OXFORD UNIVERSITY PRESS | Tree Physiology

Non-structural carbohydrates in woody plants compared among laboratories

Journal:	*Tree Physiology*
Manuscript ID:	TP-2014-394.R1
Manuscript Type:	Research Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Quentin, Audrey; CSIRO, Ecosystem Sciences Pinkard, Elizabeth; CSIRO, Ecosystem Sciences Ryan, Michael; Colorado State University, Natural Resource Ecology Laboratory; USDA Forest Service, Rocky Mountain Research Station Tissue, David; University of Western Sydney, Hawkesbury Institute for the Environment
Keywords:	non structural c compounds, methods comparison, Starch, Soluble sugar, extraction and quantification consistency, Reference Method

Non-structural carbohydrates in woody plants compared among laboratories

Audrey G. Quentin[1,2,*], Elizabeth A. Pinkard[1], Michael G. Ryan[3,4,5], David T. Tissue[2], L. Scott Baggett[5], Henry D. Adams[6], Pascale Maillard[7], Jacqueline Marchand[8], Simon M. Landhäusser[9], André Lacointe[10,11], Yves Gibon[12,13], William R.L. Anderegg[14], Shinichi Asao[3,4], Owen K. Atkin[15, 16], Marc Bonhomme[10,11], Caroline Claye[17], Pak S. Chow[9], Anne Clément-Vidal[18], Noel W. Davies[19], L. Turin Dickman[6], Rita Dumbur[20], David S. Ellsworth[2], Kristen Falk[21], Lucía Galiano[22,23], José M. Grünzweig[20], Henrik Hartmann[24], Günter Hoch[25], Sharon Hood[26], Joanna E. Jones[17], Takayoshi Koike[27], Iris Kuhlmann[24], Francisco Lloret[28,29], Melchor Maestro[30], Shawn D. Mansfield[31], Jordi Martínez-Vilalta[28,29], Mickael Maucourt[13,32], Nathan G. McDowell[6], Annick Moing[12,13], Bertrand Muller[33], Sergio G. Nebauer[34], Ülo Niinemets[35], Sara Palacio[30], Frida Piper[36], Eran Raveh[37], Andreas Richter[38], Gaëlle Rolland[33], Teresa Rosas[28], Brigitte Saint Joanis[10,11], Anna Sala[26], Renee A. Smith[2], Frank Sterck[39], Joseph R. Stinziano[40], Mari Tobias[35], Faride Unda[31], Makoto Watanabe[41], Danielle A. Way[40,42], Lasantha K. Weerasinghe[15, 43], Birgit Wild[38,45], Erin Wiley[9], David R. Woodruff[44]

[1] CSIRO Land and Water, Private Bag 12, Hobart, Tasmania 7001, Australia; [2] Hawkesbury Institute for the Environment, University of Western Sydney, Richmond, NSW 2753, Australia; [3] Natural Resources Ecology Laboratory, Colorado State University, Fort Collins, CO 80523-1499, USA; [4] Graduate Degree Program in Ecology, Colorado State University, Fort Collins, CO 80523-1401, USA; [5] USDA Forest Service, Rocky Mountain Research Station, Fort Collins, CO 80521, USA; [6] Earth and Environmental Sciences Division, Los Alamos National Laboratory, Los Alamos, NM, 87545, USA; [7] INRA, UMR 1137, Ecologie et Ecophysiologie Forestières, Centre de Nancy, F-54280 Champenoux, France; [8] INRA, UMR 1137, Ecologie et Ecophysiologie Forestières, Plateforme Technique d'Ecologie Fonctionnelle (OC 081) Centre de Nancy, F-54280 Champenoux, France; [9] Department of Renewable Resources, University of Alberta, Edmonton, AB, T6G 2E3, Canada, [10] INRA, UMR547 PIAF, F-63100 Clermont-Ferrand, France; [11] Clermont Université, Université Blaise Pascal, UMR547 PIAF, F-63100 Clermont-Ferrand, France; [12] INRA UMR1332, Biologie du Fruit et Pathologie, 71 avenue Edouard Bourlaux, F-33140 Villenave d'Ornon, France; [13] Plateforme Métabolome du Centre de Génomique Fonctionnelle Bordeaux, MetaboHUB, IBVM, Centre INRA, 71 avenue Edouard Bourlaux, F-33140 Villenave d'Ornon, France; [14] Princeton Environmental Institute, Princeton University, Princeton NJ 08540, USA; [15] Division of Plant Sciences, Research School of Biology, Building 46, The Australian National University, Canberra, ACT, 0200, Australia; [16] ARC Centre of Excellence in Plant Energy Biology, The Australian National University, Canberra, ACT, 0200, Australia; [17] Tasmanian Institute of Agriculture, School of Land and Food, Private Bag 98, University of Tasmania, Hobart, Tasmania 7001, Australia; [18] CIRAD, UMR AGAP, F-34398 Montpellier, France; [19] Central Science Laboratory, Private Bag 74, University of Tasmania, Hobart, Tasmania 7001, Australia; [20] Robert H. Smith Faculty of Agriculture, Food and Environment, the Hebrew University of Jerusalem, P.O. Box 12, Rehovot 7610001, Israel; [21] Department of Forest Ecosystems and Society, Oregon State University, Corvallis, OR 97331, USA; [22] Swiss Federal Research Institute WSL, CH-8903 Birmensdorf,

Switzerland; [23] Institute of Hydrology, Freiburg University, Fahnenbergplatz, D-79098 Freiburg, Germany; [24] Max Planck Institute for Biogeochemistry, Hans-Knöll Str. 10, 07745 Jena, Germany; [25] Department of Environmental Sciences - Botany, University of Basel, Schönbeinstrasse 6, CH-4056 Basel, Switzerland; [26] Division of Biological Sciences, University of Montana, Missoula MT-59812, USA; [27] Silviculture and Forest Ecological Studies, Hokkaido University Sapporo, Hokkaido 060-8589, Japan; [28] CREAF, Cerdanyola del Vallès E-08193 Barcelona, Spain; [29] Universidad Autònoma Barcelona, Cerdanyola del Vallès E-08193 Barcelona, Spain; [30] Instituto Pirenaico de Ecología (IPE-CSIC) Av. Nuestra Señora de la Victoria s/n, 22700 Jaca, Huesca, Spain; [31] Department of Wood Science, University of British Columbia, V6T 1Z4 Vancouver, Canada; [32] Université Bordeaux, UMR 1332, Biologie du Fruit et Pathologie, 71 avenue Edouard Bourlaux, F-33140 Villenave d'Ornon, France; [33] CIRAD, Agropolis-TA-A-108/1, Montpellier Cedex 5, 34398 France; [34] Plant Production Department, Universitat Politécnica de Valéncia, Camino de vera s.n. 46022-Valencia, Spain; [35] Department of Plant Physiology, Estonian University of Life Sciences, Kreutzwaldi 1, 51014 Tartu, Estonia; [36] Centro de Investigación en Ecosistemas de la Patagonia (CIEP) Conicyt–Regional R10C1003,Universidad Austral de Chile, Bilbao 323 of. 216, Coyhaique, Chile; [37] Department of Fruit Trees Sciences, Institute of Plant Sciences, A.R.O., Gilat Research Center, D.N. Negev 85289, Israel; [38] Department of Microbiology and Ecosystem Science, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria; [39] Forest Ecology and Forest Management Group, Wageningen University, Postbox 47, 6700 AA, Wageningen, the Netherlands; [40] Department of Biology, Western University, 1151 Richmond Street, London, ON, Canada; [41] Institute of Agriculture, Tokyo University of Agriculture and Technology Fuchu, Tokyo 183-8509, Japan; [42] Nicholas School of the Environment, Duke University. Box 90328, Duke University, Durham, NC, USA; [43] Faculty of Agriculture, University of Peradeniya, Peradeniya, 20400, Sri Lanka; [44] USDA Forest Service, Forestry Sciences Laboratory, Corvallis, OR 97331, USA [45] Department of Earth Sciences, University of Gothenburg, Guldhedsgatan 5A, 40530 Gothenburg, Sweden.

*Author for correspondence:
Audrey Quentin
Tel: +61 4 48498572
Email: Audrey.Quentin@csiro.au

Summary

Non-structural carbohydrates (NSC) in plant tissue are frequently quantified to make inferences about plant responses to environmental conditions. Laboratories publishing estimates of NSC of woody plants use many different methods to evaluate NSC. We asked if NSC estimates in the recent literature could be quantitatively compared among studies. We also asked if any differences among laboratories were related to the extraction and quantification methods used to determine starch and sugar concentration. These questions were addressed by sending sub-samples collected from five woody plant tissues, which varied in NSC content and chemical composition, to 29 laboratories. Each laboratory analyzed the samples with their laboratory-specific protocols, based on recent publications, to determine concentrations of soluble sugars, starch and their sum, total NSC.

Laboratory estimates differed substantially for all samples. For example, estimates for *Eucalyptus globulus* leaves varied from 23-116 (mean = 56) mg g^{-1} for soluble sugars, 6-533 (mean = 94) mg g^{-1} for starch and 53-649 (mean = 153) mg g^{-1} for total NSC. Mixed model analysis of variance showed that much of the variability among laboratories was unrelated to the categories we used for extraction and quantification methods (method category R^2 = 0.05-0.12 for soluble sugars, 0.10-0.33 for starch, and 0.01-0.09 for total NSC). For *Eucalyptus globulus* leaves, the difference between the highest and lowest least-squares means for categories in the mixed model analysis was 33 mg g^{-1} for total NSC, compared to the range of laboratory estimates of 596 mg g^{-1}. Laboratories were reasonably consistent in their ranks of estimates among tissues for starch (r= 0.41-0.91), but less so for total NSC (r= 0.45-0.84), and soluble sugars (r= 0.11-0.83). Our results show that NSC estimates for woody plant tissues cannot be compared among laboratories. The relative changes in NSC between treatments measured

within a laboratory may be comparable within and between laboratories, especially for starch. To obtain comparable NSC estimates, we suggest that users either adopt the Reference Method given in this publication, *or* report estimates for a portion of samples using the Reference Method, *and* report estimates for a Standard Reference Material. Researchers interested in NSC estimates should work to identify and adopt standard methods.

Keywords: non-structural carbohydrate chemical analysis, extraction and quantification consistency, particle size, soluble sugars, starch, standardisation, Reference Method.

Running head: Comparing NSC content among laboratories.

Introduction

Non-structural carbohydrates (NSC) are products of photosynthesis, provide substrates for growth and metabolism and can be stored by the plant. Consequently, NSC play a central role in plant response to the environment (Chapin et al. 1990, Kozlowski 1992). Major theories of plant defense and growth such as the "growth-differentiation balance hypothesis" (Loomis 1932), the "carbon/nutrient hypothesis" (Bryant et al. 1983), revisions to the "hydraulic limitation hypothesis" (Ryan et al. 2006), and the "carbon limitation hypothesis" (Körner 2003) all outline a role for NSC, but that role has yet to be firmly established or rejected. In more recent years, NSC of woody plants has received wider attention for understanding drought-induced mortality (Grunzweig et al. 2008, McDowell et al. 2008, Galiano et al. 2011, Muller et al. 2011, Piper 2011, Adams et al. 2013, Duan et al. 2013, Hartmann et al. 2013, Mitchell et al. 2013, Dickmann et al. 2014, Mitchell et al. 2014, O'Brien et al. 2014, Sevanto et al. 2014), altitudinal boundaries for forests (Hoch et al. 2002, Hoch and Körner 2003, Handa et al. 2005, Li et al. 2008, Fajardo et al. 2011, 2012, 2013, Fajardo and Piper 2014), growth limitation (Sala et al. 2010, Piper and Fajardo 2011, Sala et al. 2012, Palacio et al. 2014), and plant survival under poor-resource conditions (Kobe 1997, Strauss and Agrawal 1999, Haukioja and Koricheva 2000, Lusk and Piper 2007, Quentin et al. 2011, Piper and Fajardo 2014).

Several major questions about the role and regulation of stored carbohydrates in woody plants remain unanswered, such as their role in indicating plant carbon balance, helping plants cope with stress, and if control of storage and use is active, passive or more complex (Chapin et al. 1990, Sala et al. 2011, 2012, Wiley and Helliker 2012). The many uncertainties about how

NSC are involved in the regulation of whole-tree carbon metabolism make predictions of growth and productivity under environmental change difficult (Ryan 2011).

