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Analysis of Carbohydrates and Lipids in Microalgal Biomass with HPAE-MS and LC/MS

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INTRODUCTION

Biodiesel is rapidly becoming an attractive alternative to fossil-derived diesel fuel, and algae are a promising feedstock. Efficient production of biodiesel from microalgae requires analysis of all cell products, including carbohydrates, lipids, and proteins. Profiling of lipids in biomass feed stocks is critical for the production of quality biodiesel. Determination of carbon chain length and the degree of saturation is key to ensuring high quality biodiesel that delivers optimum performance during engine combustion. Lipid profiling is commonly done by GC with a flame ionization detector (FID), as specified in the ASTM D6584 and EN 14105 methods; however, this methodology has several limitations. High-boiling triglycerides often require special inlet types to preclude discrimination which can cause thermal degradation and interfere with the summative mass closure calculations typically done to determine the total composition of biomass. Recently, HPLC analysis at ambient temperatures with MS detection has emerged as the preferred method for biomass lipid profiling because it is well-suited to analysis of nonvolatile components and can handle very complex or dirty sample matrices without clogging of the LC/MS interface.

In addition to lipid profiling, a complete characterization of the carbohydrate breakdown products in the algae is essential for efficient nutrient recycle to determine which sugars are best absorbed by the algae. Carbohydrate and lipid analyses of microalgal biomass are often complex and require a high-resolution, high-sensitivity technique capable of separating and quantifying trace-level components in the presence of multiple interfering compounds. The use of high pH anion-exchange chromatography mass spectrometry (HPAE-MS) permits baseline separation of key saccharides in complex lysate mixtures.

EXPERIMENTAL

Instrument Setup

Carbohydrate analyses were performed on a Dionex ICS-3000 IC system, which includes an ICS-3000 DP gradient pump, AS autosampler, and ICS-3000 DC column compartment with electrochemical cell. Lipid analyses were performed on a Dionex UltiMate® 3000 RS system with HPG-3400 RS pump, WPS-3000 RS sampler, TCC-3000 RS column thermostat, DAD-3000 RS diode-array detector, and SofTA model 400 evaporative light scattering detector (ELSD).

Carbohydrates were detected using pulsed amperometry with a gold electrode and Ag/AgCl reference electrode using the standard quadruple waveform developed at Dionex.[1] Mass spectrometric analyses were run on an MSQ Plus™ single quadrupole mass spectrometer. The effluent of the HPAEC column was passed through a CMD™ desalter [2] (Dionex, P/N 059090), then to the MSQ Plus mass spectrometer. Pressurized water was pumped through the CMD as regenerating reagent. Postcolumn addition of 0.5 mM lithium chloride was mixed into the eluent at 0.1 mL/min using a mixing tee and AXP-MS pump (Dionex, P/N 060684). Detailed instrument setup is shown in Figure 1. The addition of lithium allowed mono- and disaccharides to form lithium adducts. The use of lithium allows detection of the sugars in the lithium form as opposed to mixed forms which may be caused by contamination of weaker adduct-forming cations, such as ammonium or sodium.

Lipids were detected using LC-UV/MS. Postcolumn addition of ammonium acetate was applied to facilitate the formation of lipid-ammonium or lipid-acetate adducts. The MSQ Plus was operated in the positive full scan mode over a scan range of *m/z* 100 to 1500 with a cone voltage fragmentor setting of 50 V.

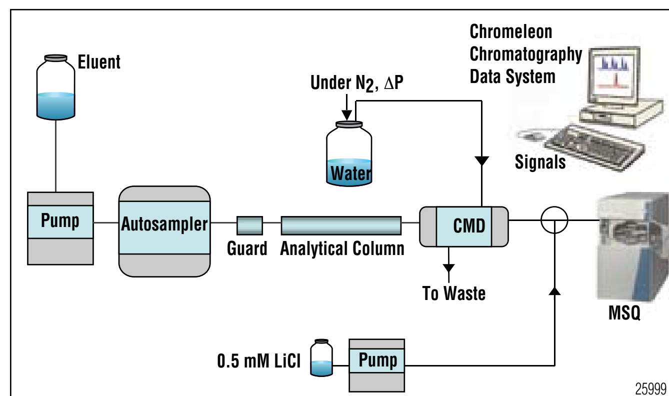

Figure 1. Instrument setup for on-line HPAE-MS analysis of carbohydrates.

