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Carotenogenesis Up-regulation in *Scenedesmus* sp. Using a Targeted Metabolomics Approach by Liquid Chromatography−High-Resolution Mass Spectrometry

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ABSTRACT: Carotenoids have potent antioxidant activity as well as therapeutic value, and their formation has been seen to be induced in algae by stress, including high-salt culture conditions. A differential profiling of carotenoids was conducted using a targeted metabolomics approach with accurate mass data generated by liquid chromatography−electrospray−time-of-flight (LC-ESI-TOF) mass spectrometry followed by postacquisition filtering based on isotope patterns and mass defects to detect carotenoids up-regulated in *Scenedesmus* sp. exposed to high-salt conditions. Algal cultures treated with high concentrations of sodium acetate or sodium chloride were found to cause an increase in various carotenoids. On the basis of differential analysis, astaxanthin and canthaxanthin increased upon salt treatment. Astaxanthin, in its free form and as fatty acid esters, was seen to increase in *Scenedesmus* sp. using accurate mass MS. A few other carotenoid compounds increased upon salt treatment, including echinenone and adonirubin, involved in the pathway of astaxanthin biosynthesis from β-carotene, as well as isomers of astaxanthin and canthaxanthin. A time course study of acetate treatment was done to observe the time-dependent up-regulation of carotenogenesis.

KEYWORDS: carotenoids, up-regulation, high-resolution mass spectrometry, electrospray−time-of-flight, *Scenedesmus*, sodium acetate, sodium chloride, stress-induced carotenogenesis, differential analysis, astaxanthin, canthaxanthin, targeted metabolomics, metabolite profiling

INTRODUCTION

Carotenoids are widespread naturally occurring compounds found as yellow-, orange-, and red-colored pigments.[1] They are present in various biological species, including algae, yeast, marine animals, plants, fruits, and vegetables.[2−6] Their large natural abundance is most likely due to their relatively simple biosynthetic pathways.[1] Most carotenoid compounds are known to have potent antioxidant activity and important therapeutic value.[7−9] These compounds have been proposed to be involved in the prevention of cancer[10−12] and also exemplify cardioprotective properties.[2,13] For example, antioxidants can neutralize free radicals in cells and tissues, thus protecting them from oxidative damage.[7,14] Among them, astaxanthin is considered to be more effective in terms of its antioxidant properties as compared to β-carotene, canthaxanthin, and lutein.[15,16] Astaxanthin has been reported to protect the skin from sunlight radiation injuries, to slow macular degeneration due to aging, and to improve functioning of the immune system.[17] Extraction and identification of carotenoids are therefore important for both pharmaceutical and food industries.

Interestingly, increased formation of carotenoids in algae has been reported as an oxidative (stress) response and therefore can be up-regulated using different culture conditions. For example, stress-induced astaxanthin accumulation has been observed in green alga *Haematococcus pluvialis* in high-light/irradiance, high-salt, high-temperature, and nutrient-deficiency environments,[18−24] as well as under elevated temperature and irradiance conditions in *Scenedesmus obliquus*.[25] Furthermore, we have recently demonstrated increased carotenogenesis under high-salt culture conditions, including sodium acetate and sodium chloride, in *Scenedesmus* sp.[26,27]

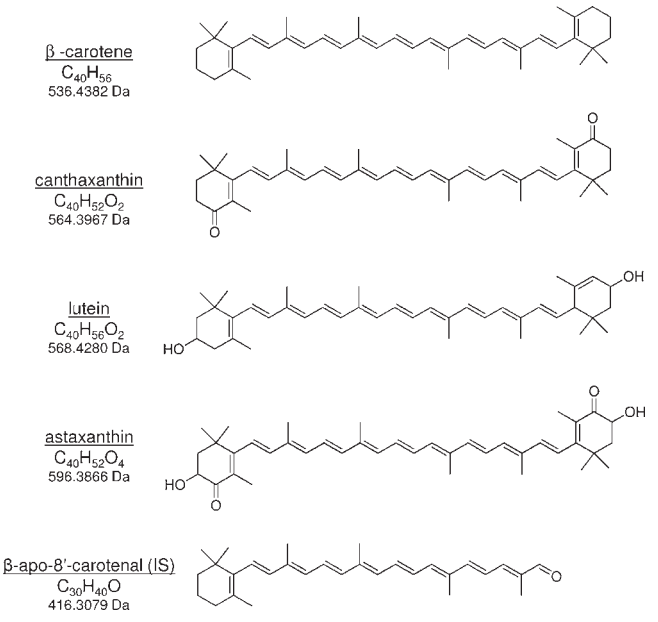

Figure 1. Structures of the carotenoid standards used in this study.

Various chromatographic techniques have been developed for carotenoid separations, using C18 or C30 reversed phase HPLC columns with either isocratic or gradient elution, with specific