Many carbohydrates can comprise NSC: monosaccharides (glucose and fructose), disaccharides (sucrose), polysaccharides (starch and fructans), oligosaccharides (raffinose), and sugar alcohols (inositol, sorbitol and mannitol) (Rastall 1990, Stick and Williams 2010). Sucrose, fructose and glucose are generally, but not always, the predominant soluble sugars, and starch is the pivotal non-soluble longer term storage compound (Mooney 1972, Chapin et al. 1990); many studies focus on these four carbohydrates when measuring plant NSC. The diversity of carbohydrates and matrices (tissue structural and biochemical characteristics), and the search for reliable and inexpensive methods that can be used for the large number of samples in environmental plant physiology studies, has led to the development of many analytical methods to determine the identity and amount of carbohydrates in plant tissue (Tables 1, S1; Gomez et al. 2003). Within any given plant species, a wide range of NSC values have been reported in different studies (Table 2). Potential explanations for these differences include plant age and growing conditions, but the extraction and quantification methods may also have a major impact on the results (Rose et al. 1991, Chow and Landhäusser 2004). For 8 to 12 month-old *Eucalyptus globulus* saplings, leaf total NSC concentration varied between 28 and 224 mg g^{-1} when measured using three different soluble sugar and starch extraction methods, and three different quantification methods (Table 2). Studies have also used the same extraction and assay methods to analyse different tissues (leaves, stems, roots) that consist of different matrices (Table 2), despite evidence that different matrices can have a profound impact on the analytical results (Smeraglia et al. 2002, Matuszewski et al. 2003, Thompson and Ellison 2005, Santiago da Silva et al. 2012). For example, the phenolics and tannins in many conifer

needles can interfere with enzymatic/colorimetric techniques (Ashwell 1957), but not all plant tissues contain these chemicals. Given such variability in NSC estimates, we believe that there is an urgent need to compare estimates of NSC of standard samples for different laboratories around the world, with the laboratories using the same methods as in their recent publications.

Several other factors suggest that a comparison of the NSC of standard samples would be worthwhile. First, such a comparison would allow plant ecophysiologists studying NSC role and regulation to assess and compare their own results. Second, the composition of NSC can vary widely among species, tissues, and seasons (Hoch et al. 2003, Landhäusser and Lieffers 2003, El Zein et al. 2011, Richardson et al. 2013, Dickmann et al. 2014), and this diversity further contributes to potential misinterpretation when comparing results from studies that use different methods. Finally, knowledge of the comparability of quantitative estimates of NSC would benefit papers that review NSC among studies to formulate hypotheses about the regulation of plant carbon regulation and growth mechanisms (Körner 2003, Ainsworth and Rogers 2007, McDowell et al. 2008). To our knowledge, no study has addressed the comparability of NSC among different laboratories.

Our primary objective was to assess if soluble sugar, starch and total NSC concentrations could be compared across the laboratories that use NSC estimates to understand plant response to a variety of biotic and abiotic factors. Many of these studies focused on NSC estimates in woody species, so our common samples were from trees. We answered the question of inter-laboratory comparability in NSC quantification by sending sub-samples of five different tissue samples (leaf, root and stem) that we hypothesised varied widely in NSC, matrix structure and chemistry,

to 29 laboratories. The laboratories evaluated the samples using their own 'in-house' protocols of NSC extraction and quantification (Tables S1 and S2).

Our second objective was to determine if estimates from an individual laboratory were consistent across the five standard samples. If a laboratory's estimates were high, low or similar relative to all laboratories for a given sample, would the same rank apply for the other four standard samples? Consistency among samples would indicate the reliability of comparing relative change within and among laboratories.

The third objective was to determine if any differences among laboratory estimates were related to the methods of extraction and/or quantification of soluble sugars and starch, and if variability among laboratories differed by sample. Because our first objective was the primary purpose for the study, our ability to test the third objective suffered by having to group extraction and quantification methods into broad categories. This grouping and our sample of laboratories precluded testing factors that may be important sources of variability because of lack of replication. These factors include the number, temperature and duration of extractions, and the gelatinization of starch. We partially addressed this issue by investigating the effect of different extraction methods on sugar estimates in a single laboratory using a common quantification method.

Material & Methods

Non-structural carbohydrate analyses of standard samples in different laboratories

We selected five samples for our standards: leaves (EGL), roots (EGR) and stem (EGS) of *Eucalyptus globulus*, *Pinus edulis* needles (PEN) and *Prunus persica* leaves (PPL). We selected

these samples because *a priori* knowledge suggested they differed in the concentration of soluble sugars and starch, and had very different structural or chemical matrices that would challenge NSC extraction. Each substrate was homogenised, irradiated at 27.8 kGy for microbiological control to meet international quarantine requirements, and homogenised. Supporting Information Method S1 describes the collection and handling of samples used.

Sub-samples of the same five dried and ground samples were sent to 29 laboratories around the world (Austria, Australia, Canada, Chile, Estonia, France, Germany, Japan, Israel, Netherlands, Spain, Switzerland and USA), where each laboratory used their own protocol to analyse the samples in triplicate (see Supporting Information Method S2, Tables S1 & S2). One laboratory (Q), only provided sugar estimates, and two laboratories (L1, L2; Z1, Z2) provided sugar estimates from two different methods. The number of estimates for starch was 28, and the number of estimates for total soluble sugars and total NSC was 30. Table 1 summarises the procedures used in this study to measure soluble sugars and starch in plant tissues and Tables S1 & S2 provide more detailed methods. All data were reported as mg g^{-1} of dry mass.

Different methods for soluble sugar extraction within a single laboratory

We selected four methods of soluble sugar extraction: 80% ethanol (80%EtOH), 70% methanol (70%MeOH), methanol-chloroform-water (MCW) at 80ºC (MCW80) and MCW at ambient laboratory temperature (MCWamb). Individual soluble sugars (glucose, fructose, sucrose) were extracted from 20 mg of dried plant tissue for each of the five samples for each of the four methods. Alcohol methods (EtOH) were derived from Gomez et al. (2002), and ternary solvent methods (MCW) from Dickson and Larson (1975). All four methods were conducted within the same laboratory (see Supporting Information Method S3).

Other Methods

We also performed an analysis of the effect of microwaving duration to halt enzymatic activity (Supporting Information Method S4), and the effect of particle size (Supporting Information Method S5) in single laboratories.

Statistical analyses

For objective one, we used a general linear mixed model analysis to determine differences in estimates among laboratories with laboratory and sample types as fixed effects and the extraction and quantification categories (below) as random effects. For objective two, we used Spearman rank correlations for laboratory ranks among all sample pairs to evaluate the consistency of laboratory estimates for samples with different chemical constituents. Correlations were estimated for total soluble sugars, starch and total NSC.

For objective three, we used a different general linear mixed model analysis, with extraction and quantification groups and sample as fixed effects, and laboratory as a random effect. We could not perform one overall test with laboratories and methods, because methods were confounded with laboratory. We grouped methods by the type of solvent for the extraction methods (EtOH, EtOH+W, MCW, W for the soluble sugars; and Acid, AA+amylo., Amylo. for starch) and by the type of quantitative assay for the quantification methods (HPLC, Enz., Spec. 490, Spec. 620 and Spec. 510). HPAEC-PAD and ^{1}H-NMR were grouped with HPLC. Both sugar and starch concentrations were log-normally distributed and all components were transformed for analysis. Least squares means were back-transformed to original units after estimation of the model parameters. Other differences in laboratory protocols (differences among the number, temperature and duration of extractions or methods used for the

gelatinisation of starch) were not considered as factors within the method because of the lack of replication. General linear mixed model analyses were done using SAS PROC GLIMMIX (SAS, 2012). The proportion of the variance explained by the method categories compared with sample and laboratory was evaluated using the method of computing R^2 for generalized linear mixed models described in Nakagawa and Schielzeth (2013). We assessed how differences among method categories compared with differences among samples and laboratories by comparing the R^2 for models with only the method category as a fixed factor with (1) R^2 for models with only sample category as a fixed factor, and (2) with the R^2 for the full model with sample and method as fixed factors and laboratory as a random factor. R^2 measures were computed using the 'R' statistical package version 3.1.2 (R Development Core Team 2014) and the *MuMIn* library.

We examined the differences between soluble sugar extraction methods on total NSC in the same laboratory with an ANOVA for each sample type (α = 0.05). For all tests and all experiments, we set α at 0.05. Participants were assured of anonymity in the experiment, and the results were coded by letters.

Results

Objective 1: Estimates for soluble sugars, starch and total NSC for the same samples varied substantially among laboratories

Estimates for individual sugars, total soluble sugars, starch and total NSC differed among laboratories ($P < 0.001$, Fig. 1), with a large range for all components. For example, in *Eucalyptus globulus* leaves (EGL), laboratory estimates ranged from 23-116 mg g^{-1} (CV 35%) for total soluble sugars, 6-533 mg g^{-1} (CV 102%) for starch, and 53-649 mg g^{-1} (CV 69%) for

total NSC (Figs. 1A, 1B). Laboratory estimates for *Prunus* leaves (PPL, average CV=87% for sugars, starch and total NSC) were more variable than those for other samples (average CV=54-69% for all NSC components). Starch estimates were more variable among laboratories (CV 87-120%) than were soluble sugars and total NSC (CV 24-71% for sugars and 44-71% for total NSC, Figs. 1A, 1B). For all samples and NSC components, 10-57% of the laboratories were within the 95% confidence intervals estimated for the means. Laboratories were most consistent for starch estimated for the *Eucalyptus* leaf, stem, and root samples (EGL, EGS, EGR, 16 of 28 laboratories were within the 95% confidence intervals), and least consistent for sugar estimates for *Eucalyptus* leaves (4 of 30 laboratories) and total NSC estimated for *Pinus* leaves (8 of 30 laboratories) and *Prunus* leaves (3 of 30 laboratories). The subset of the laboratories that identified sucrose and glucose+fructose (n=20) were relatively consistent, having an average of 51% or 10 of 20 laboratory estimates within the 95% confidence intervals (range = 7-14 laboratories, Fig. 1A). The interaction between laboratory and sample type was highly significant for sugars, starch and total NSC ($P < 0.001$), indicating that differences among laboratories differed with sample type.

The range of estimates varied substantially with method and sample types (Figs. 1 & S1). For example, NSC in the PPL sample showed high variability among laboratories (Figs. 1A, 1B, S1A), and estimates for soluble sugars varied largely within each method of extraction and quantification, except for the water extraction (W) (Fig. S1A). In comparison, NSC in the EGS sample had the lowest variability among laboratories (Fig. 1B) and estimates varied less within each method (Fig. S1B).

Objective 2: Laboratories had similar rankings for all five common samples

Laboratory rankings were consistent for most sample pairs (Table 3; Fig. 2), with higher rank correlations for starch (0.41-0.91, mean = 0.71) and total NSC (0.45-0.84, mean = 0.60) than for soluble sugars (0.11-0.83, mean = 0.44). This consistency shows that laboratories with estimates below, above or near the mean for one sample tend to have a similar ranking for that carbohydrate relative to other laboratories for other samples.

Objective 3: Extraction and quantification methods affect NSC estimates, but the effect is lower than variability among laboratories

We investigated if the methods used to extract or quantify NSC could explain the variability in NSC results among laboratories (Table 4; Fig. 3). When analyses were pooled across laboratories and samples, NSC estimates did not differ by sugar or starch extraction or quantification methods (Table 4, P=0.07-0.84, Figs. 3C, 3E, 3G, 3I: LSM). Across laboratories and samples, starch estimates were lower for ethanol+water sugar extraction than for the other three sugar extraction categories (Fig. 3B: LSM, $P < 0.05$), but did not differ by starch extraction or quantification categories (Figs. 3D, 3H: LSM). Across laboratories and samples, sugar estimates did not vary by extraction method category (Fig. 3A: LSM), but did by sugar quantification method category (Fig. 3F: LSM, $P < 0.05$), with the Spec 620 colorimetric method producing higher estimates than the HPLC, enzymatic or Spec 490 method. A PCA analysis showed that within a method, the estimates for soluble sugars were more variable than were estimates for starch (Figs. S2, S3).

An analysis of R^2 for model components showed that the differences in method category in our analysis accounted only for a small portion of differences in NSC among laboratories. R^2 for total soluble sugars with sugar extraction method category was 0.05 and 0.12 for sugar

detection method category, compared with 0.30 for sample and 0.66-0.69 for the full model. R^2 for starch with starch extraction method category was 0.10 and 0.11 for starch detection method category, compared with 0.23 for sample and 0.88 or 0.92 for the full model; sugar extraction method category had an R^2 of 0.33. R^2 for total NSC with sugar extraction method category was 0.09, 0.04 for sugar detection method category, 0.01 for starch extraction method category, and 0.09 for starch detection category compared with 0.37 for sample, and 0.79-0.84 for the full model. Additionally, differences between the highest and lowest least squares means for the overall effect of methods categories was small compared to the differences among laboratories (Compare Fig. 3 with Fig. 1).

