

# Preparation of starch and soluble sugars of plant material for the analysis of carbon isotope composition: a comparison of methods<sup>†</sup>

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Starch and soluble sugars are the major photosynthetic products, and their carbon isotope signatures reflect external versus internal limitations of CO<sub>2</sub> fixation. There has been recent renewed interest in the isotope composition of carbohydrates, mainly for use in CO<sub>2</sub> flux partitioning studies at the ecosystem level. The major obstacle to the use of carbohydrates in such studies has been the lack of an acknowledged method to isolate starch and soluble sugars for isotopic measurements. We here report on the comparison and evaluation of existing methods (acid and enzymatic hydrolysis for starch; ion-exchange purification and compound-specific analysis for sugars). The selectivity and reproducibility of the methods were tested using three approaches: (i) an artificial leaf composed of a mixture of isotopically defined compounds, (ii) a C<sub>4</sub> leaf spiked with C<sub>3</sub> starch, and (iii) two natural plant samples (root, leaf). Starch preparation methods based on enzymatic or acid hydrolysis did not yield similar results and exhibited contaminations by non-starch compounds. The specificity of the acidic hydrolysis method was especially low, and we therefore suggest terming these preparations as HCl-hydrolysable carbon, rather than starch. Despite being more specific, enzyme-based methods to isolate starch also need to be further optimized to increase specificity. The analysis of sugars by ion-exchange methods (bulk preparations) was fast but produced more variable isotope compositions than compound-specific methods. Compound-specific approaches did not in all cases correctly reproduce the target values, mainly due to unsatisfactory separation of sugars and background contamination. Our study demonstrates that, despite their wide application, methods for the preparation of starch and soluble sugars for the analysis of carbon isotope composition are not (yet) reliable enough to be routinely applied and further research is urgently needed to resolve the identified problems. Copyright © 2009 John Wiley & Sons, Ltd.

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The carbon isotope signatures ( $\delta^{13}\text{C}$ ) of primary photosynthates closely track changes in leaf gas exchange via changes in the ratio between leaf internal and atmospheric CO<sub>2</sub> concentration ( $c_i:c_a$ ), and they thus reflect the balance between foliar limitation and CO<sub>2</sub> diffusion and the rate of photosynthetic CO<sub>2</sub> fixation.<sup>1,2</sup> Leaf carbohydrates (starch, soluble sugars) rapidly display environmental fluctuations, often with a time lag of a few hours to days, while bulk leaf

material usually responds much more slowly.<sup>3</sup> The  $\delta^{13}\text{C}$  signatures of leaf carbohydrates have been studied in relation to plant stress, such as drought and salinity stress.<sup>4</sup> Sugars are also the major organic compounds for phloem export to sink tissues where they are utilized for biomass production or respiration.<sup>5,6</sup> Environmental signals are thereby quickly transferred to roots and are reflected in the  $\delta^{13}\text{C}$  signals of root respiratory  $\text{CO}_2$ .<sup>7–9</sup> This has attracted much interest especially with relation to partitioning ecosystem respiration fluxes into their heterotrophic and autotrophic components and understanding their respective controls.<sup>10–13</sup> The carbon isotope changes at the interfaces from leaf-to-phloem and phloem-to-root are not well understood, and there is increasing recognition that isotopic fractionation may occur along the transport pathway,<sup>14</sup> during stem metabolism and tree ring formation,<sup>15</sup> and during respiratory use of sugars.<sup>16,17</sup>

Despite the long interest in deciphering and understanding the controls of  $\delta^{13}\text{C}$  signatures of starch and soluble sugars (e.g.<sup>18,19</sup>), we still lack an acknowledged and reliable method for starch and sugar preparation and quantitative analysis to determine their carbon isotope signature. Any method for the determination of starch content, and for the isolation of starch from plant material for isotopic measurements, is based on a three-step procedure:<sup>20</sup> (1) removal of soluble sugars by repeated washing with water and/or hydrophilic organic solvents, (2) hydrolysis of starch by acids or enzymes, and (3) purification of the hydrolysate by precipitation of hydrolyzed starch (acid hydrolysis) or by removal of enzyme by dialysis or ultrafiltration (enzymatic hydrolysis). Both approaches, i.e. acid and enzymatic hydrolysis, are prone to several (potential) problems. First, suitable reference materials are lacking and reference methods for starch quantification are available only for commercial applications (e.g. starch content of seeds of crop species), but not for ecological samples. Second, prior to starch hydrolysis, low molecular weight compounds such as soluble sugars have to be extracted to avoid contamination. Third, it has to be demonstrated that starch is selectively hydrolyzed while other high molecular weight compounds are not. Contamination can originate from hydrolysable organic carbon compounds such as hemicelluloses, cellulose, gums (mucilage, pectin) and condensed polyphenols, but also from proteins. Fourth, through the addition of starch-degrading enzymes, the enzymatic approach has to deal with carbon blanks and/or take care to decrease this blank by efficient enzyme removal. Finally, both methods have to ensure complete hydrolysis of starch since starch granules may be isotopically inhomogeneous.

In contrast to the position with starch, several methods are available for the determination of  $\delta^{13}\text{C}$  values in soluble sugars, and reference substances (ANU Sucrose, Standard Reference Material, National Institute of Standards and Technology, Gaithersburg, MD, USA) and high-purity sugars are commercially available. The carbon isotope composition of soluble sugars can be determined in two ways: (i) after preparing a low molecular weight neutral fraction (bulk soluble sugars) through successive cation- and anion-exchange chromatography of plant extracts,<sup>1</sup> and (ii) by compound-specific isotope analysis of sucrose, glucose,

fructose and others after high-performance liquid chromatography (HPLC)<sup>21,22</sup> or gas chromatography (GC) separation (e.g.<sup>23,24</sup>). The first approach differs from the second as it also includes sugar-like substances, such as polyols (e.g. *myo*-inositol), and soluble oligo- or polysaccharides such as fructans.

Recently, it has been reported that the measurement of  $\delta^{13}\text{C}$  (and  $\delta^{18}\text{O}$ ) of starch and sugar ring-test samples showed a high degree of variation, although only commercial preparations, not real ecological samples, were used.<sup>25</sup> Therefore, a systematic comparison of the most widely used methods for starch and sugar isolation and preparation is urgently needed, in order to identify major problems and advantages for natural plant materials (leaves, roots) with low and high starch and sugar contents.

Here we report on the evaluation of such methods, focusing on a comparison between acid hydrolysis and enzymatic hydrolysis of starch, and on the preparation of sugars for the comparison of compound-specific approaches to analyze soluble sugars versus bulk sugar methods. To facilitate these comparisons we prepared and analyzed (1) an 'artificial leaf' sample, a mixture of pure substances (starch, soluble sugars and others; Table 1) with known amounts and isotopic compositions resembling an average natural leaf in composition (not in structure), (2) a  $\text{C}_4$  leaf sample spiked with  $\text{C}_3$  starch, and (3) two natural samples (root and leaf). We also analyzed marker molecules representative of possible contaminants such as hemicellulose (mannose), mucilage (uronic acids) and protein (organic nitrogen). The present study does not represent an inter-laboratory comparison but rather the comparison of six individual laboratory protocols for starch preparation (three based on HCl hydrolysis and three based on enzymatic hydrolysis) and four protocols for soluble sugar isolation (two based on the isolation of bulk sugars using ion exchangers and two using HPLC methods for the isolation of individual sugars).