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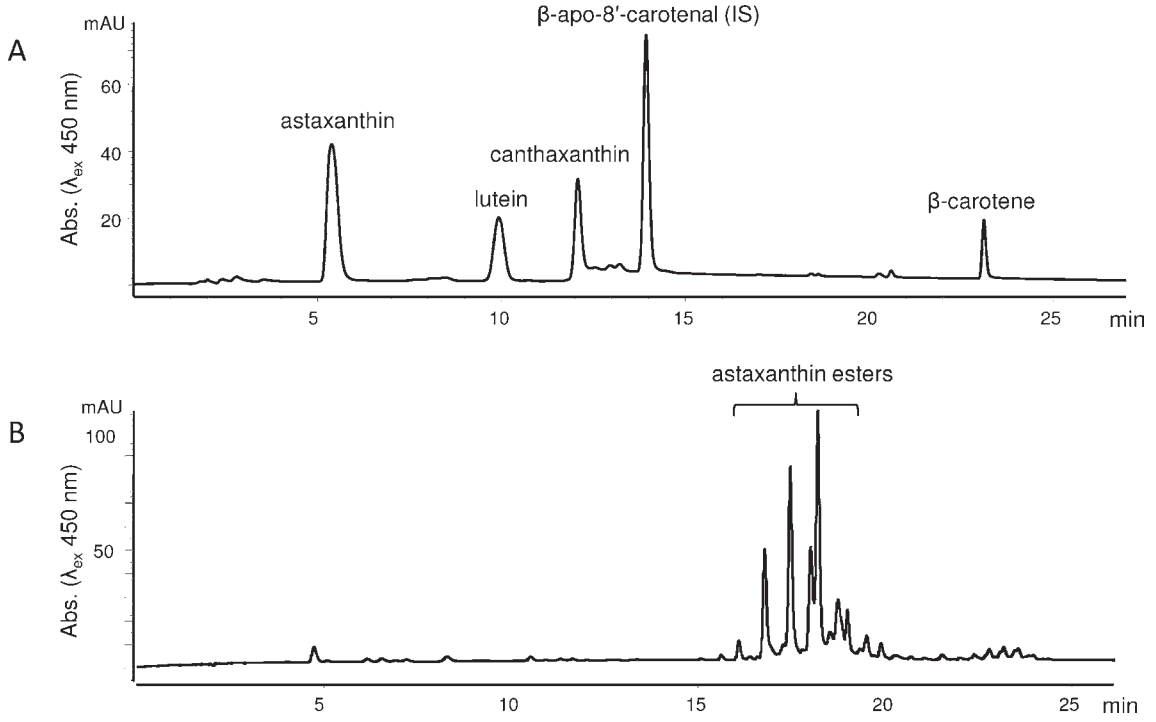

Figure 2. HPLC-UV−vis chromatogram (detected at 450 nm) for (A) a mix of standard carotenoids (concentrations of standards were as follows: astaxanthin, 0.002 mg/mL; β-carotene/canthaxanthin, 0.005 mg/mL; β-apo-8′-carotenal/lutein, 0.0008 mg/mL) and (B) Asta-Real L10 extract containing astaxanthin esters (0.32 mg/mL).

spectrophotometric (UV−vis) detection around 450 nm.[28−30] However, identification using UV−vis depends on the availability of commercial standards and remains doubtful unless very high resolution separations are achieved. The first method using liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS) was reported for studying carotenoids using a C30 reversed-phase column.[31] Atmospheric pressure chemical ionization (APCI) has also become popular for carotenoid profiling by LC-MS in biological samples.[6,32] A fairly recent paper has also indicated the usefulness of matrix-assisted laser desorption ionization (MALDI)-TOF-MS for metabolite profiling of plant carotenoids in a high-throughput manner.[33] In the present study, the analysis of complex biological samples was possible by employing LC coupled to high-resolution MS on an electrospray−time-of-flight (ESI-TOF) instrument for accurate mass-based elemental formula confirmation. Over the past decade, metabolomic approaches have commonly been used to study biologically relevant metabolite species with low molecular masses, typically below 1000 Da.[34,35] This methodology enables monitoring of different molecules involved in biosynthesis and biodegradation pathways.[34] In a recent review, metabolomics has been described as a high-throughput approach for studying carotenoids in plants and algae, using numerous analytical techniques including LC-UV, GC-MS, and NMR.[36] The present study involves the development of a new method for screening carotenoids in algal samples under different culture conditions using a targeted metabolomics approach based on accurate mass data and confirmation by carotenoid-specific absorbance at 450 nm. From the accurate mass data, various known carotenoids, such as lutein, canthaxanthin, astaxanthin, and β-carotene, were confirmed in *Scenedesmus* cultures. Furthermore, astaxanthin fatty acid esters as well as a few other carotenoid compounds involved in the biosynthetic pathway of astaxanthin were detected in the cultures. This paper describes a novel approach for monitoring the up-regulation of specific carotenoid species upon high-salt treatment in *Scenedesmus* algal cultures.

MATERIALS AND METHODS

Source of Chemicals. Dichloromethane, methanol, and methyl *tert*-butyl ether (MTBE) were all of HPLC grade and were purchased from Caledon Laboratory Chemicals (Georgetown, ON, Canada). Ultrapure water was supplied by a Barnstead NANOpure Diamond UV ultrapure water system from Thermo Scientific (Mississauga, ON, Canada) and was used throughout the experiments. Sodium acetate, ammonium acetate (ultrapure), β-carotene, canthaxanthin, β-apo-8′-carotenal, and lutein were purchased from Sigma-Aldrich (Oakville, ON, Canada). Sodium hydroxide and sodium chloride were purchased from EMD Chemicals (Gibbstown, NJ). AstaReal L10 containing 10% astaxanthin (as a mix of esters) extracted from *H. pluvialis* was purchased from BioReal Sweden (Gustavsberg, Sweden). Free astaxanthin was purchased from Chromadex (Irvine, CA).

Microalgae Incubation Conditions. The microalgal *Scenedesmus* sp. was obtained from the Canadian Phycological Culture Centre (CPCC). *Scenedesmus* sp. was grown in sterile BG11 liquid medium.[37] To obtain a high biomass, algae were cultured at 23 °C in a 2 L bottle bioreactor with bubbling air and under continuous low light intensity (50 μmol of photons/m^2/s). Green vegetative cells in exponential growth phase were harvested by centrifugation at 4000*g* for 15 min to concentrate the cultures. Algae were washed with sterile ultrapure water and suspended again in BG11 medium without added nitrogen. To induce secondary carotenoid accumulation, cultures were prepared with BG11 medium without added nitrogen (control) and having 4.5 mM N_2 (in the form of urea) with the addition of (a) 120 mM sodium acetate or (b) 600 mM sodium chloride. The induction of carotenogenesis was