Objective 3: Method effects differ by sample

Sample and method had significant interactions (Table 4, $P < 0.0001$), with the foliar samples (EGL, PEN and PPL) showing more variation among method categories than the wood samples (EGR, EGS). For example, the sugar extractions with water (W and EtOH+W) yielded lower soluble sugar and total NSC estimates for the foliar samples (EGL, PEN and PPL), while having less effect on woody samples (EGR and EGS, Figs. 3A and 3C). Starch concentration differences among extraction and quantification methods in woody samples were similar to that for foliar samples (Figs. 3B, 3D, 3H). Colorimetric quantification (Spec 490 and Spec 620) of starch and soluble sugars almost always produced higher estimates for soluble sugars, starch and total NSC than did the HPLC and or enzymatic methods (Figs. 3F, 3G, 3H, 3I).

Objective 3: Single laboratory tests of soluble sugar extraction methods, microwaving, and particle size.

Soluble sugar extraction methods influenced sugar estimates when samples were quantified in the same laboratory using the same method. Estimates of total soluble sugars were affected by extraction methods for all samples ($P < 0.05$) except EGL ($P > 0.10$). Differences among sugar extraction methods tested in the same laboratory (Fig. 4) were relatively minor compared to differences among laboratories (Fig. 1A), with the largest differences occurring for the MCW extractions at different temperatures (Fig. 4).

Microwaving small samples (< 5 g) of *Pinus edulus* at 800W required 180 s to deactivate enzymes. No microwaving or 90 s of microwaving were not effective at halting the conversion of sucrose and starch to glucose+fructose. At 300 s, starch and NSC increased, suggesting conversion of non-NSC compounds to NSC (Method S4, Fig. 5). Grinding *Pinus banksiana* tissues to a smaller particle size (< 105 μm) yielded higher starch and total NSC estimates for root tissues (but not needles or stem) compared with extractions of larger particle size (< 400 μm, Method S5, Fig. S4).

Discussion

Absolute estimates of NSC are not comparable among laboratories (Objective 1)

Results demonstrate that estimates of soluble sugar, starch and total NSC provided by different laboratories in this study cannot be compared, even if they are obtained with the same general methods. Laboratories differed substantially in estimates for sugars, starch and total NSC, and the variability across laboratories and even within a method category was unexpectedly large. Therefore, comparing values for any NSC component across studies in the literature (e.g.,

Ainsworth et al. 2002, Morgan et al. 2003, Wittig et al. 2009) should not be done, both for individual studies and for meta-analyses, unless the study accounts for laboratory effects.

Relative differences within a single laboratory can be consistent and meaningful (Objective 2)

The Spearman rank correlation analysis of sample pairs showed that laboratory ranks were fairly consistent among the five samples for starch, but less so for soluble sugars and total NSC. These results suggest that relative differences among treatments and species within a laboratory can be meaningful. While we did not explicitly test how laboratories would perform using the same substrate with two different NSC concentrations, preserving laboratory rank across such a diverse sample cohort was a significant finding in this experiment. Therefore, an assessment of relative responses of different treatments to a control may be robust, especially for starch, and meaningful within and between studies.

Method differences explained only some of the variability among laboratories, but meeting Objective 1 compromised our ability to identify these differences (Objective 3)

Differences among methods, as captured by our extraction and quantification group approaches, were generally small relative to the differences among laboratories. However, fulfilling our primary objective (to identify if NSC estimates could be compared among laboratories) compromised the ability to identify differences between methods. We can interpret these results to mean that (1) real differences among methods would exist, and variation among laboratories would be minimized if the laboratories using the same method followed the same protocols exactly for extraction and quantification; *or* (2) NSC quantification is such a highly variable and sensitive procedure that even minor differences

among laboratories' procedures not captured in an explicit protocol would cause variation among laboratories using the same method. We suspect that both explanations play a role in the low ability of 'methods' to explain laboratory differences.

Variation in protocols within a method category may have contributed to the lack of significant differences among methods. For example, the number, temperature and duration of extractions, and the method of starch gelatinization (Tables 2, S1, S2) are known to affect soluble sugar and starch estimates (Yemm and Willis 1954, MacRae et al. 1974, Rose et al. 1991, Johansen et al. 1996, Shi et al. 2002, Gomez et al. 2003, Kim et al. 2003). We were surprised at the variability among laboratories in these factors, and even laboratories using the same 'method' differed in these important factors. Variability of method application within a method category yielded little or no replication for these factors, and limited the evaluation to broad method categories. As an example of how these factors might contribute to differences among laboratories, yet not appear in our methods analysis, we found that higher temperature increased sugar concentration for MCW extracts in two of the four samples (Fig. 4).

The lack of differences among soluble sugar extraction method categories (P=0.12, Table 4), coupled with the small differences between different methods within a single laboratory (Fig. 4) suggests that variation in the application of extraction methods across laboratories was larger than the effect of the extraction solvent. However, despite laboratory differences in protocol, we could still detect an effect of soluble sugar quantification methods on sugar estimates (Fig. 3, P = 0.004). These differences may result from the fact that different methods quantify different sugars. This result suggests that systematic differences in

quantification, especially between colorimetric and HPLC-based methods, might be interpreted and possibly corrected.

We also did not assess the effect of other factors such as air temperature, level of expertise of the person conducting the analyses, or quality of the lab equipment. Such factors might contribute to the variability among laboratories, even for those using the same general method, but they have not been assessed.

Method effects differ by sample (Objective 3)

NSC components exist within a complex and varied chemical matrix and need to be extracted from this matrix for analysis. Procedures to extract NSC from the matrix can free the target compound, but also convert other compounds into the target. Maximizing the extraction while minimizing the conversion is the goal of procedures, but may not always occur (Hansen and Møller 1975, Thompson and Ellison 2005, Santiago da Silva et al. 2012, Huang and Fu 2013). In our study, soluble sugar estimates for *Eucalyptus* and *Prunus* leaves differ with the sugar quantification method (colorimetric methods generate higher estimates than do HPLC or enzymatic methods, Fig. 3; see Supporting Information Note S1). Clearing interfering compounds from the solvent might minimise these effects (Thompson and Ellison 2005), as would avoiding acid use during sugar extraction (Chow and Landhäusser 2004). The significant interactions between sample type and methods also suggest that different extraction and quantification protocols will give different results for NSC in samples with different matrices.

How can we make quantitative, comparable estimates of the true value of NSC components?

Determination of the role and regulation of NSC is governed by what we can measure (Dietze et al. 2014). Our study demonstrates that laboratories and methods produce widely different and non-comparable estimates and progress in plant science will be limited until this problem is resolved, although relative differences in NSC have been and will continue to be important for many questions. Being able to compare between and within studies and knowing the true value are essential for a mechanistic understanding of NSC pools and fluxes (Ryan 2011), especially for questions about the role of NSC in ecosystem productivity, stress responses, and plant adaptations. Relative differences within and across studies are valuable for testing many hypotheses, and this study shows that these have value, particularly for starch.

Comparability might be solved using two approaches: either adopt a standard method and report values for certified reference material, or embrace a central laboratory for all processing. A standard method would require a detailed and easily applied protocol, from sample collection to quantification, so that any laboratory can reproduce values for the certified reference material. Another solution to the comparability problem would be to establish and adopt a central laboratory for all NSC analyses, similarly to the calibration laboratories of the Global Atmosphere Watch program (http://www.wmo.int/pages/prog/arep/gaw/qassurance.html) or the U.S. National Atmospheric Deposition Program (http://nadp.sws.uiuc.edu). A central laboratory could use different methods for samples of different characteristics and still maintain comparability among samples. Both approaches can be criticized for the lack of flexibility and freedom they impose on the scientific community, and raise the practical issue of what to do with the existing costly analytical equipment. Adopting a standard method for NSC determination in plants would likely be more practical than establishing a central facility, but would impose an

investment for laboratories to comply with the selected standard. Adoption of either approach would depend on the cooperation of the science community.

Our results provide some insights into which methods might give the most homogenous results (*i.e.*, those less affected by random error). HPLC was the quantification method with the least variable results, while colorimetric assays exhibited more variability (Figs. 1A, 1B & S1). HPLC methods (including HPAEC-PAD and ^{1}H-NMR) are increasingly chosen by laboratories because of (1) their high resolution, even with a small amount of sample and (2) reproducibility due to a close control of parameters affecting the efficiency of separation and quantification (Giannoccaro et al. 2008, Raessler et al. 2010). However, the HPLC process is time-consuming, laborious and expensive—especially for carbon balance studies where only the total amount of glucose equivalents may be of interest. In addition, HPLC still relies on sugar and starch extractions that vary substantially with solvent and other method details.

Colorimetric methods are less expensive than other techniques, rapid and can detect all types of sugars, and therefore are still widely used; nevertheless, they have major drawbacks, including: (1) the necessity to prepare a calibration curve using a series of standards because different carbohydrates give different absorbance responses (see Dubois et al. 1956, Hall 2013); (2) the use of toxic and dangerous chemicals; and (3) possible interference of metabolites with the concentrated sulphuric acid (Ashwell 1957).

The enzymatic method also produced relatively consistent results and allowed for the measurement of individual sugars. This method requires expertise for timing of enzyme additions, checking for cross contamination (converting non-targeted oligosaccharides using glucose, fructose and sucrose standards), and maintenance of a precise pH for NADPH. In this

study, three laboratories using the enzymatic method reported negative results for sucrose (Figs. 1A, 1B; Table S1). Negative results are not normally reported and usually assumed to be zero, but indicate that something went wrong in the assay. This might be caused by inappropriate extraction (hydrolyzing sucrose into glucose and fructose) or too low pH (leading to NADPH degradation following the addition of invertase, the enzyme enabling the quantification of sucrose). To solve these issues, cross-validation with HPLC or NMR should be performed each time a new sample type is analyzed.

Best practice in other plant chemical analyses generally use certified reference materials (CRM) to ensure comparability of results (e.g. Quevauviller et al. 1994, Clement et al. 1996, Saunders et al. 2004). Unfortunately, CRM for carbohydrates do not currently exist. Many laboratories use pure sugar and/or starch standards (n = 15 in our study) to define recovery of known concentrations of specific sugars. However, these standards do not account for the effect of plant matrix which may generate incomplete carbohydrate extraction or yield compounds that interfere with quantification (Emons et al. 2004). A CRM is accompanied by a certificate, which specifies property values of the material: Before the certificate is delivered, a procedure establishes material traceability to an accurate realization of the unit, and for which each certified value is accompanied by an uncertainty at a stated level of confidence (Emons et al. 2004). CRM are a key element of analytical data quality assurance and are used for four main purposes: (1) instrument calibration; (2) method validation, in particular for assessment of the reliability of a method; (3) ensuring the traceability of measurement results; and (4) statistical quality control (Emons et al. 2004). Certified reference material for NSC will likely require several samples with different matrices, sugar and starch concentrations. Integration of CRMs into NSC analysis should be standard practice to improve comparability among laboratories.

In addition to the difficulty of quantitatively assessing soluble sugars and starch, studies assessing NSC may miss important components that could represent a substantial fraction of NSC. Most studies assessing NSC have focused on analysing the three "major" sugars (sucrose, glucose, fructose) and starch, and assume that this pool represents the NSC available to the plant—a reasonable assumption for most trees (Hoch et al. 2003, Hoch and Körner 2005). A few studies suggest we should sometimes look deeper. For example, sorbitol is found in high concentrations in *Prunus persica* leaves (Zhang et al. 2013) and quercitol in droughted *Eucalyptus astringens* leaves (Arndt et al. 2008), and raffinose concentration was greater than that of starch in birch buds (Ruuhola et al. 2011).

Conclusions and recommendations for the future

We conclude that absolute values of NSC, total soluble sugars, starch, and individual sugars cannot be directly compared among laboratories, even among laboratories that use a method in the same method category. Differences relative to a control may have value with a single laboratory and for comparisons among laboratories for starch—but less so for total NSC and for soluble sugars. Differences in absolute values among laboratories were poorly related to our broad method categories, but many factors that may contribute to different estimates could not be assessed in our analyses.