**Table 1.** Chemical and isotopic composition of 'artificial leaf'

Compound	Content (% dry matter)	$\delta^{13}\text{C}$ [‰] v-PDB
$\alpha$ -Cellulose	42	$-25.06 \pm 0.08$
Tannic acid	5	$-26.77 \pm 0.07$
Hemicellulose (xylan)	10	$-23.41 \pm 0.05$
Gum	10	$-24.95 \pm 0.01$
Oil	2	$-15.67 \pm 0.04$
Starch ( $\text{C}_4$ )	5	$-10.97 \pm 0.08$
Alanine	0.4	$-22.83 \pm 0.09$
Arginine	0.4	$-12.95 \pm 0.13$
Aspartate	0.4	$-24.09 \pm 0.07$
Glutamate	0.4	$-27.66 \pm 0.14$
Serine	0.4	$-25.29 \pm 0.14$
Malate	2	$-23.05 \pm 0.12$
Citrate	1	$-22.52 \pm 0.08$
Fructose	1.25	$-24.44 \pm 0.13$
Glucose	1.25	$-10.83 \pm 0.22$
Sucrose	2.5	$-25.29 \pm 0.05$
<i>myo</i> -Inositol	1	$-29.95 \pm 0.14$
$\text{KH}_2\text{PO}_4$	5	
$\text{CaSO}_4$	2.5	
$\text{MgCl}_2$	2.5	
<b>TOTAL (measured)</b>		<b><math>-22.98 \pm 0.21</math></b>

## EXPERIMENTAL

### Plant materials

One root sample, two leaf samples, one from C<sub>3</sub> plants and one from C<sub>4</sub> plants, and an artificial leaf sample mixed from major biochemical compounds originating from both, C<sub>3</sub> and C<sub>4</sub> plants, were prepared and distributed to the partner laboratories for starch and sugar preparation.

#### Artificial leaf

A range of organic carbon compounds representing the major low and high molecular weight constituents comprising plant tissues were obtained from commercial sources (Merck/VWR and Sigma-Aldrich, both Vienna, Austria; p.a. qualities), and weighed to mimic the composition of real leaves (Table 1) to produce 100 g of 'artificial' leaf material. To mix the compounds that differed widely in consistency, the oil aliquot was dissolved in chloroform and pipetted onto the  $\alpha$ -cellulose fraction, mixed and dried at 45°C. The full cocktail of substances was then well mixed, and smaller aliquots of 10 g were homogenized using a ball mill (5 min at 20 Hz). Thereafter, aliquots were combined, mixed and the milling procedure repeated twice.

#### Maize (*Zea mays*) leaf, spiked

Leaf material was collected in October 2003 from a small experimental field at Augarten, Vienna, Austria. Immediately after harvesting enzymatic activities in leaves were stopped using a microwave oven (1000 W, 2 min) and the plant material was dried in an oven at 60°C for 2 days. The dried leaf material was coarsely ground in a coffee mill and thereafter finely homogenized in a ball mill for 5 min at 20 Hz. Native potato starch (Sigma-Aldrich) was added to this powder (4%, w/w;  $\delta^{13}\text{C}$  value of  $-25.51 \pm 0.17\%$ , vs. V-PDB; Vienna PeeDee Belemnite), and homogeneously mixed by ball milling (10 min, 20 Hz). The starch content of the unspiked leaf material was  $4.4 \text{ mg g}^{-1}$  DM (dry matter), measured by a standard procedure.<sup>20</sup> We assumed that the  $\delta^{13}\text{C}$  value of the native starch was similar to the bulk leaf  $\delta^{13}\text{C}$  value ( $-11.8\%$ ) and used a mixing model to estimate that the target value for starch  $\delta^{13}\text{C}$  of the spiked leaf was  $-24.1\%$ . The  $\delta^{13}\text{C}$  value of the bulk leaf including the added starch was  $-12.39 \pm 0.06\%$ .

#### Plantago lanceolata leaf

Leaf material was collected in November 2004 from 'The Jena Experiment', Germany.<sup>26</sup> After harvest, the collected leaves were immediately frozen in dry ice on site, transported to the laboratory and freeze-dried. The plant material was finely ground in a ball mill (Retsch, Haan, Germany) for 240 s at 20 Hz. The  $\delta^{13}\text{C}$  value of the bulk leaf was  $-28.27 \pm 0.15\%$  (vs. V-PDB).

#### Spruce (*Picea abies*) root

Root material was collected from 0–10 cm soil depth in August 2002 in Wetzstein, Germany, from a pure spruce stand of 90 years of age.<sup>27</sup> Root material was hand-picked in the laboratory from the soil material and washed with deionized water. The material was oven-dried at 60°C for 2 days and ground in a ball mill for 5 min at 20 Hz. The

$\delta^{13}\text{C}$  value of the bulk root material was  $-26.68 \pm 0.18\%$  (vs. V-PDB).

No certified reference material is available for starch, either for content or for isotope ratios. The real isotopic values as well as the real content for starch for the natural samples are consequently unknown.

### Protocols for preparation of starch for isotope-ratio mass spectrometry (IRMS)

Table 2 presents a short overview of the methods used for preparation of starch for carbon isotope analysis. More elaborate descriptions are given below, and detailed descriptions (standard operating procedures) are available on request. H1–H3 and E1a,b and E2 were carried out in different laboratories, under the conditions under which these methods were developed and optimized.

#### Starch preparation by acid hydrolysis

H1: Plant powder (50 mg) was suspended in 1 mL of fresh distilled water in an Eppendorf vial, vortexed and maintained on ice for 20 min (vortexed again after 10 min on the ice). After 5 min centrifugation at 12 000 g at 5°C the supernatant was stored at  $-20^\circ\text{C}$  for further purification of the soluble sugars using a HPLC method and the pellet containing the non-soluble fraction for starch extraction using a HCl solubilization method, both described in Durancieu *et al.*<sup>21</sup> The pellets were first washed with 95% (v/v) ethanol in order to remove the pigments; 1 mL ethanol was added to the pellet and heated for 10 min at 70°C before centrifugation at 12 000 g for 10 min at 5°C. The supernatant was removed and the pellet was repeatedly washed with ethanol until the supernatant became colourless. For starch hydrolysis 1 mL 6 N HCl was added to the pellet, vortexed and transferred to a 10 mL tube. In addition, 1 mL 6 N HCl was used to completely transfer the pellet from the Eppendorf tube to the 10 mL tube. The tube containing the pellet with 2 mL 6 N HCl was kept at 5°C for 1 h. After 20 min centrifugation at 12 000 g at 5°C, the supernatant containing solubilized starch was removed to a 50 mL tube kept in ice. Then 2 mL HCl was added to the pellet and kept at 5°C for one more hour to solubilize the remaining starch. After centrifugation, the supernatant was added to the 50 mL tube and 16 mL of methanol (4 times the volume of HCl) was added and kept at 5°C overnight to precipitate starch. After centrifugation at 12 000 g for 10 min at 5°C, the supernatant was removed and the precipitated starch was freeze-dried for 48 h prior to isotopic analysis. The starch amount was determined by weighing the 50 mL tubes empty and after the starch was freeze-dried.

H2: Plant powder (50 mg) was weighed into 10 mL centrifuge tubes and suspended twice with 5 mL 30% ethanol by vortexing. The suspensions were each kept at room temperature for 60 min and were centrifuged for 10 min at 12 000 g. The combined supernatants were stored at  $-20^\circ\text{C}$  for further purification of the soluble sugars and the pellet containing the non-soluble sugar fraction processed for starch extraction using a HCl solubilization method, as described by Brugnoli *et al.*<sup>1</sup> The pellets were then washed with 5 mL 80% (v/v) ethanol to remove pigments, and kept at room temperature for 10 min before centrifugation at 12 000 g

