Non-structural carbohydrates in woody plants cannot be quantitatively compared among laboratories

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Summary

Quantifying non-structural carbohydrates (NSC) in plant tissue is frequently employed to make inferences about plant responses to environmental conditions. Laboratories recently publishing estimates of NSC of woody plants used 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 matrix, to 29 laboratories. Each laboratory analysed 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 25-125 (mean = 59) mg g^{-1} for soluble carbohydrates, 0.5-535 (mean = 94) mg g^{-1} for starch and 30-600 (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. For *Eucalyptus globulus* leaves, differences between the highest and lowest least-squares means in categories in the mixed model analysis were 33 mg g^{-1} for total NSC, compared to the range of laboratory estimates of 600 mg g^{-1} or a 90^{th}-percentile range of 154 mg g^{-1}. Laboratories were reasonably consistent in their ranks of estimates among tissues for starch (r= 0.46-0.92), total NSC (r= 0.49-0.85), and soluble sugars (r= 0.17-0.84). Our results show that NSC estimates for woody plant tissues cannot be quantitatively compared among laboratories. However, the relative changes in NSC between treatments measured within a laboratory may be comparable within and between laboratories. To obtain comparable quantitative NSC estimates, we argue that users should identify and adopt standard methods and use standard tissues as reference material.

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

Running head: Comparing NSC content among laboratories.

Introduction

Non-structural carbohydrates (NSC) are the immediate products of photosyntheis, 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). Despite their importance, the role of NSC in physiological, ecological, and evolutionary plant responses to biotic and abiotic stimuli has been one of the most challenging subjects in plant science. For example, the "growth-differentiation hypothesis" (Loomis 1932), the "carbon/nutrient hypothesis" (Bryant et al. 1983), the "hydraulic limitation hypothesis" (Ryan and Yoder 1997), and the "carbon limitation hypothesis" (Körner 2003) all outline a role for NSC. In more recent years, NSC of woody plants has received wider attention for understanding drought-induced mortality (McDowell et al. 2008, Muller et al. 2011, Piper 2011, Mitchell et al. 2013, O'Brien 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 or passive (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).

NSC consist of many carbohydrates: sugar alcohols (inositol, sorbitol and mannitol), monosaccharides (glucose and fructose), disaccharides (e.g. sucrose), oligosaccharides (raffinose) and polysaccharides (starch and fructans) (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 sugar (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 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 S1). 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 S1). Studies have also used the same extraction and assay methods to analyse different tissues (leaves, stems, roots) that consist of different matrices (see Table S1), 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, 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, interest in quantifying NSC to understand its role and regulation is growing. A recent search of ISI Web of Science© for 'non-structural carbohydrate* and (tree or forest)' found 352 publications for the past 25 years. The rate of increase since 2005 (2.4 publications/year) is four times that of 1991-2004 (0.6/year) and total publications in the past 10 years (229) are nearly double those from the period of 1991-2004. Second, such a comparison would allow plant ecophysiologists studying NSC role and regulation to assess and compare their own results. Third, 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 quantitatively compared across the laboratories that use NSC estimates to understand plant response to a variety of biotic and abiotic factors (Table 2). Most 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, which are described in recent publications (Table 2).

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 conclusively 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 and temperature 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 Methods 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 S2 & S3). Table 1 summarises the procedures used in this study to measure soluble sugars and starch in plant tissues and Tables S2 & S3 provide 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).

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 correlation to evaluate the consistency of laboratories across the different sample types 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 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).

We examined the differences between soluble sugar extraction methods on total NSC in the same laboratory with an ANOVA for each sample type ($\alpha = 0.05$). 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, even excluding outliers by restricting the range to the 5-95 percentile of the data. For example, in *Eucalyptus globulus* leaves (EGL), 5-95th percentile laboratory estimates ranged from 32-85 mg g^{-1} (CV 25%) for total soluble sugars, 7-161 mg g^{-1} (CV 52%) for starch, and 65-220 mg g^{-1} (CV 36%) for total NSC (Figs. 1A, 1B). Laboratory estimates for *Prunus* leaves (PPL, average CV=71%) were more variable than those for other samples (average CV=32-50%) for all NSC components, and