Chromatographic separations by LC/MS analyses were performed using a Dionex Acclaim® RSLC C18 2.2 µm column, in 2.1 × 150 mm format. Chromatographic separations by HPAE-MS were performed using a Dionex CarboPac® MA1 (3 × 250 mm) analytical column and CarboPac MA1 (4 × 50 mm) guard column at a flow rate of 200 µL/min at 30 ˚C under isocratic conditions. Chromatography and MS analysis were controlled by Chromeleon® Chromatography Data System (Dionex, version 6.8). Reference spectra were acquired using Xcalibur® (Thermo Fisher Scientific).

Sample Preparation

Samples of algal biomass and oil were obtained from General Atomics, San Diego, CA. To prepare biomass samples for the HPAEC-MS analysis, 2 mL of lysed microalgae sample was centrifuged at 12,000 rpm for 60 min. The supernatant was collected and centrifuged for an additional 30 min. The supernatant was collected and filtered through a 0.2 µm syringe filter to remove any remaining particles. The sample was passed through an OnGuard® RP cartridge to remove hydrophobic components. The OnGuard RP cartridge was activated first with 5 mL methanol followed by 10 mL DI water using a 10 mL syringe. Next, a 400 µL sample of supernatant was mixed with 600 µL water and passed through the activated cartridge using a 1 mL syringe. The cartridge was then washed with 0.5 mL water and the eluted washing solution was combined with the initial eluent. The sample was then passed through another activated RP cartridge. This step was repeated until the sample was free of color. All the eluted solutions (~3 mL) were combined and concentrated down to a volume of ~250 µL.

RESULTS

Carbohydrate Analysis

The separation profile of carbohydrates in microalgae samples on the CarboPac MA1 is shown in Figure 2. More than a dozen peaks were observed. On-line mass spectrometry analysis was used on the same samples to confirm the carbohydrate identity of the peaks. Note the similarities between the TIC and PAD profiles (Figure 3). The full scan spectrum of each peak is shown in Figure 4. Because many mono- and disaccharides have identical mass-to-charge ratios (*m/z*), HPAE-PAD profiles of carbohydrate standards were compared with the sample profile. Comparison of their retention times helped to identify the peaks (Figure 5). Table 1 shows the identification results based on the peak's *m/z* and comparison with PAD profiles of the standards. These data indicated that monosaccharide alditols (fucitol, arabitol, inositol), monosaccharides (glucose, mannose, and others) and disaccharides (sucrose, maltose, and others) were present in the microalgal biomass, sucrose being the major sugar component.

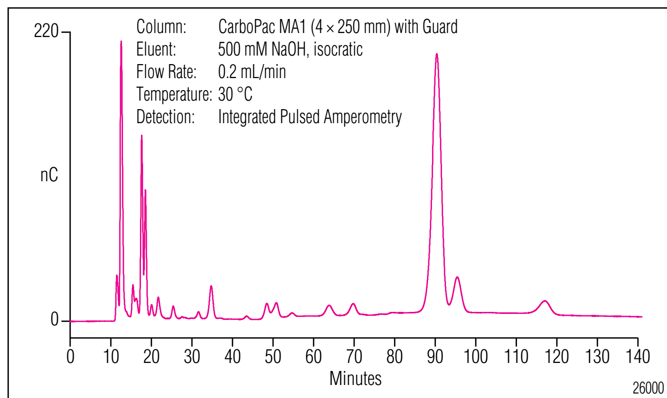

Figure 2. Separation profile of microalgae carbohydrates on the CarboPac MA1 column.

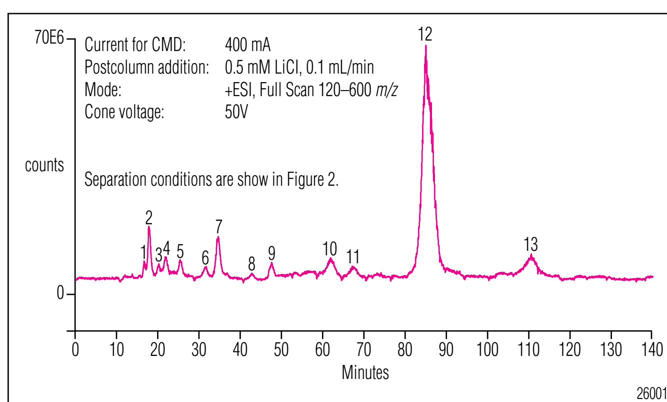

Figure 3. MS TC profile microalgae carbohydrates.

