## **Supporting Information**

Article title: Non-structural carbohydrates in woody plants compared among laboratories

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#### Method S1. Preparation of standard samples

Ten plants of *Eucalyptus globulus* were selected for destructive biomass harvesting in March 2013 from the CSIRO Ecosystem Sciences glasshouse facilities ( $42^{\circ}$  55' 0" S /  $147^{\circ}$  20' 0" E). The seedlings were obtained from the Forestry Tasmania Nursery near Perth, Tasmania. Plants were grown for 12 months in 75 L plastic bags with potting mix consisting of eight parts composted pine bark to three parts coarse river sand, and amended with a low phosphorus premium controlled-release fertiliser (N:P:K - 17.9:0.8:7.3). Plants were irrigated twice daily for 30 minutes using drip irrigation at a rate of 12 L per hour to maintain soil at field capacity. Plant biomass was harvested on the same day, and divided into leaves (EGL), stem (EGS) and coarse roots (>5 mm) (EGR). Bark was removed from the stem and root samples. Immediately after harvesting, each tissue component was placed overnight into a -4°C freezer until processing. All samples were freeze-dried at -80°C for 24 to 48 hours (LY-5-FM, Breda Scientific, Netherlands). Dried samples were ground into a fine powder. Leaves were ground and homogenised by passing through a cyclone mill (18 mesh, 1 mm; Foss Cyclotec 1093, Denmark) and a Micro Ball Mill "Lab Wizz" 320 (Laarmann Group B.V., Op het Schoor 6, 6041 AV Roermond, Netherlands) at 30 Hz for 45 seconds (270mesh, 53  $\mu$ m). Woody tissues (stem and roots) were ground and homogenised by passing through a Wiley Mill (40 mesh, 400 µm), then with a Mini Wiley Mill (60 mesh, 250 µm). Following grinding, each sample was well homogenised by thorough mixing.

Foliage for the pine standard was sampled from a mature *Pinus edulis* (PEN) at mid-day on 26/7/2010 at Mesita del Buey, Los Alamos, USA ( $35^{\circ} 51' 0" \text{ N} / 106^{\circ} 17' 12" \text{ W}$ ). The sample was collected, stored on dry ice, oven-dried at 70°C until constant weight, then ground and homogenised to a fine powder by passing it twice through a cyclone mill (Udy Corporation, Fort Collins, CO, USA). Following homogenization, the sample was stored in a plastic bag in a cool dry cabinet.

Standard foliar samples of *Prunus persica* (PPL) (SRM1547) were commercially purchased. The plant material was collected from healthy peach trees in Peach County, Georgia, USA. The leaves were dried and ground in a stainless steel mill past a 1 mm screen, and then jet milled and air classified to a particle size of ~75  $\mu$ m (200 mesh). Ground material was then homogenised by mixing in a large blender.

All standard materials were irradiated at 27.8 kGy for microbiological control to meet international quarantine requirements, homogenised, bottled, and stored at room temperature until shipment to the analytical laboratories.

**Method S2.** Summary of extraction and quantification procedures for soluble sugars and starch

For soluble sugars three types of extraction were performed: alcohol using either ethanol or methanol, water, and methanol:chloroform:water (MCW, 12:5:3, v:v:v). Quantification of soluble sugars were done on supernatant solution, with one of five methods: high-performance liquid chromatography (HPLC), High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD), enzymatic assay based on NAD-linked enzymatic reactions and monitoring reduction of NAD+ to NADH at 340 nm, anthrone-sulfuric acid method with a colorimetric determination at 620 nm, and phenol-sulfuric acid method with a colorimetric determination at 490 nm. HPLC and HPAEC-PAD quantified individual sugars: glucose, fructose and sucrose. <sup>1</sup>H-NMR profiling of ethanolic extracts was also used for targeted quantification of selected soluble sugars. The enzymatic assay quantified glucose+fructose, and sucrose as glucose equivalents. The anthrone and phenol methods measured only total soluble sugars. Total soluble sugars issued from chromatography-based and enzymatic methods were expressed as the sum of glucose, fructose and sucrose. More detailed protocols are summarised in Table S2.

Estimation of starch was performed on the pellets remaining after the sugar extraction or on a second aliquot of the same sample. Prior to the starch extraction procedure, samples were treated with buffers, an ultrasonic bath or acid to solubilise granules of starch and to better allow the extraction of starch and conversion into glucose by enzymes (see Table 1; Table S3). Two main methods were used to hydrolyse starch into glucose: acid or enzymatic. Within the enzymatic digestion, we distinguished two main methods: 1. amyloglucosidase (AMG) from *Aspergillus niger* used solely, and 2. AMG used in association with  $\alpha$ -amylase (AA+AMG). Quantification of starch content in the material was determined as a glucose equivalent (when based on two separate aliquots: minus the glucose and half of the fructose concentration of the in soluble sugar extraction). In addition to the quantification methods using anthrone (620 nm) or phenol (490 nm), a total starch assay kit (Megazyme International

Ireland Ltd, Wicklow, Ireland) was also used which was then colorimetrically assayed by using a mixture of glucose oxidase/peroxidase-o-dianisidine (GOPOD) with a determination at 510 nm (Table 1; Table S3). More detailed protocols are summarised in Table S3. Total non-structural carbohydrates (NSC) were the sum of total soluble sugars and starch.

