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Identification and quantification of Major Carotenoids in Mysis diluviana by Capillary Electrophoresis

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Abstract

Mysis diluviana is a small shrimp-like crustacean that lives in many Canadian lakes and is an important food for fish. Species of Mysis are known to contain compounds known as 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, when they feed on Mysis. Carotenoids are responsible for the orange-red colouration in the muscle tissues of trout and salmon. Carotenoids also play significant roles in animals such as antioxidants, precursors in the production of vitamin A, and photo-inhibitory molecules. Carotenoid analyses will be carried out by capillary electrophoresis (CE). CE is the chosen analytical method for the identification and quantification of carotenoids due to its high resolution, small sample volumes, and small solvent consumption. The micellar electrokinetic chromatography (MEKC) was used to quantify the carotenoids present. Several parameters were explored to achieve the optimum conditions necessary for the analysis. The optimized conditions, which include a background electrolyte containing 60 mM borate, and 20 mM sodium dodecyl sulfate at pH 9.5 and analysis at 214 nm, successfully detected the carotenoid standards within 10 minutes.

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 (1). 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 (2). 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. 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 (3,4).

Carotenoids are one of the most common types of natural pigments and can influence the colors of living organisms (5). 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. Carotenoids are also responsible for the orange-red colouration 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 (6). Diet proportions by weight were dominated by copepods, although this shifted towards cladocerans in autumn. A study that analyzed zooplankton and phytoplankton in Mysis of Okanagan Lake supported the importance of diatoms and cladocerans in the diet of Mysis (7).

A study of carotenoids in marine animals suggested that the principal carotenoid in crustaceans such as Mysis is astaxanthin (8). In crustaceans, astaxanthin exists as carotenoproteins such as crustacyanin, and exhibits purple and yellow colors. Many crustaceans can synthesize astaxanthin from beta-carotene ingested from dietary algae. Furthermore, it suggests that carotenoids found in phytoplankton such as diatoms are metabolites of fucoxanthin and alloxanthin.

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) (8, 9). 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.

Capillary electrophoresis (CE) is an ideal method for carotenoid analysis due to its high resolution and efficiency, small sample volumes, low risk of contamination, and reproducibility. An article in the Journal of Chromatographic Science discusses a CE method for the separation of photosynthetic pigments such as chlorophyll derivatives and carotenoids (10). 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 as well as specific carotenoids that affect tissue colouration in salmonids.

Carotenoids in M. diluviana will be analyzed by capillary electrophoresis (CE). CE is an analytical method that is used for the separation of analytes in solution. 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.

The carotenoid content of M. diluviana will be analyzed in samples from Okanagan Lake that had previously been collected for Dr. Gosselin's research. The wavelength of the UV detector, pH and concentration of the buffer, as well as voltage and injection time conditions on the CE will be optimized to obtain high resolution peaks for each carotenoid. The linear relationship between the concentration of the carotenoids and their corresponding peak area on the electropherogram will be used to validate the method by showing reproducibility of the peak area, migration time, precision, and accuracy.

Materials and Methods

1.1. Chemicals

Astaxanthin, beta-carotene, canthaxanthin, and lutein standards were purchased from Sigma-Aldrich. The chemicals sodium tetraborate decahydrate and sodium dodecyl sulfate (ACS reagent, purity ≥99.5%), used for the preparation of the electrolyte solution, was obtained from Sigma-Aldrich. Methanol was purchased from Caledon (Georgetown, ON, CA). All other reagents were of analytical grade, and 18 MΩ water was used to prepare the solutions.

1.2. Electrolyte and Standard Solutions

The capillary electrophoresis separations were performed with a background electrolyte solution of 60 mM borate and 20 mM sodium dodecyl sulfate, which was prepared by dissolving an appropriate amount of reagent in 18 MΩ water. The pH of the buffer was adjusted to 9.5, 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. New buffer solutions were prepared every two weeks to prevent electrolyte depletion.

Stock standard solutions of 100 ppm for astaxanthin, beta-carotene, canthaxanthin, and lutein were prepared by dissolving appropriate amounts of each carotenoid in methanol, filtered through a 0.45 µm nylon filter into an amber glass bottle, and stored at 4°C until analysis.

