Carbon cycling in benthic diatom mats: Novel applications of LC/IRMS

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Publication date
2016

Document Version
Final published version

Citation for published version (APA):

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Download date: 26 Jul 2023
A versatile method for stable carbon-isotope analysis of carbohydrates by high-performance liquid chromatography - isotope ratio mass-spectrometry

Published in Rapid Commun. Spectrom. 2008; 22: 3902-3908

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Abstract

We developed a method to analyze stable carbon isotope ($^{13}$C/$^{12}$C) ratios in a variety of carbohydrates using high-performance liquid chromatography / isotope ratio mass-spectrometry (HPLC/IRMS). Chromatography is based on strong anion-exchange columns with low strength NaOH eluents. An eluent concentration of 1 mM resulted in low background signals and good separation of most of the typical plant neutral carbohydrates. We also show that more strongly bound carbohydrates such as acidic carbohydrates can be separated by inclusion of NO$_3^-$ as an inorganic pusher ion in the eluent. Analyses of neutral carbohydrate concentrations and their stable carbon isotope ratios are shown for plant materials and marine sediment samples both at natural abundance and for $^{13}$C-enriched samples. The main advantage of HPLC/IRMS analysis over traditional gas-chromatography based methods is that no derivatization is needed resulting in simple sample treatment and an improved accuracy and reproducibility.
Introduction

Carbohydrates in their various forms take a central place in the biosphere. They are the first compounds formed during photosynthesis and are major substrates for heterotrophic organisms. Carbohydrates are found in structural materials like in the lignocellulose of the plant cell wall and in storage products such as starch. In addition, they are excreted by many organisms as extracellular polysaccharides. The extracellular release of carbohydrates by diatom biofilms on coastal sediments has major consequences for food web functioning and sediment stability (de Brouwer & Stal, 2002). Carbohydrates are also a major part of soil, sediment and dissolved organic matter pools and as such play an important role in the carbon cycle by providing labile substrates for growth and respiration and refractory components for the accumulation of organic carbon (Moers et al., 1993; Amon et al., 2001; Derrien et al., 2004). Given their many functions and wide use, it is not surprising that many different forms of monomeric carbohydrates are found in natural systems, which include neutral sugars, sugar alcohols, uronic acids and amino sugars. Stable carbon isotope (\(^{13}\text{C}/^{12}\text{C}\)) analysis of carbohydrates has been very useful not only in studying sources of carbohydrates and their turnover in the environment (Moers et al., 1993; Derrien et al., 2004) but also to detect food adultery (Cabanero et al., 2006) and to trace microbial biomass dynamics (Glaser & Gross, 2005).

Gas chromatography combustion isotope ratio mass spectrometry (GC-c-IRMS) has been the principle method for compound specific isotope analysis and several methods are available for the isotopic analysis of carbohydrates (Moers et al., 1993; van Dongen et al., 2001; Derrien et al., 2003). Carbohydrates however need to be heavily derivatized before they are amendable to isotopic analysis by gas-chromatography, and substantial corrections have to be made for the carbon atoms added during derivatization and for the kinetic isotope effects associated with some methods (Macko et al., 1998; Teece & Fogel, 2007). These corrections decrease accuracy and reproducibility of the stable isotope analysis and stringent testing of analytical procedures is needed to determine correction factors which vary among carbohydrates and possibly also with sample matrix. Moreover, GC-c-IRMS methods have a limited analytical range and are only available for neutral sugars, sugar alcohols and amino sugars (Moers et al., 1993; Glaser & Gross, 2005).