**Method S3.** Summary of extraction procedures for soluble sugars used for experiment where different extractions were applied in the same laboratory

<u>Method 1- 80%EtOH</u>: Samples were placed in 500µl of 80% aqueous ethanol (EtOH) solution in a conical tube and mixed well. The mixture was placed in an incubator (Digital Dry Bath D1100, Labtech Inc., Woodbridge, NJ, USA) at 80°C for 20 minutes, and then centrifuged at 10 000 g for 5 minutes at 5°C. The supernatant was collected and the procedure was repeated and supernatant from both extractions was combined for analysis.

<u>Method 2 – 70%MeOH</u>: Samples were placed in 650µl of 70% aqueous methanol (MeOH) solution in a conical tube and mixed well. The solution was incubated and mixed at room temperature for 10 minutes, and then centrifuged for 5 minutes at 17000 g. The supernatant was collected, and the procedure was repeated twice more, and supernatant from all extractions was combined for analysis.

<u>Method 3 – MCW80:</u> Samples were placed in 350µl of MCW solution (12/5/3) into each conical tube, and mixed well. The mixture was incubated at 80°C (Digital Dry Bath D1100, Labtech Inc., Woodbridge, NJ, USA) and mixed for 30 minutes, and then centrifuged for 3 minutes at 11 400 g at room temperature (derived from Dickson and Larson 1975). The supernatant was collected, and the procedure was repeated twice, and supernatant from both extractions was combined for analysis.

<u>Method 4 – MCWamb</u>: Samples were placed in 400µl of MCW solution (12/5/3) into each conical tube, and mixed well. The solution was incubated and mixed at room temperature (~20°C) for 30 minutes, and then centrifuged for 10 minutes at 2000 g at 15°C. The supernatant was collected, and the procedure was repeated, and supernatants from both extractions were combined for analysis.

In each method and sample, the supernatants were filtered prior to quantification. Soluble sugars from the supernatants were quantified by anthrone-sulfuric acid method with a colorimetric determination at 620 nm (Spectrophotometer UV-visible DU 640 B, Beckman Coulter, USA) as described by Hansen and Møller (1975) with glucose as standard.

**Method S4.** Enzyme de-activation treatments using microwave and effect on non-structural carbohydrate in foliar and twig samples of *Pinus edulis* 

Four foliar and twig samples were collected from six *Pinus edulis* trees at Mesita Del Buey, Los Alamos, USA ( $35^{\circ} 51' 0" \text{ N} / 106^{\circ} 17' 12" \text{ W}$ ) in June 2012. Samples were collected in liquid nitrogen and stored at -70 °C. Prior to drying, one sample from each tree was subject to one of four enzyme deactivation treatment times using a microwave at 800 Watts: 1) 0 seconds, 2) 90 seconds, 3) 180 seconds, and 4) 300 seconds. Samples were then oven-dried at 65 °C to constant weight. Leaf tissues were ball milled to a fine powder (high throughput homogenizer; VWR). Woody tissues were milled to 40 mesh (400 µm) prior to ball milling (Wiley Mini Mill; Thomas Scientific).

Samples were analysed following the protocol described by Hoch et al. (2002) with minor modifications. Approximately 12 mg of fine ground plant material was extracted in a 2 mL deep-well plate with 1.6 mL distilled water for 60 min in a 100 °C water bath (Isotemp 105; Fisher Scientific). Following extraction, an NAD-linked enzymatic assay was used to evaluate NSC content. All sugars were hydrolysed to glucose, linked to the reduction of NAD+ to NADH, and monitored at 340 nm with a spectrophotometer (Cary® 50 UV-Vis).

Data were analysed using one-way analysis of variance (ANOVA) in R. Tukey's Honest Significant Difference was used for pairwise comparison of significant effects ( $\alpha < 0.05$ ).

**Method S5.** Analyses of non-structural carbohydrates in *Pinus banksiana* samples in the experiment on grinding particle size, fine (< 105  $\mu$ m) versus coarse (> 400  $\mu$ m)

The samples were collected from leaves, stem and roots of ten jack pine (*Pinus banksiana* Lamb.) seedlings in 2013. Samples were immediately oven-dried at 70°C until constant weight, and either ground with a Wiley Mini-Mill (Thomas Scientific, Swedesboro, NJ, USA) to pass a 40 mesh (400 µm) or with a Micro Ball Mill "Lab Wizz" 320 (Laarmann Group B.V., Op het Schoor 6, 6041 AV Roermond, Netherlands) at 30 Hz for 2 minutes.

Root and stem samples from the Ball Mill had particles  $<105 \mu m$  (140 mesh), and particles from the needle sample were  $<53 \mu m$  (270 mesh).

Total soluble sugars were extracted from 50 mg of dried plant tissue in 4.7 ml 80% aqueous ethanol in a glass test tube. The mixture was placed in a water bath at 90°C for 10 mins, and then centrifuged at 2500 rpm for 4 minutes. The supernatant was collected, and the procedure was repeated two more times. Total soluble sugars were determined on the supernatant combined using a phenol–sulphuric acid colorimetric assay, and absorbance was read at 490 nm, consistent with methods described by Chow and Landhäusser (2004). Starch was determined on the remaining pellets and assayed enzymatically using a combination of  $\alpha$ -amylase and amyloglucosidase. Prior to digestion, pellets were solubilised using a 0.1 M of NaOH solution. Starch was determined using GOPOD assay, and absorbance was read at 525 nm. A Student t-test determined the effect of particle sizes on total soluble sugars, starch and NSC contents for the different tissues of *P. banksiana* ( $\alpha = 0.05$ ).