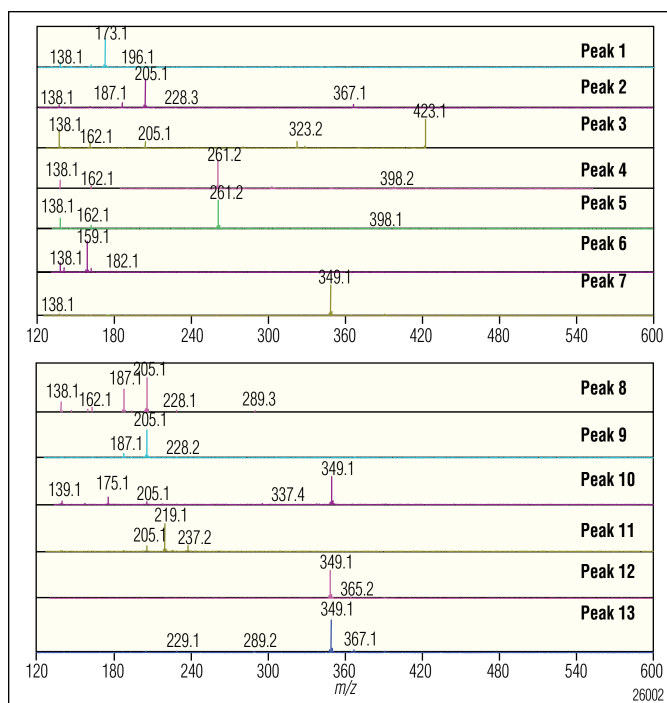

Figure 4. Background-substracted ESI mass spectra of peaks shown in Figure 3.

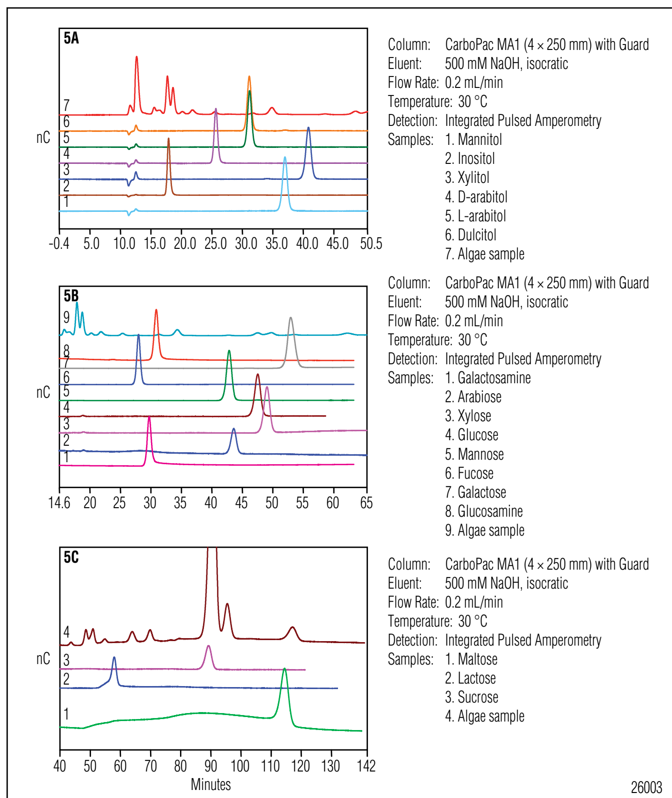

Figure 5. Overlay comparisons of algae samples with carbohydrate standards with, A: alditol standards; B: monosaccharide standards; C: disaccharide standards.

Table 1. Identification of Sample Peaks Based on Mass-to-Charge Ratios and Retention Times

Peak	*m/z*	Adduct	Possible Identification
1	173.1	166 + 7	(Fucitol + Li)$^+$
2	205.1/187.1	180 + 7, 180 + 7 + 18	(Inositol + Li)$^+$, (Inositol + Li + H_2O)$^+$
3	423.1	Unknown	Unknown
4	261.2	Unknown	Unknown
5	261.2	Unknown	Unknown
6	159.1	152 + 7	(Monoalditol + Li)$^+$ (L-Arabitol)
7	349.1	342 + 7	(Disaccharide + Li)$^+$
8	205.1/187.1	180 + 7	(Monosaccharide + Li)$^+$ (Mannose)
9	201.1/187.1	180 + 7	(Monosaccharide + Li)$^+$ (Glucose)
10	349.1	342 + 7	(Disaccharide + Li)$^+$
11	219.1	Unknown	Unknown
12	349.1	342 + 7	(Disaccharide + Li)$^+$ (Sucrose)
13	349.1	342 + 7	(Disaccharide + Li)$^+$ (Maltose)

Lipid Analysis

The oil samples were analyzed under conditions that give a simplified chromatogram where triacylglycerides (TAGs) with similar Partition Numbers or Effective Carbon Numbers[3] tend to coelute in a single peak.[4] Mass spectrometry allows us to assign total carbon and total unsaturation numbers to components within each peak. The notation x:y is used where x is the total carbon number of the acyl chains, and y is the total number of double bonds. The typical fatty acid compositions of lipids from various sources are well known in the literature, and with few exception, they have an even number of carbon atoms.[5,6] For each x:y we enumerate the *m/z* value of the $[M+NH_4]^+$ adduct, and a theoretical isotope pattern. We then assign matching x:y to the components in the chromatogram. Reference 4 shows an example of this process for soybean oil. Figure 6 shows the extracted ion chromatograms and x:y assignments for the main components of algal oil.