starch estimates were more variable among laboratories (CV 47-88%) than were soluble sugars and total NSC (CV 19-62% for sugars and 29-64% for total NSC, Figs. 1A, 1B). For all samples and NSC components, only 27% of the laboratories were within the 95% confidence intervals estimated for the 5-95th percentile means. Laboratories were most consistent for starch estimated for the *Eucalyptus* stem (EGS) sample (16 of 28 laboratories were within the 95% confidence intervals), and least consistent for starch estimates for *Prunus* leaves and total NSC estimated for *Eucalyptus* and *Prunus* leaves (only 3 of 28 laboratories were within the 95% confidence intervals). The subset of the laboratories that identified sucrose and glucose+fructose (n=20) were relatively consistent, having an average of 50% or 10 of 20 laboratory estimates within the 95% confidence intervals estimated for the 5-95 percentile means (range = 6-14 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 (Fig. S1). For example, NSC in the PPL sample showed high variability among laboratories (Fig. S1B), 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 (0.46-0.92) and total NSC (0.46-0.85) than for soluble sugars (0.17-0.84). 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, Figs. 3C, 3E, 3G, 3I). For the same pooled analysis, starch estimates were lower for ethanol+water sugar extraction than for the other three sugar extraction categories (Fig. 3B), but did not differ by starch extraction or quantification categories (Figs. 3D, 3H). Sugar estimates did not vary by extraction method category (Fig. 3A), but did by sugar quantification method category (Fig. 3F), 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).

To assess how differences among methods compared with differences among laboratories, we compared the highest and lowest least squares means for the methods from the linear mixed model analysis with the 5-95th percentile range of the data. The greatest difference between the least squares means for methods was lower than the 5-95th percentile data range in most cases, and varied with the sample that was measured. For example, the difference between the highest and lowest least squares means for the overall effect of soluble sugar extraction for starch estimates was 30 mg g^{-1}, compared to the 5-95th percentile ranges of starch of the five samples of 50-154. The difference between the highest and lowest least squares means for the overall effect of soluble sugar quantification for sugar estimates was 25 mg g^{-1}, compared to the 5-95th percentile ranges of soluble sugars of the five samples of 26-71. The highest and lowest least squares means for the overall effect of starch quantification on total NSC estimates (not significant) was 50 mg g^{-1}, compared to the 5-95th percentile ranges of total NSC estimates for five samples of 97-154. While a coefficient of variation is undefined for factors in a mixed model analysis, this comparison suggests that method differences in our analysis accounted only for a portion of differences in NSC among laboratories.

Method effects differ by sample (Objective 3)

Sample and method had strong interactions (Table 4), with the foliar samples (EGL, PEN and PPL) showing more variation among methods 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 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).

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.

Discussion

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

Results demonstrate that quantitative 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 quantitative values for any NSC component across studies in the literature may generate more confusion than insight, both for individual studies and for meta-analyses (e.g., Ainsworth et al. 2002, Morgan et al. 2003, Wittig et al. 2009).

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

The Spearman rank correlation analysis of sample pairs showed that laboratory ranks were consistent among the five samples, especially for starch. These results suggest that relative differences among treatments and species within a laboratory are 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, a *qualitative* assessment of responses or determination of relative responses of different treatments to a control, are robust and can be used 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 quantitative 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 S1, S2, S3) 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 within a method category (giving little or no replication) meant that we could not evaluate these factors, thereby limiting us to evaluation of our 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 five samples (Fig. 4).

The lack of differences among soluble sugar extraction method categories (P=0.12, Table 4), coupled with the results of different methods in 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 ($P < 0.01$). 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 both 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, 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 Document Note S1). Clearing interferring compounds from the solvent might minimise these effects (Thompson and Ellison 2005). 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. 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.

Comparability might be solved using two approaches: 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. However, given the investment laboratories have in current techniques, we suspect that selecting a standard method would not be simple. 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). 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 a huge investment for laboratories to comply with the selected standard. Adoption of either approach would depend on the cooperation of the science community.

While the aim of this study is not to give a consensus recommendation on any 'best' method, 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 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, when 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 cheap, 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. However, this method requires a specific enzyme for each sugar, which could make it relatively expensive and a lengthy process. The major limitation of the enzymatic method is that many non-targeted oligosaccharides can be converted to monosaccharides and confound the results. For instance, in this study, three laboratories using the enzymatic method reported negative results for sucrose, which is extracted in a second step of enzymatic digestion (Figs. 1A, 1B; Table S2). Negative results are not normally reported as such in the literature and usually assumed to be zero, but also indicate that the target sugar has been converted. The enzymatic method may be useful only to identify the type of carbohydrates present, but perhaps not their relative concentrations.

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) 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. However, a few studies suggest we should look deeper. For example, sorbitol is found in high concentrations in *Prunus persica* leaves (Zhang et al. 2013), and raffinose concentration was greater than that of starch in birch buds (Ruuhola et al. 2011).