High performance liquid chromatography based methods are used widely for the direct analysis of carbohydrates without derivatization (Quemener et al., 1997). Recently, the first commercially available high-
performance liquid chromatography / isotope ratio mass-spectrometry (HPLC/IRMS) system was described by Krummen et al. (2004) that holds great promises for the stable isotope analysis of specific (biological) compounds that are water soluble such as carbohydrates. This system is based on wet chemical oxidation of all eluting compounds with peroxodisulfates under acidic conditions. The carbon dioxide released is removed from the eluent flow in a miniature membrane separator with a helium flow and carried into the IRMS after water vapor removal. Carbohydrate analysis with this HPLC/IRMS system has been shown (Krummen et al., 2004; Cabanero et al., 2006; Penning & Conrad, 2006), but the available methods have limited application in environmental and biological studies due to a rather narrow analytical range as separation of only glucose, fructose and sucrose has been shown. These methods cannot be used to separate the typical plant carbohydrates. A versatile and commonly used HPLC approach for analyzing carbohydrate concentrations is by anion exchange chromatography with strong alkaline eluents and sensitive pulsed-amperometric detection (HPAEC-PAD) (Johnson & Lacourse, 1990; Panagiotopoulos & Sempere, 2005). This method can be adapted for a wide range of carbohydrates by changing eluent composition. However, the chromatographic part of this method cannot be directly transferred to HPLC/IRMS, because the strong alkaline eluents generally result in high carbon backgrounds due to carbonate inclusion in the eluent and may also interfere with the acidic wet oxidation process. In addition, the commonly used organic sodium acetate pusher cannot be used in HPLC/IRMS to elute stronger binding carbohydrates such as uronic acids.

We have adapted the HPAEC method to make it applicable to HPLC/IRMS. Principle changes were the use of a narrow bore column to adapt to the low flow limits of the HPLC/IRMS and a low strength NaOH eluent. This significantly lowered carbon backgrounds and improved separation of the major neutral sugars without interfering with the wet oxidation process. We also investigated the use of nitrate (NO$_3^-$) as an alternative pusher ion over the commonly used carbon-containing acetate to show the analysis of strongly bound carbohydrates like acidic sugars. The method yields accurate and reproducible $\delta^{13}$C and concentration data for a range of carbohydrates including the typical plant carbohydrates in natural samples with minimal sample preparation.
Experimental

**Chemicals and reagents**
All reagents were of analytical grade and were purchased from Sigma (St. Louis, USA), except NaOH solution (50%) and D(+) galacturonic acid which were purchased from Fluka (Buchs, Switzerland). The carbohydrates standards used during this study are given in table 2.1. Freshly prepared Milli-Q water (18.2 MΩ, DOC free; Millipore, Bedford, USA) was used in all experiments.

Table 2.1. Stable carbon isotope analysis of carbohydrate standards by EA-IRMS, μEA-IRMS and HPLC/IRMS. Stable isotope data are the averages and SD of five replicate analysis for EA-IRMS and μEA-IRMS (5 nmol carbohydrate injected with 50 µL loop). HPLC/IRMS data (N is 20 to 30 depending on compound) and based on replicate analysis of a concentration range from the detection limit to the maximum concentration used (20 nmol carbohydrate injected with 10 µL loop). Detection limits for HPLC/IRMS are amounts of carbohydrate injected at which reproducibility (SD) was better than 0.5 ‰ .

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>EA-IRMS δ13C (%)</th>
<th>μEA-IRMS δ13C (%)</th>
<th>HPLC-IRMS δ13C (%)</th>
<th>Detection limit (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>-26.61 ± 0.04</td>
<td>-26.48 ± 0.09</td>
<td>-26.72 ± 0.23</td>
<td>0.5</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>-24.81 ± 0.05</td>
<td>-24.75 ± 0.04</td>
<td>-24.78 ± 0.38</td>
<td>0.5</td>
</tr>
<tr>
<td>Galactose</td>
<td>-23.38 ± 0.17</td>
<td>-23.72 ± 0.07</td>
<td>-23.37 ± 0.47</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>-10.95 ± 0.17</td>
<td>-12.12 ± 0.16</td>
<td>-11.42 ± 0.41</td>
<td>0.5</td>
</tr>
<tr>
<td>Xylose</td>
<td>-21.09 ± 0.03</td>
<td>-21.60 ± 0.16</td>
<td>-20.93 ± 0.36</td>
<td>1.0</td>
</tr>
<tr>
<td>Mannose</td>
<td>-28.17 ± 0.03</td>
<td>-28.01 ± 0.07</td>
<td>-25.13 ± 1.73*</td>
<td>1.0</td>
</tr>
<tr>
<td>Fructose</td>
<td>-24.85 ± 0.03</td>
<td>-24.96 ± 0.07</td>
<td>-25.83 ± 2.32*</td>
<td>2.0</td>
</tr>
<tr>
<td>Ribose</td>
<td>-14.56 ± 0.10</td>
<td>-15.47 ± 0.16</td>
<td>-14.42 ± 1.44</td>
<td>2.0</td>
</tr>
<tr>
<td>Muramic acid</td>
<td>-19.99 ± 0.38</td>
<td>-20.59 ± 0.29</td>
<td>-19.31 ± 0.56</td>
<td>0.5</td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td>-22.84 ± 0.16</td>
<td>-23.67 ± 0.16</td>
<td>-23.32 ± 0.70</td>
<td>0.5</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>-7.24 ± 0.19</td>
<td>-8.82 ± 0.15</td>
<td>-7.77 ± 0.30</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*: Mannose and fructose were not completely separated at higher concentrations.
**HPLC/IRMS**