# Tables:

Table S	<b>1.</b> Summar	v of pr	ocedures of	extraction and	quantification	for soluble sug	ars measurements us	ed by	participati	ng laboratories.
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Lab ID	Extraction	Quantification (assay; standards used)	References
0	70% MeOH (room temperaure; 10 mins) (x3)	Spec. 620 (anthrone-sulfuric assay; GLUC)	Hansen and Møller (1975)
U	70% EtOH (55-65°C; 30 mins)	Spec. 620 (anthrone-sulfuric assay; GLUC)	Yemm and Willis (1954); Hansen and Møller (1975)
S	80% EtOH +30% EtOH + W (each 90°C; 15 mins)	Enz. (340 nm; GLUC, FRUC, SUC)	Blunden and Wilson (1985)
V	80% EtOH (60°C; 30 mins)	Spec. 490 (phenol-sulfuric assay; GLUC)	Dubois et al. (1956); Buysse and Merckx (1993); Palacio et al. (2007)
CC	80% EtOH (80°C; 20 mins)	HPAEC-PAD	Van Meeteren et al. (1995)
Q	80% EtOH + 50% EtOH + W (each 80°C; 15 mins)	<sup>x1</sup> H-NMR (GLUC, FRUC, SUC)	Adapted from Moing et al. (2004)
С	80% EtOH (x2) + W (each 95°C; 30 mins)	Spec. 620 (anthrone-sulfuric assay; GLUC)	Ebell (1969)
D	80% EtOH (x2) + 50% EtOH (each 80°C; 20 mins)	Enz. (340 nm; GLUC, FRUC, SUC)	Jelitto et al. (1992)
L	80% EtOH (80°C; 30 mins) + 80% EtOH (80°C; 15 mins) + 50% EtOH (80°C; 15 mins)	HPLC (mannitol) <sup>y</sup>	Maine et al. (1002)
	80% EtOH (80°C; 30 mins) + 80% EtOH (80°C; 15 mins) + 50% EtOH (80°C; 15 mins)	HPLC (mannitol) <sup>z</sup>	Moing et al. (1992)
В	80% EtOH (75°C; 30 mins) (x3)	HPAEC-PAD (GLUC, FRUC, SUC)	Mialet-Serra et al. (2005)
Е	80% EtOH (100°C; 2 mins) + 80% EtOH (90°C; 2 mins) (x2)	HPLC (trehalose)	Lucas et al. (1993)
J	80% EtOH (60°C; 60 mins) (x3)	HPLC (trehalose)	Lambrechts et al. (1994); Blake (1999)
Μ	80% EtOH (90°C; 10 mins) (x3)	Spec. 490 (phenol-sulfuric assay; GLUC, FRUC, GALAC)	Chow and Landhäusser (2004)
Т	80% EtOH (100°C; 10 mins) (x3)	Spec. 630 (anthrone-sulfuric assay; GLUC)	McCready et al. (1950)
X	80% EtOH (60°C; 60 mins) (x3)	HPLC (trehalose)	Lambrechts et al. (1994); Blake (1999)

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DD	80% EtOH (80°C; 20 mins) (x3)	Enz. (340 nm; GLUC, FRUC, SUC)	Hendrix (1993)
А	80% EtOH (80°C; 10 mins) (x2) + (room temperature) (x2)	Enz. (340 nm; GLUC, FRUC, SUC)	Sigma®
K	80% EtOH (room temperature; 3 mins) (x4)	Spec. 490 (phenol-sulfuric assay; GLUC)	Kabeya and Sakai (2003)
Y	80% EtOH (80°C; 20 mins) (x5)	Spec. 620 (anthrone-sulfuric assay; GLUC)	Schaffer et al. (1985)
Р	MCW (4°C; overnight) + MCW (x2)	HPLC (galactitol)	Coleman et al. (2009)
W	MCW (20°C; 5 mins) (x2)	Spec. 490 (phenol-sulfuric assay; SUC)	Haissing and Dickson (1979); Rose et al. (1991); Chow and Landhäusser (2004)
Z2	MCW (60°C; 30 mins)	HPAEC-PAD (GALAC, GLUC, FRUC, SUC)	Wanek et al. (2001)
F	W (65°C; 10 mins) (x3)	HPLC	Raessler et al. (2010)
Z1	W (85°C; 30 mins)	HPAEC-PAD (GALAC, GLUC, FRUC, SUC)	Richter et al. (2009)
G, N, R AA, BB & EE	W (steam; 60 mins)	Enz. (340 nm; GLUC, FRUC, SUC)	Wong (1979); Hoch et al. (2002)

Enz: enzymatic; EtOH: ethanol; FRUC: fructose; GALAC: galactitol; GLUC: glucose; MCW: methanol:chloroform:water; spec: spectrophotometry; SUC: sucrose; W: water.