Conclusions and recommendations for the future

Reliable NSC analyses require that the best possible techniques be employed in each step, beginning with sample collection (see Supporting Information Method S4 and Figure S4), processing (see Supporting Information Method S5 and Figure S5) and ending in final analysis (see Supporting Information Note S2 and Figure S6). We feel compelled to remind investigators that the choice of a method should not rely on its logistical aspects (costs, duration); but rather on its ability to produce consistent, reliable and accurate data across a range of samples and conditions. In this study, we highlighted pitfalls in comparing absolute values of NSC among laboratories, and suggest caution when comparing studies that use different methods and techniques. Discrepancies among laboratories only partly reflect differences in analytical methods. However, we are aware that the major differences among laboratory estimates remain largely unexplained, as many factors could not be accounted for in our analyses. Indeed, interfering compounds in plant samples and other method practices are likely another major source of uncertainty. It seems clear that standardizing methods to analyse NSC in plant tissue is a serious challenge. We recommend the following to help the research community move towards more standardized NSC analysis that is comparable both among and within laboratories:

- 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. These methods should be followed exactly as specified without modification. Research should report NSC values of the currently commercially available peach leaf standard (SRM 1547).
- Quality assurance measures such as 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.
- Researchers should implement standard procedures of internal quality control (IQC) and include a detailed description of this procedure to the method. Analytical results should therefore have an evaluation of 'measurement uncertainty' attached to them given by the CRM and an informed interpretation to help users and readers in determining the accuracy of a method for a specific type of sample.

The problem we have highlighted here, that NSC results are not quantitatively comparable among different laboratories, will likely limit crucial research into plant response to environmental stress, although we can still rely on relative responses. 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.

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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 S2 and S3.

Table 2. A sample of publications within the last 10 years that use the methods examined in this study. This is not an exhaustive list, but represents one or two publications from the participating laboratories. Many other recent publications also use the methods examined in this study.

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.

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.

Figure Legends

Figure 1. Laboratory estimates of (A) sucrose, glucose+fructose, total soluble sugar, and (B) starch and non-structural carbohydrates (NSC) for each five samples: *Eucalyptus globulus* leaves (EGL), *Pinus edulis* needles (PEN), *Prunus persica* leaves (PPL), *E. globulus* roots (EGR) and *E. globulus* stem (EGS). Samples are ranked within a soluble sugar or starch extraction category. Means (text and solid line), range, coefficient of variation (CV) and 95% confidence interval (dashed lines) are estimated from the 5-95th percentile data values for each graph to reduce outlier influence. The graphs show that estimates differed substantially among laboratories and within method categories.

Figure 2. Correlations of laboratories between sample pairs that show the worst and best correlations for soluble sugars, starch and total NSC. Plots show that laboratory rankings are reasonably consistant for the different samples. Spearman rank correlations for all pairs are in Table 2.

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. 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. * indicates significant differences among method within each tissue (a=0.05, Tukey-Kramer test). Plots show that method category generally had little effect on NSC difference, perhaps because of high within-method variance.

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 effect 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 a=0.05 according to F-protected LSD test.

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 S2 and S3.

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
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 (°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 ; 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. A sample of publications within the last 10 years that use the methods examined in this study. This is not an exhaustive list, but represents one or two publications from the participating laboratories. Many other recent publications also use the methods examined in this study.

Experiment	**Species**	**Reference**
Drought, temperature	*P. edulus*	Adams et al. (2013)
Girdling	*Hieronyma alchorneoides, Pentaclethra macroloba, Virola koschnyi, Vochysia guatemalensis*	Asao and Ryan (2015)
[CO_2], temperature	*E. saligna*	Ayub et al. (2011)
Carbohydrate distribution	*Juglans regia*	Bonhomme et al. (2010)
GM modification	*Populus alba x grandidentata*	Coleman et al. (2009)
Drought, [CO_2], temperature	*E. globulus*	Duan et al. (2013)
Seasonality	*Quercus petraea, Fagus sylvatica*	El Zein et al. (2011)
Altitude	Abies georgei var. smithii	Genet et al. (2011)
Photoperiod	*Arabidopsis thallinia*	Gibon et al. (2009)
Fungal invasion	*Pinus contorta*	Goodsman et al. (2013)
Drought	*Quercus douglasii, Quercus ithaburensis, Quercus agrifolia, Quercus calliprinos*	Grunzweig et al. (2008)
Drought	*Picea abies*	Hartmann et al. (2013)
Temperature regimes	*Larix decidua and Pinus mugo*	Hoch and Körner (2009)
Pruning after frost	*Vitis vinifera*	Jones et al. (2013)
[CO_2], drought	*Betula platyphylla var. japonica*	Kitao et al. (2007)
Drought	*P. edulus*	McDowell et al. (2008), Dickmann et al. (2014), Sevanto et al. (2014)
Frankincense tapping	*Boswellia papyrifera*	Mengistu et al. (2013)
Drought	*E. globulus, Pinus radiata*	Mitchell et al. (2014)

