PRODUCTION OF ASTAXANTHIN BY *HAEMATOCOCCUS PLUVIALIS* USING DEPROTEINIZED WHEY PERMEATE AS A NUTRIENT SOURCE

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Dedication

To Mom, Dad, Allison, Aron, Becca, Lizzie, Gretchen, Holly, Julie, Katie, Kristen, Liza, Michael Ann, Michelle, and Wendy. Thank you.

Abstract

The goal of this project was to demonstrate the feasibility of a value-added use for deproteinized whey permeate (DWP) as feed material for the algae *Haematococcus pluvialis* to produce astaxanthin, the main carotenoid contributing to salmon and shrimp pigmentation. Algae was grown under 3 mixotrophic conditions: standard WC medium, standard WC medium with 10 g/L DWP, and 10 g/L DWP alone. Algae were harvested and carotenoids extracted with a modified Mojonnier method. The extracts contained free astaxanthin and astaxanthin mono- and diesters. Extracts were treated with cholesterol esterase to facilitate total astaxanthin quantification via HPLC-UV/VIS-MS/MS. Total astaxanthin dry weight was 2.11 μg (SD=0.80), 5.98 μg (SD=1.79), and 9.97 μg (SD=3.74) for the three treatments, respectively (n=4), indicating that DWP supports growth of *H. pluvialis* and that the algae can utilize lactose in DWP without hydrolysis. This is promising for dairy processors, who could utilize DWP as a novel income stream.

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1 Introduction

Algae, such as *Haematococcus pluvialis*, can be used to produce valuable food products including the carotenoid pigment astaxanthin. Carotenoids, such as astaxanthin, are required for animals' biological function. When astaxanthin is fed to some animals, such as salmon, they produce desirable coloring (Johnson and An, 1991). The carotenoid astaxanthin is also known to have high antioxidant activity (Ambati et al., 2014; Christaki, et al., 2013) and is used as a human nutritional supplement.

Astaxanthin may be produced synthetically, by yeast fermentation, or by algae. Astaxanthin derived from yeast fermentation and algae is approved for human consumption and is currently mostly used as human nutritional supplement, due to the high costs of production, but has the advantage of being marketable as a natural form of astaxanthin (Ambati et al., 2014; Nguyen, 2013; Lorenz and Cysewski, 2000). Synthetic forms of astaxanthin are primarily used for animal feed (Ambati et al., 2014; Nguyen, 2013). Astaxanthin for human consumption is among the algae-derived products of highest economic value ($2,500-$15,000/kg) yet, due to higher production cost than via chemical synthesis, only accounts for a minor share of astaxanthin on the market (Panis and Carreon, 2016; Shah et al., 2015).

The waste stream of deproteinized whey permeate (DWP) from whey protein concentrate production and ultrafiltered milk production has high levels of lactose and nitrogen, and a relatively high ash content. DWP is one of the dairy by-products with the least current application potential. This study was performed to investigate whether DWP could be utilized by *H. pluvialis* as a feed source. The consequence of this outcome would be that low-value DWP (for which the current supply exceeds the demand in terms of value-added applications) could be used by dairy plants to start cultivating algae on-site. The dairy industry already has a vast

body of knowledge on how to handle tanks and centrifuges, both required for astaxanthin production in photobioreactors (Lorenz and Cysewski, 2000). In a photobioreactor, algae could biometabolize minerals from DWP to increase their numbers (biomass) and produce astaxanthin. The amount of biomass and astaxanthin produced by algae vary depending on the growth conditions such as nutrient type, concentration, light, and temperature conditions (Del Campo et al., 2004). Currently, glucose is added for heterotrophic and mixotrophic growth in algal production of astaxanthin, yet it was unknown whether algae would be able to utilize lactose directly without hydrolysis (Julius, 2014, personal communication). This project evaluated whether food-grade deproteinized whey permeate could be used to produce astaxanthin. We hypothesized that *H. pluvialis* can metabolize the lactose in DWP to support the algae's growth and production of astaxanthin. This work involved method development for effective algal oil extraction, astaxanthin de-esterification, and astaxanthin quantification via high performance liquid chromatography and mass spectrometry.

2 Literature review

2.1 Deproteinized whey permeate

2.1.1 Source

Deproteinized whey permeate is a by-product of cheesemaking and ultrafiltered milk production. In cheesemaking, milk, fermenting microorganisms, and the rennet enzyme are combined until coagulation occurs. The curds are cut, stirred, and cooked, and the whey is drained off leaving curds and whey. The curds are used for cheese, and the whey can undergo a variety of processes to become a variety of products. One of these processes is ultrafiltration, which isolates some of the whey proteins for use in products like whey protein concentrate. Deproteinized whey permeate is formed when the remaining material undergoes lactose removal by crystallization. In ultrafiltered milk production, the components of milk are separated by size using pressure and membrane filtration. A portion of the milk's protein and fat is separated from the lactose, ash, and water. Dewatering the latter portion results in deproteinized whey permeate (Wisconsin Center for Dairy Research, 2008).

2.1.2 Composition

Deproteinized whey permeate is mostly lactose. To be called "permeate," the FDA requires the material have at least 59% lactose, and no more than 27% ash, 10% protein, and 6% moisture (Wisconsin Center for Dairy Research, 2008). Permeate does not contain fat. Typically in industry, dried permeate contains approximately 84% lactose, 9% ash, 3% protein, and 4% moisture (Wisconsin Center for Dairy Research, 2008). Protein is calculated by nitrogen content, and could represent non-protein nitrogen (Wisconsin Center for Dairy Research, 2008).

2.1.3 Current uses

Deproteinized whey permeate is low value, and the current supply of deproteinized whey permeate exceeds the demand in terms of value-added applications. Dairy companies producing DWP dispose of it as waste, sell it for animal feed, use it for land spreading, and sell it for food applications such as a solids source in baked goods or beverages. Disposal of deproteinized whey permeate often requires extensive pretreatments, costing dairy plants money. Land spreading DWP contributes significantly to land and water pollution due to its high biological oxygen demand (40,000-48,000 mg/L) and chemical oxygen demand (80,000-95,000 mg/L) (Kushwaha et al., 2011), which causes algal blooms in waterways.

2.2 Astaxanthin

2.2.1 Structure

Astaxanthin is a large, fat-soluble, red xanthophyll compound in the carotenoid class with formula $C_{40}H_{52}O_4$ and molar mass of 596.84 g/mol (Figure 1) (Ambati et al., 2014; Bruijn et al., 2016). Astaxanthin has two stereo centers, resulting in three isomers: (3S, 3S'), (3R, 3S'), and (3R, 3R'), all of which are naturally occurring (Higuera-Ciapara et al., 2006). Astaxanthin exists in both free and esterified forms. Both monoesters and diesters of astaxanthin are formed when one or both hydroxyl groups react with a fatty acid (Ambati et al., 2014). Free astaxanthin, astaxanthin monoesters, and astaxanthin diesters are all naturally occurring in microorganisms and aquatic animals, including the green alga *Haematococcus pluvialis* (Ambati et al., 2014).

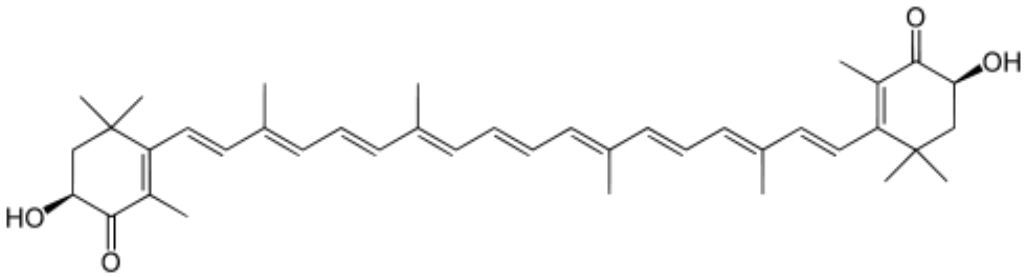


Figure 1. Free form astaxanthin (3,3′-dihydroxy-β, β′-carotene-4,4′-dione).

2.2.2 Uses

Astaxanthin has many commercial uses including use as a human nutritional supplement and use as salmon feed to give farm-raised salmon its signature red color (Ambati et al., 2014). Benefits to humans include antioxidant activity, anti-inflammatory properties, anti-diabetic activity, cardiovascular disease prevention, and immuno-modulation. Consuming a dose of 3.6 mg of astaxanthin daily can improve human health (Ambati et al., 2014).

Natural astaxanthin is reported to be the strongest known antioxidant, with antioxidant activity 100 times more effective than α-tocopherol, 65 times stronger than vitamin C, 54 times stronger than β-carotene, and 10 times more potent than lutein, canthaxanthin, and zeaxanthin (Niu et al., 2020; Shah et al., 2016). While synthetic astaxanthin also has antioxidant capacity, it is 20-fold lower than that of naturally derived astaxanthin (Shah et al., 2016). This could be because natural astaxanthin contains more monoesters and diesters than synthetic astaxanthin, and astaxanthin esters are more readily absorbed than free astaxanthin (Ambati et al., 2015). Synthetic astaxanthin is also not approved for human consumption, making natural astaxanthin the leading choice for human supplementation; natural astaxanthin has been used as a nutraceutical for over 17 years without detrimental side-effects (Shah et al., 2016).

2.2.3 Market

The global astaxanthin market was valued at $447 million in 2014, and is expected to exceed a value of $1.5 billion in 2020 (Panis and Carreon, 2016; Shah et al., 2016). Production of algae-produced astaxanthin only accounts for 1% of this (primarily due to high cost and challenges of production), but the natural astaxanthin segment demand is not met, and its growth outpaces that of synthetic astaxanthin (Shah et al., 2016). Unmet demand can be attributed to increased consumer interest in natural products and dietary supplements. While natural astaxanthin is more costly to produce than synthetic astaxanthin, it sells for 3-4 times the value of synthetic astaxanthin (Shah et al., 2016). Natural astaxanthin sells for $2,500-15,000/kg depending on purity and pigmentation, while the cost to produce it is about $1,000/kg, promising reliable income for producers (Shah et al., 2016). Recent increasing demand has prompted several companies to double their production capabilities to meet demand and cash-in on this profitable market (Shah et al. 2016). Companies producing astaxanthin via *H. pluvialis*, include Algaetech International SDN BDH, Algatechnologies Ltd., Atacama Bio Natural, Beijing Ginko Group (BGG) Biological Technology Co. Ltd., BioReal (Sweden) AB, Britannia Health Products Ltd., Cyanotech Corporation, Fuji Chemical Industry Co. Ltd., Jingzhou Natural Astaxanthin Inc., Kunming Biogenic Co. Ltd., Mera Pharmaceuticals Inc., Parry Nutraceuticals Ltd. (EID Parry), Stazen Inc., Supreme Biotechnologies NZ Ltd., and Wefirst Biotechnology Co. Ltd. (Shah et al., 2016). Cyanotech was the first producer to do in-house supercritical fluid extraction of astaxanthin from *H. pluvialis*, and is the number one astaxanthin brand in the United States (Khani, 2018). Cyanotech has an impressive 90 acre natural astaxanthin production facility in Kailua-Kona, Hawaii, USA, where they use outdoor raceway ponds to produce United States Pharmacopeia (USP®) verified BioAstin® softgels (Khani, 2018), each containing 12 mg astaxanthin from H. pluvialis. In 2020, Cyanotech's combined astaxanthin and spirulina

business saw nearly $32,000,000 in net sales, and gross profit of nearly $13,000,000 (Cysweski, 2020).