<sup>x1</sup>H-NMR profiling of ethanolic extracts was also used for targeted quantification of selected soluble sugars.

<sup>y</sup> HPLC using Metrosep Carb1 250 x 4.6 mm column (Metrohm ltd, CH-9101 Herisau, Switzerland)

<sup>2</sup> HPLC using CARBOSep COREGEL 87C column (Transgenomic Inc., NE 68164, Omaha, USA)

Lab ID	Gelatinisation or Solubilisation	Extraction/Digestion	Quantification (assay; standards used)	References
F	Ultrasound	52% HClO4 (23°C; 20 hrs) (x2)	HPLC	Raessler et al. (2010); Hartmann et al. (2013)
Р	-	H <sub>2</sub> SO <sub>4</sub> + (120°C; 3.5 mins)	HPLC (GLUC)	Coleman et al. (2009)
Т	-	35% HClO4 (23°C; 16 hrs)	Spec. 630 (anthrone-sulfuric assay; GLUC)	McCready et al. (1950)
U	Vigorous shaking	1% HCl (100°C; 30 mins)	Spec. 620 (anthrone-sulfuric assay; GLUC)	Yemm and Willis (1954); Hansen and Møller (1975)
С	AA (100°C; 6 mins)	Amylo. (50°C; 30 mins)	Spec. 515 (GOPOD assay; GLUC)	Megazyme ®; McCleary et al. (1997)
D	0.1 M NaOH (95°C; 30 mins)	AA + amylo. (37°C; 10-16 hrs)	Enz. (340 nm; GLUC)	Hendriks et al. (2003)
J	DMSO (100°C; 5 mins)	AA (100°C; 6 mins) + amylo. (50°C; 30 mins)	Spec. 515 (GOPOD assay; GLUC)	Megazyme ®;
Μ	0.1 M NaOH (50°C ; 30 mins)	AA + amylo. (50°C; 20-24 hours)	Spec. 525 (GOPOD assay; GLUC)	Chow and Landhäusser (2004)
DD	DMSO (100°C; 5 mins)	AA (100°C; 16 mins) + amylo. (50°C; 30 mins)	Spec. 510 (with GOPOD assay; GLUC)	Megazyme ®
Α	AA (100°C; 9 mins)	Amylo. (50°C; 30 mins)	Spec. 515 (with GOPOD assay; starch)	Megazyme ®
CC	AA (90°C; 30 mins)	Amylo. (60°C; 15 mins)	HPAEC	Van Meeteren et al. (1995)
Z1 Z2	AA (85°C; 30 mins)	Amylo. (55°C; 30 min)	HPLC (GLUC)	Göttlicher et al. (2006)
K	0.2 M KOH (95°C; 30 mins)	Amylo. (55°C; 30 mins)	Spec. 490 (phenol-sulfuric assay; GLUC)	Komatsu et al. (2013)
L	0.02N NaOH (120°C; 60 mins)	BA (52°C; 90 mins)	Enz. (340 nm; GLUC)	Moing et al. (1992)
В	0.02N NaOH (95°C; 90 mins)	Amylo. (50°C; 60 mins)	HPAEC (GLUC)	Mialet-Serra et al. (2005)
Ε	Water (100°C; 60 mins)	Amylo. (55°C; 3 hrs)	Spec 340 (GOPOD assay; GLUC)	Lucas et al. (1993)
Q				
S	Autoclave (120°C; 90 mins)	Amylo. (56°C; 90 mins + 100°C; 5 mins)	Enz. (340 nm; GLUC)	Thivend et al. (1965); Gomez et al. (2003b); Gomez et al.

# **Table S2.** Summary of procedures of gelatinisation, extraction and quantification for starch measurements.

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(2003a)

V	0.1 M NaOH (100°C; 60 mins)	Amylo. (50°C; 16 hours)	Spec. 490 (phenol-sulfuric assay; GLUC)	Dubois et al. (1956); Buysse and Merckx (1993); (Palacio et al. 2007)
X	0.1 M NaOH (100°C; 60 mins)	Amylo. (50°C; 16 hours)	Spec. 490 (phenol-sulfuric assay; GLUC)	Dubois et al. (1956); Buysse and Merckx (1993); Palacio et al. (2007)
Y	W + autoclave (120°C; 60 mins)	Amylo. (55°C; 22 hrs)	Spec. 620 (anthrone-sulfuric assay; GLUC)	Schaffer et al. (1985)
0	0.02 N NaOH (100°C; 60 mins)	Amylo. (50°C; 30 mins)	Spec. 620 (anthrone-sulfuric assay; GLUC)	Hansen and Møller (1975)
W	47.5% EtOH (100°C; 30 mins)	Amylo. (45°C; overnight)	Spec. 490 (phenol-sulfuric assay; GLUC)	Rose et al. (1991); Marquis et al. (1997)
G, N, R, AA, BB & EE	0.1 M NaOH	Amylo. (48°C; overnight)	Enz (340 nm; GLUC)	Wong (1979); Hoch et al. (2002)

AA: α-amylase; Amylo.: amyloglucosidase; BA.: β-amylase; DMSO : diméthylsulfoxide; Enz: enzymatic; EtOH: ethanol; GLUC: glucose; GOPOD: glucose oxidase/peroxidase-o-dianisidine; H<sub>2</sub>SO<sub>4</sub>: Sulfuric acid ; HClO<sub>4</sub>: Perchloric acid ; HPAEC: High Performance Anion Exchange Chromatography; HPLC: High-performance liquid chromatography; KOH: Potassium hydroxide ; NaOH: Sodium hydroxide; Spec: spectrophotometry; W: water.