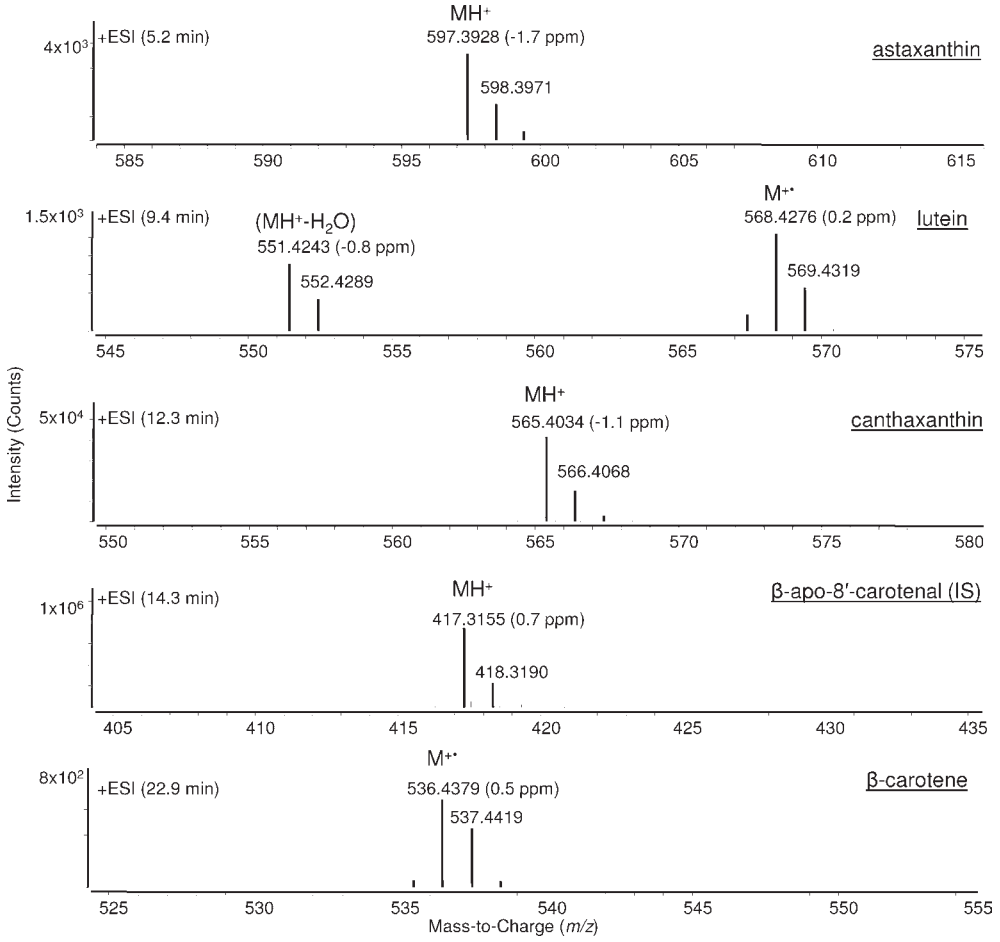

Figure 3. High-resolution mass spectra measured on ESI-TOF instrument for carotenoid standards used in this study.

Table 1. Carotenoids Found To Increase upon Salt Treatment in *Scenedesmus* sp. Cultures Following Postacquisition Filtering of Accurate Mass Data with Differential Analysis (Mass Profiler) Software

carotenoid	RT (min)	m/z (MH^+)	formula	error (ppm)
canthaxanthin	11.3	565.4033	$C_{40}H_{52}O_2$	−1.3
canthaxanthin isomer 1	6.4	565.4053	$C_{40}H_{52}O_2$	2.3
canthaxanthin isomer 2	12.6	565.4048	$C_{40}H_{52}O_2$	1.4
astaxanthin	4.9	597.3942	$C_{40}H_{52}O_4$	0.6
astaxanthin isomer 1	6.9	597.3945	$C_{40}H_{52}O_4$	1.1
astaxanthin isomer 2	7.3	597.3942	$C_{40}H_{52}O_4$	0.6
echinenone	18.3	551.4242	$C_{40}H_{54}O$	−1.0
adonirubin	7.5	581.3989	$C_{40}H_{52}O_3$	0.1

performed in 120 mL Erlenmeyers flasks with 50 mL cultures. The initial cellular density in all cultures tested was 15×10^6 cells/mL. In one set of experiments, algal cells were exposed to sodium acetate or sodium chloride (as well as under control conditions) under continuous higher light irradiance (150 μmol of photons/m^2/s) for 30 days. A second set of experiments including a time course study for acetate treatment was performed with sampling of algal cultures at 8, 15, 22, and 29 days. Five milliliter samples of algal cultures were collected and then washed with ultrapure water to remove salts followed by lyophilization.

Table 2. Accurate Mass Data for Fatty Acid Astaxanthin Esters Found To Increase upon Salt Treatment in *Scenedesmus* sp. Cultures

fatty acid chain	RT (min)	m/z (MH^+)	formula	error (ppm)
C16:0	19.4	835.6222	$C_{56}H_{82}O_5$	−1.6
C18:1	19.6	861.6381	$C_{58}H_{84}O_5$	−1.2
C18:2	18.9	859.6228	$C_{58}H_{82}O_5$	−0.8
C18:3	18.2	857.6057	$C_{58}H_{80}O_5$	−2.5

Carotenoid Extraction. To a quantity of 5 mg (lyophilized dry weight) of algae was added 40 μL of β-apo-8′-carotenal stock solution (0.1 mg/mL), serving as internal standard (IS), for relative quantitation purposes. The samples were extracted with a mixture of dichloromethane/methanol (75:25). The extraction was repeated until no color was further extracted and pellets were almost colorless (approximately 5−7 mL total). The samples were dried in a Thermo Scientific Lindberg/Blue M vacuum chamber and then reconstituted in 1.5 mL of 75% methanol/25% water prior to solid-phase extraction (SPE) using Water Oasis HLB (30 mg) cartridges (Milford, MA). Samples were loaded and then cartridges washed with methanol/water (75:25). Carotenoids were eluted with 1 mL of MTBE/methanol/water (60:37:3) containing 3 mM ammonium acetate. The samples were lyophilized using a Thermo Scientific Savant SPD131DDA SpeedVac concentrator and a UVS800DDA Universal Vacuum Source (Fisher

