Using High Performance Liquid Chromatography to Detect Carotenoid and Chlorophyll Pigments in the Peel and Flesh of North Carolina Apple Varieties: Degradation of Beta-Carotene and Implications for Sample Storage

by

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Abstract

Apples have high nutrient content in part due to their organic pigments, such as carotenoids and chlorophylls. Not found naturally in the human body, carotenoids and chlorophylls, must be consumed through the diet. The quantification of these compounds is important because they act as antioxidants and prevent multiple chronic diseases. A method was developed using reverse phase high performance liquid chromatography (HPLC) to quantify beta-carotene, zeaxanthin, chlorophyll a, and chlorophyll b in apple extracts. Method optimization steps were taken to improve peak resolution and shape. The linear range for zeaxanthin, chlorophyll a, and chlorophyll b was 1 mg/L - 10 mg/L. However, beta-carotene was not linear and often produced multiple peaks in the chromatogram that potentially are attributed to isomers or degradation products. An internal standard has been obtained, and further method validation is in progress prior to the analysis of apple samples.

Introduction

1. Organic Pigments

Organic pigments such as carotenoids and chlorophylls are important nutrients found in fruits and vegetables like apples, spinach, kale, and carrots and animal products such as salmon and eggs. Produced in the chloroplast and chromoplast organelles in plant cells, both carotenoids and chlorophylls are responsible for the color of unripe fruits and vegetables. Carotenoids alone are responsible for the color of ripe fruits.[1] Organic pigments act as antioxidants, which prevent heart disease, cataracts, cancer, and other chronic diseases.[2] Not manufactured inside of the human body, these compounds must be obtained from the diet. Apples contain lower pigment concentrations than other fruits and vegetables, but their pigments do contribute significantly to their coloration and health benefits.[1] Apples are the fourth most commercially important fruit crop and probably the most "well-known" fruit as there are thousands of varieties. The diversity seen in apples, whether it be through the cultivar, climate, harvest and storage conditions, or processing practices, leads to variation in pigment concentrations that should be measured.[1]

2. Carotenoids

a. Physical Properties

Carotenoids are a large group (>750) of organic pigments, usually C_{40} compounds, with a linear and symmetrical structure.[2, 3] Carotenoids can be classified as carotenes or xanthophylls. Carotenes are nonpolar and xanthophylls are polar due to the presence of hydroxyl groups in their structures.[4] Figure 1 shows the chemical structures of beta-carotene and the xanthophyll, zeaxanthin. Carotenoid molecules are hydrophobic and are therefore

only soluble in organic solvents with a wide range of stability therein. The stability of xanthophylls varies, however, because they are more polar.[4, 5]

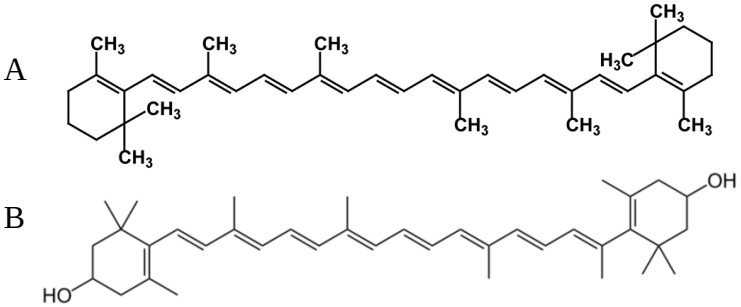

Figure 1: A: All-trans Beta-carotene ($C_{40}H_{56}$, MW=536.888 g/mol). B: zeaxanthin ($C_{40}H_{56}O_2$, MW= 568.866 g/mol) structures. Beta-carotene is non-polar while zeaxanthin, a xanthophyll, is polar due to the presence OH groups.

Carotenoids are sensitive to light, heat, air, and active surfaces. The chain of conjugated double bonds found in carotenoids contains delocalized electrons that allow for the absorption of and degradation by light.[6] For beta-carotene, this property makes it highly susceptible to isomerization and oxidation. The four major cis-isomers (15', 13', 11' and 9') of beta-carotene are shown in Figure 2. Isomerization from all-trans beta-carotene to its cis-isomers is thought to occur first, followed by oxidation, where oxygen can attack the isomer from either side of the cis-bond. A mechanism has been proposed by Penicaud et al.[6] and shown in Figure 3. Oxidation occurs by three pathways: autoxidation, photo-oxidation, or enzymatic oxidation. Homolytic substitution leads to the formation of epoxides which then form apocarotenones and apocarotenals as final products.[6] The intermediate expoxide and one of the final oxidation products is shown in Figure 4. The rate of degradation is highly dependent on temperature and, to a lesser extent, light exposure. For autoxidation, oxygen partial pressure is the main factor, however in oils and fruits, oxygen concentration is hard to obtain. The multiple isomers and numerous oxidation products of beta-carotene make it challenging to quantify. These challenges are magnified in real foods, where composition,

other oxidizable compounds, and the food matrix can affect degradation and interfere with measurements.[6]

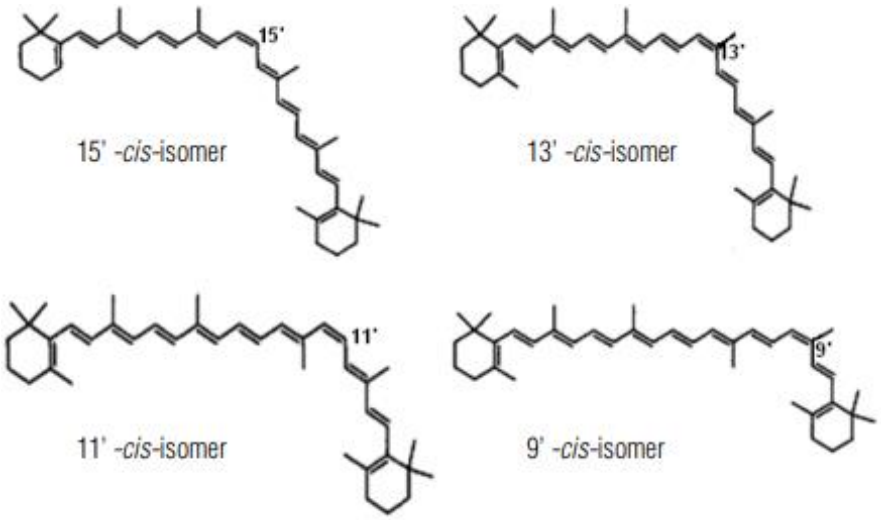

Figure 2: The four main cis-isomers of beta-carotene, from Jing et al.[12] The all-trans isomer of beta-carotene is shown in Figure 1.

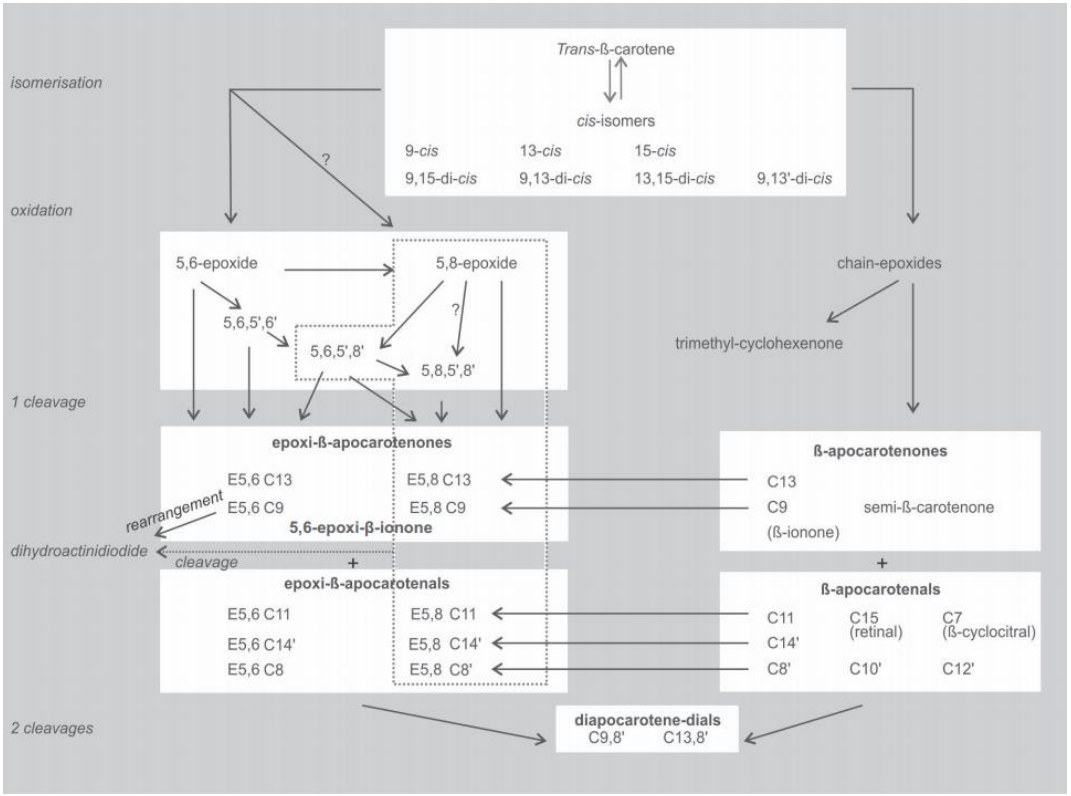

Figure 3: General overview of the degradation scheme of beta-carotene, from Penicaud et al.[6]