**Table 2.** Overview of methods of starch preparation for carbon isotope analysis

	H1	H2	H3	E1a, E1b	E2
Reference	Duranceau <i>et al.</i> <sup>21</sup> Tcherkez <i>et al.</i> <sup>17</sup>	Brugnoli <i>et al.</i> <sup>1</sup>	Wanek <i>et al.</i> <sup>20</sup> Göttlicher <i>et al.</i> <sup>28</sup>		
Amount of plant material	50 mg	50 mg	100–150 mg	50–100 mg	50–100 mg
Removal of low molecular weight compounds	+ 1 × 1 mL A.dest, 20 min, ice + 2–6 × 1 mL 95% EtOH, 10 min, 70°C	+ 1 × 5 mL 30% EtOH, 45 min, RT <sup>a</sup> + 1 × 3 mL 80% EtOH, 10 min, RT	+ 1 × 5 mL 80% EtOH, 45 min, RT + 3 × 5 mL MCW <sup>b</sup> (12:5:3), RT + 2 × 5 mL, EtOH 30%, 10 min, RT	+ 3 × 1.5 mL MCW 30 min, 70°C + 3 × 1.5 mL A.dest., RT, drying at 60°C	+ 3 × 1.5 mL MCW 30 min, 70°C + 3 × 1.5 mL A.dest., RT, drying at 65°C
Hydrolysis	2 × 2 mL 6 M HCl at 5°C, 1 hour	3 × 1 mL 6 M HCl at RT, 1 hour; filter solution through 0.45 µm	2 × 2 mL + 1 × 1 mL 20% HCl, RT, 1 h; filter solution through 0.45 µm	+ 0.75 mL A.dest. 15 min, 100°C + 250 µL α-amylase (750 U), <sup>c</sup> 120 min, 85°C	+ 0.75 mL A.dest. 15 min, 100°C + 250 µL amyloglucosidase (500 U), 120 min, 85°C —
Precipitation	4 mL solubilized starch + 16 mL 100% MeOH, 16 h at 5°C	3 mL solubilized starch + 12 mL 100% MeOH, 16 h at 4°C	5 mL solubilized starch + 20 mL 100% EtOH, 16 h at 5°C	—	—
Removal of the enzyme	—	—	—	Ultrafilter 450 µL solubilized starch (10 kDa MWCO)	Ultrafilter 450 µL solubilized starch (10 kDa MWCO)
Starch preparation	Centrifuge and freeze dry for 48 h Weigh into tin capsules	Centrifuge and freeze dry for 24 h Weigh into tin capsules	Centrifuge and freeze dry for 24 h Weigh into tin capsules	Pipette 50 µL into tin capsules	Pipette 50 µL into tin capsules
Time per sample	3 days per batch (40 samples)	3 days per batch (40 samples)	3 days per batch (40 samples)	3 day per batch (20 samples)	3 day per batch (20 samples)
Costs per sample (preparation only)	3 €	3 €	3 €	7 €	7 €

<sup>a</sup> RT, room temperature.<sup>b</sup> MCW, methanol/chloroform/water (12:5:3).<sup>c</sup> Enzyme solution was purified by ultra-filtration (MWCO 20 kDa); blank correction applied.



for 10 min. The supernatant was removed and the pellet was repeatedly washed with 80% ethanol until the supernatant became colourless. For starch hydrolysis 3 mL 6 N HCl was added to the 10 mL tube containing the pellet, vortexed and kept at room temperature for 60 min. After 20 min centrifugation at 12 000 g, the supernatant containing the solubilized starch was filtered through a 0.45 µm HPLC filter to remove coarse contaminants and transferred to a 50 mL tube kept in ice. Then 12 mL of methanol was added and the sample was kept at 5°C overnight to precipitate starch. After centrifugation at 12 000 g for 10 min at 5°C, the supernatant was removed and the precipitated starch was freeze-dried for 24 h prior to isotopic analysis. The starch amount was determined as given for H1.

H3: Plant powder (100–150 mg) was suspended in 5 mL 80% ethanol, vortexed and maintained at RT for 45 min. After 10 min centrifugation at 12 000 g at RT, the pellet containing the non-soluble sugar fraction was processed for starch extraction using a HCl solubilization method, as described by Brugnoli *et al.*<sup>1</sup>

The pellets were washed three times – or until the supernatant was colourless – with 5 mL methanol/chloroform/water (MCW, 12:5:3, v/v/v), each for 10 min at RT, and then centrifuged at 12 000 g at RT for 10 min. To remove chloroform traces the pellets were washed twice with 5 mL 30% ethanol. The supernatants were each discarded. To hydrolyze starch 2 mL 20% HCl were added to the pellet, vortexed and transferred to a 20 mL syringe. For complete transfer of the pellet to the syringe, a further 2 mL and 1 mL aliquots of 20% HCl were used. The tube containing the pellet with 5 mL 20% HCl was kept at RT for 60 min. After 20 min centrifugation at 12 000 g and RT, the supernatant containing solubilized starch was filtered with a 0.45 µm HPLC filter to remove coarse contaminants and transferred into a 50 mL beaker kept in ice. Then 20 mL 100% ethanol was added to precipitate the starch overnight at 5°C. After centrifugation at 12 000 g for 10 min at 5°C, the supernatant was removed and the precipitated starch was freeze-dried for 24 h for isotopic analysis. The starch amount was determined as given above for H1.

### Starch preparation by enzymatic hydrolysis

E1a/E1b (amylase method) and E2 (amyloglucosidase method): The starch fractions were enzymatically prepared according to the method of Wanek *et al.*,<sup>20</sup> and modified by Göttlicher *et al.*<sup>28</sup> for E1a and E1b. For all enzymatic methods ground plant material (100 mg) was weighed in 2 mL reaction vials and a glass bead was added to facilitate resuspension of the pellet. Plant material was extracted with 1.5 mL MCW (12:3:5, v/v/v) for 30 min at 70°C, centrifuged at 10 000 g for 2 min and the supernatant removed. The samples were re-extracted with 1.5 mL MCW for 10 min at 70°C and centrifuged as described above (this step was repeated twice). Thereafter, the pellets were washed three times with water (1.5 mL HPLC-grade water) and centrifuged at 10 000 g for 2 min. The samples were then dried at 60°C (E1a and E1b) or 65°C (E2) in a drying oven.

For E1a and E1b a solution of  $\alpha$ -amylase (10 mL, 3,000 U mL<sup>-1</sup>) was prepared by dissolving heat stable  $\alpha$ -amylase in water and ultrafiltration in centrifugal filter devices (Centricon Plus-20,

polyethersulfone membrane, 5000 molecular weight cutoff (MWCO), Biomax-5 Amicon Bioseparations, Millipore Corporation, Billerica, MA, USA) for 20 min at 3000 g to remove stabilizers and other low molecular weight substances. The retentate was diluted with 8 mL water and centrifuged again for 20 min at 3000 g (this step was repeated 3 times). The retentate was then made up to 10 mL with water. The purified  $\alpha$ -amylase solution was prepared fresh every day and kept at 4°C until use.

For E2 commercially available amyloglucosidase (from *Aspergillus niger* E.C. 3.2.1.3, Sigma Aldrich GmbH, Munich, Germany) solution dissolved in 3.2 M ammonium sulphate was diluted with demineralized water to a total volume of 10 mL and purified by ultrafiltration with a regenerated cellulose membrane with a 10 000 Da MWCO (Centricon Plus 20, Millipore Corporation). The filtrate was discarded and 8 mL of demineralized water were added to the retentate. The filtration step was repeated twice and the final retentate was made up to 10 mL with demineralized water. The activity of the enzyme preparation was 2000 U mL<sup>-1</sup>. For all enzymatic methods the filter devices were pre-cleaned to remove membrane stabilizers (e.g. glycerol) by spinning them five times with 10 mL water (HPLC-grade) at 3000 g in a swinging-bucket centrifuge (Beckmann J2-21 centrifuge with swing-bucket rotor JS-13.1; Beckmann Coulter, Krefeld, Germany).