High performance anion-exchange chromatography was carried out on a Thermo Surveyor system consisting of an HPLC pump (MS Pump Plus) and autoinjector (Autosampler Plus; Thermo Electron, Bremen, Germany), fitted with a CarboPac PA20 guard and narrow-bore analytical column (3 × 150mm; Dionex Benelux, Amsterdam, the Netherlands) and eluted at 300 µL min⁻¹ isocratically, either with 1 mM NaOH for analyzing neutral carbohydrates or with 1 mM NaOH and 2 mM NaNO₃ for analyzing acidic carbohydrates. The column was regularly regenerated with 200 mM NaOH. All eluents were carefully degassed in an ultrasonic bath for 30 min before NaOH and NaNO₃ were added and further degassed with helium during analysis. ‘No-Ox’ tubing (1/8” × 1.5” mm; Socochim, Lausanne, Switzerland) was used to connect the eluent bottles to the pump to prevent atmospheric gases to re-enter solvents. All pump heads were rinsed at least once a day to prevent crystallization. An in-line filter of 2 µm (Vici, Schmidlin Labor, Switzerland) was placed after the LC column to avoid any particles passing into the interface.

The HPLC system was coupled to the IRMS by an LC Isolink interface (Thermo Electron, Bremen, Germany) first described by Krummen *et al.* (2004). The technique of the Isolink interface is based on wet oxidation of organic analytes with peroxodisulfate under acidic conditions. The CO₂ produced is subsequently separated from the mobile phase in a capillary gas exchanger flushed with helium gas, dried and enters the ion source of the mass spectrometer in a helium stream via an open split interface. The temperature of the oxidation reactor was set at 99.9 °C. The flow rates of the acid (1.5 M H₃PO₄) and oxidant reagents (0.7 M NaS₂O₆) were 50 µL min⁻¹ each. Samples can also be injected directly on the LC Isolink interface, which then operates as a sensitive elemental analyzer IRMS (μEA-IRMS) (Krummen *et al.*, 2004).

Isotopic ratio measurements were carried out on a Delta V Advantage IRMS (Thermo Electron, Bremen, Germany). The control of the HPLC/IRMS system and data collection was done using Isodat 2.5 SP 1.13 software. Baseline corrections were done by the basic algorithm provided by the Isodat software and manually optimized where necessary. To calibrate the system, two pulses of CO₂ reference gas were admitted into the inlet of the IRMS for about 20 s each at the beginning of a run. The reference gas was regularly calibrated against phthalic acid (Schimmelmann, Bloomington, USA) with a δ¹³C value of -27.21 ± 0.02 ‰. Stable carbon isotope ratios are reported in the delta-notation: $\delta^{13}C (\text{‰}) = (R_{\text{sample}}/R_{\text{VPDB}}-1) \times 1000$, where $R_{\text{sample}}$ and $R_{\text{VPDB}}$ are the $^{13}C/^{12}C$-ratio in the sample and international standard (Vienna Pee Dee
Stable isotope analysis of carbohydrates

2

Belemnite), respectively. Peak identification was based on retention times in comparison with external standards. Concentration measurements were based on peak areas of the separated compounds and calibrated against external standards.