Our study shows that developing methods to produce reliable, absolute and comparable estimates of NSC and its components in plant tissue will be a serious challenge because of high variability in methods currently in use, lack of absolute standards, and little information about the causes of the high variation in estimates among laboratories. Our team discussed the benefits

and pitfalls of proposing a standard method for sample collection, storage, processing, extraction, and quantification as a first step towards achieving comparability among laboratory estimates. Team members mostly supported the publication of a standard method (although there was less agreement about the particular method), but there were also strong arguments against such an approach. The small differences among method categories and the high variability of lab processes within the method categories in this study suggest that adopting a standard method would have a higher likelihood of producing comparable estimates across studies. A standard method would at least insure that differences among studies are not because of methodological differences. However, neither this study, nor any other of which we are aware has identified a 'best' method. Arguments against proposing a standard method are (1) that we do not have the data to support selecting any particular method, (2) laboratories that change methods will lose a connection to past studies, (3) laboratories that do not adopt the proposed standard method risk having difficulty publishing their results, and (4) there was disagreement over what the proposed method should be—with the largest disagreements over sample size (50 mg samples processed in ~10 ml vials versus 10mg samples processed in standard 96 well plates) and sample storage prior to processing (to freeze or not).

Recognizing the different viewpoints of our team members, to help the research community move towards NSC analysis that is comparable both among and within laboratories, we propose:

- A Reference Method for sample collection and storage, sample processing, sugar extraction, starch extraction, and quantification. We use the term 'Reference Method' to identify the method as one that can indicate comparability among laboratory estimates,

compared to a 'Standard Method' that might imply a 'best', fully vetted method. Our data showed that water extractions gave the least variability among laboratories for soluble sugar extraction (Fig. S2A), and that the α-amylase + amyloglucosidase extractions gave the least variability for starch (Fig. S3A). Although water is the optimal extraction solvent for low molecular weight sugars and exhibited the least variability, it can also dissolve interfering hydrophilic polysaccharides and proteins. Extraction in aqueous alcohol can minimize this problem, and provide a high recovery of low molecular weight sugars. Standardization of alcohol strength and the number, temperature and duration of extractions is important to minimize variability in the results (Fig. S2A). Using these results, the discussion about methods in Supplemental Material Note S2, and the results for microwave duration and intensity (Fig. 5) and particle size (Fig. S4), we recommend the method detailed in Fig. 6 be adopted as a Reference Method. HPLC and variants showed the least variability among quantification methods because of its precision, but perhaps also because HPLC procedures incorporate filtration to remove interfering compounds. However, the Reference Method does not include a filtration or quantification step. We ended the Reference Method with extraction, because our study does not provide the data to support a recommendation for the adoption of the expensive HPLC quantification and filtration steps.

- That laboratories adopt the Reference Method for sample collection and storage, sample processing, sugar and starch extraction and filtration; *or* laboratories retain their current methods but analyze a portion of a study's samples with the Reference Method for sample collection and storage, sample processing, sugar and starch extraction and filtration. Samples selected for analysis with the Reference Method should span the

range of NSC values identified using the laboratory's current methods and results should be reported in publications. Laboratories retaining methods different from the Reference Method should provide a rationale for their use and a full description of the method. Following either of these recommendations would aid both in-house procedures and comparability among studies.

- Researchers should implement standard procedures of internal quality control and include a detailed description of this procedure to the method. Analytical results should evaluate and present 'measurement uncertainty', given by the sample replicates, starch and sugar standards, and NSC values for the peach leaf standard (SRM 1547). While SRM 1547 does not have certified estimates for NSC and its components, it is a widely available and standardized sample.

- Certified Reference Materials (CRM) and laboratory inter-calibration should be developed and applied in all NSC analyses. The development of an appropriate range of CRMs will require coordination within the research community to ensure that the CRMs represent the range of tissues and matrices of interest. Once CRMs have been developed, an indication of quality control should be published with all NSC results, to aid in more effective among-laboratory comparisons.

- The research community, including ecologists and biochemists, should work to develop a small set of standard methods that are appropriate for particular samples and questions and test the Reference Method.

The problem we have highlighted here, that NSC estimates are not comparable among different laboratories, will likely limit understanding of plant response to environmental stress. While our

study focused on NSC determination in woody vegetation, a similar range of methods is used in non-woody species (e.g., Campo et al. 2013, Jaikumar et al. 2014, Kagan et al. 2014, King et al. 2014), and our results are likely to be relevant to the broader plant science community. A more unified approach to NSC analysis and standardisation of methods will contribute to better understanding of plant responses to environment and management.

Acknowledgement

Audrey Quentin is grateful to members of the participating laboratories for their work and co-operation. We would like to thank Marc Vandame, Nicole Sonderegger, Laurianne Rouan, Mathieu Moreau, Alieta Eyles, Elena Lahoz, Leo Goudzwaard, Arjen van Peppel, Efrat Neuhaus, Julie Lavergne, Catherine Deborde, Britta Jahn-Humphrey, Dugald Close, Widad Al-Shawi for their help and contribution with the laboratory analyses. Michael Ryan was funded by McMaster fellowship (1158.C). Sara Palacio was funded by Juan de la Cierva contract (MCI project) and project ARBALMONT/786-2012 (OPAN, MAAMA, Spain). Frida Piper was funded by Fondecyt 11121175. Ülo Niinemets and Mari Tobias were funded by the Estonian Ministry of Education and Science, grant IUT-8-3. Nathan McDowell and Lee Dickman were funded by DOE-BER. Henry Adams was funded by LANL-LDRD. Jordi Martínez-Vilalta was funded by the Spanish Government (CGL 2010-16376). Sharon Hood was funded by the Montana Institute on Ecosystems' Graduate Enhancement Award from NSF EPSCoR Track-1 NSF-IIA-1443108. Valuable comments from Dr. Mauricio Mencuccini (University of Edinburgh), Dan Binkley (Colorado State University), and two anonymous reviewers were also greatly appreciated.

References

Adams HD, Germino MJ, Breshears DD, Barron-Gafford GA, Guardiola-Claramonte M, Zou CB, Huxman TE (2013) Nonstructural leaf carbohydrate dynamics of *Pinus edulis* during drought-induced tree mortality reveal role for carbon metabolism in mortality mechanism. New Phytol 197:1142-1151.

Ainsworth EA, Rogers A (2007) The response of photosynthesis and stomatal conductance to rising [CO_2]: mechanisms and environmental interactions. Plant Cell Environ 30:258-270.

Ainsworth EA, Davey PA, Bernacchi CJ, Dermody OC, Heaton EA, Moore DJ, Morgan PB, Naidu SL, Yoo Ra Hs, Zhu Xg (2002) A meta□analysis of elevated [CO_2] effects on soybean (*Glycine max*) physiology, growth and yield. Global Change Biol 8:695-709.

Anderegg WR, Anderegg LD (2013) Hydraulic and carbohydrate changes in experimental drought-induced mortality of saplings in two conifer species. Tree Physiol 33:252-260.

Arndt SK, Livesley SJ, Merchant A, Bleby TM, Grierson PF (2008) Quercitol and osmotic adaptation of field-grown Eucalyptus under seasonal drought stress. Plant Cell Environ 31:915-924.

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

Barry KM, Quentin A, Eyles A, Pinkard EA (2012) Consequences of resource limitation for recovery from repeated defoliation in *Eucalyptus globulus* Labilladière. Tree Physiol 32:24-35.

Bonhomme M, Rageau R, Lacointe A, Gendraud M (2005) Influences of cold deprivation during dormancy on carbohydrate contents of vegetative and floral primordia and nearby structures of peach buds (*Prunus persica* L. Batch). Sci Hort (Amst) 105:223-240.

Bryant JP, Chapin III FS, Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos:357-368.

Campo L, Monteagudo AB, Salleres B, Castro P, Moreno-Gonzalez J (2013) NIRS determination of non-structural carbohydrates, water soluble carbohydrates and other nutritive quality traits in whole plant maize with wide range variability. Span J Agric Res 11:463-471.

Chapin FS, Schulze E-D, Mooney HA (1990) The ecology and economics of storage in plants. Ann Rev Ecol Syst:423-447.

Cheng J, Fan P, Liang Z, Wang Y, Niu N, Li W, Li S (2009) Accumulation of end products in source leaves affects photosynthetic rate in peach via alteration of stomatal conductance and photosynthetic efficiency. J Am Soc Hortic Sci 134:667-676.

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

Clement R, Keith L, Siu K (1996) Reference Materials for Environmental Analysis. Lewis Publishers, Boca Raton, FL, USA,

Dichio B, Xiloyannis C, Sofo A, Montanaro G (2007) Effects of post-harvest regulated deficit irrigation on carbohydrate and nitrogen partitioning, yield quality and vegetative growth of peach trees. Plant Soil 290:127-137.

Dickmann LT, McDowell NG, Sevanto S, Pangle R, Pockman WT (2014) Carbohydrate dynamics and mortality in a piñon□juniper woodland under three future precipitation scenarios. Plant Cell Environ 38:729-739.

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

Dietze MC, Sala A, Carbone MS, Czimczik CI, Mantooth JA, Richardson AD, Vargas R (2014) Nonstructural carbon in woody plants. Ann Rev Plant Biol 65:667-687.

Drake PL, Mendham DS, Ogden GN (2013) Plant carbon pools and fluxes in coppice regrowth of *Eucalyptus globulus*. For Ecol Manage 306:161-170.

Duan HL, Amthor JS, Duursma RA, O'Grady AP, Choat B, Tissue DT (2013) Carbon dynamics of eucalypt seedlings exposed to progressive drought in elevated [CO_2] and elevated temperature. Tree Physiol 33:779-792.

Dubois M, Gilles KA, Hamilton JK, Rebers Pt, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350-356.

El Zein R, Maillard P, Bréda N, Marchand J, Montpied P, Gérant D (2011) Seasonal changes of C and N non-structural compounds in the stem sapwood of adult sessile oak and beech trees. Tree Physiol 31:843-854.

Emons H, Linsinger T, Gawlik B (2004) Reference materials: terminology and use. Can't one see the forest for the trees? TrAC Trends Anal Chem 23:442-449.

Escobar-Gutiérrez A, Moing A, Gaudillère J-P (1997) Time course of carbohydrates concentration in mature leaves of peach seedlings [*Prunus persica* (L.) Batsch] during the night. *In* IV International Peach Symposium 465, pp 337-344.

Eyles A, Pinkard EA, Mohammed C (2009a) Shifts in biomass and resource allocation patterns following defoliation in *Eucalyptus globulus* growing with varying water and nutrient supplies. Tree Physiol 29:753-764.

Eyles A, Pinkard EA, O'Grady AP, Worledge D, Warren CR (2009b) Role of corticular photosynthesis following defoliation in *Eucalyptus globulus*. Plant Cell Environ 32:1004-1014.

Eyles A, Pinkard EA, Davies NW, Corkrey R, Churchill K, O'Grady AP, Sands P, Mohammed C (2013) Whole-plant versus leaf-level regulation of photosynthetic responses after partial defoliation in *Eucalyptus globulus* saplings. J Exp Bot 64:1625-1636.

Fajardo A, Piper FI (2014) An experimental approach to explain the southern Andes elevational treeline. Am J Bot 101:788-795.

Fajardo A, Piper FI, Cavieres LA (2011) Distinguishing local from global climate influences in the variation of carbon status with altitude in a tree line species. Glob Ecol Biogeogr 20:307-318.

Fajardo A, Piper FI, Hoch G (2013) Similar variation in carbon storage between deciduous and evergreen treeline species across elevational gradients. Ann Bot 112:623-631.

Fajardo A, Piper FI, Pfund L, Körner C, Hoch G (2012) Variation of mobile carbon reserves in trees at the alpine treeline ecotone is under environmental control. New Phytol 195:794-802.

Galiano L, Martinez-Vilalta J, Lloret F (2011) Carbon reserves and canopy defoliation determine the recovery of Scots pine 4 yr after a drought episode. New Phytol 190:750-759.

Giannoccaro E, Wang Y-J, Chen P (2008) Comparison of two HPLC systems and an enzymatic method for quantification of soybean sugars. Food Chem 106:324-330.

Gomez L, Rubio E, Auge M (2002) A new procedure for extraction and measurement of soluble sugars in ligneous plants. J Sci Food Agri 82:360-369.

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

Gordon D, Rosati A, Damiano C, Dejong T (2006) Seasonal effects of light exposure, temperature, trunk growth and plant carbohydrate status on the initiation and growth of epicormic shoots in *Prunus persica*. J Hort Sci Biotechnol 81:421-428.