2.3 *Haematococcus pluvialis*

2.3.1 Characteristics

Haematococcus pluvialis is a green alga found in bodies of freshwater across the globe, especially small temporary pools of water (Shah et al., 2016). *H. pluvialis* can tolerate extreme conditions due to its ability to encyst (Shah et al., 2016). Under nutrient deprivation, extreme temperature, salinity, or light, the algal cell wall thickens and hardens and the algae accumulates high levels of astaxanthin (Shah et al., 2016). Stress is positively correlated with accumulation of astaxanthin in *H. pluvialis* (Shah et al., 2016).

2.3.2 Life cycle

H. pluvialis primarily undergoes vegetative reproduction. Under stress conditions such as high light, high salinity, extreme temperature, and lack of nutrients, *H. pluvialis* becomes encysted and produces the red pigment astaxanthin. The life cycle of *H. pluvialis* includes four phases: the green vegetative macrozooid, microzooid, and palmella stages, and the red encysted hematocyst phase involving astaxanthin accumulation (Shah et al., 2016). When macrozooids become stressed, they lose flagella and become larger, non-motile palmella (Shah et al., 2016). If stress conditions persist, the palmella enter the hematocyst stage and accumulate astaxanthin and other carotenoids in lipid droplets, causing the algae to turn red (Shah et al., 2016). *H. pluvialis* produces up to 3-5% astaxanthin in both free and esterified forms on a dry weight basis (Shah et al., 2016), with monoesters being the most abundant at 70%, followed by diesters at 25%, and free-form at 5% (Galarza et al., 2019; Yuan et al., 2011). A

study by Karsten Holtin et al. identified C18:3, C18:2, C18:1, and C16:0 as the most commonly occurring monoesters of astaxanthin in an oil extract derived from Haematococcus pluvialis. Extracts in this study also contained astaxanthin diesters, most commonly C18:1/C18:1, but in lower concentrations than astaxanthin monoesters (Holtin et al., 2009). If hematocysts undergo great stress such as starvation, drying out, or freezing, followed by non-stress conditions, the hematocysts will undergo gametogenesis to become microzooids. Alternatively, if hematocysts forego such extreme conditions prior to returning to normal environmental conditions, they will germinate to form flagellated macrozooids (Shah et al., 2016).

2.4 Production, harvesting, identification, and quantification of astaxanthin by *Haematococcus pluvialis* using deproteinized whey permeate as a nutrient source

2.4.1 Existing production of astaxanthin by *Haematococcus pluvialis*

Haematococcus pluvialis algae is the leading choice for production of natural astaxanthin (Shah et al., 2016). Demand for natural astaxanthin is growing, as it is approved for human consumption (Ambati et al., 2014; Shah et al., 2016). Astaxanthin is commercially produced by many manufacturers, but less than 1% of commercial production is from *H. pluvialis* (Shah et al., 2016). Commercial *H. pluvialis* astaxanthin production operations use heterotrophic, photoautotrophic, and mixotrophic conditions to support algal growth (Shah et al., 2016). These operations can be indoors in photobioreactors or outdoors in raceway ponds (Shah et al., 2016). Producers induce a stress state for *H. pluvialis* through control of light, salinity, nutrients, and temperature, triggering the production of astaxanthin (Shah et al., 2016). Heterotrophic operations—which use carbon and nutrients as

energy sources to grow algae—have the benefit of not requiring light, eliminating a large expense (Shah et al., 2016).

2.4.2 Harvesting, identification, and quantification of astaxanthin from *Haematococcus pluvialis* in commercial and laboratory settings

Harvesting *H. pluvialis* and recovering its astaxanthin is challenging (Panis and Carreon, 2016; Shah et al., 2016). Harvesting is typically done via centrifugation to de-water the algae (Shah et al., 2016). Then, manufacturers use expeller pressing or bead milling to overcome the thick encysted cell wall and release the oil containing astaxanthin (Panis and Carreon, 2016; Shah et al., 2016). Expeller pressing is a one step process that uses pressure to break through the *Haematococcus pluvialis* cell wall—this method has the benefit of being easy to execute, and has a 75% oil recovery efficiency and low rate of contamination (Shah et al., 2016). Bead milling grinds algae against glass beads to break open the algal cell walls, releasing oil into a solvent. This method is also effective, but requires use of a solvent and works best with algal cake biomass of concentration of 100-200 g/L (Shah et al., 2016).

Next, biomass is quickly dried to preserve the pigment via freeze drying, spray drying, or solar drying. While freeze drying is most effective at preserving the extracts, it is very expensive, so spray draying is the most preferred commercial drying approach (Shah et al., 2016).

Finally, astaxanthin is recovered, most commonly via supercritical fluid extraction with carbon dioxide solvent (Holtin et al., 2009; Panis and Carreon, 2016; Shah et al., 2016). However, supercritical fluid extraction equipment is expensive (Rosa and Meireles, 2004), so laboratory settings may instead use benchtop lipid extraction methods for algal oil extraction. Lipids are soluble in organic solvents such as chloroform and ethers, and are characterized by their insolubility in water (Nielsen, 2010). Different lipids have varying solubility in

different organic solvents, so it is important to find solvents that are effective for the lipids of interest (Nielsen, 2010). In the case of oil extraction from *Haematococcus pluvialis*, repeated extraction using petroleum ether and diethyl ether is effective. These solvents are commonly used for oil extraction in compositional analysis of food products, specifically via a Mojonnier extraction (Nielsen, 2010). Mojonnier extraction involves pre-hydrolysis and typically utilizes specialized flasks that facilitate separation of the ether layer (Nielsen, 2010). The procedure can be modified for smaller sample sizes by using small glass vials instead of specialized flasks. Pretreatment using chemical or enzyme mediated hydrolysis prior to extraction can reduce matrix effects if lipids are bound to other components (Nielsen, 2010). Extraction efficiency is inversely correlated with particle size, so mechanical breakdown may be used, especially for non-homogeneous samples (Nielsen, 2010). Another common method for lipid extraction, especially in food matrices, is Folch extraction (Nielsen, 2010). Folch extraction uses chloroform and methanol as solvents (Nielsen, 2010). Solvent extraction using acetone may also be used, and has shown some success when paired with hydrochloric acid pretreatment (Dong et al., 2014).

In industry, spectrophotometric methods for astaxanthin quantification are commonly used, and tend to overestimate astaxanthin by up to 20%, as other carotenoids often present in algal extracts absorb at the same wavelength as astaxanthin (Runco and Chen, 2014). While this method is simple and rapid, the overestimation can be misleading for consumers.

Astaxanthin quantification by HPLC is more accurate, but it is complex and has long analysis times (Runco and Chen, 2014). Complexity is due to *H. pluvialis* derived astaxanthin being present in numerous esterified forms, necessitating either individual standards for each astaxanthin mono- and diester (which are expensive and challenging to obtain), or pre-hydrolysis to cleave esters and allow quantification of astaxanthin using a free-astaxanthin standard paired with internal

standard with similar absorbance profile and extinction coefficient. If HPLC is used for astaxanthin quantification, C30 columns are efficient at separating groups of similar compounds (Holtin et al., 2009), making them a good choice for separation of free and esterified astaxanthin, but separation efficiency could still be improved (Miao et al., 2006).

If pre-hydrolysis to de-esterify astaxanthin esters is pursued, either saponification or enzyme mediated hydrolysis may be used, but the latter has been reported to be more effective in multiple studies (Galarza et. al., 2019). Saponification can be done with NaOH or KOH, but more studies are needed to develop effective concentrations and reaction times that minimize astaxanthin degradation (Galarza et al., 2019). Multiple studies have shown cholesterol esterase to be an effective enzyme for at de-esterification of astaxanthin esters (Galarza et al., 2019)

2.4.3 Deproteinized whey permeate as a nutrient source for *Haematococcus pluvialis*

Glucose is used as a carbon source in heterotrophic and mixotrophic growth of *Haematococcus pluvialis* for commercial astaxanthin production (Julius, 2014, personal communication). It was unknown whether *H. pluvialis* could instead utilize lactose as a carbon source without pre-hydrolysis (Julius, 2014, personal communication). Deproteinized whey permeate (DWP) from whey or milk protein concentrate production contains a high concentration of lactose, nitrogen, and a relatively high ash content that algae can use for their nutrition.

3 *Haematococcus pluvialis* growth, oil extraction and de-esterification, compound identification, and astaxanthin quantification

3.1 Introduction

The goal of this project was to demonstrate the feasibility of a value-added use for deproteinized whey permeate (DWP) as feed material for *Haematococcus. pluvialis* to produce astaxanthin. It was not known whether algae could utilize lactose directly without hydrolysis. *H. pluvialis* algae were grown under three feeding conditions—standard algal growth medium, standard growth medium with deproteinized whey permeate, and deproteinized whey permeate alone. Algal oil was extracted and analyzed via high performance liquid chromatography and mass spectrometry. This project prioritized development of non-spectrophotometric methods to more accurately quantify astaxanthin from *H. pluvialis*.

Several methods were evaluated for oil extraction, compound identification, and astaxanthin quantification from *Haematococcus pluvialis*. While in industry, super critical fluid extraction is used, benchtop methods were evaluated to maximize extraction efficiency. Evaluated oil extraction methods include Folch extraction with 2:1 chloroform:methanol, microwave extraction with 2:1 chloroform:methanol, extraction with acetone, and an adapted Mojonnier extraction with petroleum ether and diethyl ether. These methods were combined with various pretreatments including sonication, acid hydrolysis, and agitation with a tissue grinder. Several methods did not extract all of the red pigment from the algae, so they were not pursued further. Folch extraction with sonication pretreatment, acetone extraction with tissue grinder pretreatment, and the adapted Mojonnier extraction with acid pretreatment all extracted all of the red color from the *Haematococcus pluvialis* upon visual evaluation. Detailed methods are in

Appendices 1 and 2, and Section 3.3.2, respectively. Oil extraction efficiency by the three methods was quantified, and showed that the adapted Mojonnier method with acid pretreatment extracted the most oil on a weight basis (g oil/100 g biomass), as shown in Figure 2 (raw data in Appendix 3).

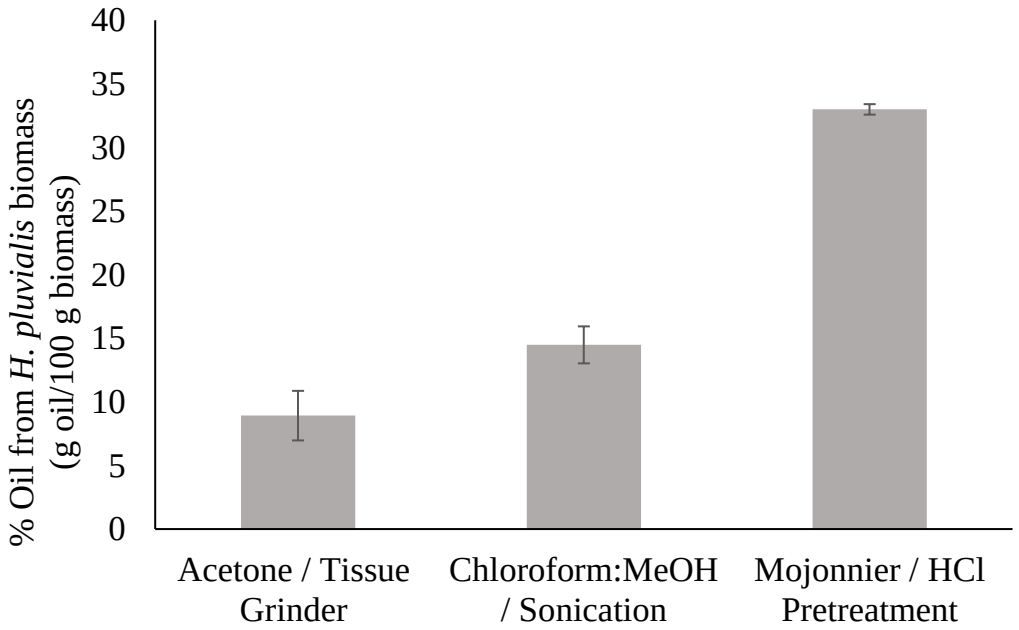


Figure 2. Efficiency of three different oil extraction methods from *Haematococcus pluvialis* on a weight basis (n=2-6).