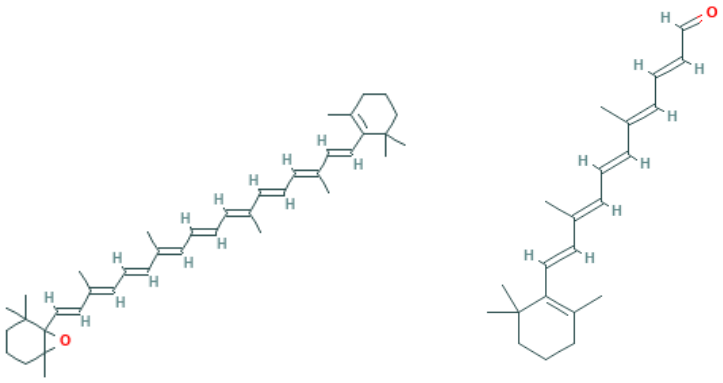

Figure 4: Sample intermediate epoxide (left, Beta-carotene 5, 6 epoxide, $C_{40}H_{56}O$, 552.887 g/mol) and final product apocarotenal (right, 14'-apo-beta-Carotenal, $C_{20}H_{30}O$, 310.481 g/mol)

To combat this instability, plants often store carotenoids both in their free and esterified forms. Figure 5 shows the esterified form of zeaxanthin. For organisms, this means that the esterified carotenoid must undergo enzyme catalyzed hydrolysis in order to be used, rather than direct absorption of the free form.[7]

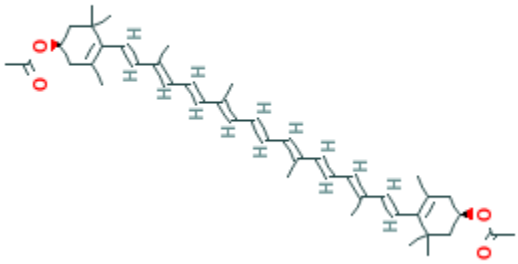

Figure 5: Structure of esterified zeaxanthin, zeaxanthin diacetate, $C_{44}H_{60}O_4$, 652.96 g/mol)

b. Biological/Health/Dietary Significance

Bioavailability refers to the fraction of any compound ingested and made available for utilization, metabolism, and/or storage by the organism. Regular or increased consumption of fruits and vegetables high in carotenoid and chlorophyll pigments increases the bioavailability in the human body of these compounds, which in turn can improve human health.[1, 11] Dietary carotenoids reduce the risk of cancer, cardiovascular disease, ulcers,

macular degeneration, and cataracts. Carotenoids are fat soluble and therefore require fat for uptake.[10, 12] Once absorbed, carotenoids act as antioxidants by quenching singlet oxygen and free radicals.[6] Carotenoids protect cellular tissues by inhibiting the oxidation or peroxidation of lipids, which make up the cell membrane. Lipid oxidation and peroxidation are a major cause of cell damage and the end products can be carcinogenic.[9] Beta-carotene, however, also acts as a pro-oxidant, and in oxidative-stressed systems, such as the lungs of chronic smokers or individuals with lung cancer, beta-carotene can contribute to lipid oxidation and peroxidation.[2, 10, 13] It is important to note that these results were achieved using beta-carotene supplements in order to control for other antioxidants that might be present in a plant-based diet.[14] Carotenoids also act as Vitamin A precursors, which is important because vitamin A is needed for human health and development.[3]

A change in diet will increase carotenoid concentrations in human plasma relatively quickly, but other characteristics such as age, sex, smoking, and alcohol consumption also affect uptake rates.[10] In a study of healthy adults, older men were unable to change their uptake rates of carotenoids through dietary changes, but young women could. Uptake rates are also affected by the retention mechanisms in human body used to store carotenoids. The concentration of beta-carotene is highest in the blood, while the concentrations of lutein and zeaxanthin are highest in macular pigments.[9] A similar retention mechanism appears to be found in apples, as pigments accumulate in the peel rather than the flesh.[1] In a high carotenoid diet, lutein and beta-carotene often compete for uptake.[10] Esterification can also play a role in the accumulation of carotenoids.[1]

The isomers of beta-carotene (Figure 2) have different biological activities and lead to different levels of bioavailability.[3, 15] In general, the trans-isomer of beta-carotene is more

prevalent and has more protovitamin A activity, generating about twice as much Vitamin A compared to the cis-isomer.[3, 6, 12] The cis-and trans- isomers vary among different species of animals, within a single species, and even within a single body. For example, the isomerization in rats and chicks is equal, while that in humans is variable from person to person.[9]

3. Chlorophyll Compounds

Chlorophyll compounds are the most abundant organic pigments. Chlorophyll a and b are found in plants, usually in a 3:1 ratio, which varies based on species, climate, harvest treatment, and processing. Chlorophyll content is usually higher than carotenoid content by up to 5:1 ratio.[11] As shown in Figure 6, chlorophyll a and b each contain a porphyrin ring, which allows electrons to migrate to other molecules and acts as the main driver for photosynthesis. Like carotenoids, chlorophyll a and b lower the risk of cancer.[11] Chlorophylls are often overlooked as preventers of chronic disease because they are generally less bioavailable in the human body. However, even a small absorption of chlorophyll or its derivatives can be significant. Chlorophylls have a long history of medicinal uses such as wound healing, anti-inflammation, and as an internal deodorizer to neutralize body odors such as bad breath or flatulence. Recent work has suggested their potential as chemopreventative agents.[11] Chlorophylls have low toxicity, suppress tumors, and reduce the bioavailability of toxins. It is thought that chlorophylls reduce cancer risk by sequestering mutagens and carcinogens through "reversible planar binding of overlapping ring systems" and inducing apoptosis in cancer cells.[11] Figure 7 shows an example overlapping ring system of aflatoxin B1 and a chlorophyll complex. Throughout food processing and digestion, natural chlorophylls lose their green color and are artificially

colored with chlorophyll complex additives. The artificial chlorophylls are surprisingly more effective at reducing cancer risks.[11]

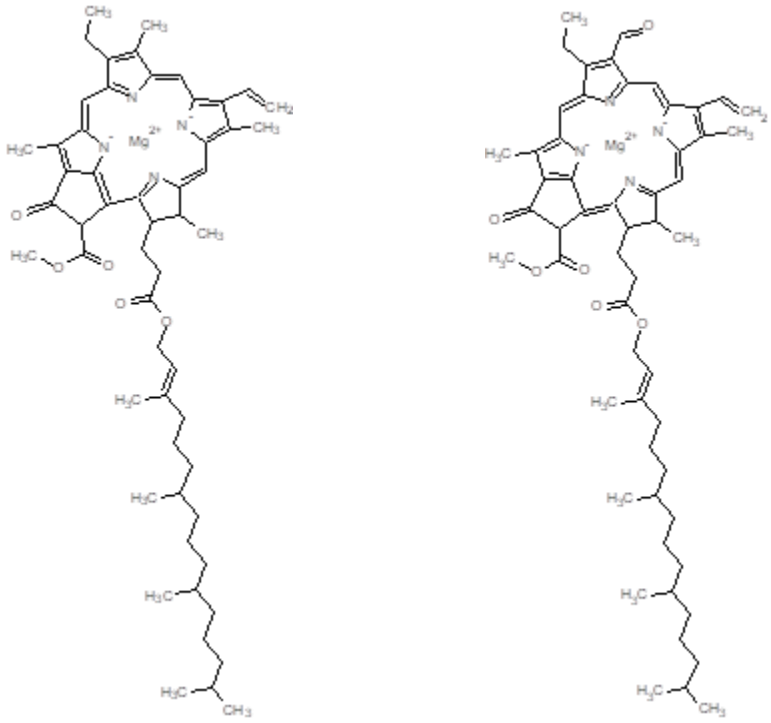

Figure 6: Left: Chlorophyll a ($C_{55}H_{72}MgN_4O_5$, MW=893.509 g/mol). Right: Chlorophyll b ($C_{55}H_{70}MgN_4O_6$, MW=907.492 g/mol) structures

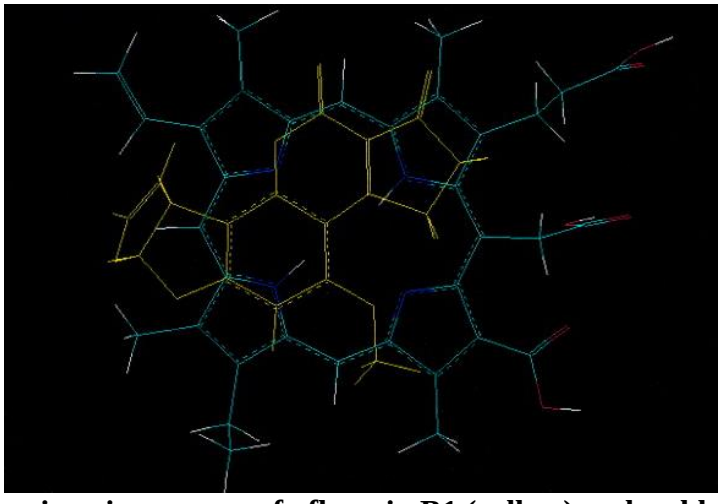

Figure 7: Overlapping ring system of aflatoxin B1 (yellow) and a chlorophyll complex (green) from Dashwood, et al.[23]

4. HPLC Analysis of Carotenoids and Chlorophylls

Carotenoids and chlorophylls have been studied with chromatography since 1906 using open column chromatography followed by spectrophotometric quantification.[16, 17] Improved technology and methodology has allowed for improved separation of various carotenoids as well as their isomers.[9, 16] Reverse-phased HPLC with a C18 column is the most common method, but normal-phased methods also exist. For separation of isomers, a C30, or "carotenoid column" is best.[18] Binary gradient elution is used, with typical mobile phases compromised of acetone, water, acetonitrile, methanol, or a mixture of those listed.[5, 17, 18]

Solvent modifiers such as butylated hydroxytoluene (BHT) are thought to improve recovery and reduce on column degradation.[16] Column temperature is generally kept at or above 20°C to prevent crystallization, and flow rates are typically 1 mL/min or 1.5 mL/min.[17] Today, many methods also couple HPLC to mass spectrometry or nuclear magnetic resonance systems. LC/NMR systems exist, but most NMR analyses occurs after separation.[5]

5. Beer's Law and Absorbance

As mentioned previously, the conjugated double bonds in carotenoids and chlorophylls make them good light absorbers; therefore they can be studied with spectrophotometry. The Beer-Lambert Law states that concentration is directly proportional to absorption, as shown in the following equation:

$$A=\varepsilon bc$$

where A is absorbance, ε is molar absorptivity (L/mol*cm), b is the path length (cm), and c is the concentration (mol/L).[20] For most species this law is followed, but there are some limitations. Beer's Law is only accurate at low concentrations, generally less than 10 mM,

due to solute-solute interactions at higher concentrations that affect the solute's ability to absorb light.[20] If a solute can exist in more than one form, for example a cis- or trans- isomer, and if there is an equilibrium between those forms, deviations from Beer's Law can also be observed, especially if the two forms have different molar absorptivity values.