1.3. Instrumentation and MEKC Method

The carotenoids were analyzed using a Beckman Coulter P/ACE^{TM} MDQ system capillary electrophoresis unit (Beckman Coulter Inc., Fullerton, CA) equipped with an ultraviolet (UV) detector. Data acquisition was done with 32Karat software. The carotenoids were detected at 214, 256, and 280 nm using a direct absorbance. A separation voltage of +15 kV was applied for 10 min. Separations were carried out in an uncoated, fused-silica capillary with an inner diameter of 50 µm. The total length of the capillary was 60 cm (effective length of 50 cm). The capillary was housed in a cartridge, and the temperature was controlled at 25°C by a liquid fluorocarbon coolant system.

New capillaries were first rinsed with methanol (20 psi, 5 min) to remove any particulates. Then they were 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). Lastly, 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, 15) 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 with injection pressure of 15 psi and injection time of 1.0 min. When 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.

1.4. MEKC Method Optimization

Wavelength of the UV detector and pH of the buffer of the CE were optimized to obtain high resolution peaks for each carotenoid. Standard carotenoid solutions were prepared in 100ppm, 200ppm, 300ppm, 400ppm, and 500ppm for astaxanthin, beta-carotene, canthaxanthin, and lutein. The Beckman Coulter P/ACE^{TM} MDQ system capillary electrophoresis unit had UV filters for 214 nm, 256 nm, and 280 nm. Wavelength was optimized to be 214 nm. The pH of the electrolyte buffer was optimized to be 9.5 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 in addition to each wavelength (214, 256, and 280 nm) to determine the maximum absorption and highest resolution.

1.5. Sample Preparation

1.6. Sample Analysis

2. Results and Discussion

The methodology discussed in this paper was used to separate and identify carotenoids in solution. With the optimized conditions, Gaussian peaks were obtained for astaxanthin and beta-carotene. Astaxanthin resulted in a strong linear relationship between the concentration of astaxanthin and peak area on the electropherogram (Figure 1). Figure 1 illustrates the proportional increase in peak area as the concentration of the known astaxanthin standards increased. Under these conditions, Figure 1 illustrates that astaxanthin has a migration time of approximately 2.15 minutes. Additionally, beta-carotene also resulted in a linear relationship between the concentration of carotenoid and the peak area on the electropherogram (Figure 2). Figure 2 shows that the concentration of beta-carotene and the peak area is highly proportional, and that beta-carotene has a migration time of approximately 2.0 minutes.