H. pluvialis produces several astaxanthin esters and other carotenoids, so efficient separation is required for quantification. Over twenty different high performance liquid chromatography (HPLC) UV-Vis methods were evaluated to optimize compound separation in the algal oil extracts. Methods screened used C18 and C30 columns for reverse-phase chromatography. Other variables that were tested include isocratic versus gradient mobile phases, mobile phase composition, column temperature, flow rate, and method duration. A C30 column with a gradient decreasing in polarity provided the best separation of the numerous esters in the *H. pluvialis* oil extracts.

Since astaxanthin present in the extracted oil of *H. pluvialis* is mainly present in esterified forms rather than as free astaxanthin, quantification is challenging. Astaxanthin ester standards are difficult or impossible to purchase, and are expensive, when available. De-esterification of astaxanthin is one way to resolve this. Two de-esterification methods were evaluated: chemical hydrolysis using 0.17 M potassium hydroxide, and cholesterol esterase enzyme mediated hydrolysis. *H. pluvialis* extracts treated with both de-esterification methods underwent identical preparation for HPLC. Resulting HPLC chromatograms demonstrated that cholesterol esterase mediated hydrolysis was more effective in converting astaxanthin esters to free astaxanthin.

Once these most efficient analysis methods were identified, *H. pluvialis* was grown in the Julius Lab at St. Cloud State University (St. Cloud, Minnesota, USA) under the three feeding conditions. Algae was then freeze-dried and sent to the Schoenfuss Lab at University of Minnesota (St. Paul, Minnesota, USA), where the algal oil was extracted, and oil extracts were de-esterified and analyzed for composition and astaxanthin quantification via HPLC UV/Vis using the selected methods. MS and MS/MS were also used for confirmation of compound identification. These methods and the results are described in detail in this chapter.

3.2 Materials

3.2.1 Growth of *Haematococcus pluvialis*

Haematococcus pluvialis was sourced from a stock culture from the Julius lab in the Biology Department at St. Cloud State University. Materials for standard WC medium (Table 1) (Guillard & Lorenzen, 1972) were sourced from the Julius Laboratory at St. Cloud State University. 20 L carboys and fluorescent lamps were used in algal growth (Figure 3). Agropur Ingredients (La Crosse, WI) provided the Crinolac DPS freeze-dried deproteinized whey permeate (DWP). Compositional

analysis methods from *Standard Methods for the Examination of Dairy Products* were used for compositional analysis of DWP, which showed the material to mainly be composed of lactose (Table 2) (Richardson, 1985).

Table 1. WC medium composition (adapted from Guillard & Lorenzen, 1972).

Component	mg/L
$CaCl_2 \cdot 2H_2O$	36.76
$MgSO_4 \cdot 7H_2O$	36.97
$NaHCO_3$	12.60
K_2HPO_4[a]	8.71
$NaNO_3$[a,b]	85.01
$Na_2SiO_3 \cdot 9H_2O$	28.42
Trace metals[c,d]	**mg/L compound**
$Na_2 \cdot EDTA$[e]	4.36
$FeCl_3 \cdot 6H_2O$[e]	3.15
$CuSO_4 \cdot 5H_2O$	0.01
$ZnSO_4 \cdot 7H_2O$	0.022
$CoCl_2 \cdot 6H_2O$	0.01
$MnCl_2 \cdot 4H_2O$	0.18
$Na_2MoO_4 \cdot 2H_2O$	0.006
H_3BO_3	1.0
Vitamins	
Thiamin·HCl	0.1 mg/L
Biotin	0.5 µg/L
B_{12}	0.5 µg/L

[a] Nitrate and phosphate should be reduced 2-10 fold for sensitive organisms
[b] Use NH_4Cl, 2.65-26.5 mg/L (50-500 µm) for organisms that cannot utilize nitrate.
[c] The more complete trace metal solution using Ferric citrate-citric acid described by Wright, 1964, can also be used.
[d] The pH of the trace metal solution is best adjusted to 4-4.5 as a compromise to retain as a compromise to retain solubility of the metals in a X 1000 stock solution yet not lower the pH of the final nutrient solution too much.
[e] Ferric citrate-citric acid 3-9 mg/L each can be used in place of ferric EDTA.

Figure 3. 20 L carboys and fluorescent lamps used in growth of *Haematococcus pluvialis.*

Table 2. Deproteinized whey permeate (DWP) composition.

Component	% of DWP Composition
Protein	3
Lactose	82
Moisture	5
Ash	10

3.2.2 Oil extraction from *Haematococcus pluvialis*

95% Ethanol and 26% hydrochloric acid, diethyl ether, and petroleum ether were sourced from Sigma Aldrich (Sigma Aldrich Inc., St. Louis, MO, USA).

3.2.3 De-esterification of *Haematococcus pluvialis* oil extracts

Cholesterol esterase, >99.9% acetone, >95% all-*trans*-canthaxanthin, 12 H_2O sodium sulfate, petroleum ether, and dimethyl sulfoxide were sourced from Sigma Aldrich (Sigma Aldrich Inc., St. Louis, MO, USA). A 0.05 M Tris(hydroxymethyl)aminomethane hydrochloride buffer pH 7 was prepared with

materials from Sigma Aldrich (Sigma Aldrich Inc., St. Louis, MO, USA). Shimadzu UV-Vis spectrophotometer was used (Shimadzu Corporation, Kyoto, Japan).

3.2.4 HPLC and MS analysis and astaxanthin quantification

Cholesterol esterase, methanol, methyl tert-butyl ether, all-*trans*-astaxanthin standard, and canthaxanthin standard were sourced from Sigma Aldrich (Sigma Aldrich Inc., St. Louis, MO, USA). Shimadzu HPLC was used with Shimadzu UV/VIS PDA (Shimadzu Corporation, Kyoto, Japan) and a 250 cm x 4.6 mm, 5 μm C30 Prontosil column (Bischoff, Leonberg, Germany). LCQ Finnegan MAT TRAP ion trap, Spectra System P4000 pump, AS3000 Sample/Column compartment were used in MS and MS/MS analysis (Thermo Separation Products, Piscataway, New Jersey, USA).

3.3 Methods

3.3.1 Growth of *Haematococcus pluvialis*

Two liters of *Haematococcus pluvialis* stock culture (ca. 25% peak density) was grown at St. Cloud State University in 20 L carboys under 24 hour light at 4000 lux under the following three feeding conditions in duplicate:

1) Standard WC medium
2) Standard WC medium + 10 g/L deproteinized whey permeate
3) 10 g/L deproteinized whey permeate

Media was sterilized by autoclaving. After the algae exhausted all the nutrients present, it entered a stress state, which it was left in for 10 days until it was harvested, dewatered by centrifuge, and freeze-dried.

3.3.2 Oil extraction from *Haematococcus pluvialis*

Oil extraction from *Haematococcus pluvialis* was conducted on each of the three growth treatments, in duplicate. For each sample, 0.8 mL ethanol was added to 0.42 g freeze-dried *H. pluvialis* and mixed to prevent particle lumping upon addition of acid. 2.4 mL of 26% hydrochloric acid was then added to sample and mixed well by inverting vial and occasionally releasing pressure. Sample was placed in 70-80°C water bath for 30 minutes with frequent mixing by vial inversion. After acid pretreatment, 0.8 mL ethanol was added to sample, and sample was allowed to cool to room temperature in fume hood. Once cooled, 6 mL diethyl ether was added to sample and sample was shaken for 1 minute to mix. Then, 6 mL petroleum ether was added to sample and sample was shaken for 1 minute to mix. Sample was then centrifuged at with 660 xg (1700 rpm, 204 mm radius) for 10 minutes at 4°C. Upper ether layer was transferred to glass collection vial via glass Pasteur pipette. A second extraction was performed by adding 6 mL diethyl ether and 6 mL petroleum ether to remaining sample, shaking for 1 minute between each addition. Sample was then centrifuged at 1700 rpm for 10 minutes at 4°C and upper ether layer was transferred to same glass collection vial as in first extraction. A third extraction was conducted using the same method as extraction two; in the third extraction the upper layer contained all of the red pigment as shown in Figure 4. After three extractions, the collection vial extract was dried under nitrogen gas at 5 psi at 35°C. Headspace of dried extract was flushed with nitrogen and extracts were stored at -20°C in darkness until use.

Figure 4. Third extraction of adapted Mojonnier extraction of oil from *Haematococcus pluvialis.* Upper layer contains algal oil, including astaxanthin.

3.3.3 De-esterification of *Haematococcus pluvialis* oil extracts

In order to simplify quantification of total astaxanthin in *Haematococcus pluvialis* oil extracts (as standards of astaxanthin mono- and diesters are numerous and expensive to obtain, if available at all), oil extracts were treated with cholesterol esterase to decrease the peak areas of astaxanthin mono- and diesters and in turn increase the peak areas of free astaxanthin peaks on HPLC. Extracts were spiked with an all-*trans*-canthaxanthin standard to calculate recovery.

Hydrolysis was conducted via cholesterol esterase, using a method adapted from Jacobs et al. (1982). Each dried oil extract was resolubilized in acetone and standardized to an absorbance of 0.8-1.2 at 475 nm on a spectrophotometer. 2 mL of a 0.05 M Tris-HCl buffer at pH 7.0 and 0.1 mL all-*trans*-canthaxanthin standard (1 mg canthaxanthin in 10 mL methyl tert-butyl ether) were added to 3 mL of each dilution. Cholesterol esterase (100 U per 22 mg solid) was dissolved in the same buffer (3.3 mg in 4.41 mL), and 0.6 mL of the enzyme solution were added to each sample. Enzyme reaction was allowed to proceed for 90 minutes at 37°C, after which 1 g of sodium sulfate was added to each sample, and the organic layer was

extracted with petroleum ether until colorless (1 x 4 mL extraction followed by 2 x 2 mL extractions). The upper ether layer was transferred via a glass Pasteur pipet to a new glass tube. Each sample's extract was then dried under nitrogen and resolubilized in 400 μL dimethyl sulfoxide and syringe filtered with 0.45 μm filter.

3.3.4. HPLC and MS analysis and astaxanthin quantification

Both algal oil extracts treated with cholesterol esterase and untreated extracts were analyzed via UV/Vis RP-HPLC using a 250 cm x 4.6 mm, 5 μm C30 Prontosil column (Bischoff, Leonberg, Germany) operated at 30°C. Mobile phase A consisted of methanol/methyl tert-butyl ether/double distilled water (83:15:2, v/v/v) and mobile phase B of methanol/methyl tert-butyl ether/double distilled water (8:90:2, v/v/v). A flow rate of 1 mL/min and the following gradient were used: 0-20 min, hold 100% A; 20-105 min, 100-49% A; 105-107 min, 49-100% A; 107-110 min, hold 100% A. This method was adapted from Holtin et al. (2009). All-*trans*-astaxanthin was quantified with UV/VIS PDA at 468 nm to develop a standard curve of all-*trans*-astaxanthin (Figure 5). The astaxanthin *cis* isomers were quantified using this same all-*trans*-astaxanthin standard curve (468 nm), as they have similar extinction coefficients (Tai and Chen, 2000). Total astaxanthin was quantified on a dry weight basis using *H. pluvialis* extracts treated with cholesterol esterase.