**Samples**

Several typical coastal marine materials were collected and analyzed for their neutral carbohydrate and isotopic composition: a marine macroalga, *Ulva* sp.; a C3 macrophyte, *Festuca rubra*; a C4 macrophyte, *Spartina anglica*, and an intertidal marine sediment, both natural and $^{13}$C-labeled. *Ulva* sp. and sediment cores (7 cm ID) were collected at a tidal flat in the Eastern Scheldt estuary (The Netherlands). The sediment was covered with a thin brown mat of benthic phototrophic microalgae mainly consisting of diatoms. One set of sediment cores was labeled with NaH$^{13}$CO$_3$ (99 % $^{13}$C, Isotec, the Netherlands) by adding 1 mL of a 10 mM solution in artificial seawater (Ca$^{2+}$ and Mg$^{2+}$ free) to the top of the sediment core and incubating the cores for 2 h at environmental temperature and light conditions. The upper 0.5 cm layers of the labeled and unlabeled sediment cores were subsequently sampled. *Festuca rubra* and *Spartina* spp. were archived samples collected at the Schiermonnikoog salt marsh (the Netherlands) from an earlier study (Bouillon & Boschker, 2006). All samples were stored frozen (-20 ºC) until analysis. Prior to analysis the samples were lyophilized for 48 hours and grinded to a fine powder in a sample mill (MM 2000, Retsch, Germany). All samples were analyzed in triplicate.

Total organic carbon content and $\delta^{13}$C ratios were determined by elemental analyzer IRMS (EA-IRMS) consisting of a Flash EA 1112 Series elemental analyzer coupled via Conflo III interface to a Delta V Advantage IRMS (Thermo Electron, Bremen, Germany) (Bouillon & Boschker, 2006).

**Sample preparation**

For neutral carbohydrates, plant tissue (25 mg) and sediment (500 mg) were hydrolyzed under acidic conditions using the sulfuric acid method (Cowie & Hedges, 1984). Samples were stirred with 0.5 mL of 11 M H$_2$SO$_4$ at room temperature for 1 h. The solution was diluted to 1.1 M H$_2$SO$_4$ and hydrolyzed for 1 h at 120 ºC. Samples were cooled in crushed ice. The hydrolysate was neutralized to pH 5.5-6.0 by adding BaCO$_3$ and the BaSO$_4$ precipitate was removed by centrifugation (15 min, 4000 g). The supernatant was collected and frozen overnight to further precipitate BaSO$_4$. Finally the samples were filtered over a 0.22 µm filter (Millex-
GV4; Millipore, Bedford, USA), placed into 1 ml glass vials and analyzed by HPLC/IRMS.
Results and discussion

Since the description of the first commercial HPLC/IRMS system (Krummen et al., 2004), several well-designed methods have been described for $^{13}$C analysis of amino acids, peptides, and volatile fatty acids (Godin et al., 2005; Heuer et al., 2006; McCullagh et al., 2006; Penning & Conrad, 2006; Schierbeek et al., 2007). Although two published methods are available for carbohydrates analysis by an HPLC/IRMS (Cabanero et al., 2006; Penning & Conrad, 2006), these have a rather limited range of carbohydrates that can be separated and for which reliable isotope data can be obtained. We have adapted a commonly used method for carbohydrate analysis by HPLC, which is based on the use of strong ion exchange columns and relatively strong NaOH eluents typically in combination with pulsed-amperometric detection (Johnson & Lacourse, 1990). The main adaptation was the use of a weak NaOH eluent, which not only improved separation of neutral carbohydrates, but also lowered background levels.

**Chromatography**

A concentration of 1 mM NaOH was found to be optimal for the separation of more than seven commonly occurring plant neutral carbohydrates (Fig. 2.1A). Only arabinose and rhamnose eluted closely together and could not be resolved on the Carbopac PA20 column. This is a problem with this type of column and can be circumvented by using other available columns such as the Carbopac PA1 column (Dionex Benelux B.V., Amsterdam, the Netherlands) that show better separation of these two carbohydrates, but have the disadvantage of limited separation of xylose and mannose. Amino sugars, commonly found in minor amounts in sediments and mainly derived from microbial biomass and zooplankton remains, are also not separated and elute in the arabinose to galactose region (data not shown). However, amino sugars normally occur in relatively low concentrations in marine sediments (Dauwe & Middelburg, 1998) and can be removed easily with cation-exchange resins. There may also be a small overlap between mannose and fructose (Fig. 2.1A), especially at high sugar concentrations and when the analytical column needs to be regenerated.