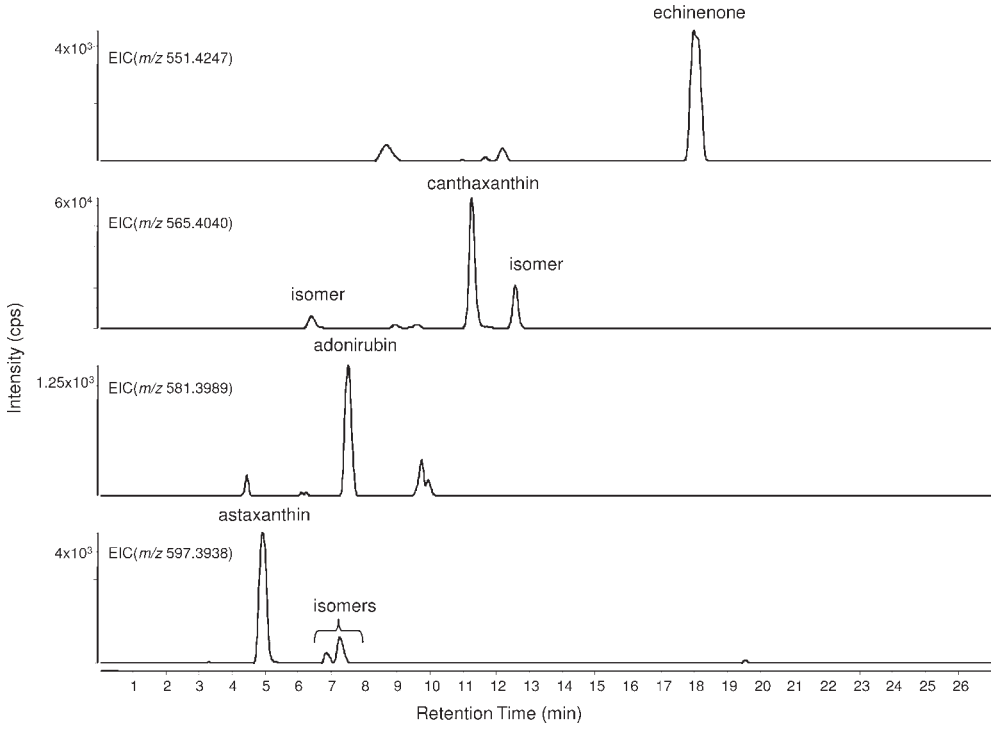

Figure 4. Extracted ion chromatograms (EICs) of selected carotenoids found to increase in *Scenedesmus* cultures during acetate treatment (day 29). Each m/z was extracted on the basis of the calculated exact mass of the protonated carotenoid (±10 ppm mass window).

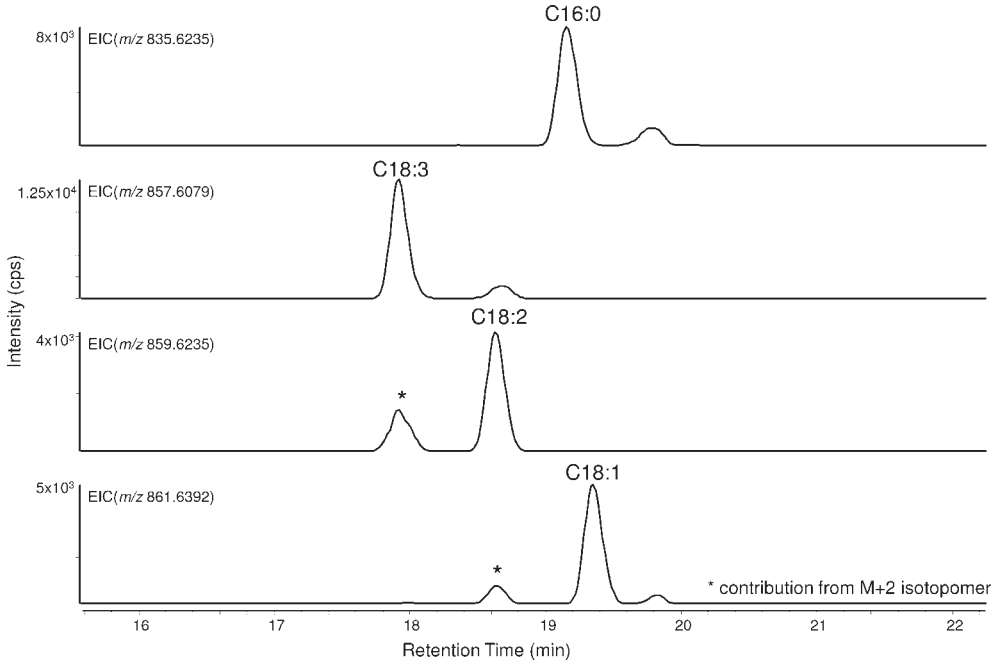

Figure 5. EICs of astaxanthin esters found to increase in *Scenedesmus* cultures following acetate treatment (day 29). Each m/z was extracted on the basis of the calculated exact mass of the protonated carotenoid (±10 ppm mass window).

Scientific, Ottawa, ON, Canada) and reconstituted with 0.5 mL of 90% MeOH, 10% H_2O, 10 mM ammonium acetate prior to sample injection. Samples were consistently stored at −30 °C between processing steps and before sample analysis.

HPLC-UV-MS Analysis of Carotenoids. Carotenoid analysis was conducted on an Agilent 1200 series high-performance liquid chromatography (HPLC) system equipped with a diode array detector (UV−vis) coupled online with an Agilent 6410 triple-quadrupole (QqQ) instrument (Santa Clara, CA). Liquid chromatography was performed and optimized on a Phenomenex Gemini-NX 150 × 2 mm C18 column (Torrance, CA) filled with 3 μm particles, and absorbance was monitored at 450 nm. The HPLC separation was performed using