Graham C (2002) Nonstructural carbohydrate and prunasin composition of peach seedlings fertilized with different nitrogen sources and aluminum. Sci Hort (Amst) 94:21-32.

Grunzweig JM, Carmel Y, Riov J, Sever N, McCreary DD, Flather CH (2008) Growth, resource storage, and adaptation to drought in California and eastern Mediterranean oak seedlings. Can J For Res 38:331-342.

Hall MB (2013) Efficacy of reducing sugar and phenol–sulfuric acid assays for analysis of soluble carbohydrates in feedstuffs. Anim Feed Sci Tech 185:94-100.

Handa IT, Körner C, Hättenschwiler S (2005) A test of the treeline carbon limitation hypothesis by in situ CO_2 enrichment and defoliation. Ecology 86:1288-1300.

Hansen J, Møller I (1975) Percolation of starch and soluble carbohydrates from plant tissue for quantitative determination with anthrone. Anal Biochem 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.

Haukioja E, Koricheva J (2000) Tolerance to herbivory in woody vs. herbaceous plants. Evol Ecol 14:551-562.

Hoch G, Körner C (2003) The carbon charging of pines at the climatic treeline: a global comparison. Oecologia 135:10-21.

Hoch G, Körner C (2005) Growth, demography and carbon relations of *Polylepis* trees at the world's highest treeline. Funct Ecol 19:941-951.

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.

Hoch G, Richter A, Körner C (2003) Non□structural carbon compounds in temperate forest trees. Plant Cell Environ 26:1067-1081.

Huang Y-B, Fu Y (2013) Hydrolysis of cellulose to glucose by solid acid catalysts. Green Chem 15:1095-1111.

Inglese P, Caruso T, Gugliuzza G, Pace L (2002) Crop load and rootstock influence on dry matter partitioning in trees of early and late ripening peach cultivars. J Am Soc Hortic Sci 127:825-830.

Jaikumar NS, Snapp SS, Flore JA, Loescher W (2014) Photosynthetic responses in annual rye, perennial wheat, and perennial rye subjected to modest source: sink ratio changes. Crop Sci 54:274-283.

Johansen HN, Glitsø V, Bach Knudsen KE (1996) Influence of extraction solvent and temperature on the quantitative determination of oligosaccharides from plant materials by high-performance liquid chromatography. J Agric Food Chem 44:1470-1474.

Kagan IA, Kirch BH, Thatcher CD, Teutsch CD, Pleasant RS (2014) Chromatographic profiles of nonstructural carbohydrates contributing to the colorimetrically determined fructan, ethanol-soluble, and water-soluble carbohydrate contents of five grasses. Anim Feed Sci Tech 188:53-63.

Kim S, Kim W, Hwang IK (2003) Optimization of the extraction and purification of oligosaccharides from defatted soybean meal. Int J Food Sci Tech 38:337-342.

King JR, Conway WC, Rosen DJ, Oswald BP, Williams HM (2014) Total nonstructural carbohydrate trends in deeproot sedge (*Cyperus entrerianus*). Weed Sci 62:186-192.

Kobe RK (1997) Carbohydrate allocation to storage as a basis of interspecific variation in sapling survivorship and growth. Oikos 80:226-233.

Körner C (2003) Carbon limitation in trees. J Ecol 91:4-17.

Kozlowski T (1992) Carbohydrate sources and sinks in woody plants. Bot Rev 58:107-222.

Landhäusser SM, Lieffers VJ (2003) Seasonal changes in carbohydrate reserves in mature northern *Populus tremuloides* clones. Trees 17:471-476.

Leite G, Bonhomme M, Lacointe A, Rageau R, Sakr S, Guilliot A, Maurel K, Pétel G, Couto-Rodriguez A (2004) Influence of lack of chilling on bud-break patterns and evolution of sugar contents in buds and stem tissues along the one-year-old shoot of the peach trees. *VII International Symposium on Temperate Zone Fruits in the Tropics and Subtropics*. Acta Hort 662:61-71.

Li M-H, Xiao W-F, Wang S-G, Cheng G-W, Cherubini P, Cai X-H, Liu X-L, Wang X-D, Zhu W-Z (2008) Mobile carbohydrates in Himalayan treeline trees I. Evidence for carbon gain limitation but not for growth limitation. Tree Physiol 28:1287-1296.

Li WD, Duan W, Fan PG, Yan ST, Li SH (2007) Photosynthesis in response to sink—source activity and in relation to end products and activities of metabolic enzymes in peach trees. Tree Physiol 27:1307-1318.

Loomis W (1932) Growth-differentiation balance *vs.* carbohydrate-nitrogen ratio. Proc Am Soc Hort Sci 29:240-245.

Lusk C, Piper F (2007) Seedling size influences relationships of shade tolerance with carbohydrate□storage patterns in a temperate rainforest. Funct Ecol 21:78-86.

MacRae JC, Smith D, McCready RM (1974) Starch estimation in leaf tissue—a comparison of results using six methods. J Sci Food Agri 25:1465-1469.

Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem 75:3019-3030.

McDowell N, Pockman WT, Allen CD, Breshears DD, Cobb NS, Kolb T, Plaut J, Sperry J, West A, Williams DG (2008) Mechanisms of plant survival and mortality during drought: why do some plants survive while others succumb to drought? New Phytol 178:719-739.

Merchant A, Peuke AD, Keitel C, Macfarlane C, Warren CR, Adams MA (2010) Phloem sap and leaf δ13C, carbohydrates, and amino acid concentrations in *Eucalyptus globulus* change systematically according to flooding and water deficit treatment. J Exp Bot 61:1785-1793.

Mitchell PJ, O'Grady AP, Tissue DT, Worledge D, Pinkard EA (2014) Co-ordination of growth, gas exchange and hydraulics define the carbon safety margin in tree species with contrasting drought strategies. Tree Physiol 34:443-458.

Mitchell PJ, O'Grady AP, Tissue DT, White DA, Ottenschlaeger ML, Pinkard EA (2013) Drought response strategies define the relative contributions of hydraulic dysfunction and carbohydrate depletion during tree mortality. New Phytol 197:862-872.

Moing A, Carbonne F, Rashad MH, Gaudillère J-P (1992) Carbon fluxes in mature peach leaves. Plant Physiol 100:1878-1884.

Mooney H (1972) The carbon balance of plants. Ann Rev Ecol Syst 3:315-346.

Morgan P, Ainsworth E, Long S (2003) How does elevated ozone impact soybean? A meta□ analysis of photosynthesis, growth and yield. Plant Cell Environ 26:1317-1328.

Muller B, Pantin F, Génard M, Turc O, Freixes S, Piques M, Gibon Y (2011) Water deficits uncouple growth from photosynthesis, increase C content, and modify the relationships between C and growth in sink organs. J Exp Bot 62:1715-1729.

Nakagawa S, Schielzeth H (2013) A general and simple method for obtaining R^2 from generalized linear mixed-effects models. Meth Ecol Evol 4:133–142.

Nii N (1997) Changes of starch and sorbitol in leaves before and after removal of fruits from peach trees. Ann Bot 79:139-144.

O'Brien MJ, Leuzinger S, Philipson CD, Tay J, Hector A (2014) Drought survival of tropical tree seedlings enhanced by non-structural carbohydrate levels. Nat Clim Change 4:710-714.

O'Grady A, Eyles A, Worledge D, Battaglia M (2010) Seasonal patterns of foliage respiration in dominant and suppressed *Eucalyptus globulus* canopies. Tree Physiol 30:957-968.

Palacio S, Hoch G, Sala A, Körner C, Millard P (2014) Does carbon storage limit tree growth? New Phytol 201:1096-1100.

Pinkard EA, Eyles A, O'Grady AP (2011) Are gas exchange responses to resource limitation and defoliation linked to source: sink relationships? Plant Cell Environ 34:1652-1665.

Piper FI (2011) Drought induces opposite changes in the concentration of non-structural carbohydrates of two evergreen *Nothofagus* species of differential drought resistance. Ann For Sci 68:415-424.

Piper FI, Fajardo A (2011) No evidence of carbon limitation with tree age and height in *Nothofagus pumilio* under Mediterranean and temperate climate conditions. Ann Bot 108:907-917.

Piper FI, Fajardo A (2014) Foliar habit, tolerance to defoliation and their link to carbon and nitrogen storage. J Ecol 102:1101-1111.

Quentin A, Beadle C, O'Grady A, Pinkard E (2011) Effects of partial defoliation on closed canopy *Eucalyptus globulus* Labilladière: Growth, biomass allocation and carbohydrates. For Ecol Manage 261:695-702.

Quentin AG, Pinkard EA, Beadle CL, Wardlaw TJ, O'Grady AP, Paterson S, Mohammed CL (2010) Do artificial and natural defoliation have similar effects on physiology of *Eucalyptus globulus* Labill. seedlings? Ann For Sci 67:203.

Quevauviller P, Astruc M, Ebdon L, Desauziers V, Sarradin P, Astruc A, Kramer G, Griepink B (1994) Certified reference material (CRM 462) for the quality control of dibutyl□and tributyl□tin determinations in coastal sediment. App Organomet Chem 8:629-637.

R Development Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org.

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. Anal Methods 2:532-538.

Rastall RA (1990) Methods in Plant Biochemistry. Volume 2. Carbohydrates. Academic Press, London, UK,

Richardson AD, Carbone MS, Keenan TF, Czimczik CI, Hollinger DY, Murakami P, Schaberg PG, Xu X (2013) Seasonal dynamics and age of stemwood nonstructural carbohydrates in temperate forest trees. New Phytol 197:850-861.

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. J Agric Food Chem 39:2-11.

Ruuhola T, Keinanen M, Keski-Saari S, Lehto T (2011) Boron nutrition affects the carbon metabolism of silver birch seedlings. Tree Physiol 31:1251-1261.

Ryan MG (2011) Tree responses to drought. Tree Physiol 31:237-239.

Ryan MG, Phillips N, Bond BJ (2006) The hydraulic limitation hypothesis revisited. Plant Cell Environ 29:367-381.

Sala A, Piper F, Hoch G (2010) Physiological mechanisms of drought□induced tree mortality are far from being resolved. New Phytol 186:274-281.

Sala A, Fouts W, Hoch G (2011) Carbon storage in trees: Does relative carbon supply decrease with tree size? In: Meinzer FC, Lachenbruch B, Dawson TE (eds) Size-and age-related changes in tree structure and function. Springer, pp 287-306.

Sala A, Woodruff DR, Meinzer FC (2012) Carbon dynamics in trees: feast or famine? Tree Physiol 32:764-775.

Santiago da Silva CM, Habermann G, Marchi MRR, 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.

Saunders S, Karduck P, Sloof WG (2004) Certified reference materials for micro-analysis of carbon and nitrogen. Microchimica Acta 145:209-213.

Sevanto S, McDowell NG, Dickman LT, Pangle R, Pockman WT (2014) How do trees die? A test of the hydraulic failure and carbon starvation hypotheses. Plant Cell Environ 37:153-161.

Shi J, Mazza G, le Maguer M (2002) Functional foods: biochemical and processing aspects. Vol. II. CRC Press UK London.

Shvaleva AL, Silva FCE, Breia E, Jouve J, Hausman J-F, Almeida MH, Maroco J, Rodrigues M, Pereira JS, Chaves MM (2005) Metabolic responses to water deficit in two *Eucalyptus globulus* clones with contrasting drought sensitivity. Tree Physiol 26:239-248.

Smeraglia J, Baldrey SF, Watson D (2002) Matrix effects and selectivity issues in LC-MS-MS. Chromatographia 55:95-S99.

Stick RV, Williams S (2010) Carbohydrates: The Essential Molecules of Life. Elsevier, London, UK, http://www.elsevier.com/books/carbohydrates-the-essential-molecules-of-life/stick/978-0-240-52118-3.

Strauss SY, Agrawal AA (1999) The ecology and evolution of plant tolerance to herbivory. Trends Ecol Evol 14:179-185.

Thompson M, Ellison SLR (2005) A review of interference effects and their correction in chemical analysis with special reference to uncertainty. Accred Qual Assur 10:82-97.

Tworkoski T, Glenn D, Welker W (1997) Carbohydrate and nitrogen partitioning within one-year shoots of young peach trees grown with grass competition. HortScience 32:1174-1177.

Weibel A, Reighard G, Rajapakse N, DeJong T (2008) Dormant carbohydrate reserves of two peach cultivars grafted on different vigor rootstocks. *In* IX International Symposium on Integrating Canopy, Rootstock and Environmental Physiology in Orchard Systems 903, pp 815-820.