Before hydrolysis of the starch by  $\alpha$ -amylase or amyloglucosidase, 750 µL water was added to the washed and dried plant material, shaken thoroughly and incubated in a water-bath at 100°C for 15 min to gelatinize starch. After cooling to room temperature 250 µL of the purified  $\alpha$ -amylase (E1a, E1b) or amyloglucosidase solution (E2) were added to each sample. For each batch of samples (between 18 and 25) three blanks (750 µL water and 250 µL  $\alpha$ -amylase or amyloglucosidase solution) were prepared. Samples and blanks were incubated in a water-bath at 85°C for 120 min to hydrolyze starch. After cooling to RT the samples were centrifuged, the supernatants transferred to new reaction vials and stored at –20°C if necessary. To separate the enzyme protein from the starch hydrolysate each 450 µL of sample or blank were ultrafiltered through pre-cleaned centrifugal filter devices (E1a and E1b: Microcon YM-10, Regenerated Cellulose, 10 000 Da MWCO, Amicon Bioseparations, Millipore Corporation; E2: Vivaspin 500, regenerated cellulose membrane, 10 000 Da MWCO, Sartorius, Göttingen, Germany) at 12 000 g for 50 min in a fixed-angle rotor centrifuge (Beckmann Avanti 30 with fix-angle rotor F2402H). The retentate was discarded and 50 µL of the filtrate was pipetted into smooth tin capsules and dried in a vacuum concentrator (Savant Speed Vac SC110 or Christ RVC 2-25; Christ GmbH, Osterode, Germany) at approximately 100 mbar before isotope analysis.

### Protocols for preparation of sugars for IRMS

A short overview of the methods used for the preparation of sugars for carbon isotope analysis is presented in Table 3.

#### Bulk sugar approaches

Bulk sugar method I1: Soluble sugar extraction and purification were performed according to Brugnoli *et al.*<sup>1</sup> with slight modifications. Finely ground plant material (100 mg)

**Table 3.** Overview of methods of soluble sugar preparation for carbon isotope analysis

	T1		T2		C1	C2
Reference	Brugnoli <i>et al.</i> <sup>1</sup>		Wanek <i>et al.</i> <sup>20</sup> Göttlicher <i>et al.</i> <sup>28</sup>		Duranceau <i>et al.</i> <sup>21</sup>	
Amount of plant material	100 mg		50 mg		50 mg	
Extraction of low molecular weight compounds	+ 2 × 5 mL A.dest., 30 min, RT Centrifuge Combine supernatants		+ 1.0 mL MCW 30 min, 70°C Centrifuge Phase separation Dry and redissolve aqueous upper phase		+ 1 × 1 mL A.dest, 20 min, ice Centrifuge Deproteinize by heating to 100°C and cooling	
Preparation of bulk soluble sugar fraction	Ion-exchange separation by Dowex-50 (H <sup>+</sup> form) and Dowex-1 (Cl <sup>-</sup> form) Rinse with 10 mL A. dest. and collect flow-through		Ion-exchange separation by Dowex-50W (H <sup>+</sup> form) and Dowex-1 (HCOO <sup>-</sup> form) Rinse with 23 mL A. dest. and collect flow-through		—  —	
HPLC separation	—		—		Sugar-Pak1 column, 0.5 mL min <sup>-1</sup> , A.dest., 90°C Refractometry	
IRMS preparation	Freeze dry through-flow for 24 h Weigh into tin capsules		Dry on rotary evaporator. redissolve and transfer into tin capsules		Collect sugar peaks Freeze dry for 24 h, redissolve and transfer into tin capsules	
					PL Hi-Plex Pb column, 0.6 mL min <sup>-1</sup> , A.dest., 80°C Light scattering detection Collect sugar peaks Dry on rotary evaporator Redissolve and transfer into tin capsules	

was weighed into reaction vials and suspended in 5 mL water at RT, after stopping enzymatic activities using a microwave oven (1000 W, 2 min). The samples were vortexed for 30 min and then centrifuged at 12 000 g for 10 min. The supernatant was collected and the pellet was re-suspended into 5 mL of water and extracted a second time. After centrifugation, the two supernatants were combined and used for soluble sugar purification. The extracts were passed through Dowex-50 ( $\text{H}^+$ ) and Dowex-1 ( $\text{Cl}^-$ ) columns in series. The former resin was used for the separation of amino acids from organic acids and sugars, and the latter was used to separate organic acids from soluble sugars. The columns were rinsed with 10 mL of water and the eluate collected. The sugar-containing samples were freeze-dried and stored dry prior to carbon isotope analysis.

**Bulk sugar method I2:** The soluble sugars were extracted and prepared according to the method of Wanek *et al.*<sup>20</sup> as modified by Göttlicher *et al.*<sup>28</sup> Finely ground plant material (50 mg) was weighed into 2 mL reaction vials, extracted with 1 mL MCW (12:3:5, v/v/v) for 30 min at 70°C and after cooling to RT centrifuged at 10 000 g for 2 min. An aliquot of the supernatant (800  $\mu\text{L}$ ) was transferred into a 2 mL reaction vial. For every five samples one blank was processed (800  $\mu\text{L}$  MCW, no sample). To induce phase separation 800  $\mu\text{L}$  water and 250  $\mu\text{L}$  chloroform were added to the supernatant and mixed vigorously. The chloroform and aqueous-methanol phases were separated by centrifugation at 10 000 g for 2 min. An aliquot of the upper aqueous phase (1.2 mL) was again mixed vigorously with 500  $\mu\text{L}$  chloroform, and the phases separated by centrifugation. Then 1 mL of the upper phase was dried in a vacuum concentrator at approximately 100 mbar (Savant Speed Vac SC110). After being re-dissolved in 1 mL water, the sample was applied to a ion-exchange cartridge (6 mL, i.d. 13 mm, Supelco) made of 2.1 mL cation-exchange resin (DOWEX 50W X 8, 50–100 mesh,  $\text{H}^+$ -form) above 3.2 mL anion-exchange resin (DOWEX 1 X 8, 50–100 mesh,  $\text{HCOO}^-$  form), separated by a low-density polyethylene frit. The solid-phase extraction (SPE) cartridges were mounted on a SPE manifold. The columns were rinsed with 3 mL and then 2  $\times$  10 mL water and the eluate (neutral fraction consisting mainly of soluble sugars) was collected. The samples were then taken to complete dryness on a rotary evaporator, and re-dissolved in 1 mL water. Volumes of 50  $\mu\text{L}$  of each sample were pipetted into smooth tin capsules, and dried in a vacuum concentrator (Speed Vac) at approximately 100 mbar.

#### *Off-line compound-specific approaches (HPLC based)*

**Compound-specific method C1:** Soluble sugar extraction and purification were performed according to the method of Duranceau *et al.*<sup>21</sup> The water-soluble fraction (extracted as described in H1) was heated at 100°C for 3 min and then kept in ice for 20 min to precipitate the heat-denatured proteins, which were then removed by centrifugation at 12 000 g for 5 min at 5°C. The de-proteinized water-soluble fraction (supernatant) was filtered (filter HV 0.45  $\mu\text{m}$  type, Nihon Millipore Kogyo K.K., Tokyo, Japan). Contents of individual soluble sugars (i.e. sucrose, glucose and fructose) in filtered extracts were determined by HPLC analysis of 20  $\mu\text{L}$  aliquots applied to a Sugar-Pak1 column (6.5 mm diameter and

300 mm length, Waters, Milford, MA, USA). The flow rate was maintained at 0.5 mL min<sup>-1</sup>, the pressure at 1700 psi and the temperature of the column at 90°C. The sugar peaks were detected by refractometry (IOTA-2 refractometer, Precision Instruments, Marseilles, France). The same process was used for preparing pure fractions of the different sugars for isotope analysis. In this case, in order to obtain enough material, 100  $\mu\text{L}$  of filtered extracts were applied to the sugar column (50  $\mu\text{L}$  only for *Plantago lanceolata* samples, because of their high sugar concentration). The individual sugar peaks were collected in Eppendorf vials manually at the end of the column, taking into account the dead-volume. After lyophilization, the purified sugars were suspended in 300  $\mu\text{L}$  distilled water and transferred to tin capsules (Courtage Analyse Service, Mont Saint-Aignan, France) and dried in an oven (50°C) prior to isotope analysis.