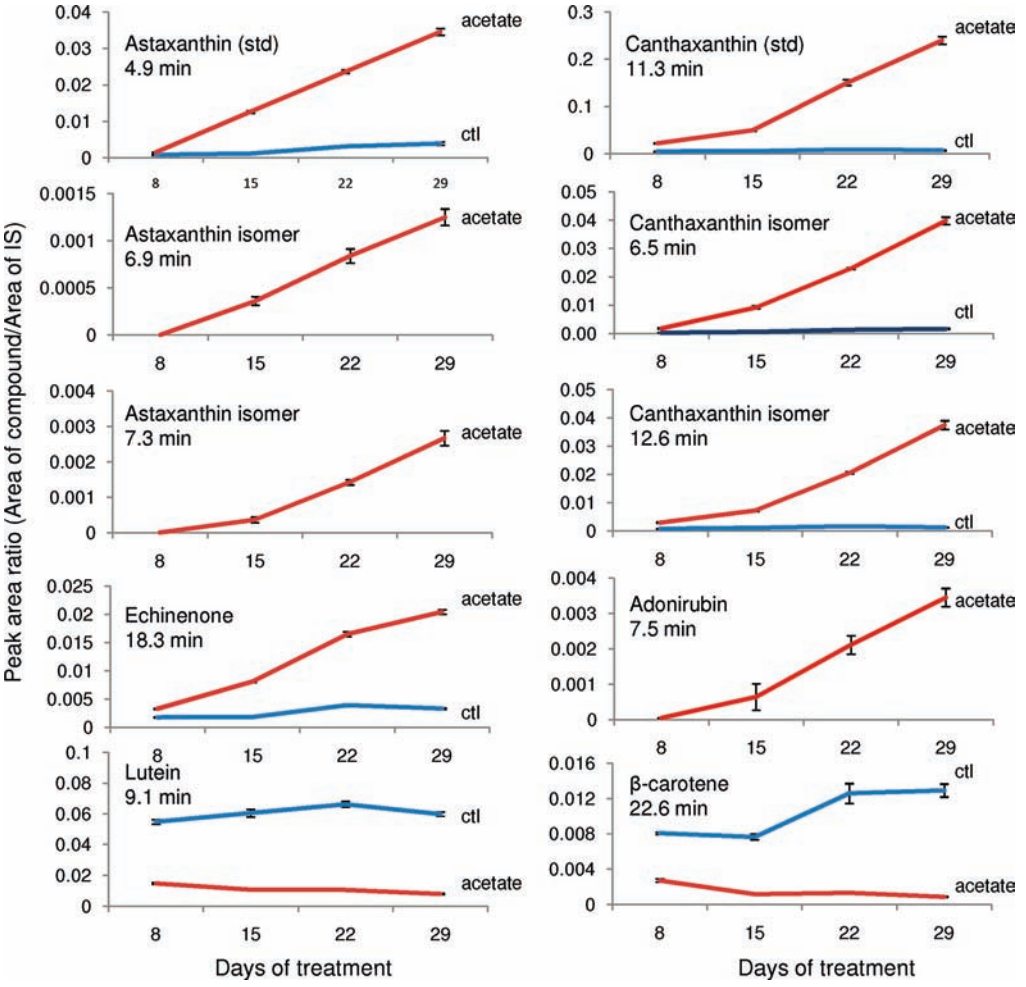

Figure 6. Time course study of carotenoids in *Scenedesmus* sp. treated with 120 mM sodium acetate (8, 15, 22, and 29 days of treatment). Peak area ratios (normalized ratio with peak area of IS) were used to represent carotenoid increase over time, with error bars indicating three triplicate injections of each sample.

the mobile phases A, 90% MeOH, 10% H_2O, 10 mM ammonium acetate; and B, 85% MTBE, 15% MeOH. The following elution gradient (min/% B) was used: 0−2/5%, 20/65%, 22−27/85%, at a flow rate of 0.2 mL/min and column temperature of 30 °C. The volume of injection was 10 μL.

Nominal mass spectra (unit resolution) for the carotenoids and free astaxanthin were obtained online with UV detection (above) by positive electrospray ionization (ESI) using a multimode (MMI) source, with a scan range of m/z 400−650 and the following source parameters: gas temperature, 300 °C; vaporizer, 200 °C; gas flow, 5 mL/min; nebulizer pressure, 60 psi; capillary voltage, 3000 V; fragmentor, 100 V.

High-Resolution Mass Spectrometry (ESI-TOF). Accurate mass data were obtained on an Agilent 6210 electrospray−time-of-flight (ESI-TOF), using the same HPLC conditions as above with an injection volume of 15 μL. The dual ESI source was operated with the following source parameters: gas temperature, 350 °C; flow, 11.5 L/min (ultrapure nitrogen); nebulizer pressure, 35 psi; capillary voltage, 4000 V; fragmentor, 100 V; skimmer voltage, 60 V. Data were acquired from m/z 100 to 1000 with internal calibration using the reference masses m/z 121.050873 and 922.009798, sprayed continuously through the reference electrospray needle. Raw ESI-TOF data were processed by molecular feature extraction (MFE) in MassHunter Qualitative Analysis software (version B.02.00) for compounds eluting from 2 to 25 min with m/z 300−1000 and a signal threshold of 200 counts. The resulting MHD files, containing compound IDs (based on neutral mass and retention times), were then amenable to further filtering and comparison of samples. Agilent MassHunter Mass Profiler software version B.02 was then employed for comparison of differentially expressed carotenoids in treated samples compared to controls.

Carotenoid identification was performed by comparing retention times with available standards: astaxanthin (free and ester forms), canthaxanthin, lutein, and β-carotene. Tentative identifications were made for other carotenoid compounds based on accurate mass data and molecular formula assignments as well as elution order.

RESULTS AND DISCUSSION

Previous studies showed that carotenogenesis increased substantially in *Scenedesmus* algal cultures upon treatment with sodium acetate and sodium chloride.[26,27] Orange−red pigments were observed in the treated samples compared to mostly green pigments seen in the control samples (data not shown). These color changes indicate a change in chemical composition of the algal cultures due to carotenoid formation, which was verified by spectrophotometric methods for quantifying total carotenoids versus total chlorophylls (UV, fluorescence).[26,27] To characterize which carotenoid species are being produced in these algal cultures, a targeted metabolomics approach has been employed