Molar absorptivity is an intrinsic, but solvent dependent property of a compound. It is a crucial value needed for Beer's Law, but has proved to be tricky to determine for carotenoids. Literature values are widely varied, and in multi-laboratory studies using the same procedure and solvent, different values were still found.[17] Further, some reported values are scaled or include a correction coefficient. However, molar absorptivity has the least amount of variance at λ_{max}, so HPLC detection occurs at these values.[20] The absorption spectra of carotenoids can be used to reveal evidence of isomerization.[12] As shown in Figure 8, all trans-beta-carotene has absorption maxima near 450 nm and 478 nm, characteristic for most carotenoids. The cis-isomer, however, shows blue-shifted maxima near 444 nm and 467 nm with the addition of a new "cis-peak" peak between 330 nm and 345 nm.

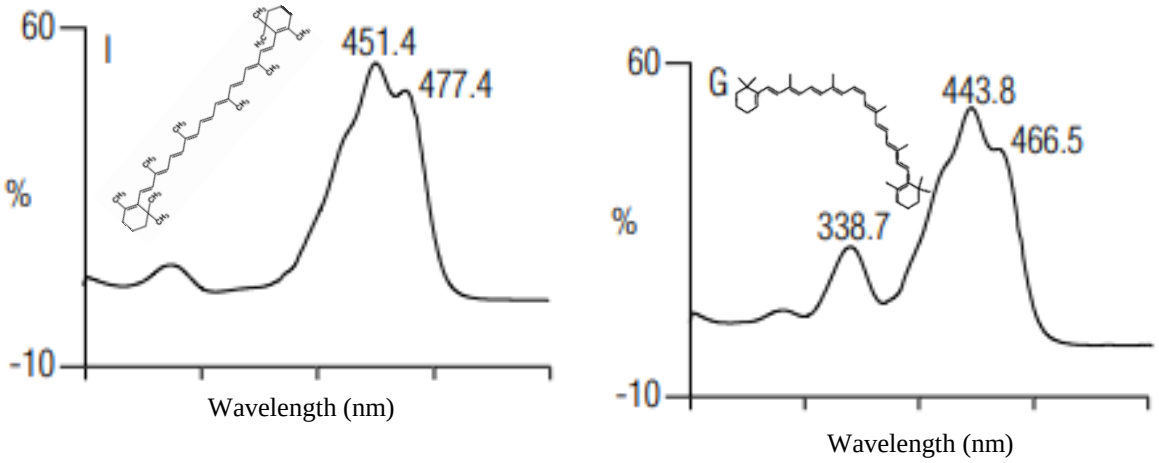

Figure 8: UV-Vis absorption spectra of all trans-beta-carotene (left) and the 15' cis-isomer (right). Using a C30 column, Jing et al. were able to separate, identify, and show the absorption spectra of beta-carotene isomers.[12]

6. Purpose

The goal of this research is to validate a method to quantify the two carotenoids, zeaxanthin and beta-carotene, along with chlorophyll a and b in the peel and flesh of North Carolina apple varieties. Reverse phase high performance liquid chromatography (HPLC) is used to analyze the standards and apple extracts. The apple extracts are prepared by Dr. Root's lab in the nutrition department.

In previous work by chemistry majors, Mathew Kelley and Dillon Carns, a method using reverse-phase HPLC with a C18 column and an acetone-water mobile phase was developed.[1, 19] The method's linear range was 2-50 mg/L, and calibration curves (r^2>0.9864) were created. Percent recoveries of QC solutions and method detection limits (0.0047-0.4366 mg/L) were also determined.[19] However, after running this method a few times, the HPLC experienced low-pressure errors, but obvious leaks in the system could not be found. Technical support at Thermo suggested that outgassing occurred when acetone and water eluents mixed in the mixing valve, leading to low pressure errors. Therefore, the next student to work on this project, Sarah McMahan, set out to re-develop the HPLC method using more conventional solvents like methanol, acetonitrile, and THF. This thesis describes the work started by Sarah, and continued by me, to optimize the separation of zeaxanthin, chlorophyll a, chlorophyll b, and beta-carotene using HPLC, a C8 column (recommended by a product specialist at Phenomenex), and a methanol-water mobile phase. As the research continued, the stability of beta-carotene was also explored.

Experimental

1. Reagents

Ethanol (Pharmco-Aasper, CAS: 64-17-5), butylated hydroxytoluene (BHT, Spectrum Chemical, CAS: 128-37-0), methanol (HPLC grade, VMR, CAS: 67-56-1), ammonium acetate (OmniPur, CAS: 631-61-8), and DI water were used throughout. Zeaxanthin (CAS: 144-68-3), chlorophyll a (CAS: 479-61-8), chlorophyll b (CAS: 519-6-20), and beta-carotene (CAS: 7235-40-7) were purchased from Sigma-Aldrich. Class A volumetric glassware and Hamilton syringes were used.

2. Standard Preparation

Most of the analytes were expensive—zeaxanthin cost over $450 for 1 mg—so a microbalance (Perkin elmer AD 6 Autobalance controller, 070382) was used to weigh small masses as accurately as possible.[22] Single-component stock solutions (100 mg/L) were prepared by diluting 1 mg of each compound in ethanol fortified with 0.1% (w/v) BHT to a total volume of 10 mL. BHT was added to prevent analyte degradation.[16] Difficulties with transferring such small masses and using the microbalance resulted in stock concentrations that deviated from the desired 100 mg/L. For example, only 0.470 mg zeaxanthin and 0.567 mg chlorophyll a were obtained, resulting in stock concentrations of 47.0 mg/L and 56.7 mg/L, respectively. The stock solutions were used to make multicomponent calibration standards of concentrations 1, 3, 5, and 7 ppm, and single component 10 ppm calibration standards, also diluted with ethanol and 0.1% BHT. Following initial runs, the calibration curve for beta-carotene was not linear, so single-component calibration standards were prepared with concentrations of 1, 2, 3, 4, 5, 7, 10, 15, 20, and 40 ppm to monitor its behavior.

3. HPLC conditions

A Dionex Ultimate 3000 Quarternary Rapid Separation LC system was used with a C8 column (Kinetex 2.6µm, 100 x 4.6 mm) and guard column (Phenomenex, AJO-9000). Chromeleon 6.80 chromatography management system software was used to analyze and interpret the data. Detection occurred at 450, 645, and 666 nm using a photodiode array detector (Dionex, PDA-3000) based on literature values for the λ_{max} of each compound. Acquisition of absorption spectra using UV-Vis confirmed these as maxima or very close to that. Zeaxanthin and beta-carotene are detected at 450 nm, chlorophyll a at 666 nm, and chlorophyll b at 645 nm. Gradient elution consisting of solvents 70:30 methanol: 1M ammonium acetate (Solvent A) and pure methanol (Solvent B) was used with a flow rate of 1.00 mL/min and an injection volume of 20.00 µL. An overview of the gradient is shown in Figure 9. The solvents were vacuum filtered through a 0.2 µm nylon membrane filter (Whatman, 7402-004) and sonicated before use. The HPLC was equilibrated with 40:60 solvent A:B at 1 mL/min until the PDA baseline was stable (20-30 min). This method is based on an application note from Phenomenex.[21]

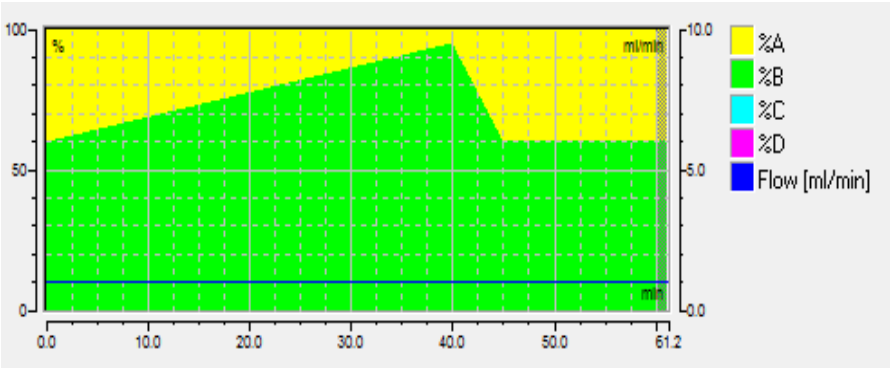

Figure 9: The gradient elution of 70:30 methanol: 1M ammonium acetate (A) and methanol (B).