Experiment	**Species**	**Reference**
Girdling	*Citrus sinensis*	Nebauer et al. (2011)
Browsing	*Betula pubescens*	Palacio et al. (2007)
Tree age, climate	*Nothofagus pumilio*	Piper (2011)
Defoliation	*Eucalyptus globulus*	Quentin et al. (2011) Pinkard et al. (2011)
Tree age	*P. ponderosa*	Sala and Hoch (2009)
Budbreak	*Carpinus betulus, Fagus sylvatica, Picea abies, Pinus sylvestris*	Schadel et al. (2009)
Tree height	*Pseudotsuga menziesii*	Woodruff (2014)

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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.

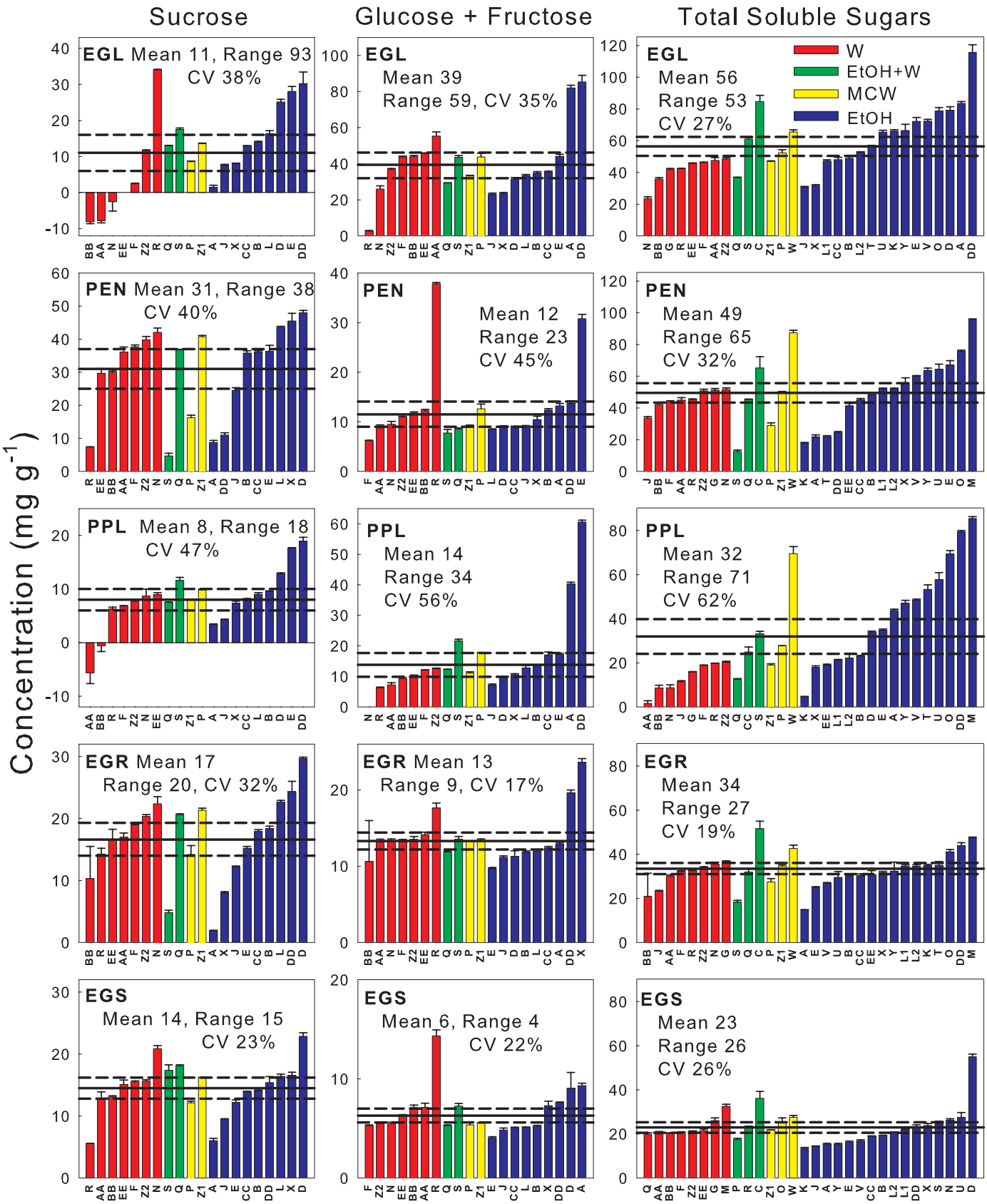
	EGL	EGR	EGS	PEN	PPL
A. Soluble sugars					
EGL					
EGR	0.33				
EGS	0.11	0.73**			
PEN	0.29	0.52**	0.41*		
PPL	0.83**	0.39*	0.37*	0.41*	
B. Starch					
EGL					
EGR	0.69**				
EGS	0.59**	0.87**			
PEN	0.47*	0.84**	0.91**		
PPL	0.41*	0.68**	0.84**	0.82**	
C. Total NSC					
EGL					
EGR	0.59**				
EGS	0.49**	0.69**			
PEN	0.47*	0.84**	0.63**		
PPL	0.50**	0.54**	0.55**	0.71**	