Wiley E, Helliker B (2012) A re□evaluation of carbon storage in trees lends greater support for carbon limitation to growth. New Phytol 195:285-289.

Wittig VE, Ainsworth EA, Naidu SL, Karnosky DF, Long SP (2009) Quantifying the impact of current and future tropospheric ozone on tree biomass, growth, physiology and biochemistry: a quantitative meta□analysis. Global Change Biol 15:396-424.

Yemm E, Willis A (1954) The estimation of carbohydrates in plant extracts by anthrone. Biochem J 57:508.

Zhang CH, Shen ZJ, Zhang YP, Han J, Ma RJ, Korir NK, Yu ML (2013) Cloning and expression of genes related to the sucrose-metabolizing enzymes and carbohydrate changes in peach. Acta Physiol Plant 35:589-602.

Table Captions

Table 1. Summary of the primary solvents and assays used for extraction and quantification methods to estimate soluble sugars (A) and starch (B) in five plant materials. The method categories also vary in the number of extractions, duration, temperature and standards. For further details on each specific method, please refer to Tables S1 and S2.

Table 2. Procedures for soluble sugar, starch measurements, and non-structural carbohydrate (NSC) concentrations and mean values for *Eucalyptus globulus* (A) and *Prunus persica* (B) and for *Pinus edulis* (C) for various environmental response studies.

Table 3. The Spearman rank correlation indicates correlations for laboratories between sample pairs of 0.11-0.83 (mean = 0.44) for soluble sugars (A), 0.41-0.91 (mean = 0.71) for starch (B) and 0.45-0.84 (mean = 0.60) for total non-structural carbohydrates (NSC; C). These results suggest starch has the most consistency among laboratory ranks for the different samples.

Table 4. The general linear mixed model analysis with laboratory as a random factor showed some differences for extraction and quantification methods for sugar and starch concentrations and interactions between extraction and quantification methods and sample for sugars, starch, and total NSC. The interactions suggest that a method performs differently for different samples.

Figure Legends

Figure 1. Laboratory estimates of (A) sucrose, glucose+fructose, total soluble sugar, and (B) starch and non-structural carbohydrates (NSC) for five samples: *Eucalyptus globulus* leaves (EGL), *Pinus edulis* needles (PEN), *Prunus persica* leaves (PPL), *E. globulus* roots (EGR) and *E. globulus* stem (EGS), with means (text and solid line), range, coefficient of variation (CV) and 95% confidence interval (dashed lines). Estimates are ranked by sugar extraction category: W = water, EtOH+W = Ethanol water mixture, MCW = methanol-chloroform-water, EtOH = Ethanol. Estimates differed substantially among laboratories and within method categories.

Figure 2. Correlations of laboratory ranks among all sample pairs that show the worst and best correlations for soluble sugars, starch and total NSC. Plots show that laboratory rankings can be consistent for the different samples. Spearman rank correlations for all sample pairs are in Table 3. Solid lines are the 1:1 line.

Figure 3. Differences in least squares means for all samples (LSM) and for individual samples (EGL, PEN, PPL, EGR, EGS) for the extraction and quantification methods for soluble sugars, starch and total NSC show that method category generally had little effect on NSC difference, perhaps because of high within-method variance. Error bars are standard errors for the least square means. Total soluble sugars results are grouped by sugar extraction (A) and quantification (F) method. Starch results are grouped by sugar (B) and starch (D) extraction method, and starch quantification method (H). Total NSC results are grouped by sugar (C) and starch (E) extraction methods, and for sugar (G) and starch (I) quantification methods. Significant differences (*) among methods within each tissue were assessed with Tukey-Kramer test (α=0.05).

Figure 4. Means and standard errors for soluble sugars by extraction method for samples processed in one laboratory and using the same quantification method. Results show that extraction method can affect estimates especially for PEN and PPL samples. In all samples MCW-based methods produced consistently lower estimates than alcohol-based methods. Different letters indicate significant difference at α=0.05 according to F-protected LSD test.

Figure 5. Effect of microwaving samples < 5 g at 800W on amount of glucose + fructose (Gluc+Fruc), sucrose (Suc), starch and total non-structural carbohydrate (NSC) for foliar (A) and twig (B) samples of *Pinus edulis*. See Method S4 for details on the method. At 0 and 90 s microwaving time, sucrose hydrolyzing and starch debranching enzymes are still active, leading to lower sucrose levels, higher glucose + fructose levels, and higher starch levels because debranching enzyme make starch more accessible to the enzymatic assay. At 180 s and above, enzymes are deactivated, yielding consistent sucrose and glucose + fructose. At 300 s, starch starts to gelatinize, again making it more accessible to the assay. Orthoginal contrasts for trend with microwaving time: glucose+fructose, quadratic for leaf and twig, $P < 0.05$; sucrose, linear for leaf and twig, $P < 0.001$; starch, quadratic for leaf and twig, $P < 0.01$; total NSC, quadratic for leaf and twig, $P < 0.01$.

Figure 6. Instructions for sample collection, handling, preparation, and sugar and starch extraction for Reference Method.

Table 1. Summary of the primary solvents and assays used for extraction and quantification methods to estimate soluble sugars (A) and starch (B) in five plant materials. The method categories also vary in the number of extractions, duration, temperature and standards. For further details on each specific method, please refer to Tables S1 and S2.

A. Soluble sugars

Extraction methods

	Strength	No. extraction	Combination	Duration (mins)	Temperature (^{o}C)	No. Laboratories
EtOH or MeOH	70-80%^{x}	1 to 5	EtOH or W	2 to 60	60 to 100	19
W	-	1 to 3	-	10 to 60	65 to 100	8
MCW	-	1 to 3	-	5 to overnight	4 to 60	3

Quantification methods

	Absorbance	Reagents	Standards	No. Laboratories
HPLC	-	-	Trehalose or mannitol	8
HPAEC-PAD	-	-	GLUC, FRUC, SUC	3
^{1}H-NMR	-	-	GLUC, FRUC	1
Enzymatic	340	G6PDH+HK+PGI+Invertase	GLUC, FRUC, SUC	10
Colorimetric	620	Anthrone	GLUC	5
	490	Phenol	GLUC	4

B. Starch

Gelatinisation methods

	Duration (mins)	Temperature (^{o}C)	No. Laboratories
None	-	-	4
NaOH	30 to 180	50 to 100	8
DMSO	5	100	2
KOH	30	95	1

EtOH	30	100	1
AA	30	85-90	2
Others[y]	NA - 90	120	5

Digestion/Extraction methods

	Reagent/enzyme	No. extraction	Temperature (^{o}C)	Duration (mins/hrs)	No. Laboratories
Acid	$HClO_4$	1	room temperature	16 to 20 hrs	2
	H_2SO_4		autoclave	3.5 mins	1
	HCl		100	6 mins	1
Enzymatic	Amylo.	1 or 2	45 to 100	30 mins to 24 hrs	16
	AA + amylo.	2	55 to 100 (1) 37 to 100 (2)	3 to 30 mins (1) 1 min to 16 hrs	8

Quantification methods

	Absorbance	Reagent	Standard	No Laboratories
HPLC	-	-	GLUC	4
HPAEC	-	-	GLUC	2
Enzymatic	340	G6PDH+HK	GLUC	10
Colorimetric	620-630	Anthrone	GLUC	4
	490	Phenol	GLUC	4
	510-525[z]	GOPOD	GLUC	5

[x] strength used for the first extraction. When more extraction, strength varied between 30 and 80% for ethanol, and 0% when water is used

[y] includes: shaking, autoclaving, boiling, ultrasound

[z] method using the Megazyme® kit.

AA: α-amylase; Amylo.: amyloglucosidase; DMSO: Dimethyl sulfoxide ; EtOH: ethanol; FRUC: fructose; G6PDH: glucose-6-phosphate dehydrogenase; GHK: Glucose Hexokinase; GLUC: glucose; GOPOD: glucose oxidase/peroxidase-o-dianisidine; H_2SO_4: Sulfuric acid ; HCl: hydrochloride acid; $HClO_4$: Perchloric acid ; ^{1}H-NMR: Proton Nuclear Magnetic Resonance; HPAEC: High Performance Anion Exchange Chromatography; HPLC: High-performance liquid chromatography; KOH: Potassium hydroxide; NaOH: Sodium hydroxide; MCW: methanol:chloroform:water; PGI : phosphoglucose-isomerase; SUC: sucrose

Note: Soluble sugar methods include 31 laboratories and starch methods 28 laboratories. Two laboratories have used two methods to estimates the soluble sugars, while one laboratory did not estimate starch.

Table 2. Procedures for soluble sugar, starch measurements, and non-structural carbohydrate (NSC) concentrations and mean values for *Eucalyptus globulus* (A) and *Prunus persica* (B) and for *Pinus edulis* (C) for various environmental response studies.

References	Age	Tissue	Sample weight (mg)	Soluble sugars		Starch		Concentration (mg g^{-1}) in the literature					
				Extr.	Quant. (assay)	Dig.	Quant. (assay)	GLUC	FRUC	SUC	TSS	St	Total NSC
A. *Eucalyptus globulus*													
Shvaleva et al. (2005)	~12 mo	L	20	EtOH	Spec. (anthrone)	HCl	Spec. 620				72-83	49-56	115-117
		R	50								32-45	29-32	78-88
Eyles et al. (2009a)	11 mo	L	50	EtOHx1	Spec. 490 (phenol)	Amylo.	Spec. 490 (phenol)				105	94	199
		S									40	79	118
		R									33	100	132
Eyles et al. (2009b)	~16 mo	L	50	EtOHx1	Spec. 490 (phenol)	Amylo.	Spec. 490 (phenol)				46	93	140
Merchant et al. (2010)	~12 mo	L	40	MCW	GC			5	4	2	12		
O'Grady et al. (2010)	>6yo	L (at 7m high)	50	EtOHx1	Spec. 490 (phenol)	Amylo.	Spec. 490 (phenol)				56	64	120
		L (at 15m high)									19	37	56
Quentin et al. (2010)	~8 mo	L	50	EtOHx1	Spec. 490 (phenol)	Amylo.	Spec. 490 (phenol)				93-106	37-39	130-145
Pinkard et al. (2011)	~3-4 mo	L	50	EtOHx1	Spec. 490 (phenol)	Amylo.	Spec. 490 (phenol)				142	93	187
Quentin et al. (2011)	> 6yo	L	50	EtOHx1	Spec. 490 (phenol)	Amylo.	Spec. 490 (phenol)						145
		S											60
		R											63
Barry et al. (2012)	18 mo	L	50	EtOHx1	Spec. 490 (phenol)	Amylo.	Spec. 490 (phenol)				60	16	76
		S									24	9	32
		R									28	40	67
Drake et al. (2013)	?	S	100	EtOHx2	Spec 630						6-14		

		R (tap)			(anthrone)						7-16		
		L									83-90	33-140	117-224
Duan et al. (2013)	8 mo	S	20	EtOHx2+W	Spec 620 (anthrone)	AA + amylo.	Spec. 515				32-60	2-8	35-62
		R									10-24	1-2	12-25
Eyles et al. (2013)	7 mo	L	50	EtOHx1	UPLC	Amylo.	Spec. 490 (phenol)	18	22	1	54	92	146
		L									85	120	206
Mitchell et al. (2013)	6 mo	S	20	EtOHx2+W	Spec 620 (anthrone)	AA + amylo.	Spec. 515 (GOPOD)				20	13	33
		R									46	30	76
(Gauthier et al. 2014)[x]	<6 mo	L	5	EtOHx3	Enz.	Amylo.	Spec 515 (GOPOD)				7	10	17
B. *Prunus persica*													
Moing et al. (1992)	2 mo	L	?	EtOHx2	HPLC	Amylo.	HPLC	11.1	5.69	36.7	95	89	184
Nii (1997)	37-38 yo	L	?	EtOH	Spec (anthrone)						78	77	155
Tworkoski et al. (1997)	5-6 yo	L	200	EtOH	HPLC	Amylo.	Spec.				38-158	33-48	86-191
		S									44-77	39-45	83-122
Escobar-Gutiérrez et al. (1997)	2.5 mo	L	?	EtOHx2	HPLC	Amylo.		39	10	53	215	135	350
Inglese et al. (2002)	3 yo	R	150	EtOH	Enz.	Amylo.	Enz.					6 - 9	
		R						16	9	9	57	52	109
Graham (2002)	2 mo	S	50	MCW	HPLC	Amylo.	Spec. 450	11	3	7	54	33	88
		L						20	7	15	106	26	132
Leite et al. (2004)	11 yo	S (Oct)	10	EtOHx2	HPLC	Amylo.	Enz.	5		27	69	65.5	134
		S (Feb)						14		35	74	16	90
Bonhomme et al. (2005)	4 yo	S	10	EtOHx2	HPLC	Amylo.	Enz.	15		28	72	12	84
Gordon et al. (2006)	2 yo	R	?	?	HPLC	Amylo.	?						150