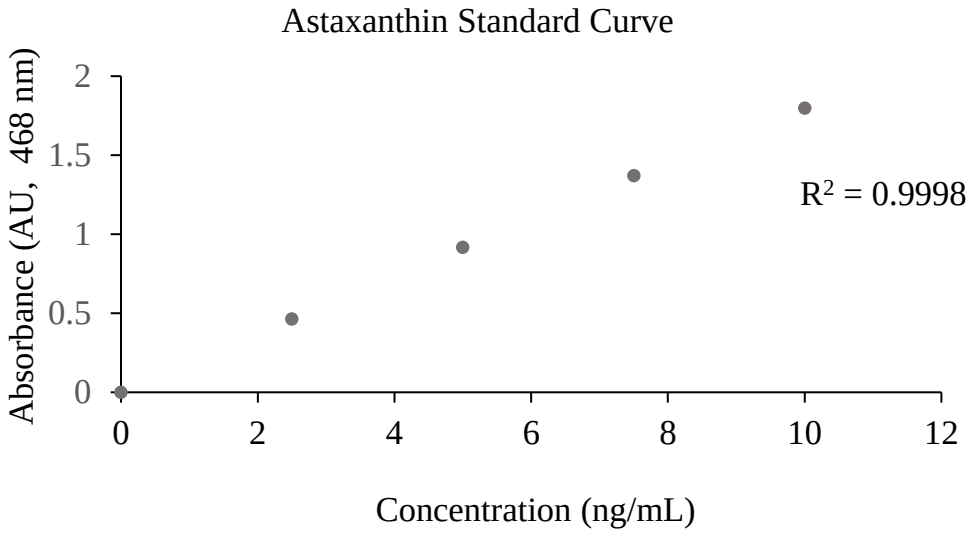


Figure 5. Absorbance (AU) at 468 nm as a function of concentration of an all-*trans*-astaxanthin standard.

Extracts were also analyzed via RP-HPLC with detection via UV/Vis PDA as well as APCI MS and MS/MS (Thermo Separation Products, Piscataway, New Jersey, USA) using the same C30 column and mobile phases as described in previous paragraph, but at room temperature. The gradient used was also slightly different: 0-20 min, hold 100% A; 20-120 min, 100-40% A; 120-125 min, 40-100% A; 125-130 min, hold 100% A. UV/Vis PDA detection was between 200-800 nm and the ion trap detected positive ions between 200-1200 m/z for MS and 400-1300 m/z for MS/MS. For both MS and MS/MS, the following conditions were used: sheath gas flow rate, 80; capillary temperature, 225°C; aux. gas rate, 10, S-lens RF level, 55%; source heater temperature, 475°C; capillary voltage, 29.00 V; tube lens offset, -20.00 V; and discharge current, 5 or 9 uA (both were used in analysis). The resulting MS and MS/MS data was used to identify mono- and diesters of astaxanthin and to verify the identity of all-*trans*-astaxanthin.

Additional MS and MS/MS analysis was conducted on extracts treated with cholesterol esterase to verify identity of *cis* isomers of astaxanthin. This analysis

used the same conditions as described in the immediate previous paragraph except the gradient was the following: 0-20 min, hold 100% A; 20-105 min, 100-49% A; 105-107 min, 49-100% A; 107-110 min, hold 100% A.

3.4 Results

3.4.1 Growth of *Haematococcus pluvialis*

Table 3 displays the biomass on a dry weight basis of each of the feeding conditions in duplicate. Biomass was different between the three feeding conditions, with DWP alone resulting in the most biomass, and WC medium alone resulting in the least biomass.

The algae successfully grew under standard WC medium, standard WC medium with DWP, and DWP alone. This confirmed that *H. pluvialis* are able to use lactose in DWP, so lactose hydrolysis is not necessary.

Table 3. Freeze-dried biomass (g) of *Haematococcus pluvialis* under various growth conditions.

Growth condition	Freeze-dried biomass (g/20 L)
Standard WC medium (Guillard & Lorenzen, 1972) - Sample A, Duplicate 1	1.92[a]
Standard WC medium - Sample A, Duplicate 2	1.82[a]
Standard WC medium - Sample B, Duplicate 1	1.39[a]
Standard WC medium - Sample B, Duplicate 2	1.39[a]
Standard WC medium + 10 g/L DWP - Sample D, Duplicate 1	3.33[b]
Standard WC medium + 10 g/L DWP - Sample D, Duplicate 2	3.33[b]
Standard WC medium + 10 g/L DWP -Sample E, Duplicate 1	2.48[b]
Standard WC medium + 10 g/L DWP -Sample E, Duplicate 2	2.48[b]
10 g/L DWP - Sample F, Duplicate 1	4.77[c]
10 g/L DWP - Sample F, Duplicate 2	4.77[c]
10 g/L DWP - Sample G, Duplicate 1	4.42[c]
10 g/L DWP - Sample G, Duplicate 2	4.42[c]

DWP = deproteinized whey permeate

3.4.2 Oil extraction from *Haematococcus pluvialis*

Table 4 displays the percent oil extracted on a dry weight basis for each of the feeding conditions in duplicate. A higher percent of oil was extracted from samples grown with WC medium alone than from samples grown under the other two feeding conditions.

Table 4. Percent oil extracted on a dry weight basis of *Haematococcus pluvialis* under various growth conditions.

Growth condition	% oil extracted
Standard WC medium (Guillard & Lorenzen, 1972) - Sample A, Duplicate 1	38.75[a]
Standard WC medium - Sample A, Duplicate 2	39.40[a]
Standard WC medium - Sample B, Duplicate 1	23.33[a]
Standard WC medium - Sample B, Duplicate 2	38.76[a]
Standard WC medium + 10 g/L DWP - Sample D, Duplicate 1	15.50[bc]
Standard WC medium + 10 g/L DWP - Sample D, Duplicate 2	17.29[b]
Standard WC medium + 10 g/L DWP -Sample E, Duplicate 1	14.80[bc]
Standard WC medium + 10 g/L DWP -Sample E, Duplicate 2	16.97[b]
10 g/L DWP - Sample F, Duplicate 1	11.44[c]
10 g/L DWP - Sample F, Duplicate 2	15.91[b]
10 g/L DWP - Sample G, Duplicate 1	12.11[c]
10 g/L DWP - Sample G, Duplicate 2	14.27[c]

3.4.3 De-esterification of *Haematococcus pluvialis* oil extracts, HPLC and MS analysis, and astaxanthin quantification

HPLC and MS evaluation show that for *H. pluvialis* oil extracts, general compound elution order began with free astaxanthin followed by triacylglycerols, then monoesters of astaxanthin, and lastly diesters of astaxanthin. An HPLC chromatogram of algae grown with standard WC medium and its corresponding MS profile are used to demonstrate this for all growth conditions, as HPLC and MS compound-type elution order was constant regardless of growth condition (Figure 6, Table 5). Within compound type (for triacylglycerols, monoesters of

astaxanthin, and diesters of astaxanthin), shorter chains eluted before longer chain fatty acids among compounds with the same level of unsaturation (Table 5). Within compound type (for triacylglycerols, monoesters of astaxanthin, and diesters of astaxanthin), among chains of the same length, less saturated compounds eluted before more saturated compounds (Table 5). The fatty acids of the triacylglycerols and diesters of astaxanthin were primarily C16 and C18 chain fatty acids (Table 5). Two isomers of pheophytin were present in the *H. pluvialis* extracts (Figure 6, Table 5). The carotenoids lutein, canthaxanthin, and zeaxanthin (commonly produced by *H. pluvialis*), were not identified in the extracts.

Cholesterol esterase treatment of extracts from algae grown under all three feeding conditions caused a decrease in peaks of mono- and diesters of astaxanthin and an increase in the free astaxanthin peak. This allowed for quantification of astaxanthin by area under the free astaxanthin peak in samples treated with cholesterol esterase. Figures 7-18 show extracts grown under all three feeding conditions without cholesterol esterase treatment.

HPLC chromatograms of cholesterol esterase treated algal extracts can be divided into two categories: those that were analyzed prior to HPLC instrument maintenance (Figures 19-20, 22-25, and 27-29), and those that were analyzed after HPLC instrument maintenance (Figures 21 and 26). There is no chromatogram for the cholesterol esterase treated Sample E, Duplicate 1. HPLC instrument maintenance caused a shift in retention times of all-*trans*-astaxanthin and all-*trans*-canthaxanthin standards, so extracts analyzed pre- and post-instrument maintenance used Figure 30 and Figure 31, respectively, to determine astaxanthin peak location to quantify free astaxanthin.

For each 20 L carboy algae grown with standard WC medium, standard WC medium with 10 g/L DWP, and 10 g/L DWP alone; total astaxanthin dry weight was 2.11 μg (SD=0.80), 5.98 μg (SD=1.79), and 9.97 μg (SD=3.74), respectively (n=4) (Figure 32). All astaxanthin isomers were included in this quantification.

Both *H. pluvialis* growth treatments that included DWP produced more astaxanthin than those grown with standard WC medium alone. Total astaxanthin dry weight produced by algae grown with standard WC medium with 10 g/L DWP versus algae grown with 10 g/L DWP alone were not different.