Throughout later runs, a high-pressure issue was encountered. To correct this, two extensive column cleaning procedures were performed, shown in Figures 10 and 11. For the

first, from the column manual, 10-20 column volumes of each solution in the following order were run through the column at 0.5 mL/min: 5:95 methanol: water, 95:5 methanol: water, tetrahydrofluron (THF), 95:5 methanol: water, and 5:95 methanol: water. A 15 minute ramp occurred for each transition to prevent outgassing and bubbles. The column was then equilibrated with the mobile phase. The second method started with 5:95 methanol: water and the ratio was stepwise increased to 95:5 methanol: water using a flow rate of 0.1 mL/min. This method was provided by a Phenomenex specialist. The issue was corrected short-term, but soon returned. Eventually, the C8 column was unusable, and final experiments were performed using a C18 column (Kinex 2.6 µm, 100 x 4.6 mm).

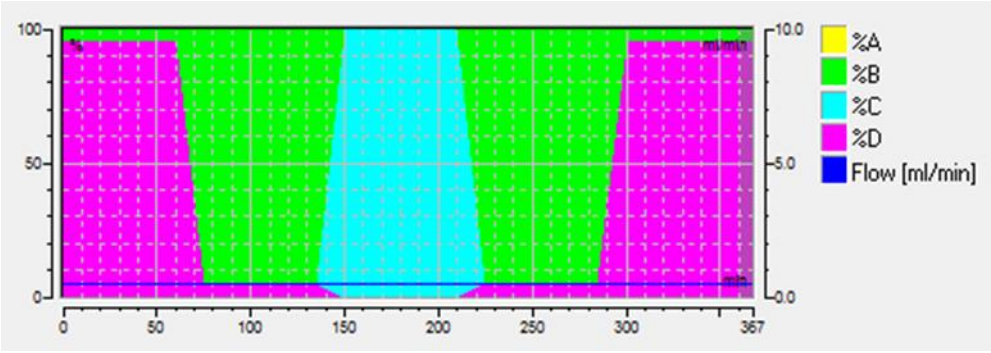

Figure 10: Cleaning 1 with solvents methanol (B), THF (C), and water (D)

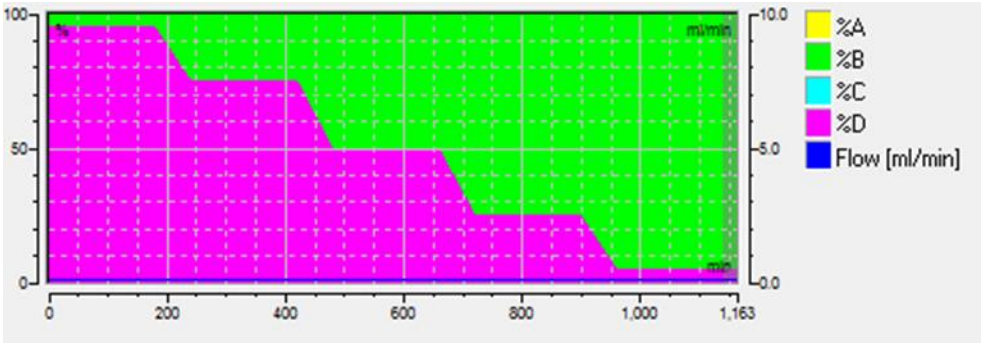

Figure 11: Cleaning 2 with solvents methanol (B) and water (D)

4. Visible Spectroscopy

The beta-carotene standards were also run on a UV-Vis (without chromatography) in order to determine the nature of its non-linear behavior. A Shimadzu UV-2401PC was used

with a wavelength range of 800-200 nm, medium scan speed, a 1.0 nm sampling interval and slit width, and reference standard of ethanol with 0.1% BHT. Experiments were also performed to determine the concentration of the beta-carotene using absorption values and molar absorptivities from the literature.

Results and Discussion

1. Using UV-Vis and ε-values to confirm the concentrations of stock solutions

As mentioned previously, it was challenging to transfer small masses of solute from the microbalance to the volumetric flask, so we wanted to confirm that the concentrations of the stock solutions using Beer's Law. However, it was difficult to find agreement in the literature on the molar absorptivity of beta-carotene. Literature values varied based on solvent, solvent-interaction corrections, error propagation methods, stability in solvents, scaling values, and λ_{max} values.[9, 13] There seemed to be an overall consensus that the molar absorptivity value was highly variable depending on experimental conditions. When literature values were used to calculate the concentrations of the standards, the results differed from the expected concentrations by multiple orders of magnitude. Table 1 shows a summary of the results from these experiments.

Further, with the high variability, it is unlikely this would be a reliable method to determine concentrations in apple samples as the molar absorptivity of beta-carotene in solvent may be different than that of beta-carotene in the apples. It was determined finding concentrations of the organic pigments using Beer's Law would be complex and particularly uncertain and/or unreliable.

Table 1: Calculated standard concentrations based on literature values of ε

Solute	Solvent	Expected conc. (mg/L)	ε (L/mol*cm)	Calculated conc. (mg/L)
Beta-carotene	Ethanol	12.7	135,800	0.90
	Ethanol	103.3	135,800	5.41
	Hexane	11.56	139,000	2.34

2. Initial experiments using the C8 column

Initial experiments were performed to separate the four compounds using the C8 column and the gradient shown in Figure 9. Figure 12 shows the whole chromatogram at 450 nm. As shown in Figures 13-16, multiple peaks were observed for each compound, retention times shifted during the run, signals were small, and linearity was poor (r^2 0.8497-0.9606). Peak height was used to prepare these calibration curves because of the poor peak shape, but in later work, peak area was used. A Granny Smith apple pulp sample was also run with little success. (In retrospect, acquiring the entire UV-Vis spectrum with the PDA would have been useful to determine if isomerization was responsible for the multiple peaks.)

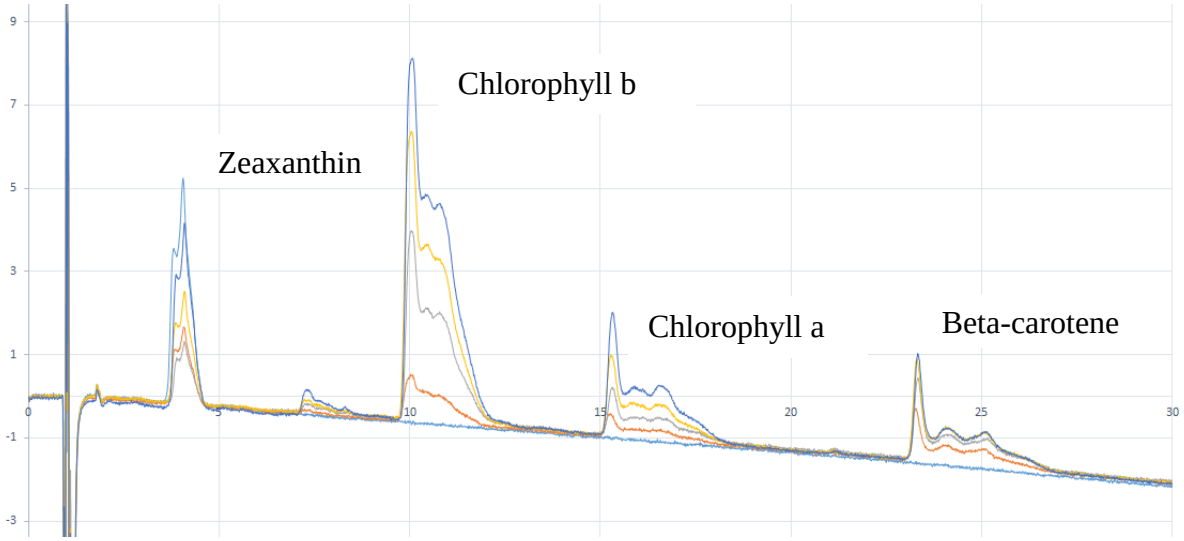

Figure 12: Full chromatogram at 450 nm from initial runs on C8 column. This run occurred in October 2017 with standards prepared 3 weeks prior.

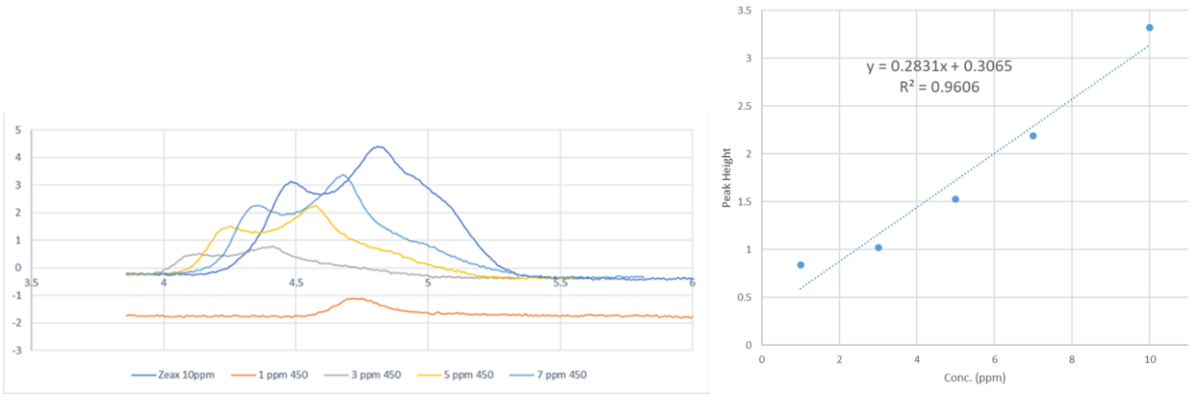

Figure 13: Zeaxanthin chromatogram at 450 nm (left) and calibration curve (right, line of best fit: y= 0.2831x+0.3065, r^2=0.9606)