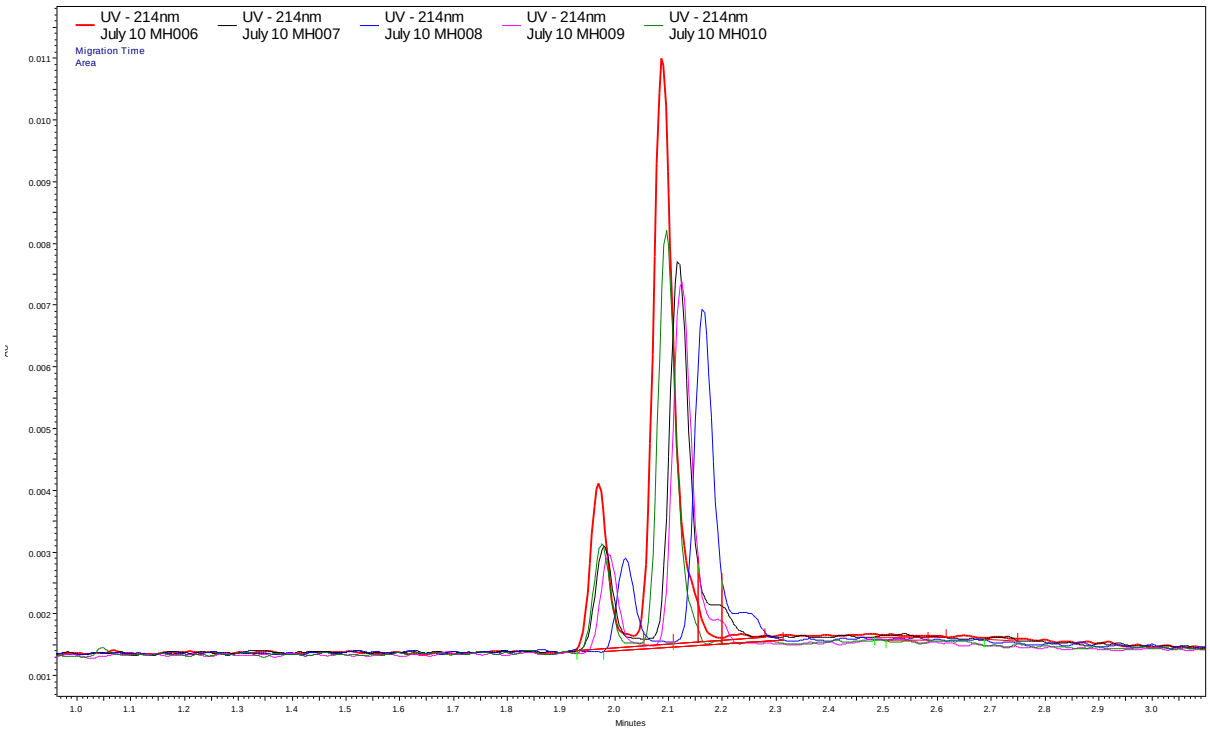

Figure 1. Electropherograms of astaxanthin standards from 100-500 mg L^{-1} obtained at 214 nm.

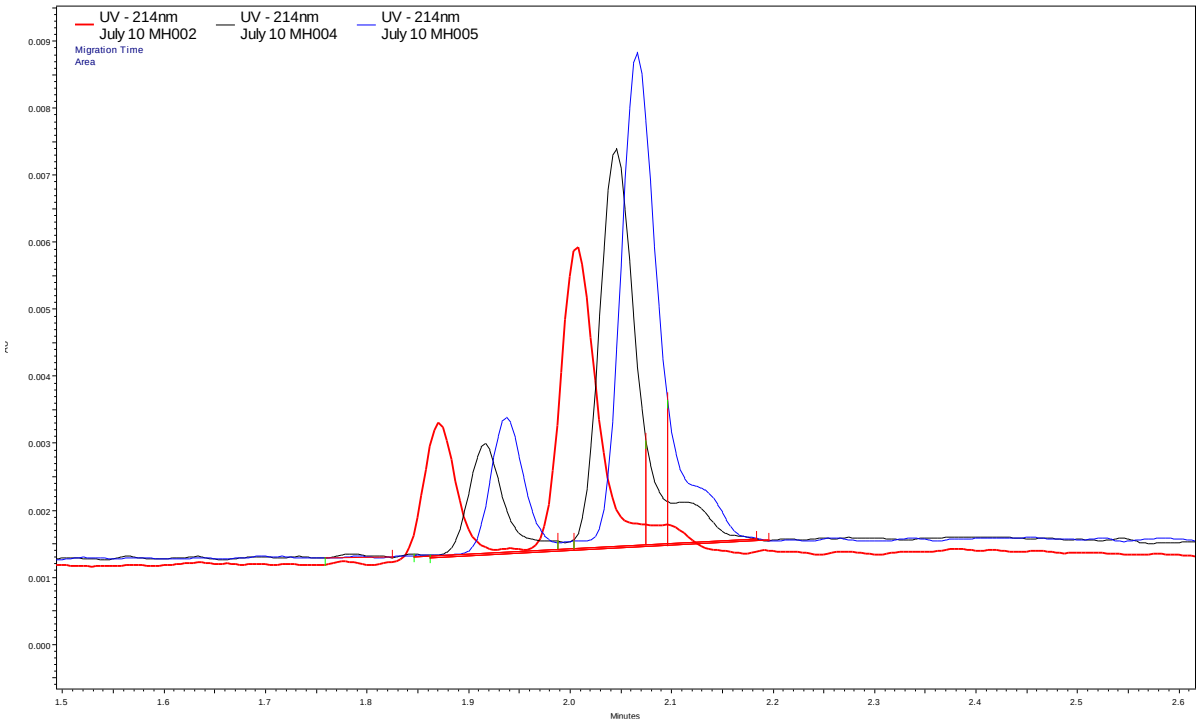

Figure 2. Electropherograms of beta-carotene standards from 100-500 mg L^{-1} obtained at 214 nm.