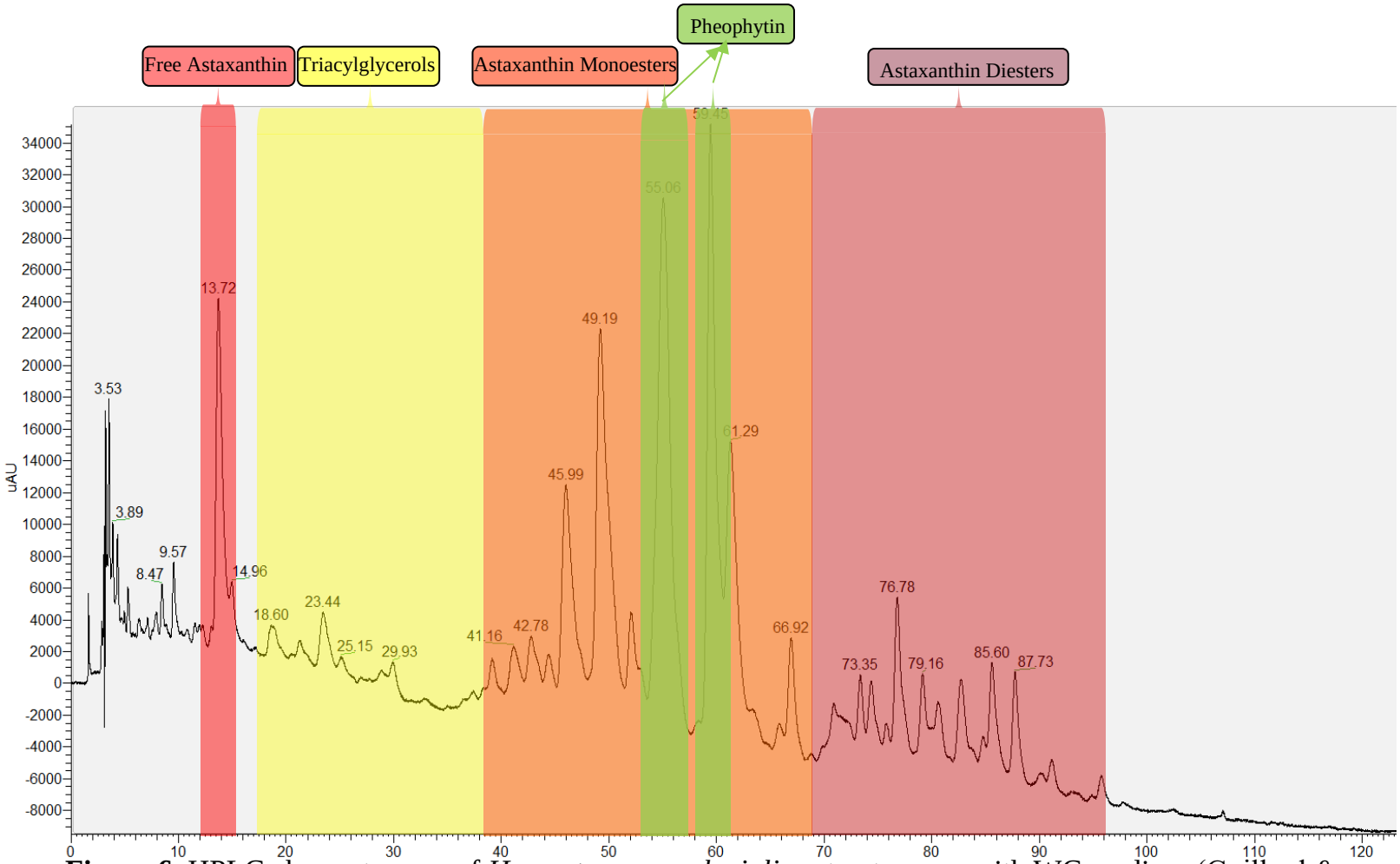


Figure 6. HPLC chromatogram of *Haematococcus pluvialis* extract grown with WC medium (Guillard & Lorenzen, 1972), and spiked with all-*trans*-astaxanthin. Banded regions represent compound type identification of included peaks based on analysis by HPLC-APCI-MS (PDA, 400-500 nm).

Table 5. Time of elution and positive ion data used for identification of compounds in *Haematococcus pluvialis* oil extract grown with WC medium (Guillard & Lorenzen, 1972), spiked with all-*trans*-astaxanthin, and analyzed by HPLC-APCI-MS (PDA, 400-500 nm).

Retention time	Mass-to-charge ratio (m/z)	Identification
13.73	597.2	Free Astaxanthin
19	841.46	Triacylglycerol (16:1,17:1,18:2)
19	849.5	Triacylglycerol (12:0,18:2, 22:5)
21.25	843.47	Triacylglycerol (16:1,17:0,18:2)
21.25	865.45	Triacylglycerol (17:1,18:1,18:3)
21.25	868.5	Triacylglycerol (17:1,18:2, 18:2)
23.44	851.55	Triacylglycerol (16:0,18:3,18:3)
23.44	843.47	Triacylglycerol (16:1,17:0,18:2)
25.15	845.42	Triacylglycerol (16:0,17:0,18:2)
25.15	869.41	Triacylglycerol (17:1,18:2,18:2)
28.91	853.49	Triacylglycerol (16:1,18:2,18:2)
28.91	871.42	Triacylglycerol (18:3,18:3,18:4)
29.93	847.52	Triacylglycerol (16:0,17:0,18:1)
29.93	869.48	Triacylglycerol (17:0,18:2,18:2)
33.2	849.89	Triacylglycerol (16:1, 18:4,18:2)
33.2	855.47	Triacylglycerol (16:0,18:2,18:2)
36.06	875.51	Triacylglycerol (16:0,18:3,20:5)
37.43	849.5	Triacylglycerol (16:1,18:3,18:3)
37.3	851.44	Triacylglycerol (16:1,18:2,18:3)
37.43	901.43	Triacylglycerol (18:2,18:2,20:5)
38.33	853.51	Triacylglycerol (16:1,18:2,18:2)
38.33	901.27	Triacylglycerol (18:2,18:2,20:5)
39.16	827.47	Astaxanthin Monoester C16:4
41.43	821.41	Astaxanthin Monoester C15:0
42.59	877.37	Astaxanthin Monoester C20:7
42.59	829.49	Astaxanthin Monoester C16:3
43.47	851.38	Triacylglycerol (16:1,18:2,18:3)
46	857.26	Astaxanthin Monoester C18:3
46	823.47	Astaxanthin Monoester C15:1

46.57	879.52	Astaxanthin Monoester C20:6
49.18	859.26	Astaxanthin Monoester C18:2
49.48	851.48	Triacylglycerol (16:1,18:2,18:3)
50.49	885.29	Astaxanthin Monoester C20:3
52.05	841.26	Astaxanthin Monoester C17:4
53.01	853.52	Triacylglycerol (16:1,18:2,18:2)
55.06	885.38	Astaxanthin Monoester C20:3
55.1	885.29	Astaxanthin Monoester C20:3
55.34	871.36	Pheophytin (isomer)
59.43	871.36	Pheophytin (isomer)
61.29	835.29	Astaxanthin Monoester C16:0
69.84	1089.35	Astaxanthin Diester C16:2/16:4
70.93	1115.29	Astaxanthin Diester C18:4/18:3
72.57	1091.35	Astaxanthin Diester C16:1/18:4
73.35	1117.3	Astaxanthin Diester C18:3/18:3
74.81	1143.26	Astaxanthin Diester C18:4/20:2
75.78	1119.36	Astaxanthin Diester C18:3/18:2
76.78	1119.36	Astaxanthin Diester C18:3/18:2
79.16	1121.35	Astaxanthin Diester C18:2/18:2
82.73	1123.33	Astaxanthin Diester C18:2/18:1
82.73	1093.43	Astaxanthin Diester C16:0/18:4
84.76	1095.33	Astaxanthin Diester C16:0/18:3
87.73	1097.4	Astaxanthin Diester C16:0/18:2
91.15	1099.47	Astaxanthin Diester C16:0/18:1

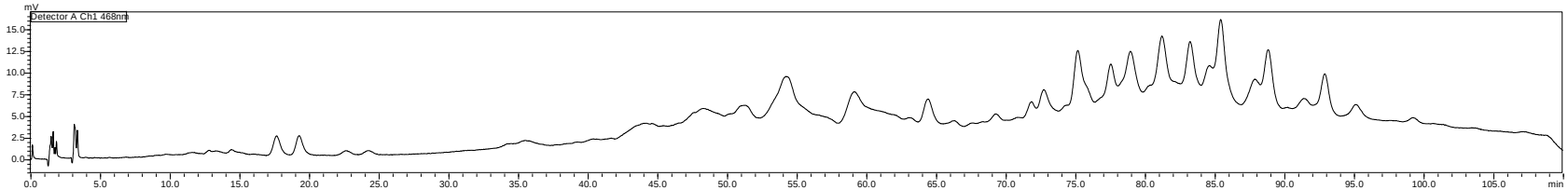


Figure 7. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972); sample A, duplicate 1.

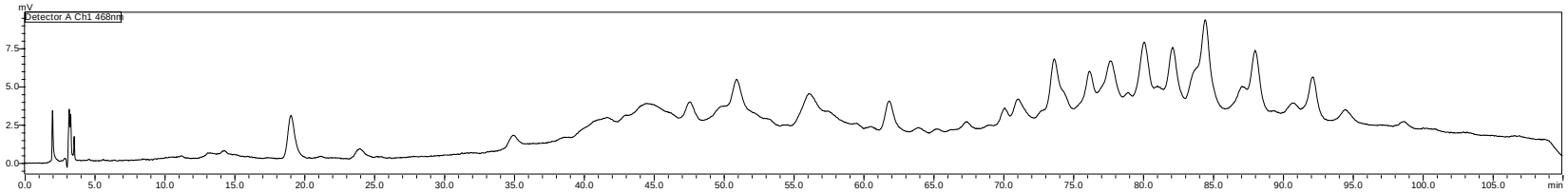


Figure 8. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972); sample A, duplicate 2.

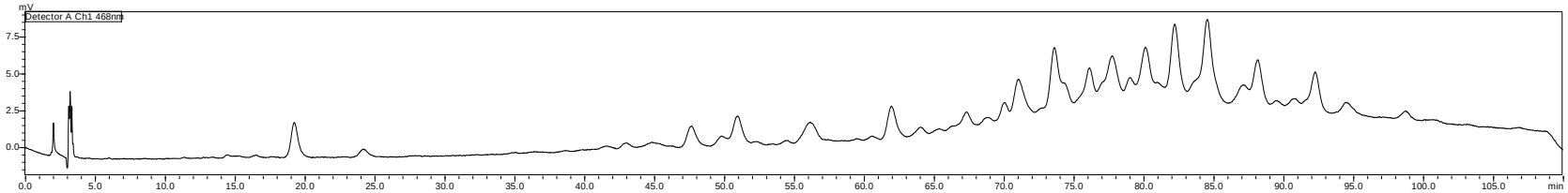


Figure 9. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972); sample B, duplicate 1.

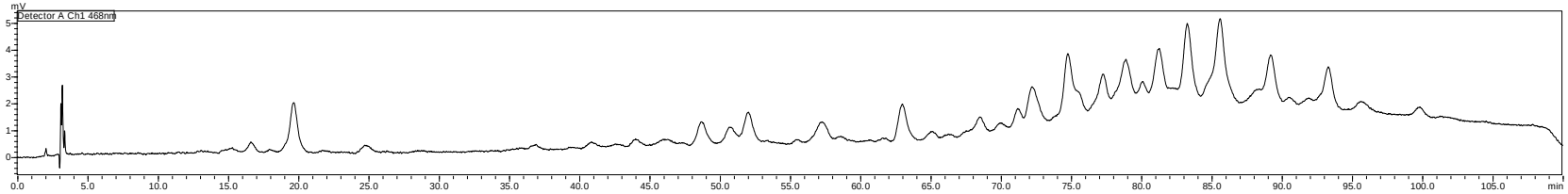


Figure 10. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972); sample B, duplicate 2.

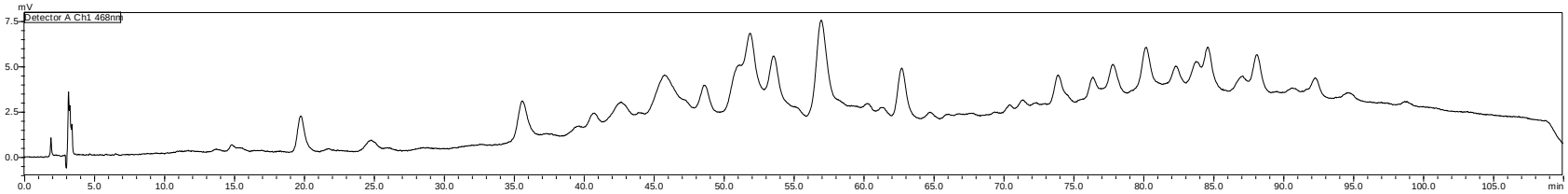


Figure 11. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972) + 10 g/L deproteinized whey permeate; sample D, duplicate 1.