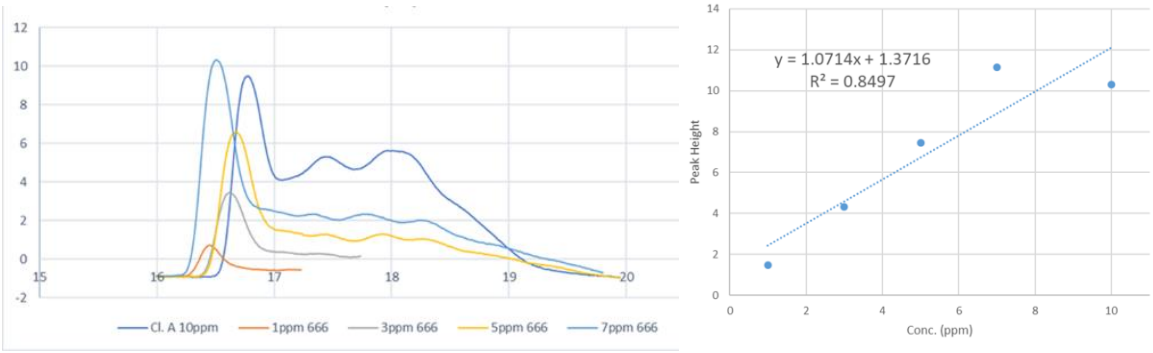

Figure 14: Chlorophyll a chromatogram at 666 nm (left) and calibration curve (right, line of best fit: y= 1.0714x+1.3716, r^2=0.8497)

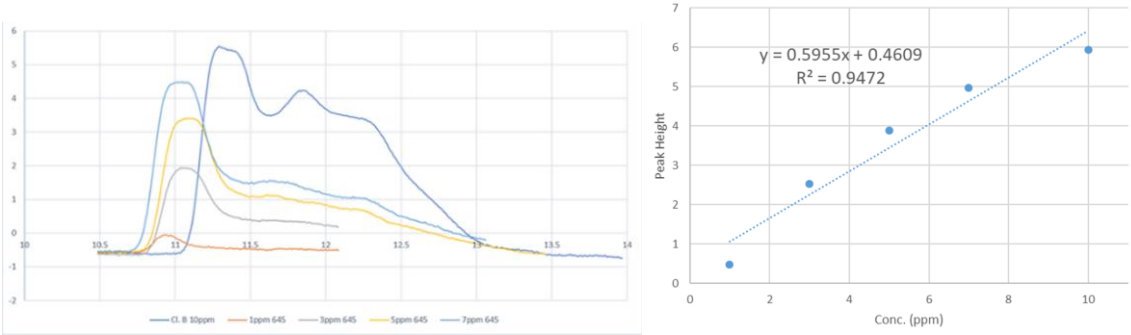

Figure 15: Chlorophyll b chromatogram at 645 nm (left) and calibration curve (right, line of best fit: y= 0.5955x+0.4609, r^2=0.9472)

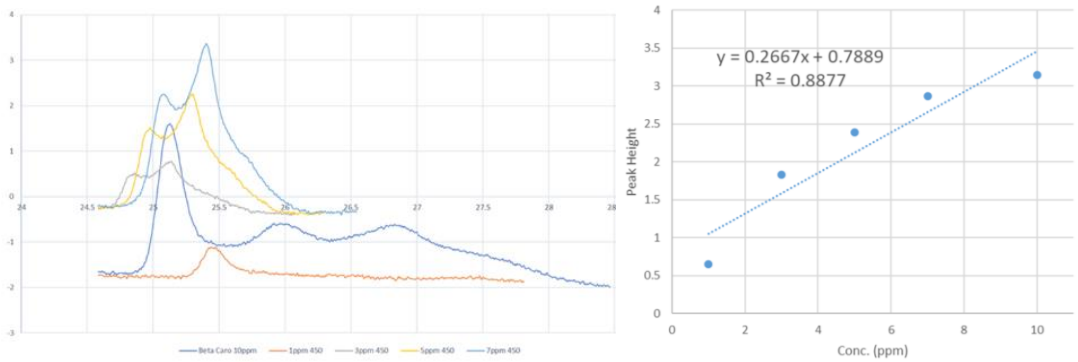

Figure 16: Beta-carotene chromatogram at 450 nm (left) and calibration curve (right, line of best fit: y= 0.2667x+0.7889, r^2=0.8877)

Suspecting that the compounds had either degraded or isomerized during storage, fresh standards were prepared. Fifteen minutes was also added at the end of the gradient to allow more time for the column to re-equilibrate with the mobile phase in between samples. As shown in Figures 18-21, this resulted in greatly improved chromatograms and linearity (r^2>0.998) for zeaxanthin, chlorophyll a, and chlorophyll b. Figure 17 gives the full chromatogram. Most notably, peak areas increased and the tails of the peaks decreased. The retention times of the compounds were determined using the single component 10 ppm standards as approximately 4, 16, 11, and 24 minutes for zeaxanthin, chlorophyll a, chlorophyll b, and beta-carotene, respectively. The linear range for zeaxanthin, chlorophyll a, and chlorophyll b was 0mg/L to 10 mg/L; however, the linear range of beta-carotene appeared to plateau at 7 mg/L.

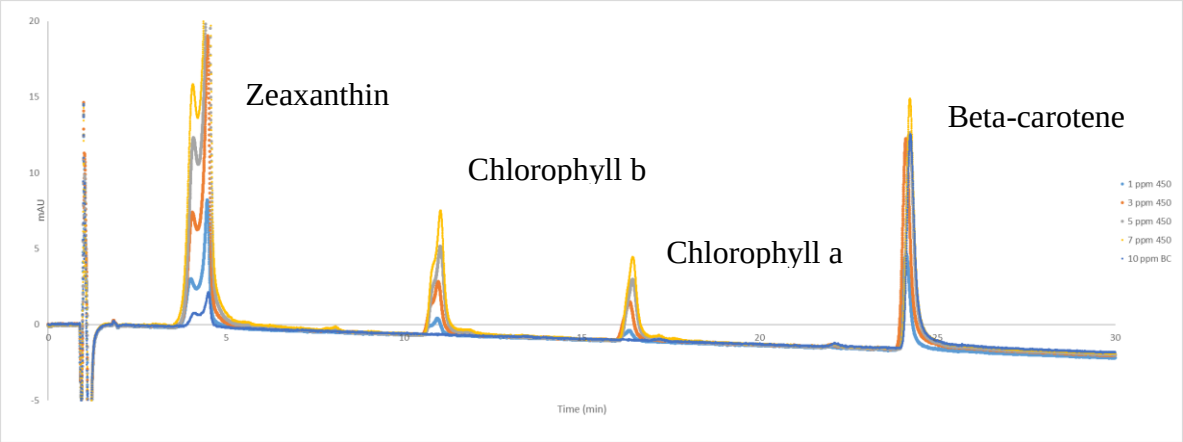

Figure 17: Full chromatogram at 450 nm for second C8 run. This run occurred in July 2018 with standards prepared 1 day prior.

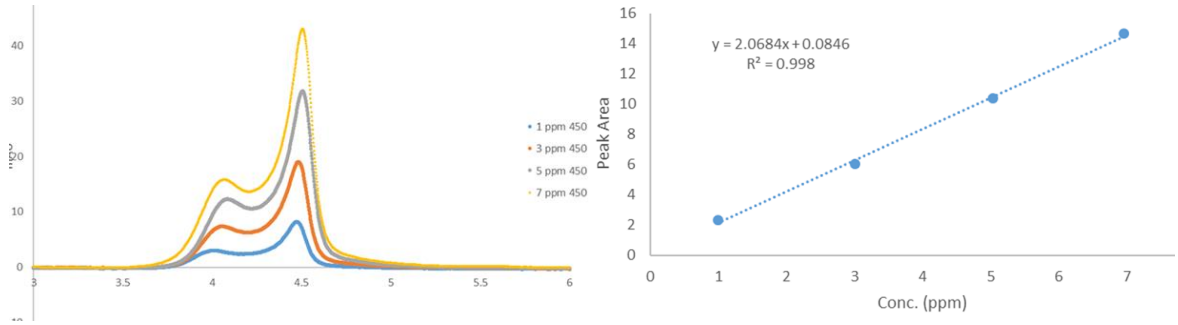

Figure 18: Zeaxanthin chromatogram at 450 nm (left) and calibration curve (right, line of best fit: y=2.0684x + 0.0846, r^2=0.998)

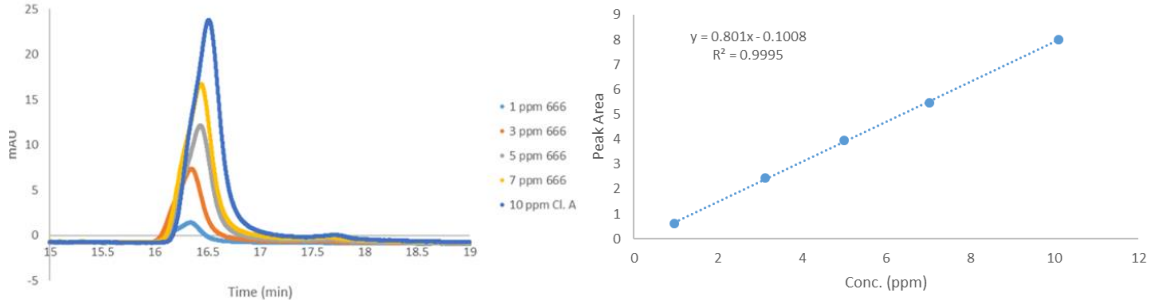

Figure 19: Chlorophyll a chromatogram at 666 nm (left) and calibration curve (right, line of best fit: y=0.801x-0.1008, r^2=0.9995)