**Compound-specific method C2:** Aliquots of powdered, freeze-dried material (300–600 mg) were extracted using an accelerated solvent extraction system (ASE 200, Dionex, Idstein, Germany) with an ethanol/water mixture (1:9) under a pressure of 1450 psi. The extraction was conducted twice at 80°C and once at 100°C; the resulting extracts were combined for each sample and centrifuged for 10 min at 3000 g. The supernatant was stored at -20°C for further analysis. For pre-purification, the supernatants were thawed and applied to a column, containing 1.7 mL cation-exchange resin (AG<sup>®</sup> 50W-X8, 200–400 mesh,  $\text{H}^+$ -form; Bio-Rad Laboratories, Munich Germany), which had been equilibrated with 12 mL 0.1 M HCl solution. The fraction containing organic acids and sugars was eluted with 12 mL (6  $\times$  2 mL) doubly distilled water, whereas amino acids and other cationic substances were retained on the column. The resulting eluate was neutralized with 1 M NaOH to pH 7. This fraction was applied to a column filled with 1.7 mL anion-exchange resin (AG<sup>®</sup> 1-X8, 200–400 mesh,  $\text{Cl}^-$ -form; Bio-Rad Laboratories, Hercules, CA, USA), equilibrated with 12 mL 1 M NaCl. The sugar fraction was eluted with 12 mL of doubly distilled water. The resulting sugar fraction was filtered (Chromafil PET 20/15 MS Pore 0.2  $\mu\text{m}$ , Macherey-Nagel, Düren, Germany) and the sugar content was determined by HPLC on a PL Hi-Plex Pb column (Polymer Laboratories, Varian Inc., Palo Alto, CA, USA; particle size 8  $\mu\text{m}$ , 300  $\times$  7.7 mm) at a flow rate of 0.6 mL min<sup>-1</sup> at 80°C with purified water as the eluent. The peaks were detected using a light scattering detector (PL-ELS 1000, Polymer Laboratories) at 90°C nebulizer and 120°C evaporator temperature. In order to determine the isotope ratios of the individual sugars, the individual sugar peaks were collected four to five times at the end of the column using a switch-over valve. After water evaporation (Büchi Rotavapor R-114, Büchi, Essen, Germany) and re-dissolution in 1 mL of water an aliquot of 300  $\mu\text{L}$  was transferred into tin capsules (IVA Analysentechnik e.K., Meerbusch, Germany) and dried at 70°C in a drying oven. The isotope ratios were determined by elemental analyzer (EA) IRMS.

#### **IRMS analysis of sugar and starch preparations**

All the starch samples and most of the sugar samples were analyzed at ETH Zürich to ensure the best possible comparison of results.

Measurement of the  $\delta^{13}\text{C}$  values was performed using a Flash EA 1112 Series elemental analyzer (Thermo Italy, former CE Instruments, Rhodano, Italy) coupled to a Finnigan MAT Delta<sup>plus</sup> XP isotope ratio mass spectrometer (ThermoFinnigan MAT, Bremen, Germany) via a six-port valve<sup>29</sup> and a universal interface for EA-IRMS coupling (ConFlo III,<sup>30</sup> ThermoFinnigan). The Flash EA was additionally equipped with a home-made Nafion trap backed by a conventional  $\text{Mg}(\text{ClO}_4)_2$  trap followed by a four-port valve<sup>31</sup> between the reduction tube and the GC column to avoid frequent renewal of the  $\text{Mg}(\text{ClO}_4)_2$  and to allow easy maintenance of the oxidation and reduction tubes without closing the isolation valve of the mass spectrometer. The positioning of the samples, blanks and (laboratory) standards (Identical Treatment principle) in a measurement series for carbon isotope ratio measurements followed the scheme described by Werner and Brand.<sup>32</sup> Post-run off-line calculations such as the blank-, offset- and possibly the drift-corrections for assigning the final  $\delta^{13}\text{C}$ -values on the V-PDB scale were performed according to Werner and Brand.<sup>32</sup> Calibration of the laboratory standards was carried out periodically by comparison of the laboratory standards with international reference materials provided by the IAEA (Vienna, Austria). The long-term precision ( $\sim 1.5$  years) of our quality control standard (caffeine) for  $\delta^{13}\text{C}$  was 0.09‰. Consequently, the variation in  $\delta^{13}\text{C}$  between samples originated mainly from the preparation methods.

In addition to the analysis described above, some of the sugar samples were also analyzed at the Max Plank Institute for Biogeochemistry in Jena. To avoid any bias between these two laboratories, laboratory and quality control standards were exchanged between Jena and Zürich.

### Determination of concentrations of hemicellulose sugars

Starch preparations ( $\sim 5$  mg) from artificial leaf material of E1a and H3 were suspended in 1 mL of a neutral detergent and boiled for 60 min.<sup>33</sup> To remove possible contaminations by soluble proteins and lipids the supernatant was discarded after centrifugation (20 000 g, 10 min, RT) and the pellet was washed ( $3 \times$  distilled water and  $2 \times$  acetone) and dried under reduced pressure. Hemicelluloses were extracted with 1 mL of acid detergent<sup>34</sup> for 60 min at 95°C. After centrifugation (20 000 g for 10 min, RT) aliquots of the supernatant were diluted 20-fold and sugar monomers from hemicellulose were separated by HPLC on a Carbowac PA20 column (Dionex) and detected with a pulsed amperometric detector (Dionex ICS3000 chromatography system). Fructose, rhamnose and arabinose were separated by elution with 20 mM NaOH (30°C; flow rate:  $0.5 \text{ mL min}^{-1}$ ), while separation of galactose, glucose, xylose and mannose was achieved with distilled water only.

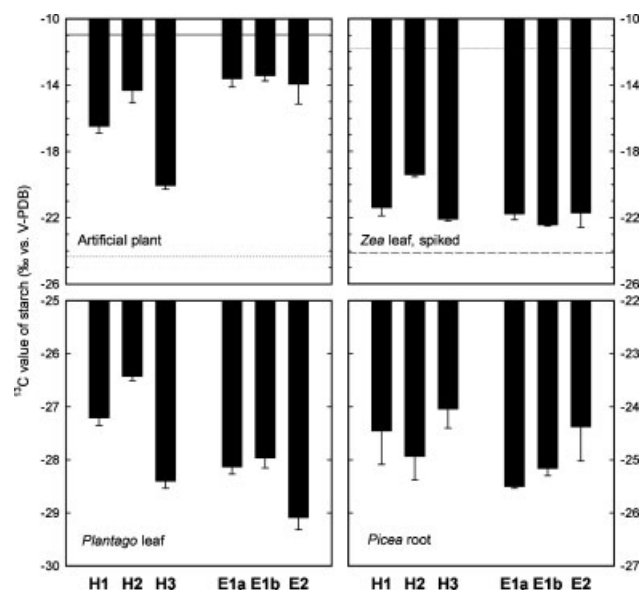
## RESULTS AND DISCUSSION

### Starch

In order to test the selectivity of the different starch extraction methods we used both the carbon isotope composition and the isolated starch content. The 'artificial leaf' contained most of the relevant plant components (cellulose, hemicellulose, pectin, and others) from  $\text{C}_3$  sources, but starch from a  $\text{C}_4$

source (Table 1). The maize leaf ( $\text{C}_4$  plant) was also spiked with potato starch ( $\text{C}_3$  plant). This design allowed the detection and quantification of possible contamination of the different starch preparations by other materials. In addition, we used two unmodified plant tissues, a leaf sample from *Plantago lanceolata* and a root sample from *Picea abies*, to determine the reproducibility with natural, unmodified plant materials.