#### Notes:

#### Note S1. "Matrix effect"

Signals from many metabolites present in plant tissue can be influenced by other interfering metabolites through several different mechanisms, collectively known as matrix effects. For example, sulphuric acid used in colorimetric assays can break down structural carbohydrates such as cellulose and hemicelluloses and could potential be converted into glucose. The gravity of this problem is easily realized when considering a situation where the signal of a given metabolite is affected by the concentration of a different metabolite with fluctuating concentration. In this scenario, the first metabolite will appear to change even if its concentration is stable. In this study, our samples had *a priori* different levels of NSCs and different compounds that may interfere with analysis. P. persica leaves have a high level of sugar-alcohol sorbitol (Loescher 1987), which may not be assessed as sugar by colorimetric methods, but identified in HPLC (Manuscript Fig. 3F). In this study, HPLC and enzymatic methods only targeted glucose, fructose and sucrose, which are supposed to be the major sugars present in plant material, and could be easily targeted by those methods, whereas colorimetric assays were non-specific. Foliage of E. globulus contains phenolics (Macauley and Fox 1980, Rapley et al. 2008), and foliage in P. edulis has terpenes (Cobb et al. 1997). These secondary compounds may interfere with extraction and/or quantification (Ashwell 1957, Hendrix and Peelen 1987), which may explain the high variability of the EGL results (Manuscript Fig. 3). Purification treatment using charcoal can increase the recovery rate of soluble sugars from tissues containing phenolics (Hendrix and Peelen 1987). The presence of matrix effects is also an important reason why quantification of an authentic standard is very different from untargeted analysis of metabolites in an actual biological sample.

Matrix components present in biological samples, such as ligneous *versus* non-ligneous components, can also affect the response of the analyte of interest. These phenomena, termed generally as matrix effects, can lead to inaccurate quantification (Silva et al. 2012), and therefore are important to be addressed in analytical method development and validation (Thompson and Ellison 2005). Here, ligneous or woody samples (*i.e.* EGR and EGS) showed least variability in soluble sugar results compared to starch results following acid hydrolysis (Manuscript Fig. 3A) or colorimetric assays (Manuscript Fig. 3F). In woody tissues, starch exists in small concentrations that are locked within a matrix of structural polysaccharides (e.g. cellulose and hemicellulose) and organic polymers (e.g. lignin). Acid hydrolysis may

not be suitable for wood matrix because not all acid methods have a selective step, and can result in low selectivity because both structural carbohydrates and starch are hydrolysed by concentrated acids into monomeric sugars which could result in high interference (Ebell 1969, MacRae et al. 1974, Marshall 1984, Rose et al. 1991). In contrast, enzyme methods rely on the inherent specificity of the catalyst, which is superior to the precipitation selectivity (Rose et al. 1991). To the best of our knowledge, no research has been devoted to measuring the matrix effect on extraction of NSC in woody plants. Appropriate matrix management could gear toward minimizing or correcting these effects. Therefore, in order to fully extract NSC from tissues, it is necessary to use solvents that readily dissolve the NSC, but also overcome the interactions between the NSC and the tissue matrix.

#### Note S2. Standardisation of sample preparation

The validity and usefulness of a plant analysis hinges on the care and method used to obtain the required plant material (Westerman 1990). If the sample taken is not representative of the general population, all the careful and costly work put into the subsequent analysis will be wasted because the results will be invalid. Unfortunately, less research has been devoted to sampling and sample preparation procedures than to other aspects of plant analysis techniques. Yet sampling and sample preparation, if not properly done, can contribute to errors which may have a cumulative effect on the final reported results.

The elemental content of a plant is not a fixed entity, yet it is crucial to obtain a representative sample from a particular plant species. Plant part selected and time of sampling must correspond to the best relationship between element concentration and physical appearance of the plant (Westerman 1990). In establishing sampling procedures for plant analysis, the researcher and user must be aware that the concentrations of non-structural carbohydrates, as well as other elements, change rather rapidly with time, particularly in leaves, and physiological maturity, and may also vary more greatly between organs (Palacio et al. 2007).

The most difficult logistical problem facing plant analysis is the preservation of fresh material during transport from field to the laboratory. Delays and adverse conditions during transport can cause substantial respiratory losses in weight or enhanced enzymatic activity (Handreck 1972). Deinum and Maasen (1994) showed that pre-freezing treatment prior to

drying did not influence the outcome, suggesting that snap-freezing using liquid nitrogen or dry ice may keep the attributes of the samples.