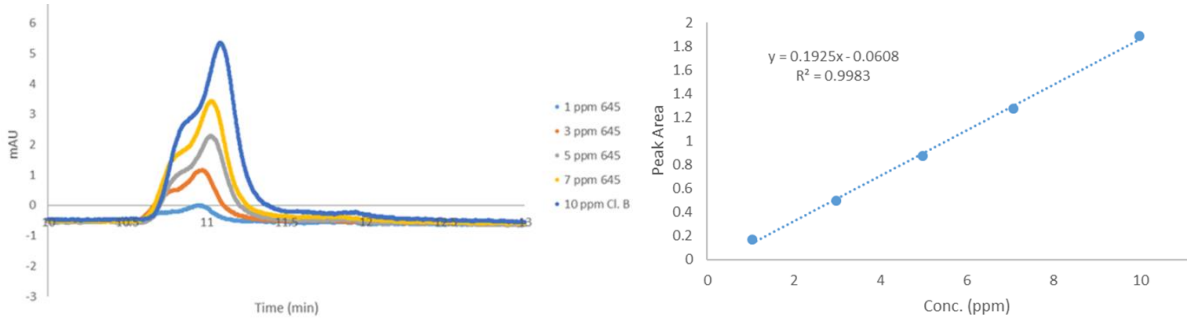

Figure 20: Chlorophyll b chromatogram at 645 nm (left) and calibration curve (right, line of best fit: y=0.1925x-0.0608, r^2=0.998)

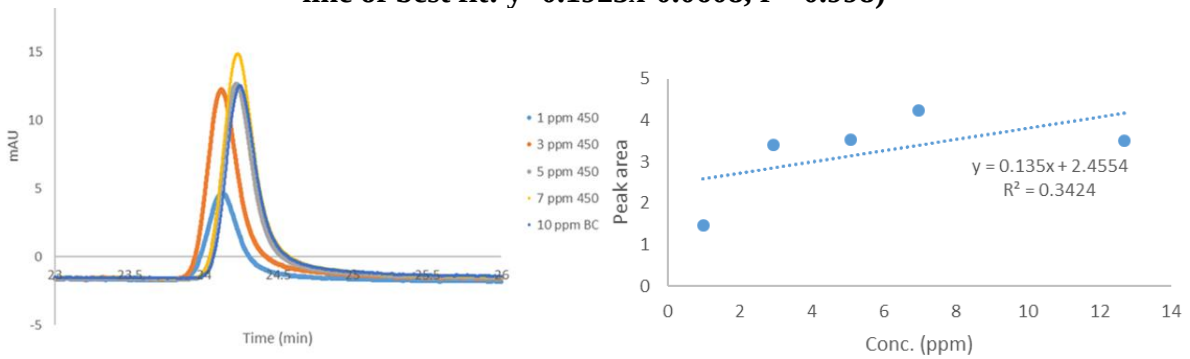

Figure 21: Beta-carotene chromatogram at 450 nm (left) and calibration curve (right, line of best fit: y=0.135x+2.4554, r^2=0.342)

Zeaxanthin, chlorophyll a, and chlorophyll b provided favorable results as they exhibited high linearity (r^2>0.998). The success of these calibration curves indicates that the method developed has potential, but other figures of merit including precision, bias, sensitivity, detection limit, dynamic range, and sensitivity must be found in order for the method to be validated. While these results are promising, the lack of linearity exhibited by beta-carotene was not expected and caused the focus of the project to shift to why beta-carotene did not behave linearly. Since beta-carotene is often regarded as the most commercially utilized carotenoid, it is of great importance to the project.[12]

3. Experiments with Beta-Carotene using the C8 Column

To confirm that the linear range of beta-carotene did in fact plateau at 7 ppm, additional (fresh) standards with concentrations below, between, and above the previous multicomponent standards (0.5 ppm, 2ppm, 4ppm, 15 ppm, 20 ppm) were prepared. Since

beta-carotene has two absorption maxima, detection occurred at both 450 nm and 453 nm to see if this played a role. Peak areas remained non-linear, although they had improved from the previous run. Surprisingly, the chromatograms showed substantial shifts in retention time between runs, shown in Figures 22 and 23. The retention time appeared to increase with concentration. Beta-carotene is not very soluble in methanol.[13] Perhaps as methanol increases during the gradient, beta-carotene falls out of solution and remains on the column, making the column more retentive towards beta-carotene in subsequent runs.

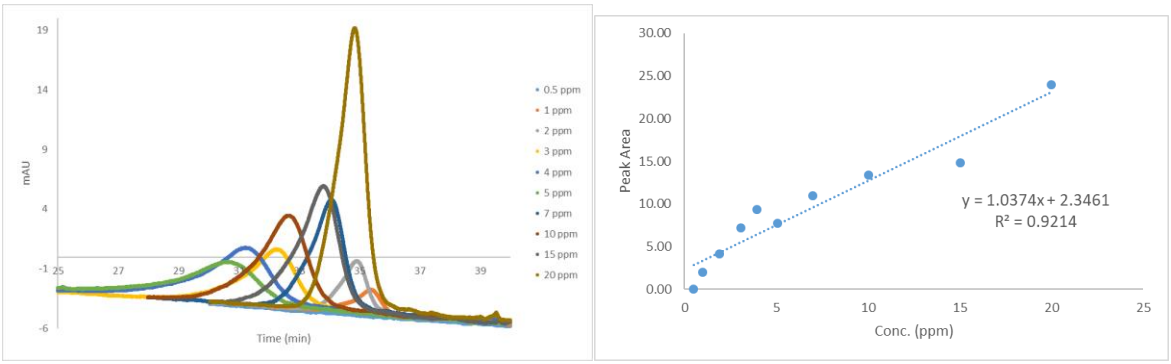

Figure 22: Beta-carotene chromatogram at 450 nm (left) and calibration curve (right, line of best fit: y= 1.0374x+2.3461, r^2=0.9214)

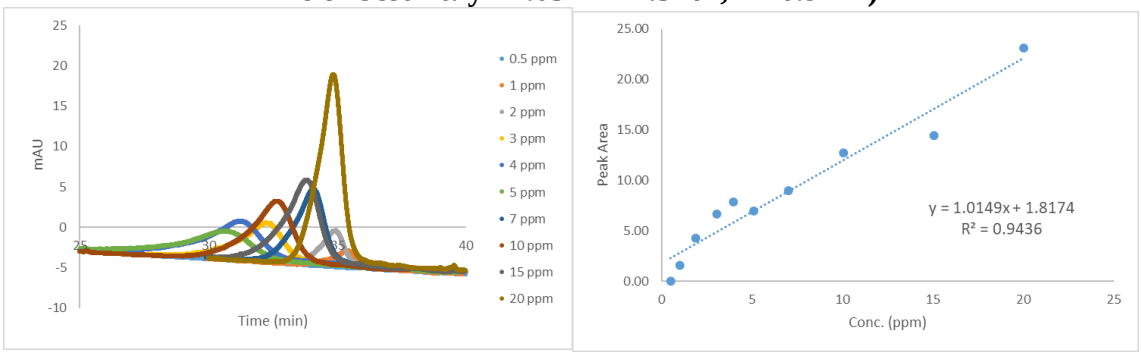

Figure 23: Beta-carotene chromatogram at 453 nm (left) and calibration curve (right, line of best fit: y= 1.0149x+1.8174, r^2=0.9436)

Fresh single-component beta-carotene standards at each of the concentrations in addition to 40 ppm were prepared and run a second time with detection only at 450 nm. Peaks had split and the areas remained non-linear, as shown in Figure 24. The non-linear nature and split peaks of beta-carotene indicated degradation had occurred (and occurred

rapidly) or that the C8 column was on the verge of failure. We still are not sure how to explain the results in Figure 24.

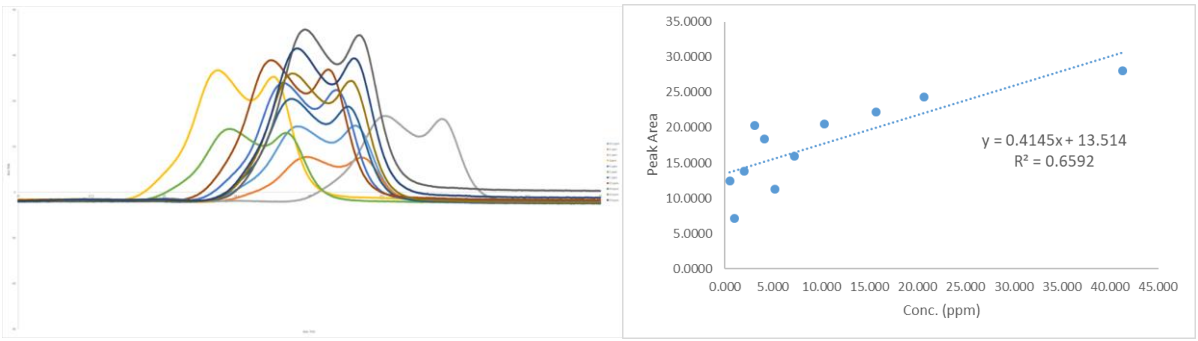

Figure 24: Beta-carotene chromatogram at 453 nm (left) and calibration curve (right, line of best fit: y= 0.4145x+13.514, r^2=0.6592). The standards were prepared on the same day of the run in October 2018.

A large area of trouble-shooting for this project was dealing with pressure issues across the column. High backpressure was encountered throughout some of the experiments, likely due to buffer (ammonium acetate) on the column. The buffer salts remaining on the column can precipitate out and clog the column. While the pressure was regulated following extensive cleaning procedures and switching columns, it is still an important factor to consider as work continues. The C8 column was determined unusable after roughly 100 injections, which is neither normal nor ideal. For optimal column health and preservation, the column should be cleaned regularly and an end of run cleaning or rinse should be used. However, it is unknown whether or not the C18 column will exhibit the same problems.