A disadvantage of using low NaOH concentrations is that the analytical column slowly loses activity probably because stronger binding anions like carbonate from the eluent or salts in the injected samples are not completely washed out and compete with the carbohydrates for binding sites. This results in a gradual decrease in retention times (Fig.
2.2) and a resulting decrease in separation. The column can be regenerated with 200 mM NaOH, and a 5 min regeneration step is sufficient after each run. However, it took about 30 min before IRMS background signals stabilized after regeneration, and our currently preferred, timesaving approach is to run the machine isocratically until separation deteriorates too far, which typically occurs after 15 to 25 runs with clean samples. The column is subsequently regenerated with 200 mM NaOH for 20 min followed by a 30 min equilibration at 1 mM NaOH. The shifting retention times cause little problems with identifications based on retention times as run-to-run shifts are mostly small and approximately linear with the number of injections (Fig. 2.2). In addition, chromatograms from samples are usually rather simple (e.g. Fig. 2.1C) and standards can be injected along with problematic samples.

Another major advantage when 1 mM NaOH was used as an eluent was a strong decrease in carbon dioxide background to about 200 mV (Fig. 2.1A and B), which is at the lower end of the reported range for other HPLC/IRMS methods (Cabanero et al., 2006; Heuer et al., 2006; Penning & Conrad, 2006). The commonly used 10 to 200 mM NaOH concentrations in combination with this type of analytic column, resulted in backgrounds of 1 to 3 V (data not shown) even though eluents were prepared with minimal carbonate impurities by carefully degassing freshly prepared MilliQ water before adding NaOH from a carbonate free 50% stock. Such high backgrounds would seriously affect detection limits and the reproducibility of the $^{13}$C analysis. The LC Isolink interface uses a wet oxidation method under acidic conditions and it has been suggested that HPLC/IRMS is therefore only possible with eluents having a pH lower than 8 (Krummen et al., 2004). However, we did not observe any problems with the efficiency of the wet oxidation process even with 200 mM NaOH eluents. The amounts of acid added in the interface are in principle sufficient to neutralize up to approximately 750 mM NaOH at a column flow of 300 $\mu$L min$^{-1}$.

High concentrations of sodium acetate are commonly used as a pusher for compounds that are more strongly retained such as oligomeric and acidic carbohydrates. An organic pusher cannot be used in HPLC/IRMS due to the design of the interface and would lead to very high backgrounds. However, other weakly bound anions can also be used instead of sodium acetate (Wong & Jane, 1995). We tested NaNO$_3$ as an alternative inorganic pusher and figure 2.1B shows the excellent separation of three common acidic carbohydrates namely muramic acid and two uronic acids in an isocratic run with 1 mM NaOH and 2 mM NaNO$_3$. This finding widens the analytical window to all carbohydrates.
currently analyzed by HPLC-PAD including oligomeric carbohydrates and possibly also sulfated and phosphorilated carbohydrates by varying the NaNO$_3$ concentration similarly as done with sodium acetate.
Fig. 2.1. HPLC/IRMS example chromatograms showing the separation of neutral carbohydrate standard mixture on the Carbopac PA20 column with 1 mM NaOH eluent (A) and acidic carbohydrate standard mixture with 1 mM NaOH and 2 mM NaNO₃ eluent (B). Injected concentrations of were 1000 μM for neutral carbohydrates and 500 μM for acidic carbohydrates. Also shown is the chromatogram (C) of a hydrolyzed Ulva sample analyzed for neutral carbohydrates.
Fig. 2.2. Shift in retention time (Rt, seconds) of the glucose peak during consecutive runs. Other carbohydrates showed similar shifts. The arrow indicates column regeneration with 200 mM NaOH as described in the text.
Stable carbon isotope analysis of carbohydrate standards

We injected a range of concentrations of all different carbohydrates used in this study (Table 2.1; Fig. 2.1) to determine detection limits and linearity for both carbon isotopic ratios and peak area. Peak areas as a measure of carbohydrate concentration were highly linear at all concentrations tested (5 to 2000 μM (equals 50 pmol to 20 nmol carbohydrate injected with a 10 μL loop), R$^2$ higher than 0.995, data not shown), showing that the method is also suited to determine carbohydrate concentrations. Stable carbon isotope ratios remained within acceptable limits (SD < 0.5 ‰) from about 500 pmol (36 ng C) carbohydrate injected for glucose to 2 nmol (150 ng C) for late eluting carbohydrates and up to the highest concentrations tested (Table 2.1; Fig. 2.3). Typically a peak height of 500 mV or more was needed for accurate isotope ratio analysis, below which large deviations from the expected isotopic ratio occurred, possibly due to errors in baseline correction. When using a 10 μL injection loop, this translates to a detection limit of 50 to 200 μM. The sensitivity can be increased even further to 10 to 40 μM by using 50 μL injections providing ample scope for the carbon isotope analysis of carbohydrates in natural materials.