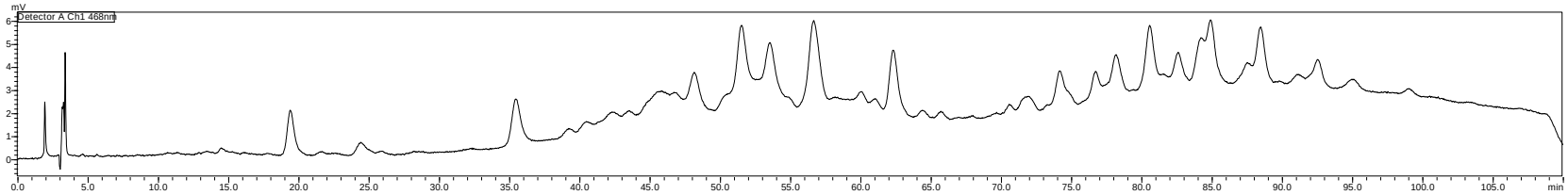


Figure 12. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972) + 10 g/L deproteinized whey permeate; sample D, duplicate 2.

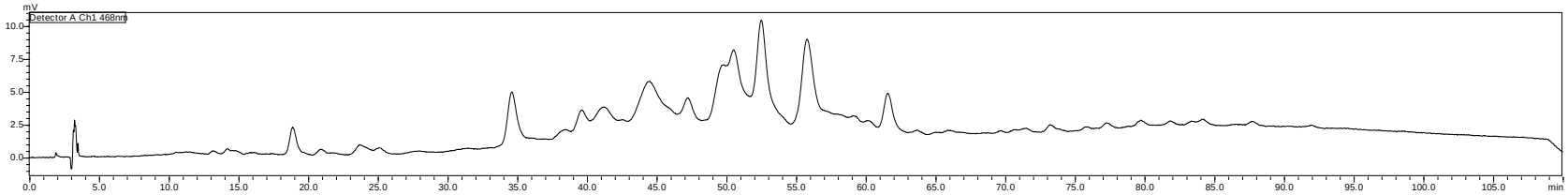


Figure 13. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972) + 10 g/L deproteinized whey permeate; sample E, duplicate 1.

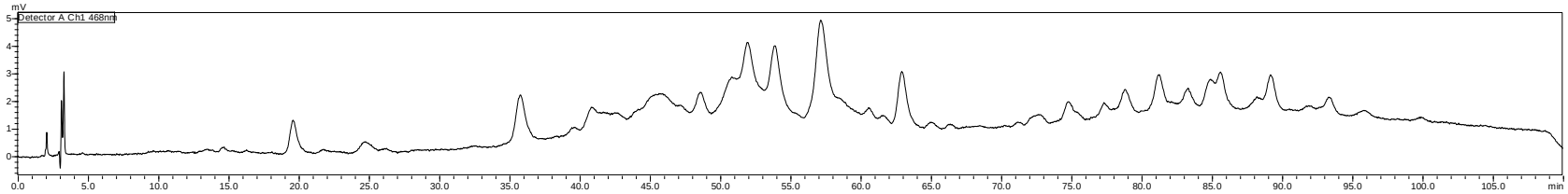


Figure 14. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (20 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972) + 10 g/L deproteinized whey permeate; sample E, duplicate 2.

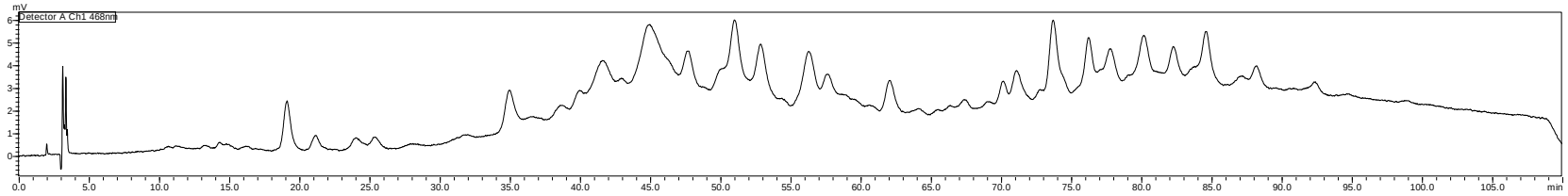


Figure 15. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with 10 g/L deproteinized whey permeate; sample F, duplicate 1.

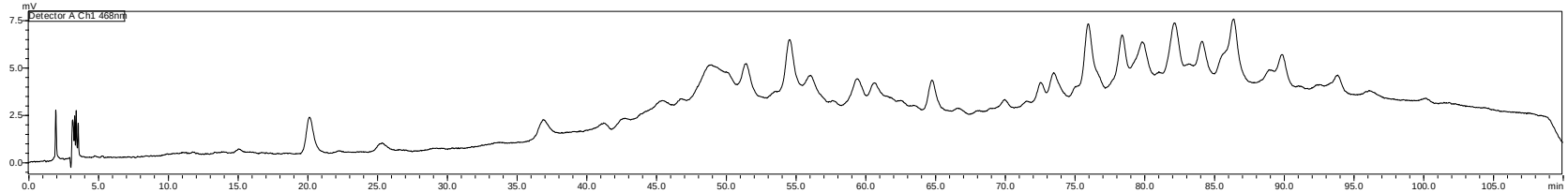


Figure 16. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with 10 g/L deproteinized whey permeate; sample F, duplicate 2.

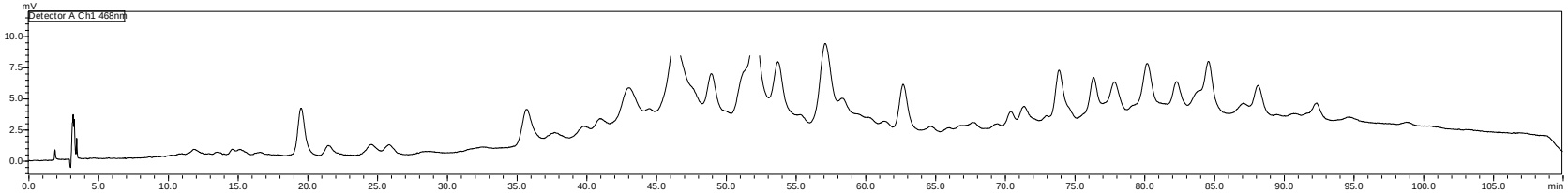


Figure 17. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with 10 g/L deproteinized whey permeate; sample G, duplicate 1.

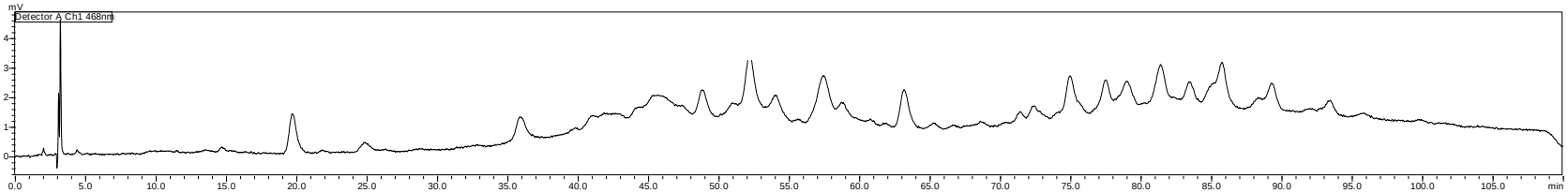


Figure 18. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (20 µL injection) grown with 10 g/L deproteinized whey permeate; sample G, duplicate 2.

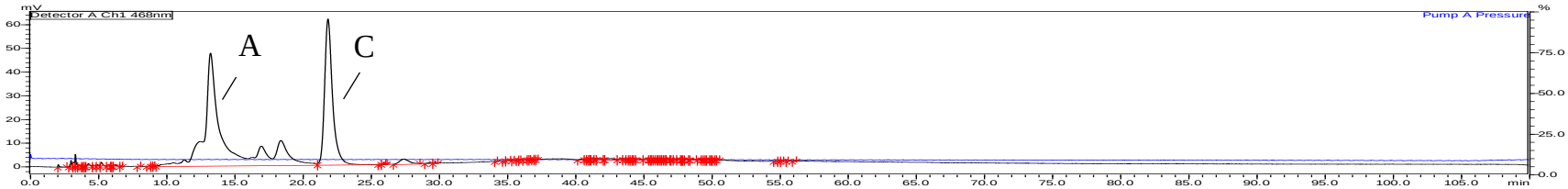


Figure 19. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972), treated with cholesterol esterase, and spiked with canthaxanthin; peak A is free astaxanthin, peak C is canthaxanthin; Sample A, Duplicate 1.

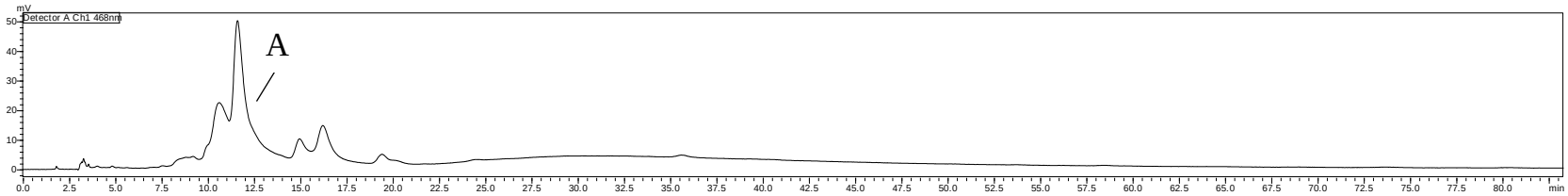


Figure 20. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972), and treated with cholesterol esterase; peak A is free astaxanthin; Sample A, Duplicate 2.

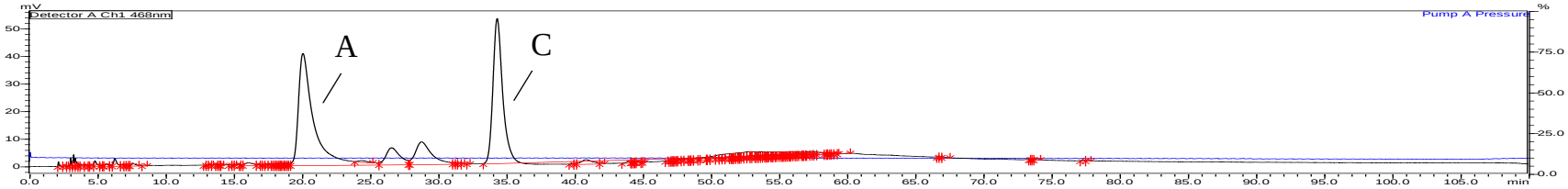


Figure 21. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972), treated with cholesterol esterase, and spiked with canthaxanthin; peak A is free astaxanthin, peak C is canthaxanthin; Sample B, Duplicate 1.

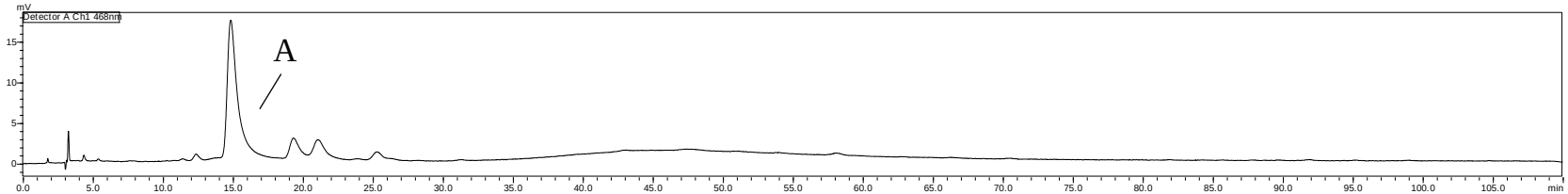


Figure 22. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (20 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972), and treated with cholesterol esterase; peak A is free astaxanthin; Sample B, Duplicate 2.