The measured starch  $\delta^{13}\text{C}$  values of the artificial leaf significantly deviated from the target  $\delta^{13}\text{C}$  value ( $-10.97\text{‰}$ ), irrespective of the methods tested. The isotope signatures resulting from enzymatic hydrolysis were closer (overall mean of  $-13.67 \pm 0.74\text{‰}$ ) to the target value than those of acid hydrolysis (overall mean  $-17.37 \pm 2.42\text{‰}$ ) (Fig. 1). While the variability within the two enzymatic methods was similar, the different methods utilizing the acid hydrolysis step yielded very different results (from  $-14.34\text{‰}$  to  $-20.07\text{‰}$ ). Small differences in the acid hydrolysis protocols may therefore strongly influence the carbon isotope composition of the final product, e.g. hydrolysis at 4°C or RT (H1 vs. H2/H3), solvents for removal of low molecular weight compounds (H1/H2 vs. H3) or starch precipitation by methanol or ethanol (H1/H2 vs. H3). In contrast, application of the same protocol for enzymatic hydrolysis in two different laboratories (E1a, E1b) yielded almost the same



**Figure 1.** Carbon isotope composition ( $\delta^{13}\text{C}$ ) of starch prepared from artificial plant material, maize leaves spiked with  $\text{C}_3$  starch, *Plantago lanceolata* leaves and spruce (*Picea abies*) roots by acid hydrolysis (H1–H3) or enzymatic hydrolysis (E1a,b and E2). Solid and dashed lines indicate the target  $\delta^{13}\text{C}$  value of added  $\text{C}_4$  starch in artificial  $\text{C}_3$  plant material and of 'target'  $\delta^{13}\text{C}$  value calculated from mixed potato starch with endogenous starch (9:1, w/w) in spiked maize leaf, respectively. Dotted lines represent the  $\delta^{13}\text{C}$  signatures of background  $\text{C}_3$  contaminants (average of cellulose, tannins, xylan and gum in artificial leaf material) or  $\text{C}_4$  contaminants (bulk material in spiked maize leaf) that are potentially extracted with starch. Error bars denote 1 standard deviation of the mean ( $n = 5$ ).

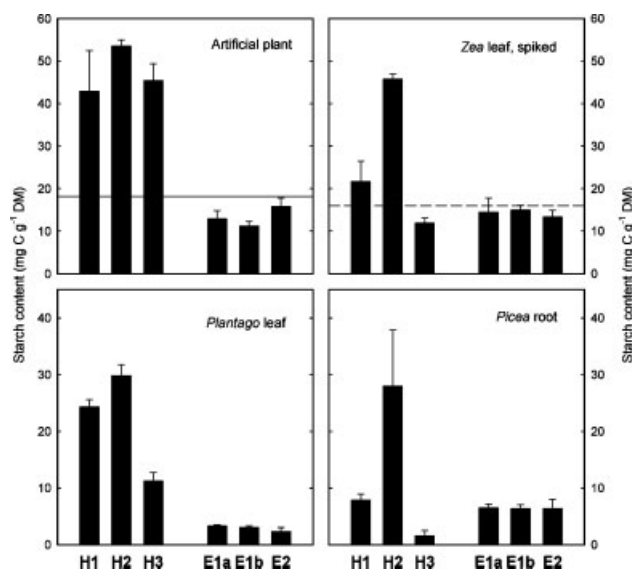


concentrations and isotopic compositions of starch. The utilization of different enzyme types (see Experimental section) for the enzymatic degradation of starch did not lead to significant differences in the  $\delta^{13}\text{C}$  values of starch (Fig. 1). Both, the enzymatic and the acid hydrolysis method also failed to provide a correct estimate for the starch  $\delta^{13}\text{C}$  values of *Zea mays* leaves spiked with potato starch (Fig. 1). The deviation from the target values was between 2.0 and 4.7‰ for the acid method and between 1.6 and 2.4‰ for the enzymatic method. In this case the overall means for the two types of hydrolysis were significantly different ( $P = 0.029$ , t-test) and the variation within each type of method (enzymatic vs. acid hydrolysis) was again higher for acid hydrolysis.

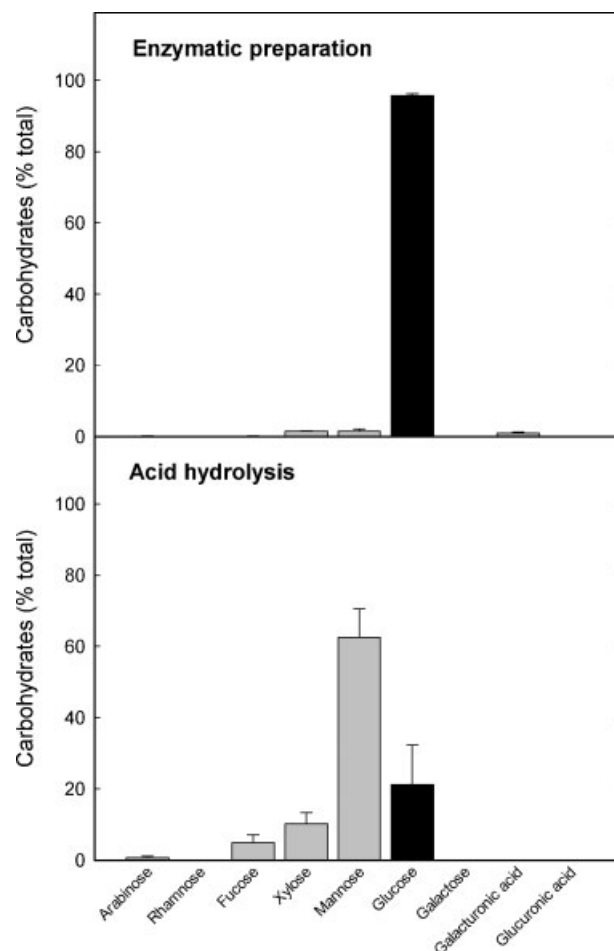
When applied to unaltered plant tissues we observed similar variations both between the two types of hydrolysis ( $\sim 1\%$ ) and within each method ( $\sim 1\%$  for *P. abies* and 1–2‰ for *P. lanceolata*) (Fig. 1). However, we found a greater variation in the starch carbon isotope composition of spiked leaves, where we added a  $\text{C}_3$  or  $\text{C}_4$  starch to a  $\text{C}_4$  or  $\text{C}_3$  matrix. The observation that the variation of the unaltered plant tissue was smaller than that of spiked leaves may be because the isotopic content of plant carbohydrates such as cellulose, hemicellulose and starch is usually similar,<sup>35</sup> and, therefore, contamination of the starch preparations by such constituents may not necessarily lead to large alterations of the measured  $\delta^{13}\text{C}$  values of starch from natural samples.

The rather small differences in the isotope composition of the natural samples between the different methods (Fig. 1) were accompanied by marked differences (up to 5-fold) in starch content (Fig. 2). Huge differences in the starch content were also found between the two types of hydrolysis for the

artificial plant: the enzymatic methods underestimated the starch content on average by 28%, while the acid hydrolysis methods overestimated it by 121% (Fig. 2). These marked overestimations raised questions on the nature of the carbon extracted in the acid hydrolysis samples. Although recently a combined dilute sulphuric acid hydrolysis with subsequent gluco-amylase incubation was applied to isolate starch-glucose from plant material for isotope analysis,<sup>36</sup> it was shown that dilute sulphuric acid is less suitable for starch digestion than enzymatic hydrolysis as the acid also breaks down structural carbohydrates, resulting in overestimates of starch content.<sup>37</sup> To address this, we subjected starch preparations from artificial leaf by H3 and E1a to an analysis of their monosaccharide composition (Fig. 3). Glucose comprised up to 95.8% of the enzymatic starch preparation, while in acid hydrolysis preparations the glucose made up only 22.9% of the monosaccharide fraction. This clearly indicates that acid hydrolysis yields other carbohydrates and/or contains other EtOH-precipitable compounds in addition to the products deriving from reaction with starch; these are probably products derived from hydrolysis of



**Figure 2.** Starch concentrations (mg starch-C g<sup>-1</sup> DM) from artificial plant material, maize leaves spiked with  $\text{C}_3$  starch, *Plantago lanceolata* leaves and spruce (*Picea abies*) roots derived from methods using acid hydrolysis (H1–H3) or enzymatic hydrolysis (E1a,b and E2). Solid line represents the target content of starch in artificial leaf material. Dashed line indicates the calculated 'target' content of starch of spiked maize leaf. Error bars indicate 1 standard deviation of the mean ( $n = 5$ ).