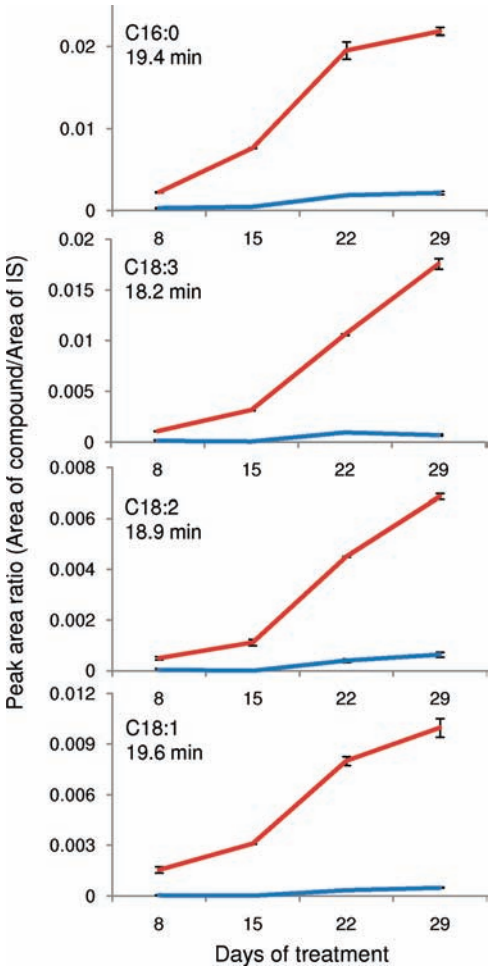

Figure 7. Comparison of peak area ratios for astaxanthin esters (16:0, 18:1, 18:2, 18:3), which increase in *Scenedesmus* cultures upon treatment with 120 mM sodium acetate over time course study, with error bars indicating replicate injections ($n = 3$) of each sample.

to compare sample treatments using accurate mass data obtained by LC-ESI-TOF-MS analysis. Extraction of carotenoids and HPLC separation were initially optimized using carotenoid standards including β-carotene, canthaxanthin, lutein, and astaxanthin, the structures of which are shown in Figure 1, as well as the internal standard chosen for relative quantitation purposes, β-apo-8′-carotenal. The UV−vis chromatogram at 450 nm for a mix of these standards is presented in Figure 2A. An astaxanthin standard extract (AstaReal L10) was also analyzed, containing multiple astaxanthin esters from *H. pluvialis*, shown in Figure 2B. By using UV−vis detection in-line with a triple-quadrupole mass spectrometer, we were able to simultaneously detect the standard carotenoids by absorbance at 450 nm as well as characterize their mass spectra in positive electrospray mode. The low-resolution MS data collected were useful for the subsequent identification of known and unknown carotenoids by high-resolution TOF measurements for analysis of the up-regulation of carotenogenesis following high-salt treatment. The high-resolution ESI-TOF-MS data for each standard are shown in Figure 3. It is important to note that the m/z values of all standards were measured within a mass error of 2 ppm, allowing elemental formula confirmation. All standards were detected in positive electrospray mode with the protonated molecule (MH^+) as the base peak, except for β-carotene and lutein, which were detected as radical molecular ions ($M^{\bullet+}$), as previously described for the electrospray behavior of these two carotenoid species.[31] Lutein was also seen to be detected as the in-source fragment of its protonated molecule ($MH^+ - H_2O$), similar to previous reports of using APCI-MS.[6]

Preparation of algal extracts consisted of liquid extraction of lyophilized cultures using 75% CH_2Cl_2/25% MeOH, followed by a further cleanup step with SPE prior to analysis. The IS, β-apo-8′-carotenal, was added prior to carotenoid extraction and used for relative quantitative purposes. Using accurate mass data generated by LC-ESI-TOF and processed by differential analysis software (Mass Profiler), algal cultures were found to contain various carotenoid components, which increased upon salt treatment. With the use of the internal standard, the changes in carotenoid concentration are measured on the basis of the changes in ratio of peak areas between the extracted ion chromatogram (EIC) at the m/z of the targeted compound (±10 ppm) and that of the EIC corresponding to the m/z of the internal standard (±10 ppm). The use of an IS ensures that any losses during sample preparation or fluctuations in instrument sensitivity over the course of the sample analysis will be normalized by using the ratio instead of absolute EIC area for each carotenoid.

To identify carotenoids present in the algal extracts for which formation was up-regulated upon salt treatment, specific data filtering parameters were used within Mass Profiler processing software. For the detection of β-carotene, canthaxanthin, lutein, and free astaxanthin as well as other structurally similar compounds, an isotope ratio of $(M + 1)/M$ (where M is the monoisotopic mass) of 0.44 ± 0.15 and a mass defect of 0.41 ± 0.05 Da were used to filter the results. These carotenoid components have molecular weights between 400 and 600 and contain 40 carbons in their structure (Figure 1). Therefore, the isotope pattern selected was based on the fact that each carbon atom has a natural ^{13}C isotope abundance of 1.1%; therefore, 40 carbons account for a total of 44% (or 0.44). In the case of mass defect filtering, a range of 0.36−0.46 was used on the basis of the measured m/z from the available carotenoid standards. Using these parameters, several carotenoids were found to increase upon salt treatment of algal cultures. Table 1 lists the species that rose in the treated samples, including canthaxanthin and astaxanthin, and several other carotenoid compounds tentatively identified as canthaxanthin and astaxanthin isomers, as well as echinenone and adonirubin. The increased production of astaxanthin in incubated samples represents an interesting result because its free radical antioxidant activity is several fold stronger than that of β-carotene and its efficacy in preventing mitochandria peroxidation can be as much as 100 times that of vitamin E, a well-known antioxidant.[38] In the case of β-carotene and lutein, we did not see any significant increases in the same time course study with acetate treatment compared to controls. In Figure 4, the EICs of each carotenoid compound found to increase in salt-treated cultures are shown with a mass extraction window of ±10 ppm. Tentative identification of echinenone and adonirubin was based on accurate mass measurement and elution order, as is often done for carotenoids,[39,40] in comparison to the commercially available standards that were tested in this study. Two additional isomers of each astaxanthin and canthaxanthin were detected in the algal cultures and also increased with salt treatment. Previous papers have described multiple isomers for these two carotenoid compounds based on cis/trans isomerization, including 9-cis, 13-cis, and all-trans isomers.[41−43]

Because astaxanthin is known to be present in its free form as well as in fatty acid ester forms, new isotope patterns and mass