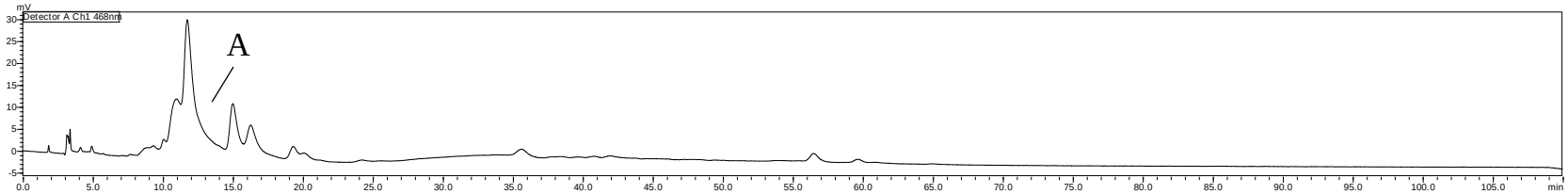


Figure 23. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972) + 10 g/L deproteinized whey permeate, and treated with cholesterol esterase; peak A is free astaxanthin; Sample D, Duplicate 1.

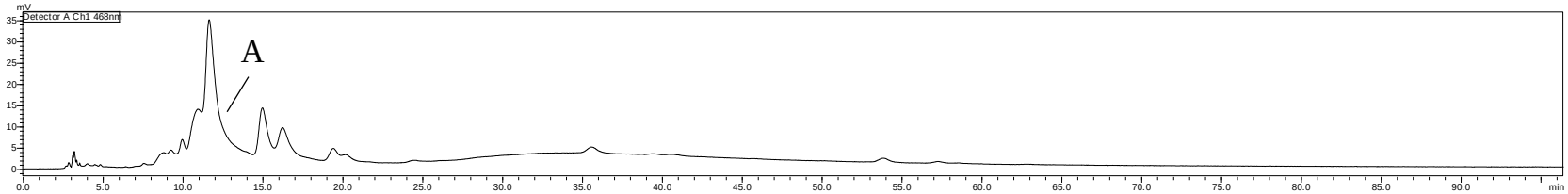


Figure 24. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972) + 10 g/L deproteinized whey permeate, and treated with cholesterol esterase; peak A is free astaxanthin; Sample D, Duplicate 2.

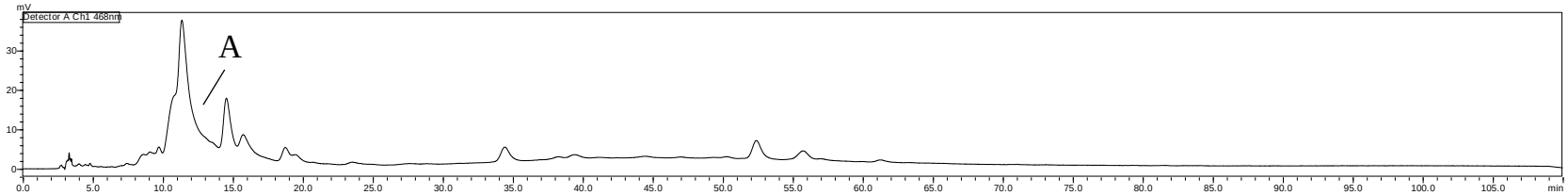


Figure 25. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (20 µL injection) grown with standard WC medium (Guillard & Lorenzen, 1972) + 10 g/L deproteinized whey permeate, and treated with cholesterol esterase; peak A is free astaxanthin; Sample E, Duplicate 2.

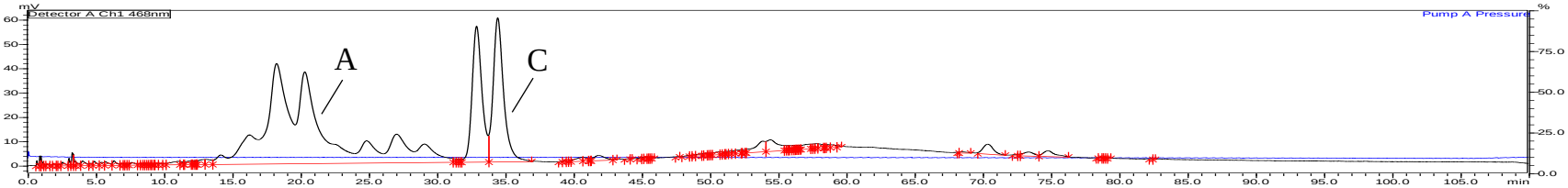


Figure 26. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with 10 g/L deproteinized whey permeate, treated with cholesterol esterase, and spiked with canthaxanthin; peak A is free astaxanthin, peak C is canthaxanthin; Sample F, Duplicate 1.

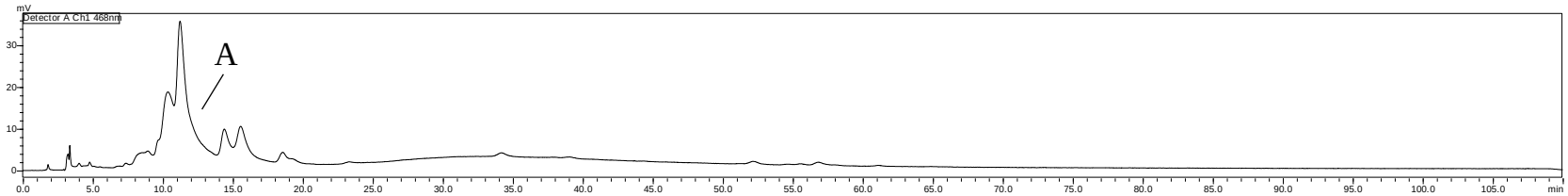


Figure 27. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with 10 g/L deproteinized whey permeate, and treated with cholesterol esterase; peak A is free astaxanthin; Sample F, Duplicate 2.

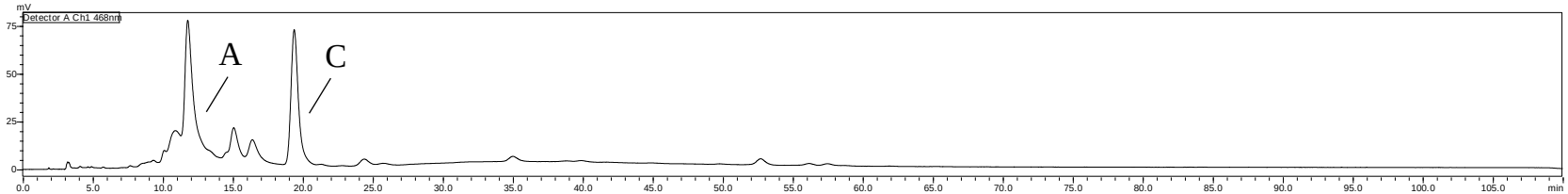


Figure 28. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (40 µL injection) grown with 10 g/L deproteinized whey permeate, treated with cholesterol esterase, and spiked with canthaxanthin; peak A is free astaxanthin, peak C is canthaxanthin; Sample G, Duplicate 1.

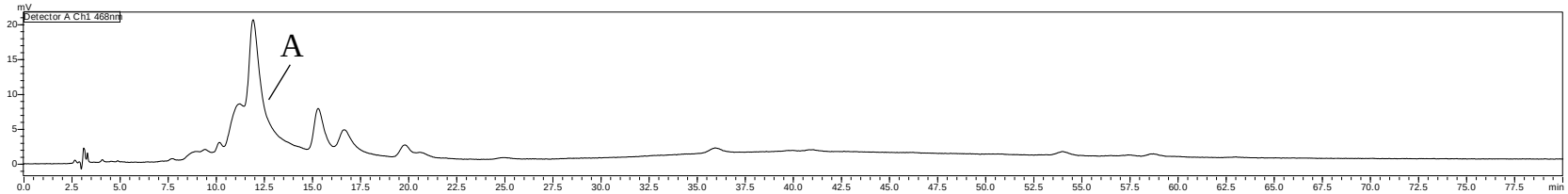


Figure 29. HPLC chromatogram (UV, 468 nm) of *Haematococcus pluvialis* oil extract (20 µL injection) grown with 10 g/L deproteinized whey permeate, and treated with cholesterol esterase; peak A is free astaxanthin; Sample G, Duplicate 2.

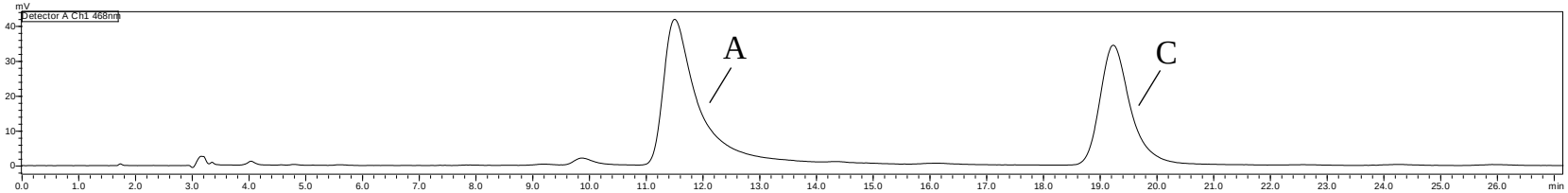


Figure 30. HPLC chromatogram (UV, 468 nm) of all-*trans*-Astaxanthin (peak A) and all-*trans*-Canthaxanthin (peak C) analytical standards prior to HPLC maintenance.

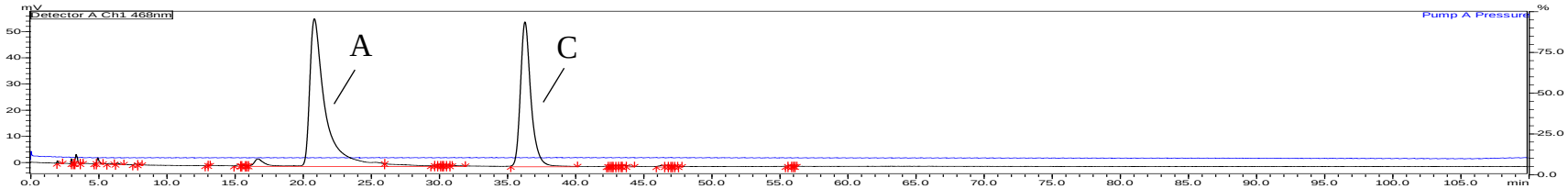
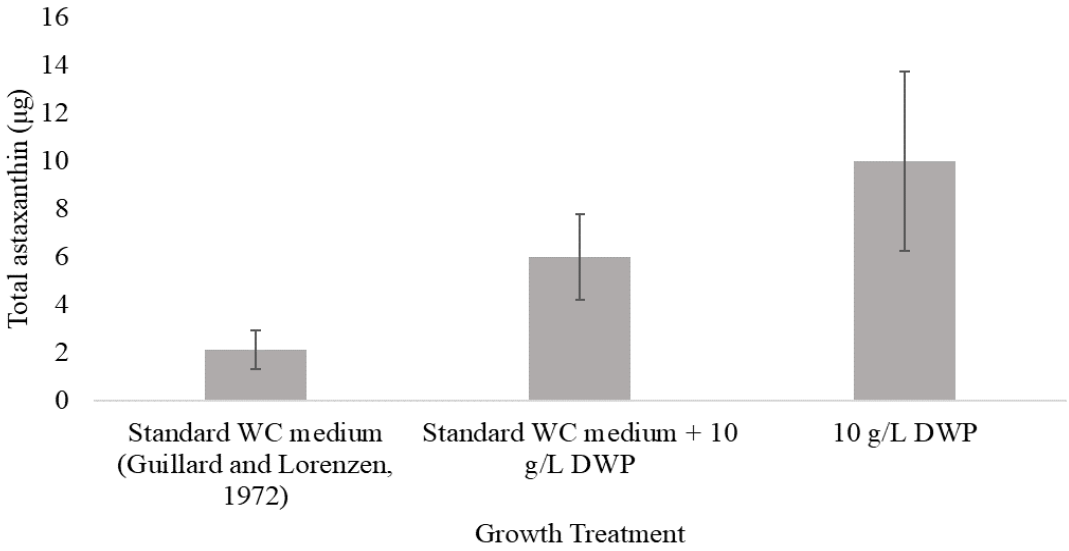


Figure 31. HPLC chromatogram (UV, 468 nm) of all-*trans*-Astaxanthin (peak A) and all-*trans*-Canthaxanthin (peak C) analytical standards after HPLC maintenance.



n = 4
DWP = deproteinized whey permeate
Figure 32. Total astaxanthin dry weight produced by *Haematococcus pluvialis* in each 20 L carboy growth treatment.