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

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				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


Sucrose
Glucose + Fructose
Total Soluble Sugars
Concentration (mg g-1)
EGL Mean 11, Range 93 CV 38%
EGL Mean 39 Range 59, CV 35%
EGL Mean 56 Range 53 CV 27%
W
EtOH+W
MCW
EtOH
PEN Mean 31, Range 38 CV 40%
PEN Mean 12 Range 23 CV 45%
PEN Mean 49 Range 65 CV 32%
PPL Mean 8, Range 18 CV 47%
PPL Mean 14 Range 34 CV 56%
PPL Mean 32 Range 71 CV 62%
EGR Mean 17 Range 20, CV 32%
EGR Mean 13 Range 9, CV 17%
EGR Mean 34 Range 27 CV 19%
EGS Mean 14, Range 15 CV 23%
EGS Mean 6, Range 4 CV 22%
EGS Mean 23 Range 26 CV 26%

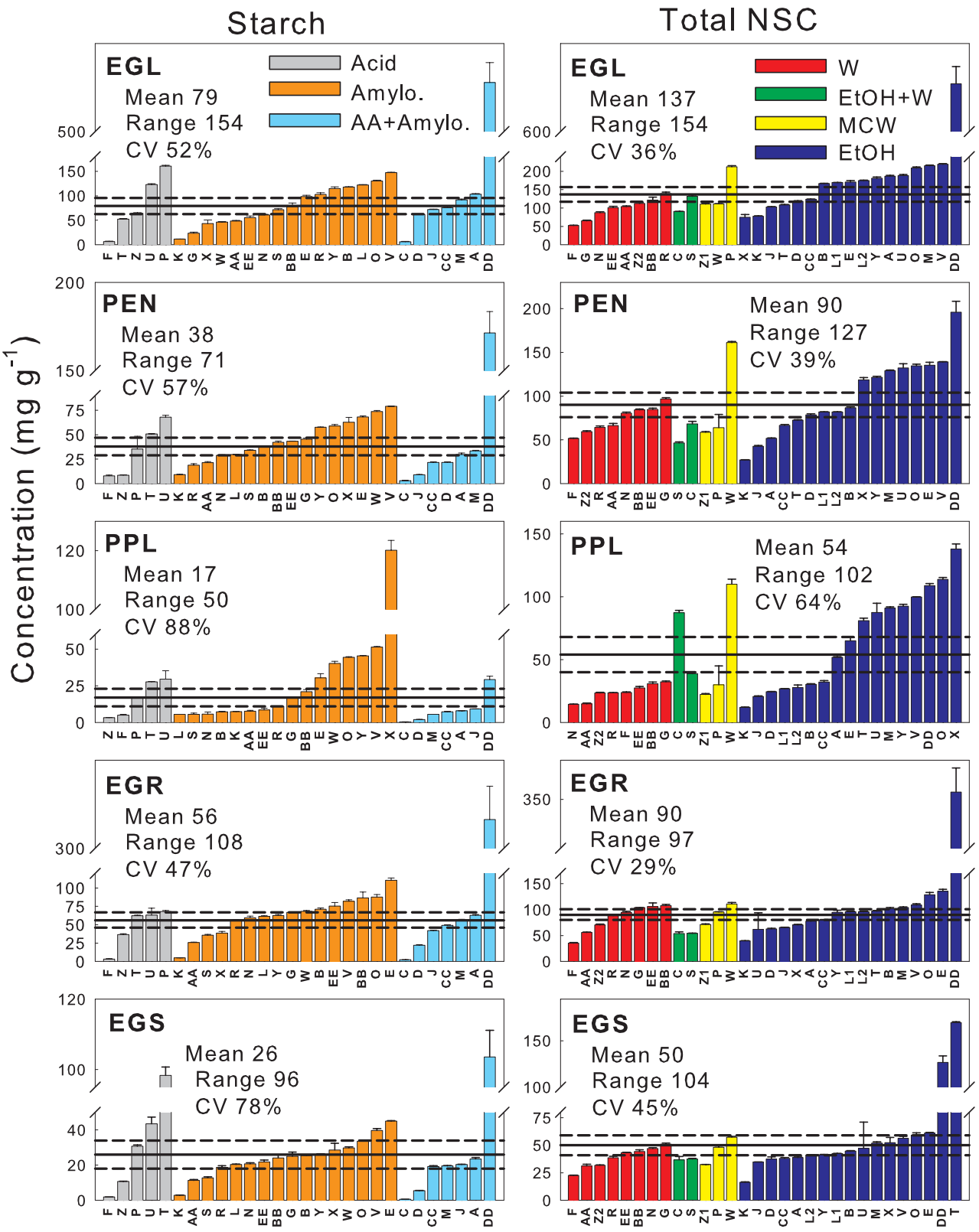


Figure 1. Laboratory estimates of (A) sucrose, glucose+fructose, total soluble sugar, and (B) starch and non-structural carbohydrates (NSC) for each five samples: *Eucalyptus globulus* leaves (EGL), *Pinus edulis* needles (PEN), *Prunus persica* leaves (PPL), *E. globulus* roots (EGR) and *E. globulus* stem (EGS). Samples are ranked within a soluble sugar or starch extraction category. Means (text and solid line), range, coefficient of variation (CV) and 95% confidence interval (dashed lines) are estimated from the 5-95th percentile data values for each graph to reduce outlier influence. The graphs show that estimates differed substantially among laboratories and within method categories.