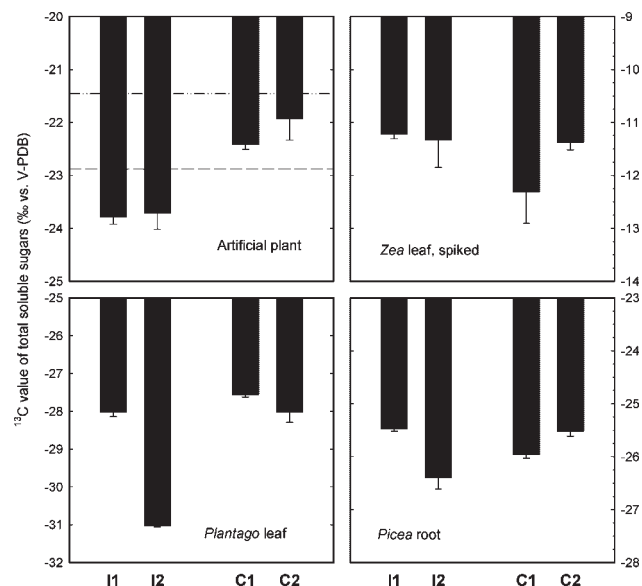
**Figure 3.** Sugar and uronic acid composition of starch preparations E1a (enzymatic hydrolysis) and H3 (acid hydrolysis) from the artificial leaf sample. Dry pellets of the starch preparations were subjected to trifluoroacetic acid hydrolysis and resulting sugars analyzed by HPLC-pulsed amperometric detection. Error bars indicate 1 standard deviation of the mean ( $n = 5$ ).

hemicelluloses, pectin and gums, as indicated by the high amounts of mannose and xylose that were found in these preparations (Fig. 3). We also found a relatively high nitrogen content in the starch preparations after acid hydrolysis ( $H_3$ :  $1.08 \pm 0.08\%$ ; data not shown), although these preparations should theoretically be free of N, indicating the presence of amino acids and peptides or proteins. We used a two-membered mixing model to calculate the percentage contamination of the starch preparation for the artificial leaf. The two end-members were the  $\delta^{13}\text{C}$  of  $\text{C}_4$ -starch and the  $\delta^{13}\text{C}$  of  $\text{C}_3$ -background (cellulose, hemicellulose, protein, etc.). The starch precipitates after acid hydrolysis were contaminated by 25.5 to 68.8% with non-starch material, while the enzymatically derived starch contained between 18.7 and 22.5% of contaminants. The latter finding is surprising, as  $\alpha$ -amylase is known to be highly specific for the cleavage of the  $\alpha$ -1,4 glycosidic linkages in amylose and amylopectine.<sup>38,39</sup> Amyloglucosidase on the other hand is known to exhibit a low side-activity on cellulose or xylans.<sup>40</sup> It is, however, possible that the deviation from the target  $\delta^{13}\text{C}$  values for the methods based on the enzymatic hydrolysis was due to isotopic inhomogeneities within the starch granules and incomplete degradation of the starch causing apparent isotope fractionation.

The combined data provide evidence that all the tested methods exhibited deviations from the expected isotopic values, and that these deviations were higher in the methods utilizing acidic hydrolysis than those utilizing enzymatic methods. The acid hydrolysis method had a lower specificity for starch, and it could be shown that other plant constituents (e.g. hemicelluloses) have been hydrolyzed. The reasons for the deviation from the target isotope values for the methods based on enzymatic hydrolysis are, however, less clear and need further investigation. This may or may not affect the estimation of the carbon isotope composition of starch, depending on the physiology of the plant and the environmental conditions.  $\delta^{13}\text{C}$  values integrate different time-spans in plant tissues: for example, cellulose and hemicellulose integrate over the whole growth period of a leaf, but stay mostly unaltered thereafter; starch, on the other hand, usually reflects much lower time-spans of hours to weeks and may change concentration and  $\delta^{13}\text{C}$  in a diurnal cycle (transitory starch<sup>41</sup>). Plant metabolic fractionation leads to small but systematic differences in the carbon isotope compositions of different plant carbohydrates.<sup>14</sup> Thus, when environmental conditions during the biosynthesis of starch are different from those of structural compounds, such as cellulose (e.g. under drought stress conditions), the isotopic offset between starch (or sugars) and structural carbohydrates is expected to change.<sup>6</sup> In such a case, i.e. if the offset between starch and structural carbohydrates is large (e.g. in case of short-term  $^{13}\text{C}$ -labelling experiments), even a small contamination of the starch preparation with, e.g., cellulose or other long-lived compounds may lead to large errors in the estimate of starch  $\delta^{13}\text{C}$  values.

## Sugars

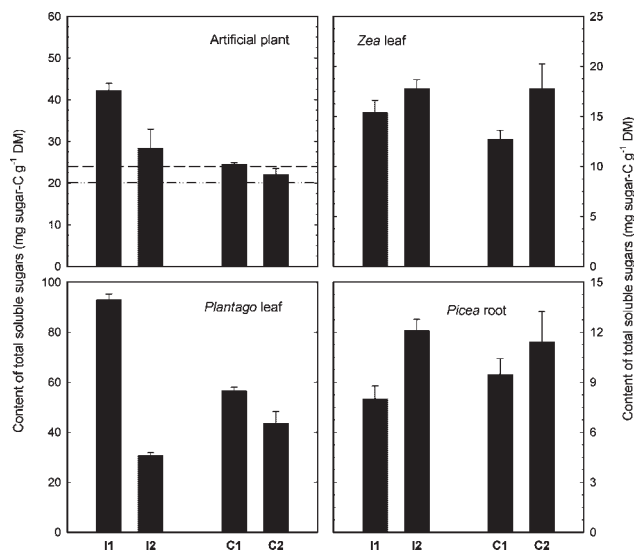
The differences in the carbon isotope signatures of soluble sugars between the methods (Fig. 4) were in general smaller than those observed for starch (Fig. 1). Significant isotopic



**Figure 4.** Carbon isotope composition ( $\delta^{13}\text{C}$ ) of total soluble sugars, i.e. bulk soluble sugar fractions or summed individual sugars (mass balance approach) (glc, frc, suc), prepared from artificial plant material, maize leaves spiked with  $\text{C}_3$  starch, *Plantago lanceolata* leaves and spruce (*Picea abies*) roots. Soluble sugars were prepared by ion-exchange chromatography (I1, I2) or via HPLC for compound-specific isotope analysis (C1, C2). Dashed line represents the target  $\delta^{13}\text{C}$  value of total sugars including *myo*-inositol for bulk soluble sugars; the dash-dot line shows the target  $\delta^{13}\text{C}$  value of total sugars without *myo*-inositol for compound-specific isotope analysis. Error bars indicate 1 standard deviation of the mean ( $n = 5$ ).

differences between bulk sugar and compound-specific methods were mainly found for artificial leaf material. Differences were also observed between I2 and the other methods, the former showing much more negative  $\delta^{13}\text{C}$  values in *Plantago* and partly in *Picea* roots, while in maize leaves C1 produced carbon isotope signatures more depleted than when using the other methods.