Oven drying is often used as sample preparation before sugar and starch analyses, even though results have shown that considerable losses may occur (Deinum and Maassen 1994, Pelletier et al. 2010). With oven drying, thick specimens (e.g., big roots) or large sample volumes can be problematic because the complete drying process may last for many hours and rapid removal of moisture is necessary (Udén 2010). Evidence implicates respiratory loss, metabolic interconversions, and deleterious effects of heat as factors that may alter plant carbohydrate composition during conventional sampling and drying procedures (Deinum and Maassen 1994). Low temperatures (below 50°C) allow time for enzymatic conversions and respiratory losses, whereas high temperatures (above 80°C) can cause thermochemical degradation (Smith 1973). For those reasons, sampling and drying procedures that rapidly inhibit enzyme activity while preserving chemical attributes are preferred. Freeze-drying is generally recognized as the best method for preserving the labile metabolites, including NSC (Pelletier et al. 2010, Raessler et al. 2010). Pre-treatment with a microwave before drying at 55°C gave similar values as for freeze drying by denaturing proteins that cause enzymatic conversions and respiratory losses (Pelletier et al. 2010). However, sucrose concentration were consistently higher in microwave pre-treated samples than in freeze-dried samples due to rapid deactivation of all plant enzyme, thereby minimising respiratory weight losses and interconversions between carbohydrate fractions (see Manuscript Fig. 5; Popp et al. 1996, Pelletier et al. 2010, Raessler et al. 2010). The drawbacks of freeze-drying include access to expensive equipment and difficulty in application to large samples under field conditions.

Dried samples are customarily ground to facilitate the preparation of homogeneous samples for chemical analysis, and should be ground to an appropriate and consistent fineness (Greub and Wedin 1969). There is a paucity of information in the literature regarding the effect of particle size on NSC values and the impact of the grinding technique, which is accomplished in a variety of mills. In our study, there was consistently higher starch concentration in finely ground root and stem samples compared with coarser ground samples, presumably due to more efficient breakdown of xylem structure and exposure of more parenchyma cells to enzymes (Fig. S4). The lack of differences in NSC between fine- and coarse-ground foliar samples of *P. banksiana* indicates that particle size of ground material does not impede extraction of NSC in these leaf tissues, most likely due to the lack of woody tissues. It

appears that a standardized sample preparation would be advisable to allow for comparisons of absolute values and that consistent sample grinding methods are necessary to evaluate NSC patterns in plant tissues as samples may pulverise at different rates and segregate differently which could have an impact on starch concentrations. Finally, dried tissue should be stored under conditions that allow minimal changes (Smith 1973). The longer the storage time, the larger the changes are likely to be, therefore carbohydrate analyses should be done as soon as possible after tissue-drying (Steyn 1959).

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**Figure S1.** A on this page, B on next. Estimates of total soluble sugar, starch and total NSC by method of extraction and quantification of soluble sugars and starch for the two samples with the highest and the lowest variability among methods and laboratories: (A) *Prunus persica* leaves (PPL, high variability), and (B) *Eucalyptus globulus stem* (EGS, lower variability). Total soluble sugars results are shown by sugar extraction and quantification methods. Starch results are shown by sugar extraction method and starch extraction methods. Total NSC results are shown for sugar and starch extraction methods, and for sugar and starch quantification methods. The linear mixed model analysis showed some differences among methods (Fig. 3), but these differences were lower than the 90-percentile range of the data (Fig. 4).



Figure S1B.



**Figure S2.** Principal component analysis on total soluble sugar values for each laboratory as a function the extraction method (A), and the quantification methods (B). Panel A shows that the water (W, red) extraction method had the most homogeneous results. Ethanol (EtOH, blue) and EtOH+W (green) extraction yields highly variable results. Panel B showed that total soluble sugar quantified using colorimetric Spec 490 (pink) and Spec 620 (grey) methods were more variable than using HPLC (turquoise) and Enz. (purple) methods. Principal component analysis (PCA) was performed on centred and scaled data using the R Software (http://www.r-project.org/) and the package FactomineR (Lê et al. 2008).



**Figure S3.** Principal component analysis on starch values for each laboratory as a function the starch extraction methods (A) and quantification methods (B). Panel A shows that the AA+ amylo. extraction method (light red) yields less variable results than the other methods. Panel B shows that all methods mostly yielded similar results. Principal component analysis (PCA) was performed on centred and scaled data using the R Software (http://www.r-project.org/) and the package FactomineR (Lê et al. 2008).



**Figure S5.** Particle size slightly affected the starch concentration of stem and root tissues of *Pinus banksiana* samples (Method S4). Different letters within the same tissue type indicate significant difference at a=0.05 using the Student t-test.

### **References:**

Ashwell G (1957) Colorimetric analysis of sugars. Methods in Enzymology 3:73-105.

Blake MR (1999) Carbohydrate Partitioning and Developmental Physiology of Nerine Bowdenii Will. Watson. University of Tasmania.

Blunden CA, Wilson MF (1985) A specific method for the determination of soluble sugars in plant extracts using enzymatic analysis and its application to the sugar content of developing pear fruit buds. Anal Biochem 151:403-408.

Buysse J, Merckx R (1993) An improved colorimetric method to quantify sugar content of plant tissue. J Exp Bot 44:1627-1629.

Chow P, Landhäusser S (2004) A method for routine measurements of total sugar and starch content in woody plant tissues. Tree Physiology 24:1129-1136.