The linear relationship between the concentration of the carotenoids (astaxanthin and beta-carotene) and their corresponding peak area on the electropherogram was used to generate a standard curve for each carotenoid. Figure 3 shows the linear relationship between the concentration of astaxanthin and peak area on the electropherogram on a standard curve. The coefficient of determination was 0.9887 which shows that the data is highly correlated and linear. Figure 4 shows the linear relationship between peak area and concentration for beta-carotene. This relationship is more highly correlation as shown by the coefficient of determination of 0.9966. Figure 3 and 4 indicate that the optimized conditions for this methodology could be used to identify and quantify the amount of astaxanthin and beta-carotene in Mysis samples.

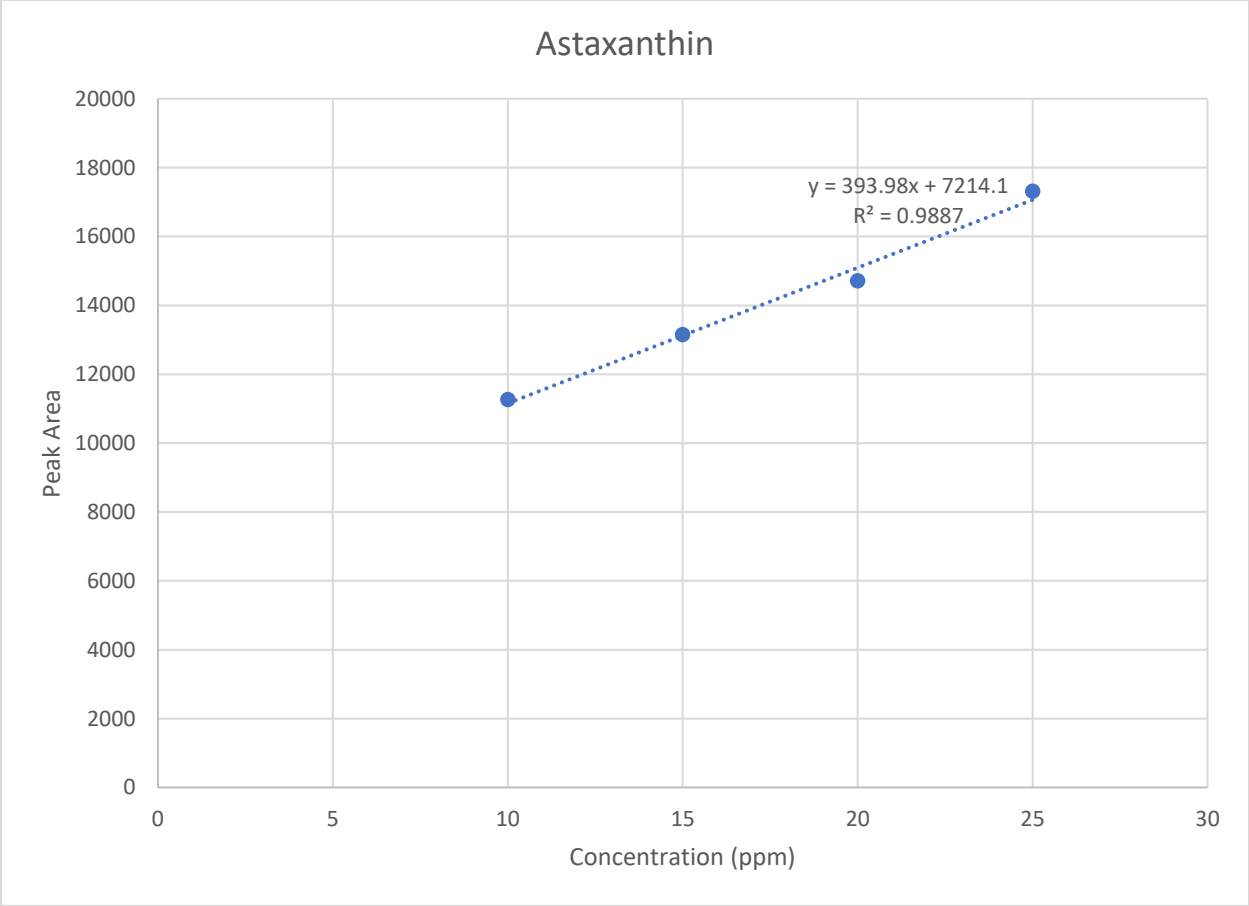

Figure 3. Calibration curve for astaxanthin at pH 9.5 and analyzed at 214 nm (n=8).

