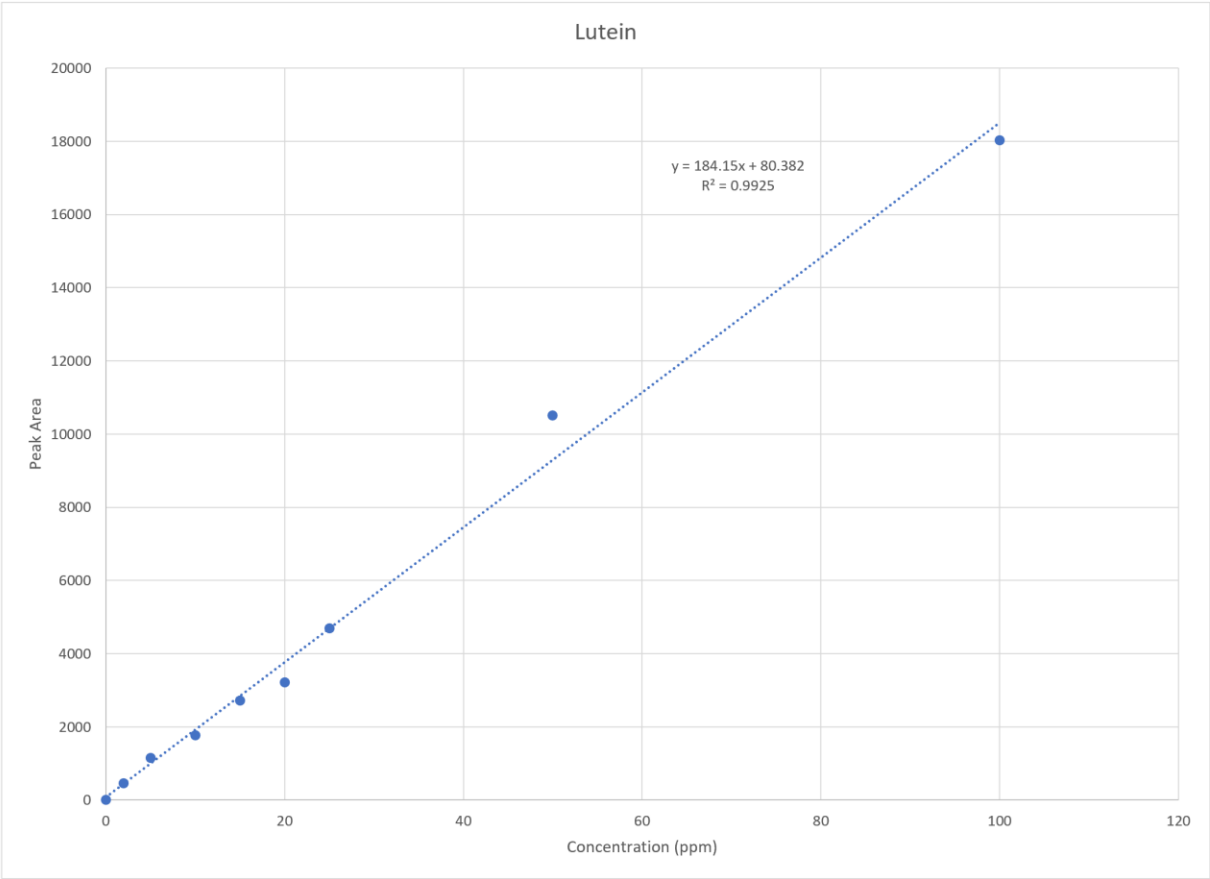

Figure 7.2c. Calibration curve generated from lutein stock solution using optimized conditions.