We compared δ$^{13}$C ratios as analyzed by HPLC/IRMS, μEA-IRMS and traditional EA-IRMS for all carbohydrates to determine reliability of the HPLC/IRMS data (Table 2.1; Fig. 2.4). Carbon isotope ratios based on μEA-IRMS corresponded well with those based on conventional EA-IRMS (Table 2.1), but there was a tendency for a small negative bias for heavier isotope values (Fig. 2.4A). Carbohydrates were dissolved in milli-Q water when analyzed by μEA-IRMS and the observed offset is most likely due to a small isotopically-depleted carbon blank in the water. Unfortunately, the amount of carbon in this blank was too low (about 1.5 nmol C) for accurate δ$^{13}$C ratio measurement and no corrections could accordingly be made. Reproducibility of HPLC/IRMS measurements was very good and HPLC/IRMS and EA-IRMS were in excellent agreement (Table 2.1; Fig. 2.4B), except for mannose and fructose which were not always completely separated at higher concentrations and the late eluting ribose. The small bias observed with μEA-IRMS was not observed with HPLC/IRMS, because HPLC separated the blank contamination from the sugars. It should be noticed that the HPLC/IRMS data were based on a range of concentrations from the detection limit of 50 μM to 200 μM, depending on carbohydrate analyzed, to the maximum concentration of 2000 μM. Our assessment therefore represents a worst case scenario. As expected, standard deviations of isotope measurements were lower if samples were analyzed
over a more restricted range of concentrations (Table 2.2). A $^{13}$C-enriched glucose (IAEA-309A, certified at $\delta^{13}$C = 93.9 ± 1.0 ‰) was also analyzed by HPLC/IRMS using a two point calibration with our un-labeled laboratory glucose reference ($\delta^{13}$C = -10.95 ± 0.17 ‰) and the international glucose reference IAEA-309B ($\delta^{13}$C = 535 ± 5 ‰). The IAEA-309A glucose reference gave an isotope ratio of 90.8 ± 1.8 ‰ (N=5, AVG ± SD) when analyzed in this way, which is close to the certified value. These results show that stable isotope ratios both at natural abundance and enriched in $^{13}$C of more than nine commonly observed carbohydrates can be analyzed reproducibly and accurately by HPLC/IRMS if complete separation of the compounds of interest can be achieved.

![Graph showing the effect of injected amount of carbohydrate on the stable isotopic ratio of glucose and galactose.](image)

**Fig. 2.3.** Effect of injected amount of carbohydrate on the stable isotopic ratio of glucose and galactose. A 10 μl injection loop was used and other carbohydrates showed similar results if they were fully separated from other components.
Fig. 2.4. Comparison of stable isotope ratios of individual carbohydrates as by μEA-IRMS versus traditional EA-IRMS (A) and HPLC/IRMS versus traditional EA-IRMS (B). Mannose and fructose were not completely separated and are indicated separately in figure 2.4B, as is ribose the latest eluting compound. The 1:1 line is also indicated in both figures.

Stable isotope analysis of carbohydrates in environmental materials

We analyzed several typical materials from coastal marine environments for neutral carbohydrate stable isotope content (Table 2.2) and concentrations (Table 2.3). The H$_2$SO$_4$ method was used to hydrolyze the samples as it is a commonly used for environmental samples (Cowie & Hedges, 1984) and the neutralized hydrolyzates could be directly analyzed by HPLC/IRMS without further sample treatment even for salt-containing marine sediments. This suggests that salt concentrations were sufficiently low probably as a result of the tenfold dilution during the second step of the hydrolysis with 1.1 M sulfuric acid. Carbohydrate standards dissolved in seawater could only be analyzed without a collapse of chromatography when diluted to a similar extent. The H$_2$SO$_4$ method cannot be used for the analysis of acidic carbohydrates as they are lost during hydrolysis or during subsequent neutralization with BaCO$_3$. Two other methods of hydrolysis were therefore also tested namely using trifluoracetic acid (TFA) alone or in combination with methanolysis (de Ruiter et al., 1992). These hydrolysis methods yield higher recoveries for some types of carbohydrates such as uronic acids and sample neutralization is easier as
TFA can be removed by evaporation. However, we found that these two hydrolysis methods were incompatible with HPLC/IRMS as chromatography degraded completely even when samples were diluted similarly as with the H$_2$SO$_4$ method. This collapse in chromatography is probably due to either higher inorganic salt concentrations as some anions are not removed with BaCO$_3$ precipitation or possibly the presence of organic ions like amino acids in the TFA hydrolysates. Further sample treatment will therefore be essential for TFA and probably also HCl based hydrolysis procedures. Fortunately, impurities disturbing chromatography were apparently removed in the H$_2$SO$_4$ method either during sample neutralization with BaCO$_3$ or diluted far enough enabling a very simple and fast sample treatment.