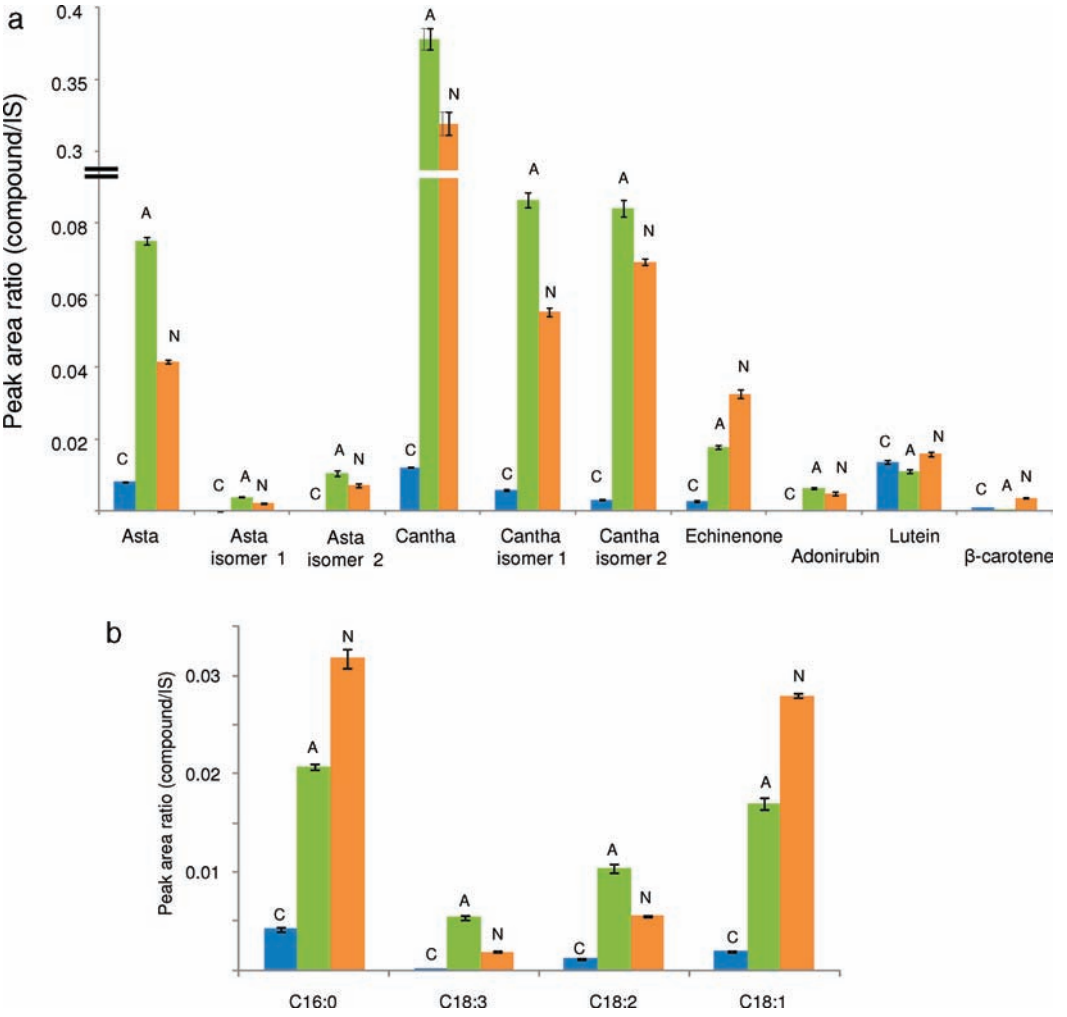

Figure 8. Comparison of control algal samples to cultures following 30 days of treatment with sodium acetate (120 mM) and sodium chloride (600 mM). Peak area ratios of carotenoids/IS for selected carotenoids (a) and for astaxanthin esters (b) are shown. Sample treatments are noted by C for control, A for acetate, and N for NaCl, with error bars indicating replicate injections (n = 3) of each sample.

defect parameters were used to filter the MS data, with larger mass defect values and isotope ratios for the higher molecular weight ester compounds. Four astaxanthin fatty acid esters were found to increase upon salt treatment using the differential analysis software (Mass Profiler) using mass filters of m/z 800−900 with a mass defect range of 0.62 ± 0.07 Da and isotope ratio $(M + 1)/M$ of 0.58 ± 0.15. Astaxanthin esters containing C16:0, C18:1, C18:2, and C18:3 fatty acyl chains increased in salt-treated *Scenedesmus* sp. algal samples. These esters were identified on the basis of accurate mass measurement and elution order as well as comparison to the astaxanthin esters present in the AstaReal L10 standard extract from *H. pluvialis*. The measured molecular weights of these esters were all within 3 ppm mass accuracy of their corresponding theoretical exact mass (Table 2). The elution order corresponded to the polarity of these fatty acids; for instance, C16 elutes earlier than C18, and the degree of unsaturation correlates with elution order (C18:3 < C18:2 < C18:1), as shown in Figure 5. Some of these esters were previously reported but without accurate mass data. For example, various astaxanthin fatty acid esters, including C16:0, C18:0, C18:1, C18:2, C18:3, and C20:4, were found in *H. pluvialis*.[17] In *Scenedesmus* sp., a fatty acid profile containing C16:0, C16:1, C18:1, C18:2, C18:3, and C20:4 was previously found.[44] In this study, we were able to detect C16:1 in our algal samples as well; however, due to its small signal intensity, it was not possible to accurately quantify if it was indeed up-regulated or not. As for the arachidonic ester of astaxanthin (C20:4), we detected this species in the Asta-Real standard but not in our algal samples (data not shown).