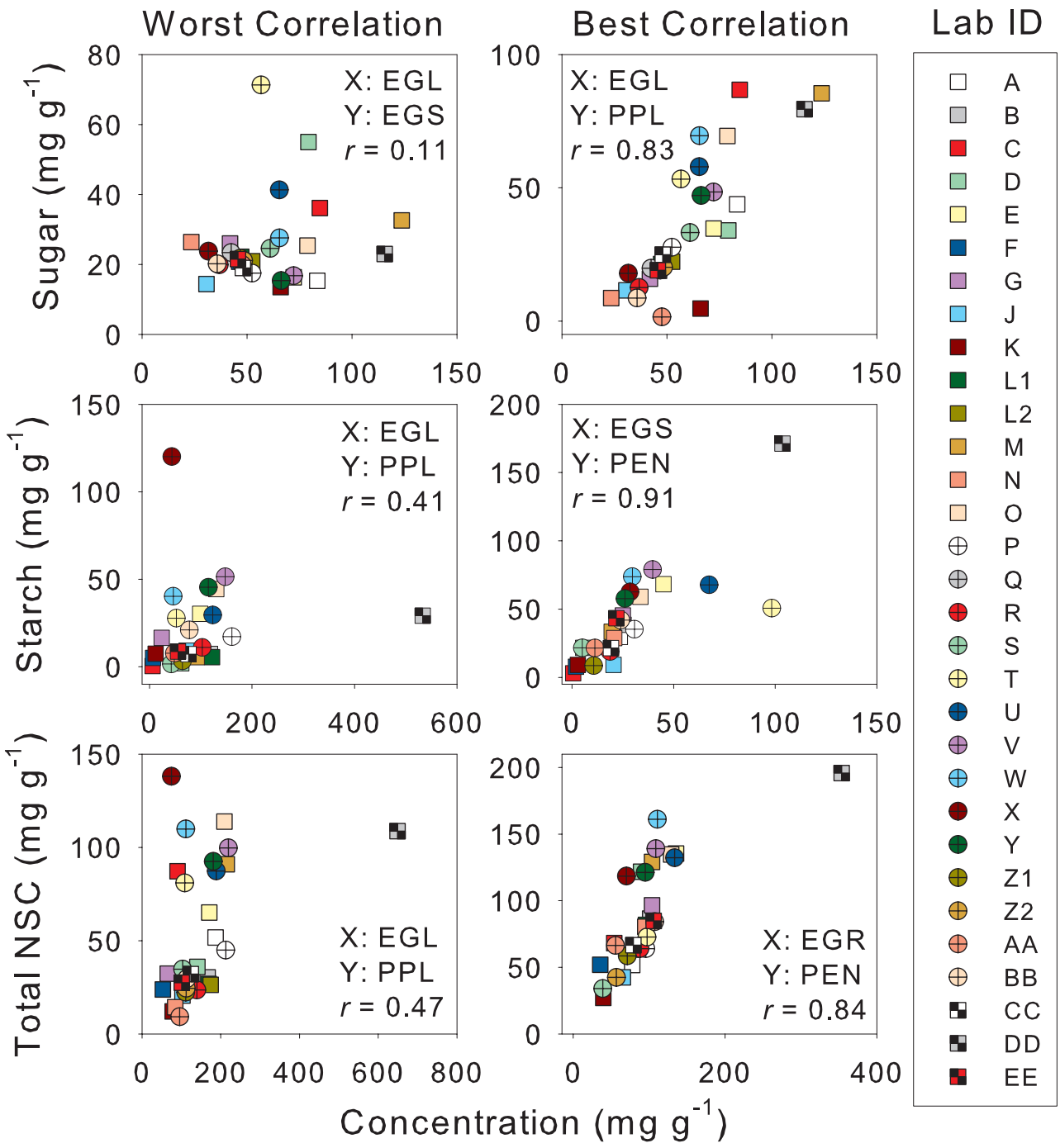


Figure 2. Correlations of laboratories between sample pairs that show the worst and best correlations for soluble sugars, starch and total NSC. Plots show that laboratory rankings are reasonably consistant for the different samples. Spearman rank correlations for all pairs are in Table 2.