Stable isotope ratios for the individual neutral carbohydrates in the plant materials typically showed standard deviations of 0.4 ‰ or less (Table 2.2). Data for the unlabeled sediment sample appeared somewhat more variable though standard deviations were still low. This higher variability is probably a result of natural variability as three different sediment cores were used as replicates. Natural variation in the stable isotope ratios of sediment carbohydrates was apparently higher than our analytical precision. Results from the $^{13}$C-labeled sediment cores were also more variable. Again this can be attributed to small differences in carbohydrate synthesis rates in the individual sediment cores incubated. This pilot experiment clearly shows that our technique provides large potential to trace carbohydrate dynamics in natural ecosystems. Individual carbohydrates were typically enriched in $^{13}$C-ratio in comparison with bulk organic carbon ratios in unlabeled samples (Table 2.2) and enrichment levels fall well within the range reported for the individual carbohydrates (Macko et al., 1990; Moers et al., 1993; van Dongen et al., 2002; Teece & Fogel, 2007). In fact, isotopic enrichment factors in four out of five carbohydrates were rather similar for the two macrophytes analyzed and much less variable among samples (Fig. 2.5) than previously reported (Moers et al., 1993; van Dongen et al., 2002; Teece & Fogel, 2007). This could be due to the similarity in growth conditions of the macrophytes as both plant species were sampled from the same salt marsh at the same time. In addition, the results for the macroalgae Ulva were mostly in line with the macrophyte data except for the combined arabinose/rhamnose peak, which may be due a dominance of the pentose arabinose in macrophytes (Cowie & Hedges, 1984) whereas the hexose rhamnose is more abundant than arabinose in Ulva (Lahaye & Jegou, 1993). The hexoses glucose and galactose were isotopically lighter than the pentoses arabinose and xylose for the two macrophyte samples, as has
been shown before (van Dongen et al., 2002; Teece & Fogel, 2007). The sediment labeling experiment showed major differences in $^{13}$C-labeling of individual carbohydrates suggesting that they were synthesized at different rates. Labeling was especially high in glucose, which is not surprising as it is the first sugar that is synthesized during photosynthesis and glucose in the form of chrysolaminaran is also a major storage compound in diatoms (Granum et al., 2002). The other carbohydrates were far less labeled and are mainly found in cell walls and extracellular polysaccharides (de Brouwer & Stal, 2002; Granum et al., 2002), which are apparently synthesized at lower rates during the short incubation time used in this initial study.