3.5 Discussion

Haematococcus pluvialis can metabolize lactose in deproteinized whey permeate (DWP) without any hydrolysis of lactose being necessary. DWP alone provides sufficient nutrients for *H. pluvialis* growth and production of astaxanthin, and astaxanthin production is not enhanced by the addition of standard WC medium (Guillard & Lorenzen, 1972) when DWP inclusion is constant at 10 g/L. Growing *H. pluvialis* with only 10 g/L DWP produces more astaxanthin than growing *H. pluvialis* with standard WC medium alone (Figure 32).

Although a higher percentage of oil was extracted from samples grown with WC medium alone than from samples grown with both WC medium and DWP, or grown with DWP alone (Table 4), total astaxanthin dry weights produced by *H. pluvialis* growth treatments that included DWP were greater than treatments grown with WC medium alone (Figure 32). This may be attributed to higher freeze-dried biomass produced in the samples grown with DWP alone, so while samples grown with WC medium alone contained a higher percentage of oil than samples grown with DWP, the oil in samples grown with DWP amounted to a higher total mass of astaxanthin, indicating DWP may promote astaxanthin production (especially astaxanthin monoester production; Table 5, Figures 7-18) and that inclusion of standard WC medium in growth of *H. pluvialis* is not necessary, nor does it enhance growth if sufficient DWP is used. While algal biomass quantities and percent astaxanthin confirmed that DWP enhanced algal growth and astaxanthin production, percent astaxanthin on a dry weight basis produced in this study was still dramatically lower for all growth conditions (1.2-2.2% x 10^{-4}) than that which *Haematococcus pluvialis* has reportedly produced in other settings (up to 3-5%) (Shah et al., 2016). This difference may be due in part, but likely not entirely, to other studies' overestimation of astaxanthin via spectrophotometric methods. Optimization of growth conditions to improve

astaxanthin yield (i.e. pH, nutrient concentration, light intensity and cycling) would be a worthy pursuit, regardless of whether growth is with WC medium, DWP, or both. Additionally, further efforts to limit degradation of carotenoids—such as limiting exposure to light and heat—could improve astaxanthin yields.

Due to astaxanthin produced by *H. pluvialis* being composed of free astaxanthin and many astaxanthin esters, identification and quantification of astaxanthin is difficult. Spectrophotometric absorption methods are used to quantify astaxanthin in commercial settings, but these methods overestimate astaxanthin due to other naturally occurring carotenoids such as lutein, canthaxanthin, and zeaxanthin absorbing at the same wavelength (Runco and Chen, 2014). This work prioritized accuracy of astaxanthin quantification through development of effective methods for oil extraction and compound identification and quantification. Oil extraction and de-esterification methods reported in other literature, such as acetone extraction and saponification by KOH, were relatively ineffective in this study (Dong et al., 2014; Galarza et al., 2019) (Figure 2). Saponification with KOH may have been unsuccessful in this study due to the process degrading astaxanthin, as was reported by Galarza et al. (2019). Algal sample preparation for analysis of astaxanthin produced by *H. pluvialis* is optimized by a modified Mojonnier oil extraction with acid pretreatment to overcome the encysted algal wall (Figure 2), followed by cholesterol esterase mediated hydrolysis to convert astaxanthin esters into free astaxanthin. This method converted 26 astaxanthin esters to free astaxanthin (Figures 6-29, Table 5), allowing HPLC to be used to quantify total free astaxanthin with a single free astaxanthin standard. Interestingly, lutein, canthaxanthin, and zeaxanthin were not identified in our extracts, though two isomers of pheophytin were present (Figure 6, Table 5). This may be attributed to the strain of *H. pluvialis* or the algal growth conditions. While further optimization of algal growth conditions would be needed, the $2,500-15,000/kg value of algal derived astaxanthin (Panis and

Carreon, 2016; Shah et al., 2015) makes this work promising for dairy processors, who may utilize this application for DWP as an alternative revenue stream.

4 Concluding remarks

With US and global cheese production still on the incline, there is an immediate need for finding novel uses for some by-products such as deproteinized whey permeate (DWP). While there have been attempts to use DWP in foods, most of its current use is as animal feed and fertilizer. However, due to its high biological oxygen demand, the latter application is restricted (Kushwaha et al., 2011). A higher-value use of DWP is as feed material for the algae *Haematococcus pluvialis* to produce astaxanthin, the main carotenoid contributing to salmon and shrimp pigmentation.

Dairy processors can use deproteinized whey permeate as a novel income stream. Apart from photobioreactors, equipment and expertise for producing algae is already present in mid- to large scale dairies (tanks, decanter- and desludging centrifuges). Harvested algae could be shipped to facilities specializing in natural product extraction. The current market value of astaxanthin ($2,500-15,000/kg) would make this a financially rewarding undertaking (Panis and Carreon, 2016; Shah et al., 2015).

Future work should include growth of *Haematococcus pluvialis* with lactose alone and glucose alone, to understand impact of vitamins, minerals, and metals on algal growth and production of astaxanthin and to determine if these sugars alone can support growth. Additionally, modified Mojonnier oil extraction efficiency should be compared to that of supercritical fluid extraction and quantification of astaxanthin via HPLC should be compared to quantification of astaxanthin via spectrophotometric absorption.

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6 Appendix

Appendix 1: Chloroform:Methanol Folch algal oil extraction with sonication pretreatment

Materials:

Freeze dried *Haematococcus pluvialis*
Weigh paper
Spatulas
Scale
15 mL plastic centrifuge tubes
HPLC grade chloroform
HPLC grade methanol
Vortex
Double distilled water
Centrifuge
Glass pipettes
Sonicator: Fisher Scientific 40:0.15:4C
Sonicator Probe: 4C15
12 mL glass collection vials
Turbovap Automated Evaporation System (Biotage, Uppsala, Sweden)
Nitrogen gas
Tinfoil

Procedure:

1) In 15 mL Corning plastic centrifuge tube, mix 0.25 g freeze dried *Haematococcus pluvialis* with 5 mL 2:1 chloroform:methanol.
2) Sonicate sample according to the following conditions:
   Depth of Probe: ½ cm from bottom of tube
   Temperature: 4ºC
   Mode: Pulse—Alternating 1 minute with sonication and 1 minute without sonication. Sonication amplitude: 50%.
   Duration: 20 minutes
3) Vortex sample and hold at -20ºC in darkness for at least 1 hour or overnight.
4) Add 1 mL dd H_2O to sample and vortex.
5) Centrifuge sample at 1700 rpm at 4ºC for 10 minutes.
6) Transfer lower chloroform phase into a new pre-washed glass collection tube.
7) Add 2 mL chloroform to the remaining upper phase.

8) Vortex sample and hold at -20ºC in darkness for at least 1 hour or overnight.
9) Vortex sample and repeat steps 5-6.
10) Add 2 mL chloroform to the remaining upper phase.
11) Vortex sample and hold at -20ºC in darkness for at least 1 hour or overnight.
12) Vortex and repeat steps 5-6.
13) Dry sample in Turbovap under 5 psi Nitrogen gas at 35ºC.
14) Flush headspace of dried extracts with Nitrogen and store at -20ºC in darkness (achieve darkness by wrapping vial in tinfoil).

Appendix 2: Acetone algal oil extraction with tissue grinder pretreatment

Materials:

Freeze dried *Haematococcus pluvialis*
Weigh paper
Spatulas
Scale
Tissue Grinder
HPLC grade acetone
Small beaker
10 mL graduated cylinder
Glass pipettes
Separatory funnel and mount
12 mL glass collection vials
Turbovap Automated Evaporation System (Biotage, Uppsala, Sweden)
Nitrogen gas
Tinfoil

Procedure:

1) Add 0.42 g freeze dried *Haematococcus pluvialis* to tissue grinder and homogenize thoroughly.
2) Add 7 mL acetone to tissue grinder and grind sample.
3) Carefully pour contents into glass vial and add more acetone. Re-grind and empty into vial until tissue grinder is empty. Use a total of about 30 mL acetone during this step, in 3-10 mL portions. Cap collection vial and cover with tinfoil to maintain darkness.
4) Centrifuge sample at 4ºC and 1700 RPM for 10 minutes.
5) Pour or pipette off top acetone layer into 12 mL glass collection vial.
6) Dry sample in Turbovap under 5 psi Nitrogen gas at 35ºC.
7) Flush headspace of dried extracts with Nitrogen and store at -20ºC in darkness (achieve darkness by wrapping vial in tinfoil).

Appendix 3: Figure 2 raw data (Efficiency of three different oil extraction methods from *Haematococcus pluvialis* on a weight basis (n=2-6))

Chloroform:Methanol Folch algal oil extraction with sonication pretreatment

Sample #	Algae Weight (g)	Collection Vial Weight (g)	Collection Vial + Sample Weight after Drying (g)	Oil Weight (g)	% Oil Extracted from Algae
1	0.2510	11.5090	11.5490	0.0400	15.936
2	0.2510	11.4633	11.4960	0.0327	13.028

Average % oil extracted: 14.482 (SD = 1.454)

Acetone algal oil extraction with tissue grinder pretreatment

Sample #	Algae Weight (g)	Collection Vial Weight (g)	Collection Vial + Sample Weight after Drying (g)	Oil Weight (g)	% Oil Extracted from Algae
1	0.4198	11.4786	11.5233	0.0447	10.648
2	0.4199	11.4740	11.5090	0.0350	8.355
3	0.4198	11.4769	11.5099	0.0330	7.861
4	0.4202	11.4400	11.4713	0.0313	7.449
5	0.4199	11.3933	11.4276	0.0343	8.169
6	0.4202	11.4843	11.5307	0.0464	11.045

Average % oil extracted: 8.918 (SD = 1.949)

Modified Mojonnier algal oil extraction with HCl pretreatment

Sample #	Algae Weight (g)	Collection Vial Weight (g)	Collection Vial + Sample Weight after Drying (g)	Oil Weight (g)	% Oil Extracted from Algae
1	0.4196	11.5040	11.6379	0.1339	31.911
2	0.4202	11.4105	11.5478	0.1373	32.659
3	0.4201	11.4784	11.6170	0.1386	32.992
4	0.4194	11.4548	11.5932	0.1384	33.000
5	0.4198	11.4421	11.5823	0.1402	33.397
6	0.4196	11.3938	11.5365	0.1427	34.009

Average % oil extracted: 32.995 (SD = 0.413)