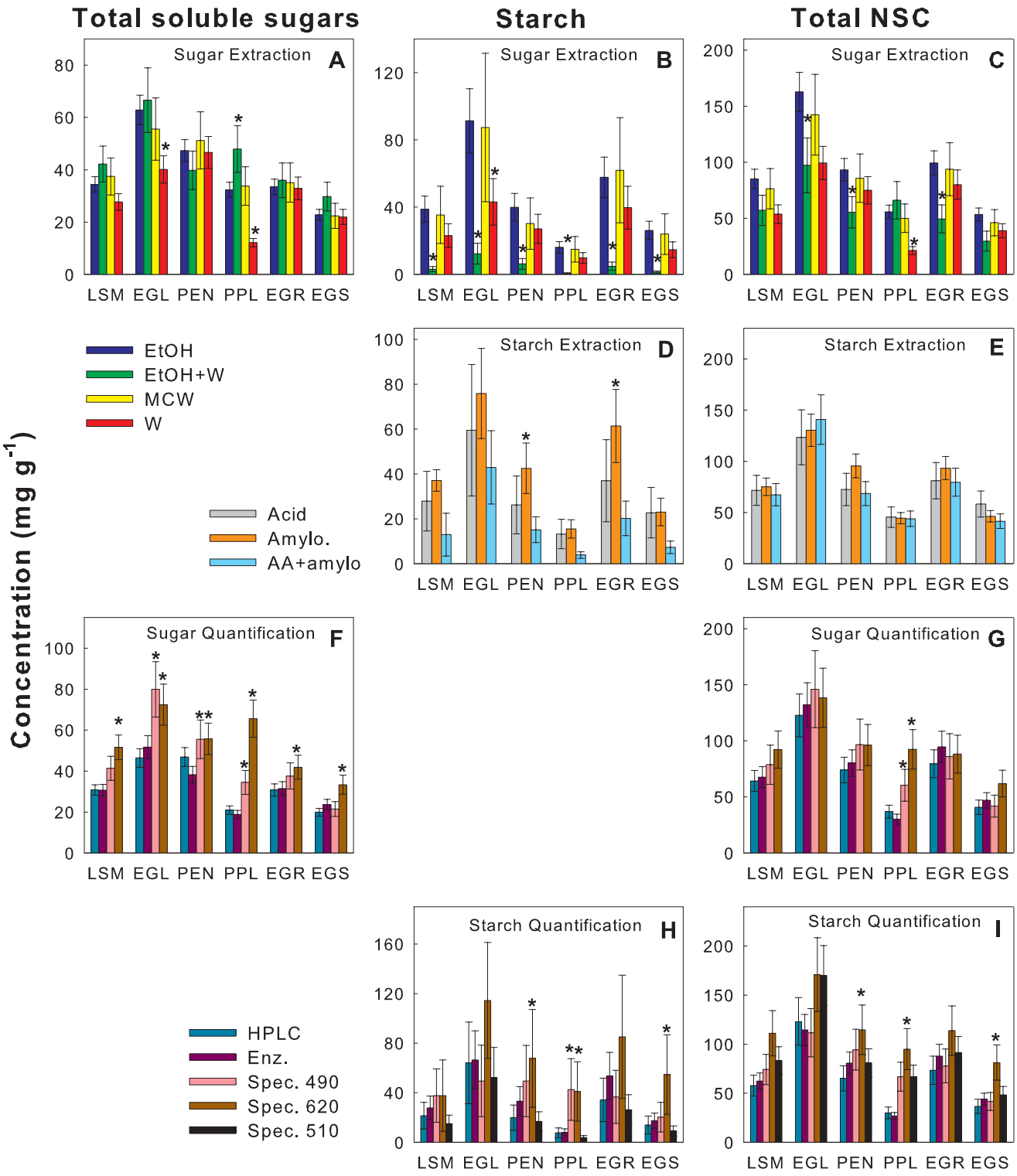


Figure 3. Differences in least squares means for all samples (LSM) and for individual samples (EGL, PEN, PPL, EGR, EGS) for the extraction and quantification methods for soluble sugars, starch and total NSC. Error bars are standard errors for the least square means. Total soluble sugars results are grouped 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. * indicates significant differences among method within each tissue (a=0.05, Tukey-Kramer test). Plots show