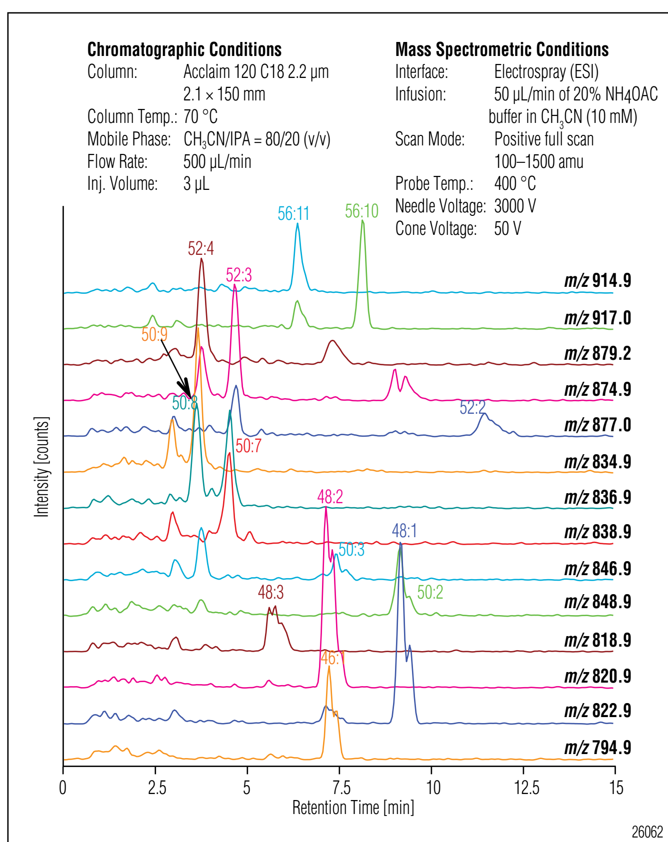

Figure 6. Extracted MS chromatograms of lipids in algae oil.

Table 2 lists the masses, peak assignments, and a partial list of probable TAGs in algal oil. The possible combinations of known fatty acids to make any given x:y can be large; the list of probable TAGs was established according to reported fatty acid composition in algal oil.[7] Each list is isobaric and the constituents cannot be distinguished by a single-stage mass spectrometer. To make a complete assignment of TAGs found in the sample would require MS/MS. Compared to typical plant-derived oils, there are some unusual/characteristic features to this algal oil. First, no 54-carbon TAGs were observed in this study. Second, the presence of highly unsaturated TAGs (unsaturations from eicosapentaenoic acid, 20:5) was confirmed and this observation agrees with reported data.[7]

Table 2. TAGs in Algal Oil

Retention time	$M+NH_4$ Adduct *m/z*	Assignment	Probable TAGs	
6.4	914.9	56:11	16:1/20:5/20:5	18:3/18:3/20:5
8.1	917.0	56:10	18:2/18:3/20:5	
3.8	872.9	52:4	16:0/18:1/18:3	16:0/18:2/18:2
4.7	874.9	52:3	16:0/18:0/18:3	16:0/18:1/18:2
11.5	877.0	52:2	16:0/18:0/18:2	16:0/18:1/18:1
3.7	834.9	50:9	16:2/16:3/18:4	16:3/16:3/18:3
3.6	836.9	50:8	14:0/16:3/20:5	16:2/16:3/18:3
4.5	838.9	50:7	14:0/16:2/20:5	16:1/16:3/18:3
7.4	846.9	50:3	14:0/18:0/18:3	14:0/18:1/18:2
9.1	848.9	50:2	14:0/18:0/18:2	14:0/18:1/18:1
5.7	818.9	48:3	14:0/16:0/18:3	14:0/16:1/18:2
7.2	820.9	48:2	14:0/16:0/18:2	14:0/16:1/18:1
9.2	822.9	48:1	14:0/16:0/18:1	14:0/16:1/18:0
7.3	794.9	46:1	12:0/16:0/18:1	12:0/16:1/18:0

CONCLUSION

Using simple sample preparation procedures, the carbohydrates in lysed microalgal biomass and lipids in algal oil were chromatographically separated respectively and analyzed using on-line mass spectrometry. Peaks were identified based on their molecular weights and retention times.

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