In the 'artificial leaf', the preparations for bulk sugars (by ion exchange) yielded identical isotope values for total sugars. The same was true for the two compound-specific approaches, but the compound-specific methods yielded  $\delta^{13}\text{C}$  values that were about 1.5‰ more positive than those obtained by the bulk sugar preparations. The main reason for this was the different contribution of *myo*-inositol to the preparations. *myo*-Inositol was depleted in  $^{13}\text{C}$  by more than 4.7‰ compared with the other sugars (Table 1). In bulk sugar methods, all non-charged metabolites such as *myo*-inositol contributed to the  $\delta^{13}\text{C}$  values of the preparation. Therefore, the target  $\delta^{13}\text{C}$  value for bulk sugar methods was  $-22.9\text{‰}$ , while that for the compound-specific methods (i.e. without *myo*-inositol) was  $-21.5\text{‰}$ . Both approaches yielded slightly  $^{13}\text{C}$ -depleted isotope signatures compared with the target values. The carbon isotope signatures of soluble sugars for *Picea* root, a natural plant sample, and spiked *Zea* leaf, were nearly identical for both the compound-specific and the bulk approaches of isotope analysis. Moreover, the known isotopic differences between plant samples belonging to  $\text{C}_3$  and  $\text{C}_4$  photosynthetic pathways were reproduced



**Figure 5.** Concentrations of total soluble sugars, i.e. bulk neutral fraction or summed single sugars ( $\text{mg sugar-C g}^{-1} \text{DM}$ ), prepared from artificial plant material, maize leaves spiked with  $\text{C}_3$  starch, *Plantago lanceolata* leaves and spruce (*Picea abies*) roots isolated by ion-exchange chromatography (I1, I2) or compound-specific analysis via HPLC (C1, C2). Dashed line represents the target content of total sugars including *myo*-inositol for bulk soluble sugars; the dash-dot line shows the target content of total sugars without *myo*-inositol for compound-specific isotope analysis. Error bars indicate 1 standard deviation of the mean ( $n = 5$ ).

correctly in soluble sugars (Fig. 4). In contrast, for *Plantago* leaf we found a 3‰ difference between the bulk sugar methods (I1 and I2), and the same was true for *Picea* root samples but the difference was smaller here (only 1‰). Re-

analysis of *Plantago* leaf material by method I2 again showed this  $^{13}\text{C}$  depletion in bulk sugars compared with I1. Although we cannot conclusively explain this large deviation, 3-fold differences in leaf 'sugar' content between I1 and I2 (Fig. 5) point to different retention and elution of non-ionic and ionic solutes that may differ isotopically. Clearly isotope fractionation during ion exchange can be rejected as an explanation of this difference since ion-exchange preparation of pure sugars (e.g. sucrose, glucose) resulted in no isotope fractionation (I-2, data not shown).

For both methods, the recovery of soluble sugars in artificial leaf was generally slightly higher than the target, suggesting a possible contribution of unretained (charged) or other neutral compounds in the ion-exchange step (bulk sugar methods), or 'bleeding' of the HPLC column (compound-specific approaches). However, the deviation was much smaller for the compound-specific methods. For natural plant samples no systematic differences in sugar content could be observed between bulk and compound-specific approaches (Fig. 5). For *Plantago* samples, however, large differences between the I1 and I2 methods were observed, I1 giving much higher sugar contents (3-fold compared with I-2 and 2-fold compared with both compound-specific methods).

In contrast to the generally good agreement of  $\delta^{13}\text{C}$  values of total soluble sugars with their target  $\delta^{13}\text{C}$  values, individual sugars purified by HPLC showed deviations of between 0.1 and 5.4‰ in artificial leaf (Table 4). There was no systematic difference between the methods with (C2) and without (C1) the pre-purification step. The highest deviation from the target was observed for the  $\text{C}_4$ -derived glucose, suggesting either (1) background contamination, (2) co-elution with  $\text{C}_3$ -derived sucrose, or (3) contamination with  $\text{C}_3$ -derived glucose from sucrose breakdown during

**Table 4.** Content and compound-specific carbon isotope composition of individual soluble sugars from artificial plant material, maize leaves spiked with  $\text{C}_3$  starch, *Plantago lanceolata* leaves and spruce roots. Values are means  $\pm$  1 standard deviation ( $n = 5$ )

	Sucrose		Glucose		Fructose	
	C1	C2	C1	C2	C1	C2
Artificial leaf						
$\delta^{13}\text{C}$ (‰)	−24.48 ±0.15	−23.17 ±0.56	−14.62 ±0.96	−16.21 ±0.68	−26.08 ±0.38	−24.53 ±0.55
Target values		−25.29		−10.83		−24.44
Content ( $\text{mg g}^{-1} \text{dm}$ )	11.43 ±1.10	8.08 ±1.31	6.20 ±0.42	5.48 ±0.68	5.33 ±1.39	8.45 ±0.51
Target values		10.00		5.00		5.00
<i>Zea mais</i> leaves						
$\delta^{13}\text{C}$ (‰)	−12.70 ±0.37	−11.44 ±0.16	−12.15 ±0.26	−11.18 ±0.29	−12.34 ±0.31	−11.36 ±0.21
Content ( $\text{mg g}^{-1} \text{dm}$ )	9.41 ±0.17	10.17 ±1.07	1.66 ±0.04	2.80 ±0.72	1.97 ±0.04	4.83 ±0.93
<i>Plantago</i> leaves						
$\delta^{13}\text{C}$ (‰)	−26.58 ±0.24	−26.53 ±0.28	−28.29 ±0.10	−29.38 ±0.46	−27.79 ±0.30	−28.19 ±0.48
Content ( $\text{mg g}^{-1} \text{dm}$ )	19.06 ±0.54	12.32 ±2.25	21.93 ±0.96	15.88 ±2.69	15.16 ±0.29	14.26 ±1.10
<i>Picea abies</i> roots						
$\delta^{13}\text{C}$ (‰)	−25.23 ±0.08	−24.40 ±0.51	−27.02 ±0.15	−26.66 ±0.37	−25.41 ±0.12	−25.34 ±0.37
Content ( $\text{mg g}^{-1} \text{dm}$ )	3.01 ±0.31	3.30 ±0.36	3.57 ±0.55	3.75 ±0.42	2.90 ±0.12	4.37 ±1.16

sugar preparation. The isotopic differences between individual sugars were generally much smaller in the natural plant material (Table 4). Individual sugars from natural plant sources differed by only 0.1 and 1.3‰ when determined by the two compound-specific methods (fructose from *Picea abies* and sucrose from *Zea mays*, respectively).

The artificial leaf sample, which consisted of a mix of C<sub>3</sub> and C<sub>4</sub> sugars, clearly demonstrates the limitation of compound-specific isotope methods: a unsatisfactory separation of individual sugars such as fructose and glucose (or *myo*-inositol) or peak broadening (by collection or by on-line digestion of the HPLC effluent) may lead to significant differences between the measured carbon isotope compositions of sugars and their target values. These problems are not automatically solved by the newly available techniques such as on-line HPLC-IRMS interfaces<sup>22,42</sup> as they rely to the same extent on a baseline separation of the target substances. However, new HPLC columns with a higher capacity for sugar separation may help to overcome these problems.

## CONCLUSIONS

### Starch

First, from the results of this study we conclude that the methods based on acid hydrolysis and on enzymatic hydrolysis are not comparable with regard to starch carbon isotope composition and starch content. Second, the specificity (selectivity) of the methods based on the acidic hydrolysis was low, and we therefore suggest terming these preparations as HCl-hydrolysable carbon, rather than starch. The methods based on enzymatic hydrolysis at the moment provide the only feasible way for a compound-specific analysis of isotopes in starch. Despite being more specific, the enzyme-based methods also showed offsets from the target  $\delta^{13}\text{C}$  values and these methods therefore need to be further optimized and adequate standards are needed.

### Sugars

In contrast to starch, sugar isotope analysis from plant material is facilitated by an internationally certified standard (sucrose, ANU) and by recent technical developments in compound-specific isotope analysis (i.e. HPLC/IRMS). The analysis of sugars by the bulk sugar approach has to be applied with caution when used for the fast screening of carbon isotopes in sugars. Problems can be encountered when other neutral plant compounds (e.g. *myo*-inositol or pinitol) are present in amounts that are equal to or even higher than those in sugars and when these compounds are isotopically different from the sugars. The most critical points in obtaining reliable  $\delta^{13}\text{C}$  values of individual sugars by the compound-specific approach were co-eluting substances and background contaminations. Both problems have to be properly addressed, irrespective of whether off-line or on-line compound-specific isotope methods are used.

Taken together, the variation in starch and sugar isotope analyses from plant material, which has been found in this study, is evidence of the need for a range of carbon isotope standards from natural materials certified for both starch and sugars. Ideally, these standards should encompass different

plant tissues (e.g. leaves and roots) and plant material of different metabolic pathways (i.e. C<sub>3</sub> and C<sub>4</sub> plants).

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