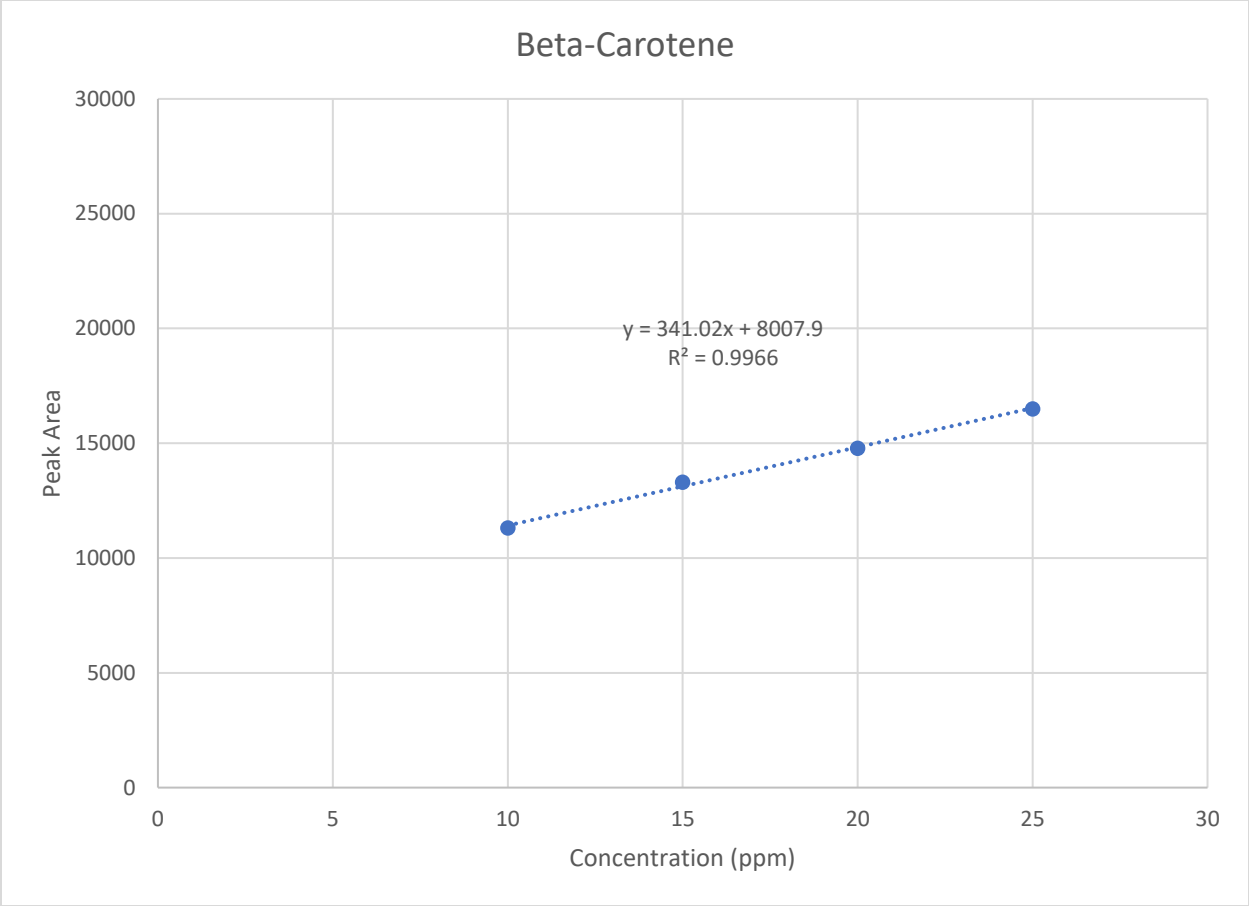

Figure 4. Calibration curve for beta-carotene at pH 9.5 and analyzed at 214 nm (n=8).

Conclusion

In this paper, a sensitive, and inexpensive MEKC method is being developed for the identification and quantification of carotenoid in Mysis feed. This method will offer a short analysis time while maintaining high resolution. This paper was able to optimize the concentration, pH and wavelength of the buffer (60 mM borate and 20 mM borate at pH 9.5 and the analysis was run at 214 nm). In addition, further optimizations are required identify canthaxanthin and lutein, and to separate each individual carotenoid. Once optimized, the MEKC method will still require validation to evaluate its precision, accuracy, linearity, and limits of detection and quantification.

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