Dichio et al. (2007)	>3 yo	L S R	?	EtOH	Spec. 625 (anthrone)	Amylo.	Spec. 425 (GOPOD)				120 100 240	5 10 60	125 110 300
Li et al. (2007)[y]	5 yo	L	15000	EtOHx3	HPLC	Amylo.	Spec. (GOPOD)	4	2	9	51	25	76
Cheng et al. (2009)[y]	8 yo	L	15000	EtOHx3	HPLC	Amylo.	Spec. (GOPOD)	4	4	11	43	23	65
Weibel et al. (2008)	4-5 yo	R S R	?	EtOH	Spec. (anthrone)								260 160 190
C. *Pinus edulis*													
Adams et al. (2013)[z]	15-25 yo	L	12	W	Enz.	Amylo.	Enz.				10-56	0-185	19-216
Anderegg and Anderegg (2013)	10-15 yo	L R	?	EtOH+MCW	Spec 595	BA + amylo.	Spec. 595	5-10 10-28		0-30 8 -21		30-60 45-95	
Dickmann et al. (2014)[z]	?	L (2007) L (2008) L (2009)	12	W	Enz.	Amylo.	Enz.	5 4 4		1 1 0	6 5 4	10 3 12	16 8 16
Sevanto et al. (2014)[z]	15-25 yo	L	12	W	Enz.	Amylo.	Enz.	13-27		1-19	27-36	6-31	36-59

[x] values reported in g m^{-2}.
[y] estimations were made on fresh weight.
[z] no fertiliser used.
AA: α-amylase; Amylo : amyloglucosidase ; BA.: β-amylase; DMSO : dimethylsulfoxide ; Dig.: digestion; Enz: enzymatic; EtOH: ethanol; Extr.: extraction; FRUC: fructose; GLUC: glucose; GOPOD: glucose oxidase/peroxidase-o-dianisidine; HCl: hydrochloride acid; L: leaf; MCW: methanol:chloroform:water; mo: month-old; Quant.: quantification; R: roots; spec: spectrophotometry; S: stem; St: starch; SUC: sucrose; TSS: total soluble sugars; W: water; yo: year-old.

Table 3. The Spearman rank correlation indicates correlations for laboratories between sample pairs of 0.1-0.8 for soluble sugars (A), 0.4-0.9 for starch (B) and 0.5-0.8 for total non-structural carbohydrates (NSC; C). These results suggest consistency among laboratories for the different samples.

	EGL	EGR	EGS	PEN	PPL
A. Soluble sugars					
EGL					
EGR	0.33				
EGS	0.11	0.73**			
PEN	0.29	0.52**	0.41*		
PPL	0.83**	0.39*	0.37*	0.41*	
B. Starch					
EGL					
EGR	0.69**				
EGS	0.59**	0.87**			
PEN	0.47*	0.83**	0.91**		
PPL	0.41*	0.68**	0.84**	0.81**	
C. Total NSC					
EGL					
EGR	0.59**				
EGS	0.49**	0.69**			
PEN	0.45*	0.84**	0.64**		
PPL	0.49**	0.54**	0.55**	0.72**	

*$P<0.05$
**$P<0.01$

Table 4. The general linear mixed model analysis with laboratory as a random factor showed some methods differences for extraction and quantification methods for sugars and starch concentrations and interactions between extraction and quantification methods and sample for sugars, starch, and total NSC. The interactions suggest that a method performs differently for different samples.

	Soluble sugars (SS)				Starch				Total NSC			
	Num. *d.f.*	**Den. *d.f.***	**F**	***P*-value**	**Num. *d.f.***	**Den. *d.f.***	**F**	***P*-value**	**Num. *d.f.***	**Den. *d.f.***	**F**	***P*-value**
Sample	4	426	63.4	<0.0001	4	387	152	<0.0001	4	386	122	<0.0001
SS extraction	3	28	2.1	0.123	3	25.01	9.2	0.0003	3	25.01	2.6	0.074
SS quantification	3	27.95	5.6	0.004	-	-	-	-	3	25.01	25.0	0.443
Starch extraction	-	-	-	-	2	26.01	3.1	0.064	2	26.02	0.12	0.837
Starch quantification	-	-	-	-	4	24	1.3	0.306	4	24.01	1.9	0.141
Sample x SS extraction	12	426	11.6	<0.0001	12	387	5.1	<0.0001	12	386	11.7	<0.0001
Sample x SS quantification	12	426	7.54	<0.0001	-	-	-	-	12	386	386	<0.0001
Sample x Starch extraction	-	-	-	-	8	391	4.7	<0.0001	8	390	3.5	0.0007
Sample x Starch quantification	-	-	-	-	16	383	15.0	<0.0001	16	382	10.7	<0.0001

df: degree of freedom
Num.: numerator
Den.: denominator

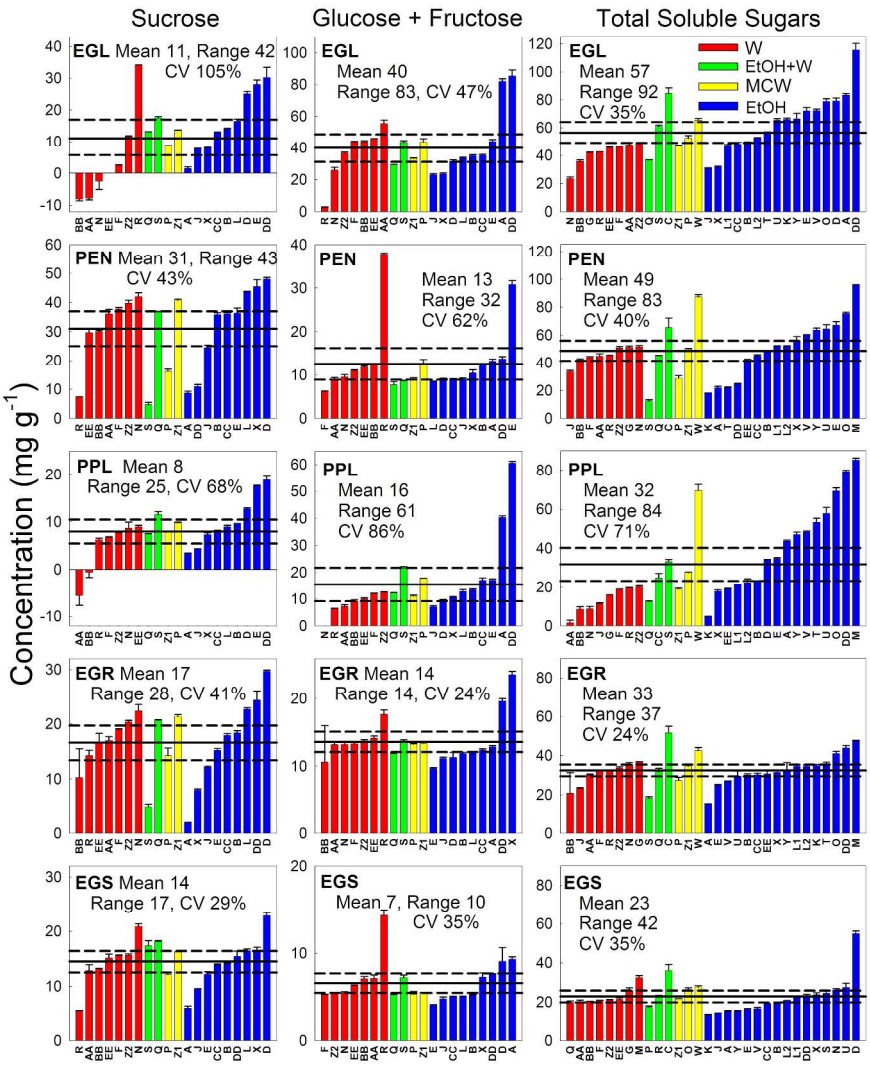
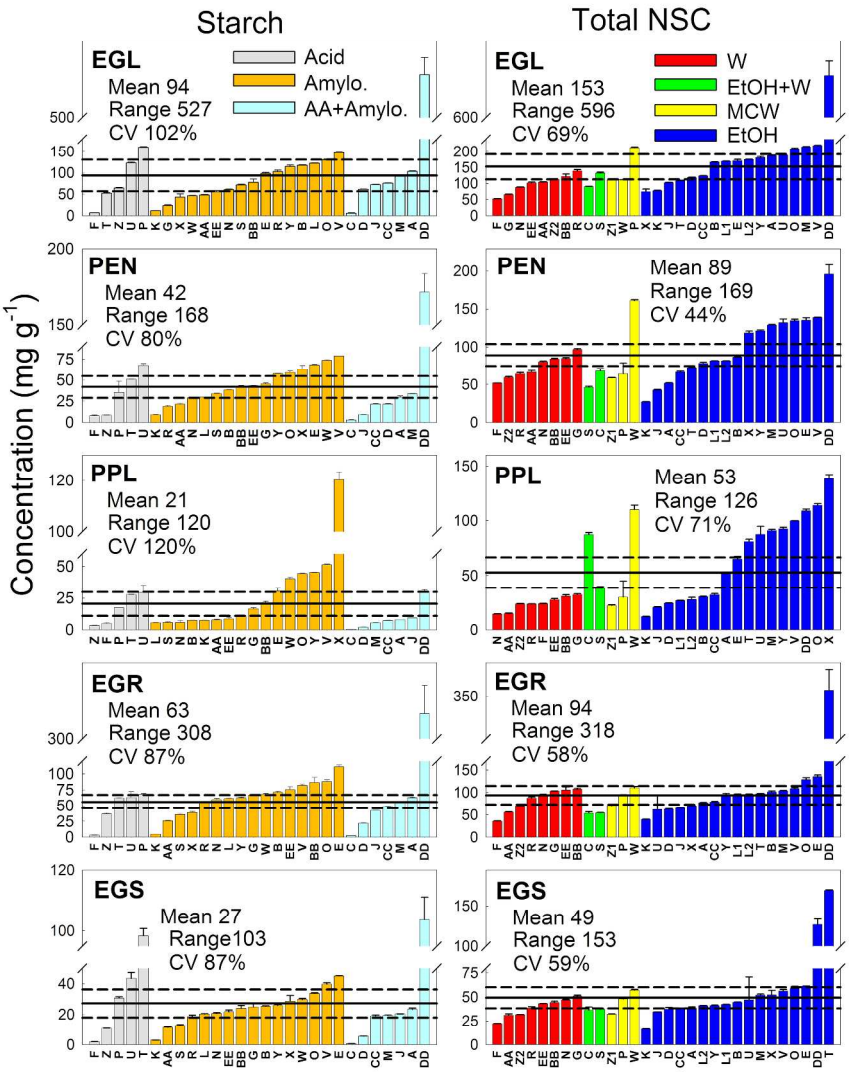


Figure 1. Laboratory estimates of (A) sucrose, glucose+fructose, total soluble sugar, and (B) starch and non-structural carbohydrates (NSC) for five samples: Eucalyptus globulus leaves (EGL), Pinus edulis needles (PEN), Prunus persica leaves (PPL), E. globulus roots (EGR) and E. globulus stem (EGS), with means (text and solid line), range, coefficient of variation (CV) and 95% confidence interval (dashed lines). Estimates are ranked by sugar extraction category: W = water, EtOH+W = Ethanol water mixture, MCW = methanol-chloroform-water, EtOH = Ethanol. Estimates differed substantially among laboratories and within method categories.
279x361mm (300 x 300 DPI)



279x361mm (300 x 300 DPI)