4. Experiments using the C18 column

Throughout the beta-carotene runs, high pressure issues were encountered. Changing the guard column and extensive cleaning procedures only fixed the problem temporarily, so we switched to a C18 column. Again, fresh, single-component beta-carotene standards of the original concentrations were prepared and run along with the old multi-component standards containing the other compounds. Because of the longer C18 chain, the compounds remained

on the column longer, and retention times increased. The new retention time were approximately 12, 35, 27, and 30 minutes for zeaxanthin, chlorophyll a, chlorophyll b, and beta-carotene, respectively, and a full chromatogram is shown in Figure 25. Figure 26 shows the chromatogram and calibration curve for the new beta-carotene standards. The peak shape improved, but tails were still observed. The calibration curve, while still not linear, improved (r^2=0.8726). This indicates that standards and samples need to be run very soon after preparation, and that storage conditions must be an important consideration in future work.

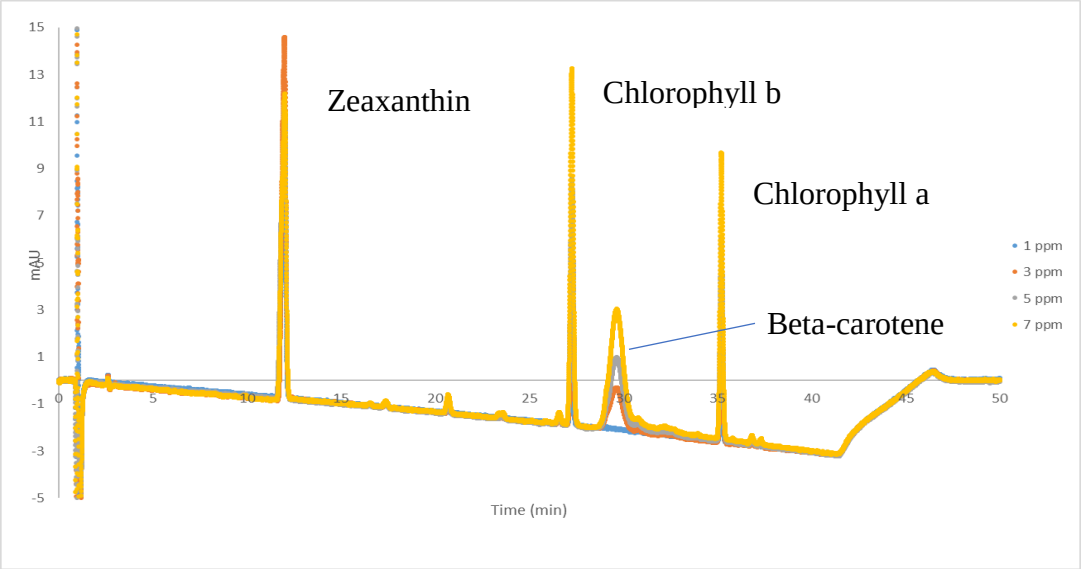

Figure 25: Full chromatogram at 450 nm from C18 run. This run occurred in March 2019 with beta-carotene standards prepared the same day and multicomponent standards from July 2018.

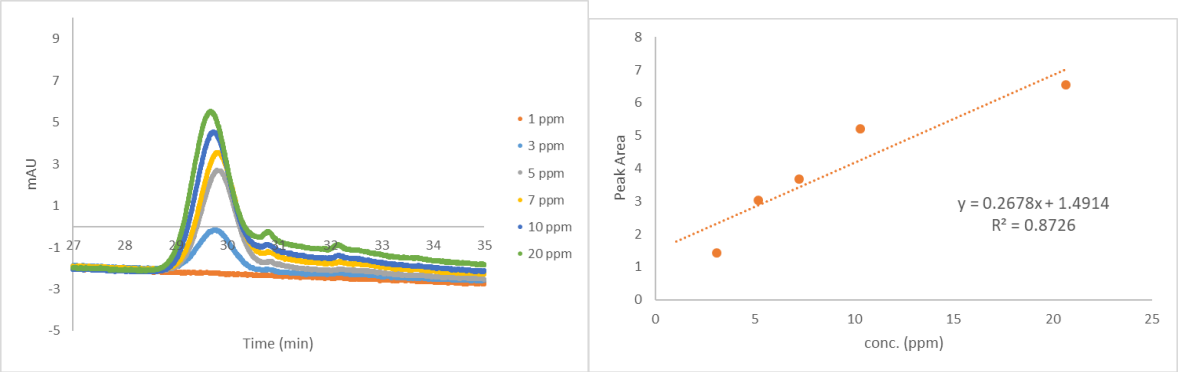

Figure 26: Beta-carotene chromatogram at 450 nm (left) and calibration curve (right, line of best fit: y=0.2678x+1.4914, r2=0.8726)

The chromatograms and calibration curves of zeaxanthin, chlorophyll a, and chlorophyll b are shown in Figures 27-29, respectively. (Beta-carotene was only detected in 3 of the 5 old standards and therefore not included.) The peak shapes of zeaxanthin, chlorophyll a, and chlorophyll b remained good, however, the calibration for zeaxanthin was no longer linear. The calibration curve resembles that of the beta-carotene results in initial runs (see Figure 21). Since zeaxanthin is also a carotenoid, this could indicate it is degrading similarly to beta-carotene, but at a slower rate.

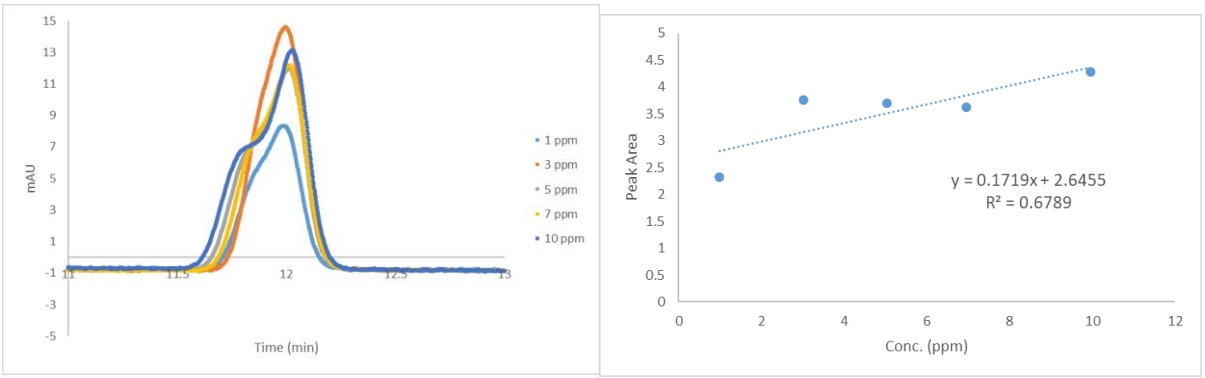

Figure 27: Zeaxanthin chromatogram at 450 nm (left) and calibration curve (right, line of best fit: y=0.1719x+2.6455, r^2=0.6789)

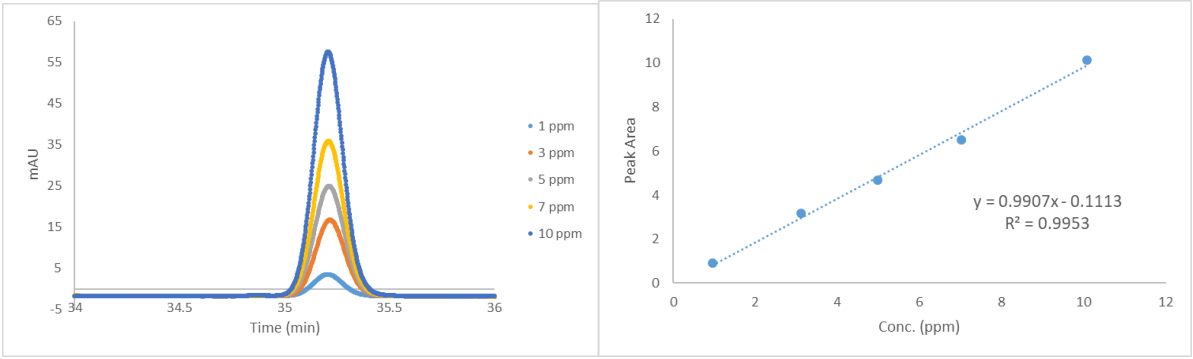

Figure 28: Chlorophyll a chromatogram at 666 nm (left) and calibration curve (right, line of best fit: y=0.9907x-0.1113, r^2=0.9953)

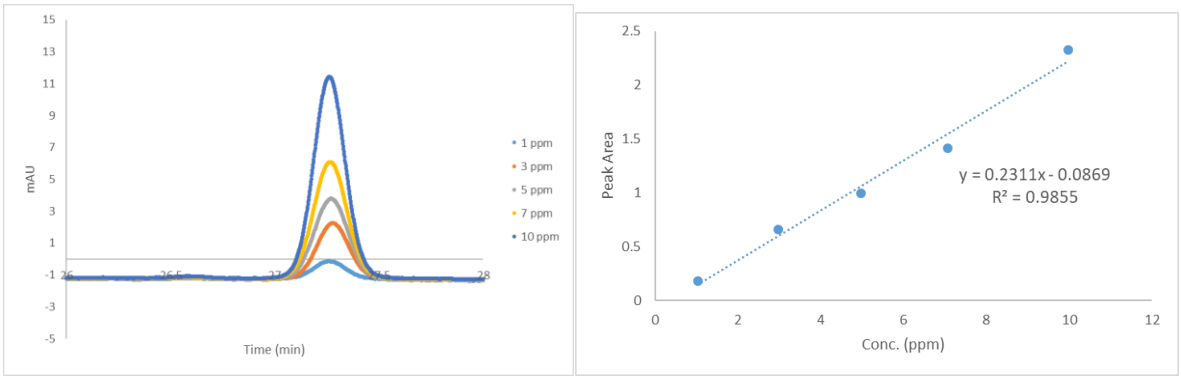

Figure 29: Chlorophyll b chromatogram at 645 nm and calibration curve (right, line of best fit: y=0.2311x-0.0869, r^2=0.9855)