A time course for acetate treatment was studied with samples analyzed at 8, 15, 22, and 29 days of treatment together with a control sample (no acetate) at each time point. The up-regulation of carotenoid formation was noted for several compounds, as previously described. The time-dependent increase for each carotenoid is represented in Figure 6, where we can easily see that astaxanthin and canthaxanthin together with their isomers, as well as echinenone and adonirubin, were seen to rise in concentration in a similar manner over the 29 day treatment. Also, it was clearly observed that lutein and β-carotene were not increased over the course of treatment and that the levels of these two species were actually lower in the treated samples than in the control. There could be a decrease of these two compounds due to the shift in metabolism of the algal cells upon treatment; however, no specific conclusion is meant to be shown through these data. The main importance here is to show a time-

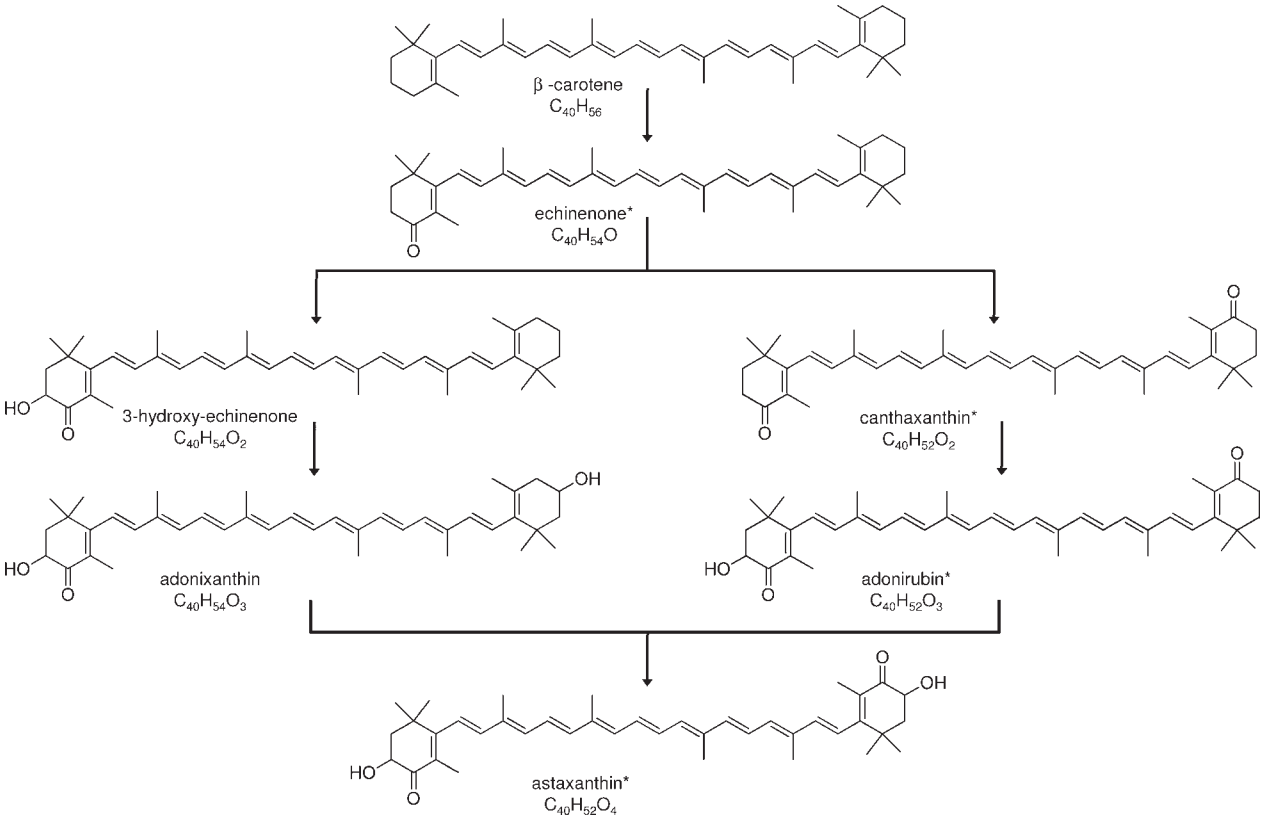

Figure 9. Biosynthetic pathway of astaxanthin from β-carotene (peaks labeled with * were shown to increase upon treatment with high salt concentrations in this study). Adapted from ref 40.

dependent increase of specific secondary carotenoids upon treatment with acetate over the time course studied. Because β-carotene is the starting point in the biosynthetic pathway of astaxanthin, it is potentially not seen to increase because it is being biotransformed into secondary carotenoids downstream in the pathway. Also, it is important to note that lutein is not present as a compound in the biosynthetic pathway of astaxanthin,[40] and therefore our results indicate a specific pathway up-regulation toward astaxanthin biosynthesis. Similarly, the time-dependent increase in concentration of four astaxanthin esters detected in our samples is shown in Figure 7, normalized with the IS peak area. Again, we see a similar effect from acetate treatment for each of the ester forms of astaxanthin detected. In a separate study, the effects of sodium acetate (120 mM) and sodium chloride (600 mM) were compared following a 30 day treatment. The results of this comparison are shown in Figure 8. Peak area ratios of carotenoids/IS are shown to increase for canthaxanthin, astaxanthin, adonirubin, and echinenone (Figure 8a), as well as several astaxanthin esters (16:0, 18:1, 18:2, 18:3) (Figure 8b). There are slight differences between acetate and NaCl treatments, but no consistent trend indicating one salt treatment as giving higher induction of carotenogenesis. According to Yuan et al.,[40] astaxanthin present in alga *Chlorococcum* was biosynthesized from "stepwise" hydroxylations of β-carotene, echinenone, canthaxanthin, and adonirubin. We detected several of the compounds in the scheme represented in Figure 9, which increased during the time course treatment of acetate as well as in sodium chloride treated cultures.

A novel LC-MS-based carotenoid screening method was developed for analyzing algal cultures using a targeted metabolomics approach based on accurate mass data. This semitargeted strategy is useful for detecting both known and unknown carotenoid species. Algal cultures with different salt treatments, including sodium acetate and sodium chloride, were analyzed and were found to contain various carotenoid compounds as well as several fatty acid esters of astaxanthin. The time-dependent up-regulation of carotenogenesis caused by sodium acetate treatment was studied, and several carotenoid species involved in the biosynthetic pathway of astaxanthin were found to increase over the course of the treatment. Further work will include a study involving quantitation of carotenoids of specific interest, for example, astaxanthin and canthaxanthin in algal cultures upon treatment with high salt concentrations as well as a time course analysis of the production of these carotenoids.

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