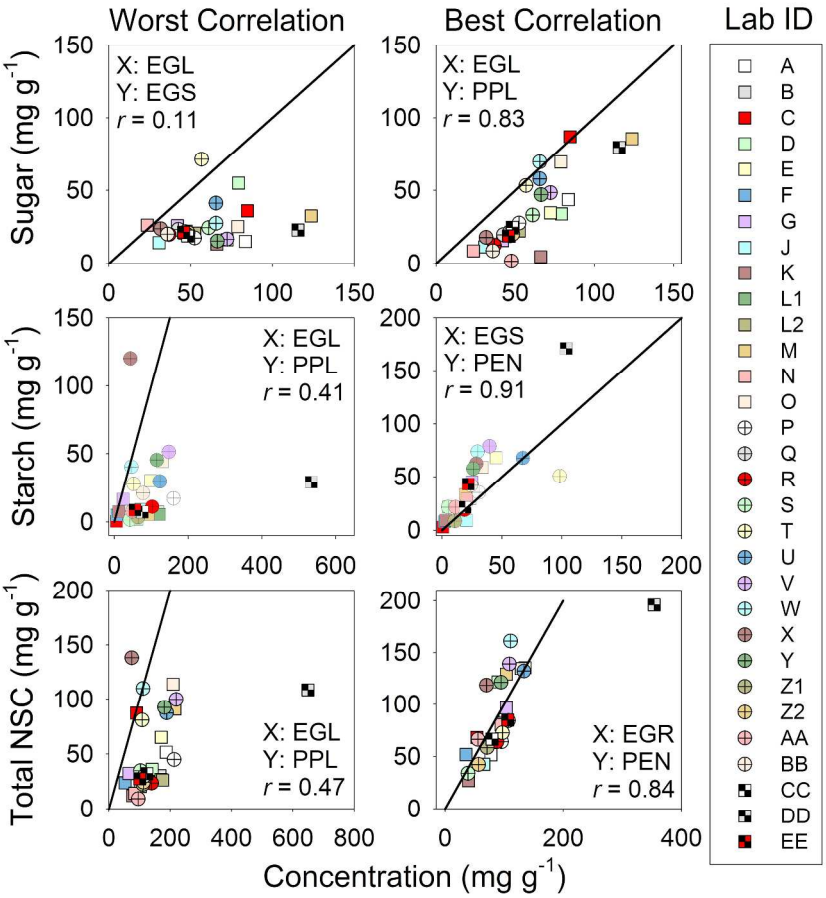


Figure 2. Correlations of laboratory ranks among all sample pairs that show the worst and best correlations for soluble sugars, starch and total NSC. Plots show that laboratory rankings can be consistent for the different samples. Spearman rank correlations for all sample pairs are in Table 3. Solid lines are the 1:1 line.
279x361mm (300 x 300 DPI)

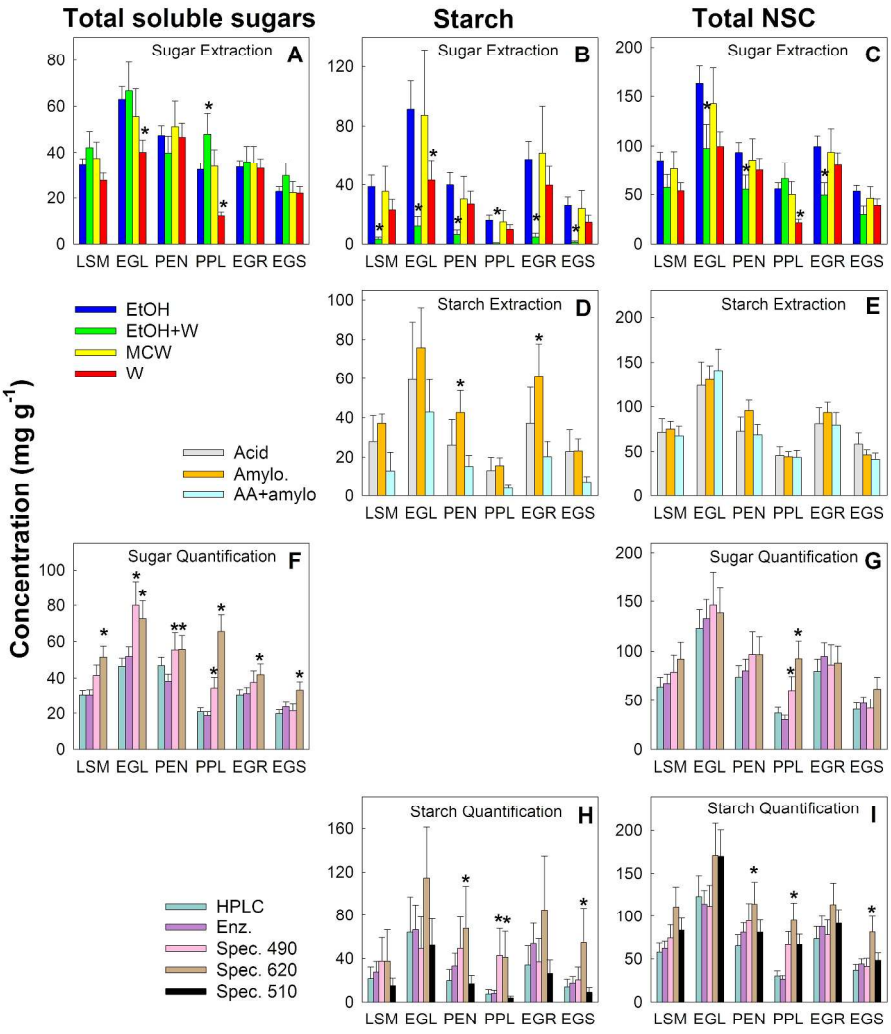


Figure 3. Differences in least squares means for all samples (LSM) and for individual samples (EGL, PEN, PPL, EGR, EGS) for the extraction and quantification methods for soluble sugars, starch and total NSC show that method category generally had little effect on NSC difference, perhaps because of high within-method variance. Error bars are standard errors for the least square means. Total soluble sugars results are grouped by sugar extraction (A) and quantification (F) method. Starch results are grouped by sugar (B) and starch (D) extraction method, and starch quantification method (H). Total NSC results are grouped by sugar (C) and starch (E) extraction methods, and for sugar (G) and starch (I) quantification methods. Significant differences (*) among methods within each tissue were assessed with Tukey-Kramer test (α=0.05).
431x508mm (300 x 300 DPI)

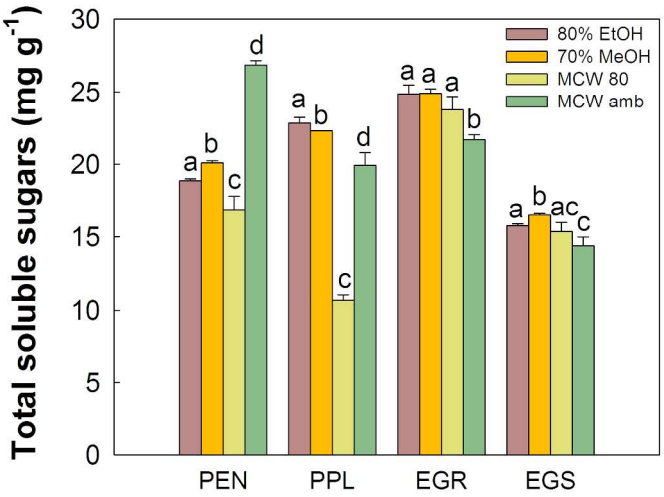


Figure 4. Means and standard errors for soluble sugars by extraction method for samples processed in one laboratory and using the same quantification method. Results show that extraction method can affect estimates especially for PEN and PPL samples. In all samples MCW-based methods produced consistently lower estimates than alcohol-based methods. Different letters indicate significant difference at α=0.05 according to F-protected LSD test.
215x279mm (300 x 300 DPI)

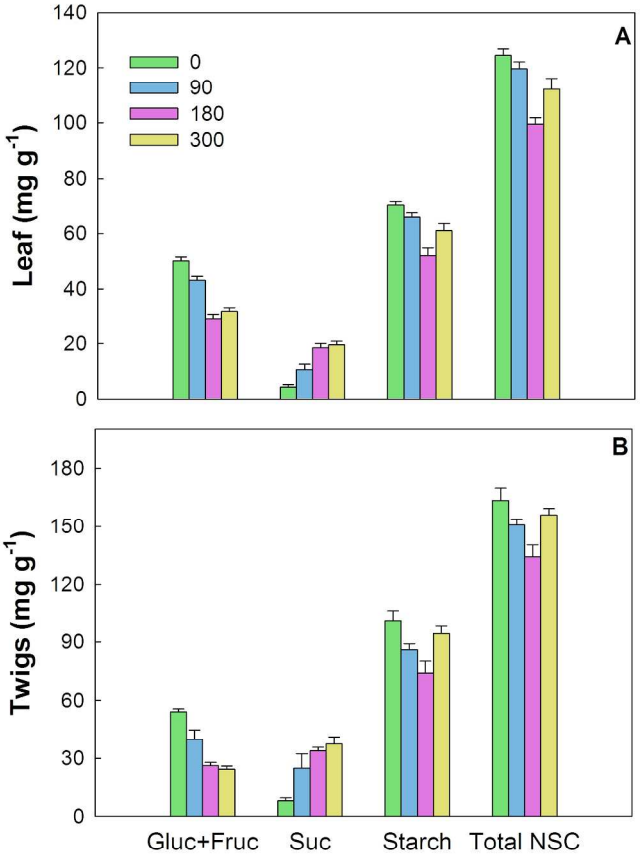


Figure 5. Effect of microwaving samples < 5 g at 800W on amount of glucose + fructose (Gluc+Fruc), sucrose (Suc), starch and total non-structural carbohydrate (NSC) for foliar (A) and twig (B) samples of Pinus edulis. See Method S4 for details on the method. At 0 and 90 s microwaving time, sucrose hydrolyzing and starch debranching enzymes are still active, leading to lower sucrose levels, higher glucose + fructose levels, and higher starch levels because debranching enzyme make starch more accessible to the enzymatic assay. At 180 s and above, enzymes are deactivated, yielding consistent sucrose and glucose + fructose. At 300 s, starch starts to gelatinize, again making it more accessible to the assay. Orthoginal contrasts for trend with microwaving time: glucose+fructose, quadratic for leaf and twig, $P < 0.05$; sucrose, linear for leaf and twig, $P < 0.001$; starch, quadratic for leaf and twig, $P < 0.01$; total NSC, quadratic for leaf and twig, $P < 0.01$.
215x279mm (300 x 300 DPI)

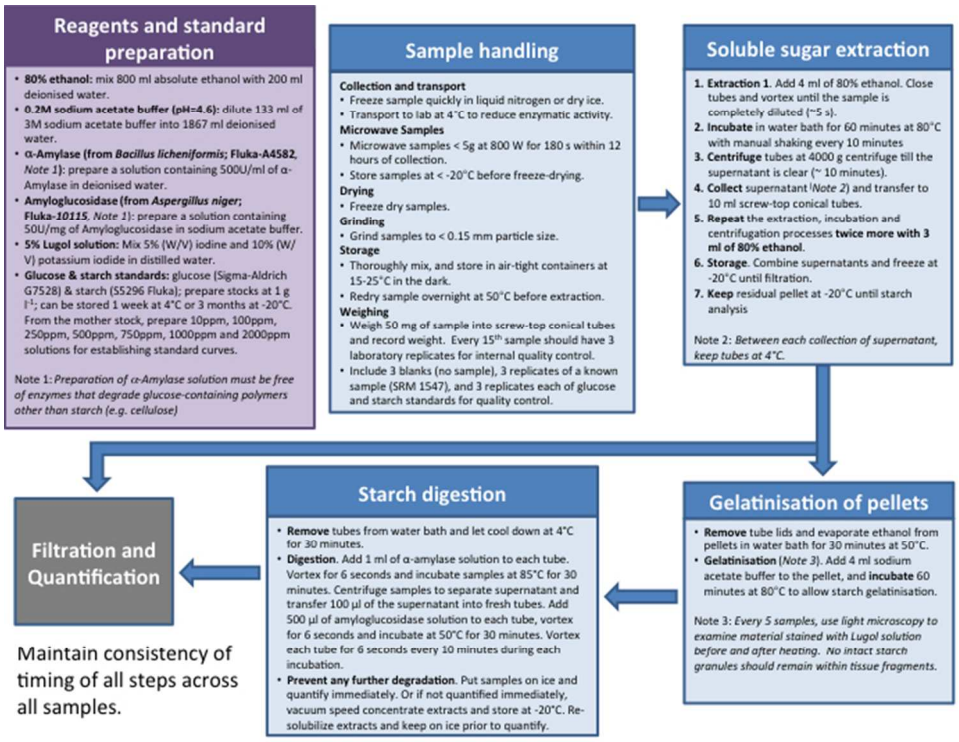


Figure 6. Instructions for sample collection, handling, preparation, and sugar and starch extraction for Reference Method.
254x190mm (72 x 72 DPI)