**Table 2.2.** Stable carbon isotope compositions of neutral carbohydrates as detected in H$_2$SO$_4$ hydrolyzates of typical coastal marine materials. Stable isotope data are the average and SD of three replicate samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fucose</th>
<th>Rham/Arab.</th>
<th>Galactose</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Mannose</th>
<th>TOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spartina</td>
<td>-9.8 ± 0.3</td>
<td>-7.2 ±0.1*</td>
<td>-12.6 ± 0.1</td>
<td>-11.3 ± 0.2</td>
<td>-8.0 ± 0.2</td>
<td>nd</td>
<td>-14.1 ± 0.0</td>
</tr>
<tr>
<td>Festuca</td>
<td>-16.9 ± 0.4</td>
<td>-20.3 ± 0.2*</td>
<td>-25.6 ± 0.1</td>
<td>-24.5 ± 0.0</td>
<td>-21.8 ± 0.2</td>
<td>nd</td>
<td>-26.1 ± 0.0</td>
</tr>
<tr>
<td>Ulva</td>
<td>-12.4 ± 0.3</td>
<td>-12.4 ± 0.1</td>
<td>-13.9 ± 0.2</td>
<td>-12.4 ± 0.2</td>
<td>-9.7 ± 0.4</td>
<td>nd</td>
<td>-13.4 ± 0.1</td>
</tr>
<tr>
<td>Sediment</td>
<td>0.20 ± 0.6</td>
<td>-18.2 ± 0.8</td>
<td>-19.9 ± 0.3</td>
<td>-17.3 ± 0.4</td>
<td>-15.9 ± 0.4</td>
<td>-18.2 ± 0.3</td>
<td>-20.0 ± 0.2</td>
</tr>
<tr>
<td>Sediment $^{13}$C labeled</td>
<td>48.1 ± 4.6</td>
<td>-1.3 ± 2.1</td>
<td>51.1 ± 8.2</td>
<td>7966 ± 16.2</td>
<td>5.5 ± 7.7</td>
<td>8.9 ± 3.0</td>
<td>-4.7 ± 1.7</td>
</tr>
</tbody>
</table>

*: Predominantly arabinose, as rhamnose concentrations are generally low in higher plants

**Table 2.3.** Neutral carbohydrates concentrations as detected in H$_2$SO$_4$ hydrolyzates of typical coastal marine materials. Concentration data are the average and SD of three replicate samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fucose (μmol/g DW)</th>
<th>Rham/Arab.</th>
<th>Galactose</th>
<th>Glucose</th>
<th>Xylose</th>
<th>Mannose</th>
<th>TOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spartina</td>
<td>18 ± 1</td>
<td>213 ± 21</td>
<td>76 ± 8</td>
<td>832 ± 52</td>
<td>703 ± 35</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Festuca</td>
<td>41 ± 5</td>
<td>175 ± 26</td>
<td>74 ± 11</td>
<td>849 ± 115</td>
<td>634 ± 89</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Ulva</td>
<td>3.8 ± 0.2</td>
<td>373 ± 46</td>
<td>38 ± 5</td>
<td>493 ± 63</td>
<td>169 ± 20</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Sediment</td>
<td>0.37 ± 0.07</td>
<td>0.61 ± 0.21</td>
<td>1.26 ± 0.15</td>
<td>3.42 ± 0.06</td>
<td>0.67 ± 0.19</td>
<td>1.03 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>Sediment $^{13}$C labeled</td>
<td>0.36 ± 0.09</td>
<td>0.71 ± 0.03</td>
<td>1.01 ± 0.08</td>
<td>3.06 ± 0.22</td>
<td>0.70 ± 0.06</td>
<td>0.41 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

*: Predominantly arabinose, as rhamnose concentrations are generally low in higher plants
Fig. 2.5. The difference ($\Delta \delta^{13}C$) in $\delta^{13}C$ ratios of neutral carbohydrates and bulk TOC (Table 2.1) for the three plant materials analyzed. Shown are averages ± SD for 3 replicates.
Conclusions

We successfully developed a method to analyze stable carbon isotope ratios in carbohydrates by HPLC/IRMS using the LC Isolink interface that is based on ion exchange chromatography in combination with low strength alkaline eluents. We show the separation of a variety of neutral and acidic carbohydrates. The analytical window of the method can probably be easily extended to oligomeric carbohydrates and sulfate- or phosphorous-bound carbohydrates by varying the concentration of the NaNO₃ pusher. We successfully analyzed neutral plant carbohydrate in typical marine materials both at the natural $^{13}$C-abundance level and for $^{13}$C-labeled material to study carbohydrate synthesis. Further applications of the method include the use of muramic acid as a biomarker to study bacterial dynamics, physiological studies on carbohydrate metabolism such as fermentation research and the use of carbohydrate substrates by various organisms, biogeochemical studies to determine the sources and fate of carbohydrates in natural ecosystems and food adultery studies.

Acknowledgements

This research was partly funded by the Netherlands Organization for Scientific Research (NWO) VIDI grant to HTSB. We thank Cees Bruggink of Dionex Benelux BV, Amsterdam, the Netherlands for advice on chromatographic conditions and the editor and two anonymous reviewers for constructive feedback. This is publication number 4391 of the Netherlands Institute of Ecology (NIOO-KNAW).