- Cobb NS, Mopper S, Gehring CA, Caouette M, Christensen KM, Whitham TG (1997) Increased moth herbivory associated with environmental stress of pinyon pine at local and regional levels. Oecologia 109:389-397.
- Coleman HD, Yan J, Mansfield SD (2009) Sucrose synthase affects carbon partitioning to increase cellulose production and altered cell wall ultrastructure. Proceedings of the National Academy of Sciences 106:13118-13123.
- Deinum B, Maassen A (1994) Effects of drying temperature on chemical composition and in vitro digestibility of forages. Anim Feed Sci Tech 46:75-86.

Dickson RE, Larson PR (1975) Incorporation of 14C-photosynthate into major chemical fractions of source and sink leaves of cottonwood. Plant Physiology 56:185-193.

- Dubois M, Gilles KA, Hamilton JK, Rebers Pt, Smith F (1956) Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28:350-356.
- Ebell L (1969) Variation in total soluble sugars of conifer tissues with method of analysis. Phytochemistry 8:227-233.
- Gomez L, Rubio E, Lescourret F (2003a) Critical study of a procedure for the assay of starch in ligneous plants. J Sci Food Agri 83:1114-1123.

Gomez L, Jordan MO, Adamowicz S, Leiser H, Pagès L (2003b) Du prélèvement au dosage: réflexions sur les problèmes posés par la mesure des glucides non structuraux chez les végétaux ligneux. Cahiers Agricultures 12:369-386.

Göttlicher S, Knohl A, Wanek W, Buchmann N, Richter A (2006) Short-term changes in carbon isotope composition of soluble carbohydrates and starch: from canopy leaves

to the root system. Rapid Communications in Mass Spectrometry 20:653-660. Greub L, Wedin W (1969) Effects of fineness of grind and periodic agitation on total available carbohydrate values obtained by enzyme saccharification. Crop Sci 9:691-692.

Haissing BE, Dickson RE (1979) Starch measurement in plant tissue using enzymatic hydrolysis. Physiol Plant 47:151-157.

Handreck K (1972) Sulphur status of plants by sulphate-sulphur: Sample handling as a source of misinterpretation. Plant Soil 37:203-207.

Hansen J, Møller I (1975) Percolation of starch and soluble carbohydrates from plant tissue for quantitative determination with anthrone. Analytical Biochemistry 68:87-94.

Hartmann H, Ziegler W, Trumbore S (2013) Lethal drought leads to reduction in nonstructural carbohydrates in Norway spruce tree roots but not in the canopy. Funct Ecol 27:413-427.

Hendriks JH, Kolbe A, Gibon Y, Stitt M, Geigenberger P (2003) ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of *Arabidopsis* and other plant species. Plant Physiol 133:838-849.

- Hendrix DL (1993) Rapid extraction and analysis of nonstructural carbohydrates in plant tissues. Crop Sci 33:1306-1311.
- Hendrix DL, Peelen KK (1987) Artifacts in the analysis of plant tissues for soluble carbohydrates. Crop Sci 27:710-715.
- Hoch G, Popp M, Körner C (2002) Altitudinal increase of mobile carbon pools in *Pinus cembra* suggests sink limitation of growth at the Swiss treeline. Oikos 98:361-374.
- Jelitto T, Sonnewald U, Willmitzer L, Hajirezeai M, Stitt M (1992) Inorganic pyrophosphate content and metabolites in potato and tobacco plants expressing *E. coli* pyrophosphatase in their cytosol. Planta 188:238-244.
- Kabeya D, Sakai S (2003) The role of roots and cotyledons as storage organs in early stages of establishment in *Quercus crispula*: a quantitative analysis of the nonstructural carbohydrate in cotyledons and roots. Ann Bot 92:537-545.
- Komatsu M, Tobita H, Watanabe M, Yazaki K, Koike T, Kitao M (2013) Photosynthetic downregulation in leaves of the Japanese white birch grown under elevated CO<sub>2</sub> concentration does not change their temperature-dependent susceptibility to
  - photoinhibition. Physiol Plant 147:159-168.

Lambrechts H, Rook F, Kolloffel C (1994) Carbohydrate status of tulip bulbs during coldinduced flower stalk elongation and flowering. Plant Physiol 104:515-520.

- Lê S, Josse J, Husson F (2008) FactoMineR: an R package for multivariate analysis. Journal of Statistical Software 25:1-18.
- Loescher WH (1987) Physiology and metabolism of sugar alcohols in higher plants. Physiol Plant 70:553-557.
- Lucas WJ, Olesinski A, Hull RJ, Haudenshield JS, Deom CM, Beachy RN, Wolf S (1993) Influence of the tobacco mosaic virus 30-kDa movement protein on carbon metabolism and photosynthate partitioning in transgenic tobacco plants. Planta 190:88-96.
- Macauley BJ, Fox LR (1980) Variation in total phenols and condensed tannins in *Eucalyptus*: leaf phenology and insect grazing. Aust J Ecol 5:31-35.
- MacRae JC, Smith D, McCready RM (1974) Starch estimation in leaf tissue—a comparison of results using six methods. Journal of the Science of Food and Agriculture 25:1465-1469.
- Marquis R, Newell E, Villegas A (1997) Non-structural carbohydrate accumulation and use

in an understorey rain-forest shrub and relevance for the impact of leaf herbivory.