that method category generally had little effect on NSC difference, perhaps because of high within-method variance.

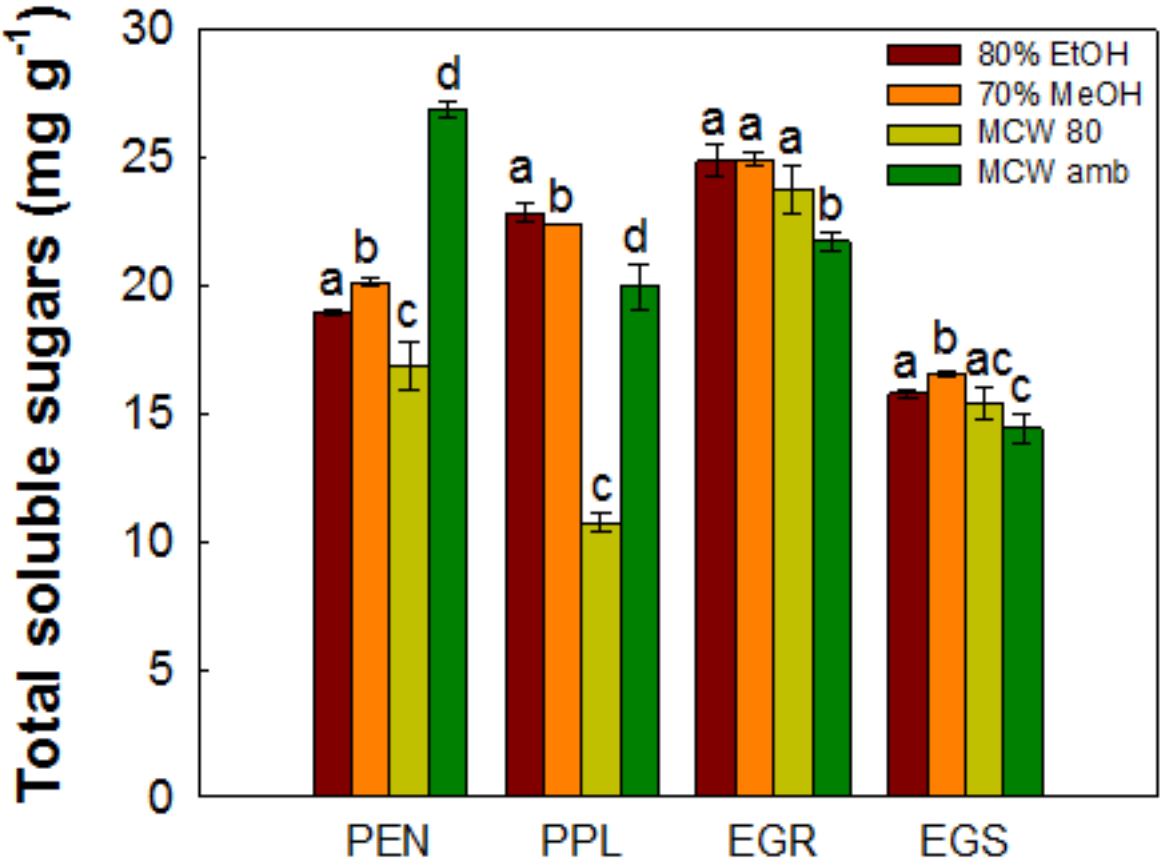


Figure 4. Means and standard errors for soluble sugars by extraction method for samples processed in one laboratory and using the same quantification method. Results show that extraction method can effect estimates especially for PEN and PPL samples. In all samples MCW-based methods produced consistently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