5. UV-Vis measurements without chromatography

To determine if the non-linear behavior of beta-carotene was caused by the separation conditions—for example, beta-carotene not eluting completely from the column and impacting subsequent runs—the beta-carotene standards, old and new, were analyzed by UV-Vis alone. The solvent used was ethanol with 0.1% BHT. The absorption spectra of the old and new standards are shown in Figure 30. A preliminary explanation for the lack of linearity of beta-carotene was isomerization, and the multiple peaks in the chromatograms for beta-carotene could have been isomers. All-trans beta-carotene can transform to have some of the cis-isomer present, which absorbs between 330 and 345 nm rather than 450 nm (see Figure 8)[12]. However, the absorption spectra of the beta-carotene standards did not exhibit this peak, as shown in Figure 30, so the lack of linearity must be attributed to something else. Another possible explanation is that the peaks are artifacts caused by solvent and mobile phase that impact the solubility of the analyte.

Figure 31 shows the calibration curve using absorption values of the new standards. The r^2 was slightly higher but similar to the HPLC results. A calibration curve for the old standards was constructed but not shown because the r^2 value was 0.0054. The difference in linearity results indicates degradation and time are playing a larger role than chromatography

methods on the lack of linearity. Looking back, however, we should have performed these experiments in the same solvents used for HPLC (70:30 MeOH: 1M ammonium acetate, MeOH).

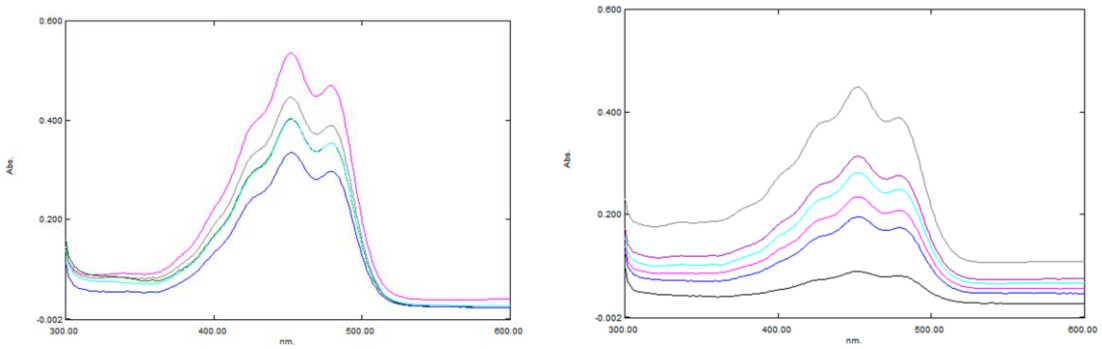

Figure 30: Absorption spectra of old (left) and new (right) beta-carotene standards. Old and new standards were prepared in October 2018 and March 2019, respectively.

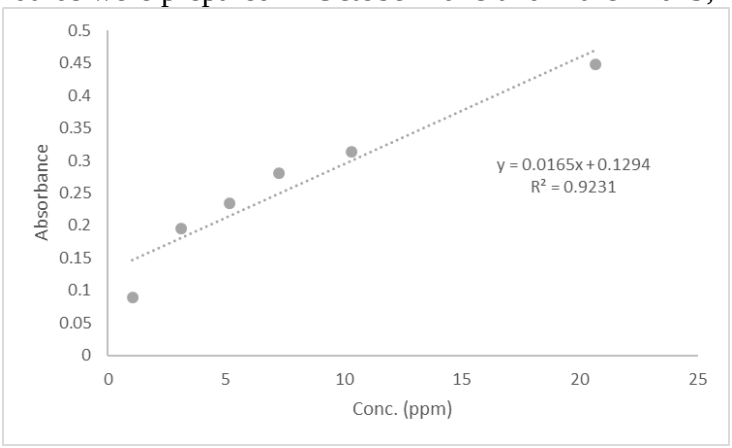

Figure 31: Beta-Carotene calibration curve using absorption values (line of best fit: $y=0.0165x+0.1294$, $r^2=0.9231$)

6. Future work

There are many directions this project could go. It is anticipated, however, that future work will focus on separation conditions. While the current method could be validated and apple samples run, we looked back on Mathew Kelley's chromatograms, and he had much cleaner peaks as well as a stronger signal. An example of his chromatograms is given in Figure 32. His work used acetone as a solvent, which is still not desirable, so a switch back to the old method would not be ideal. Our pressure issues were likely caused by buffer

remaining on the column, and most methods in the literature do not even use a buffer system. It could be investigated whether or not it is needed.

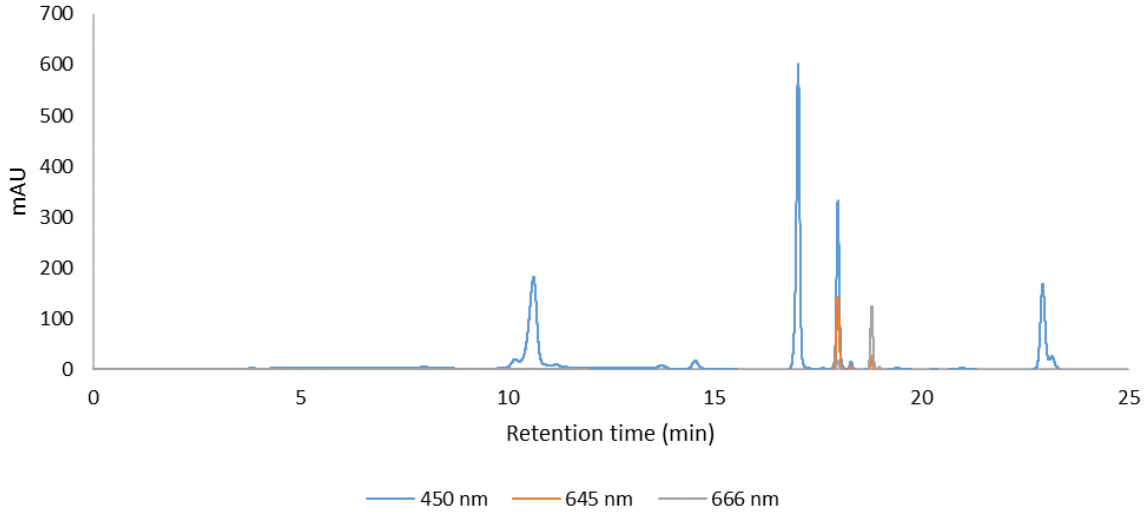

Figure 32: Chromatogram from Mathew Kelley's work, using acetone.[19]

Since beta-carotene degraded at an unexpected rate, it is possible that the BHT added to slow degradation is not at an optimal concentration. Varying the concentration of BHT in standards and monitoring storage effects could give insight into this. The full spectrum should always be acquired when using HPLC moving forward to monitor λ_{max} or the presence of cis-peaks. To additionally study isomerization of the compounds, the isomers could be iodine-induced or separated by saponification and analyzed this way rather than as a summation. However, to study isomers, a C30 column would likely be more efficient.[12] The rate of degradation could be rapid and standards should be run soon after their preparation. To prevent degradation, the standards could be nitrogen flushed. In a collaborative study between 12 laboratories of beta-carotene supplements, it was suggested that a 2-step standard purity process should take place at the time of injection using spectrophotometric and LC methods.[15] Something like this could be used to determine the standard concentrations are accurate. An internal standard would also help with this, and apocarotenal (CAS: 1107-26-

2), shown in Figure 33, has been obtained and will be used to improve accuracy and reduce error in the method.

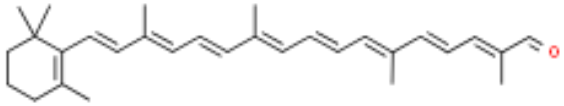

Figure 33: Structure of apocarotenal ($C_{30}H_{40}O$, MW: 416.64 g/mol), the compound to be used as the internal standard, as used by Delgado.[1]

The apple samples have been in the freezer for a couple of years, so the effect of storage on them should also be considered. A fresh apple could be extracted and compared to the old samples. The method could be expanded to include other varieties of apples or other fruits and vegetables. For example, a carrot or spinach could also be extracted. Lastly, this project is currently a collaboration between the chemistry and nutrition departments. It would be interesting to include the geography department to see if there is a geographical trend across North Carolina among the apple varieties and their pigment quantities.

Conclusion

Carotenoids and chlorophylls are compounds of importance to human health as they provide many benefits but are not generated naturally by the human body. The difficulty of analysis of carotenoids and chlorophylls indicates a need for a new and robust method to analyze these compounds. This work has allowed a method using reverse-phased HPLC to be developed with three of the four analytes of interest showing linearity. Further validation steps could allow the quantification of these pigments in North Carolina apple varieties, however, after switching to a C18 column and research into the effects of storage conditions, it is suggested the solvent system be reevaluated. Results from this experiment will be significant as they will indicate the nutrient value of the different types of apples, how the

values range throughout apple varieties, and whether or not a choice in apple type could contribute to one's nutritional health. While the scope of this project is currently limited to North Carolina apples, it could easily be expanded post-validation.

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