- Funct Ecol 11:636-643.
- Marshall JD (1984) Physiological control of fine root turnover in Douglas-fir. Oregon State University, p 98.
- McCleary BV, Gibson TS, Mugford DC (1997) Measurement of total starch in cereal products by amyloglucosidase-α-amylase method: Collaborative study. Journal of AOAC International 80:571-579.
- McCready R, Guggolz J, Silviera V, Owens H (1950) Determination of starch and amylose in vegetables. Anal Chem 22:1156-1158.
- Megazyme (2011) Total starch assay procedure. Bray Business Park, Bray, Co. Wicklow, Ireland.
- Mialet-Serra I, Clement A, Sonderegger N, Roupsard O, Jourdan C, Labouisse J-P, Dingkuhn M (2005) Assimilate storage in vegetative organs of coconut (*Cocos nucifera*). Experimental Agriculture 41:161-174.

- Moing A, Carbonne F, Rashad MH, Gaudillère J-P (1992) Carbon fluxes in mature peach leaves. Plant Physiol 100:1878-1884.
- Moing A, Maucourt M, Renaud C, Gaudillère M, Brouquisse R, Lebouteiller B, Gousset-Dupont A, Vidal J, Granot D, Denoyes-Rothan B (2004) Quantitative metabolic profiling by 1-dimensional 1H-NMR analyses: application to plant genetics and functional genomics. Funct Plant Biol 31:889-902.
- Palacio S, Maestro M, Montserrat-Martí G (2007) Seasonal dynamics of non-structural carbohydrates in two species of Mediterranean sub-shrubs with different leaf phenology. Env Exp Bot 59:34-42.
- Pelletier S, Tremblay GF, Bertrand A, Bélanger G, Castonguay Y, Michaud R (2010) Drying procedures affect non-structural carbohydrates and other nutritive value attributes in forage samples. Anim Feed Sci Tech 157:139-150.
- Popp M, Lied W, Meyer AJ, Richter A, Schiller P, Schwitte H (1996) Sample preservation for determination of organic compounds: microwave versus freeze-drying. J Exp Bot 47:1469-1473.
- Raessler M, Wissuwa B, Breul A, Unger W, Grimm T (2010) Chromatographic analysis of major non-structural carbohydrates in several wood species—an analytical approach for higher accuracy of data. Analytical Methods 2:532-538.
- Rapley LP, Allen GR, Potts BM, Davies NW (2008) Constitutive or induced defences how does *Eucalyptus globulus* defend itself from larval feeding? Chemoecology 17:235-243.
- Richter A, Wanek W, Werner RA, Ghashghaie J, Jäggi M, Gessler A, Brugnoli E, Hettmann E, Göttlicher SG, Salmon Y (2009) Preparation of starch and soluble sugars of plant material for the analysis of carbon isotope composition: a comparison of methods. Rapid Communications in Mass Spectrometry 23:2476-2488.
- Rose R, Rose CL, Omi SK, Forry KR, Durall DM, Bigg WL (1991) Starch determination by perchloric acid vs enzymes: evaluating the accuracy and precision of six colorimetric methods. Journal of Agricultural and Food Chemistry 39:2-11.
- Schaffer AA, Goldschmidt EE, Goren R, Galili D (1985) Fruit set and carbohydrate status in alternate and non-alternate bearing *Citrus* cultivars. Journal of American Society for Horticultural Science 110:574-578.
- Sigma® FA-20 Fructose Assay kit. Sain Louis, Missouri 63103, USA.
- Silva CMSd, Habermann G, Marchi MyRR, Zocolo GJ (2012) The role of matrix effects on the quantification of abscisic acid and its metabolites in the leaves of *Bauhinia variegata* L. using liquid chromatography combined with tandem mass spectrometry. Brazil J Plant Physiol 24:223-232.
- Smith D (1973) Influence of drying and storage conditions on nonstructural carbohydrate analysis of herbage tissue—a review. Grass and Forage Science 28:129-134.
- Steyn W (1959) Plant tissue analysis, errors involved in the preparative phase of leaf analysis. J Agric Food Chem 7:344-348.
- Thivend C, C. M, Guilbot A (1965) Dosage de l'amidon dans les milieux complexes. Annales de Biologie Animale, Biochimie et Biophysique:513-526.
- Thompson M, Ellison SLR (2005) A review of interference effects and their correction in chemical analysis with special reference to uncertainty. Accreditation and Quality Assurance 10:82-97.
- Udén P (2010) The influence of sample preparation of forage crops and silages on recovery of soluble and non-structural carbohydrates and their predictions by Fourier transform mid-IR transmission spectroscopy. Anim Feed Sci Tech 160:49-61.

- Van Meeteren U, Van Gelder H, Van De Peppel A (1995) Aspects of carbohydrate balance during floret opening in Freesia. *In* VI International Symposium on Postharvest Physiology of Ornamental Plants 405, pp 117-122.
- Wanek W, Heintel S, Richter A (2001) Preparation of starch and other carbon fractions from higher plant leaves for stable carbon isotope analysis. Rapid Communications in Mass Spectrometry 15:1136-1140.

Westerman R (1990) Sampling, handling, and analyzing plant tissue samples

- Wong S (1979) Elevated atmospheric partial pressure of CO<sub>2</sub> and plant growth. Oecologia 44:68-74.
- Yemm E, Willis A (1954) The estimation of carbohydrates in plant extracts by anthrone. Biochemical Journal 57:508.