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**A high throughput method using Ultra Performance Liquid Chromatography to
determine water soluble carbohydrate concentrations in pasture plants**

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Abstract

The determination of water soluble carbohydrate (WSC) concentrations in forages is important for quantifying forage-feed quality as WSC are not only a major source of energy but are also associated with the palatability of forages and subsequent intake by livestock. This paper describes a high-throughput Ultra Performance Liquid Chromatography (UPLC) method to analyse WSC concentrations of various forage samples. The application of the new method is demonstrated by investigating the WSC concentrations in (1) temperate forages at different tiller heights and (2) summer forage crops grown under rain-fed and irrigated conditions. The UPLC method provided a more descriptive, specific and precise analysis of WSC concentration compared to the colorimetric method. Grass tiller height had a significant effect on total WSC concentration and individual sugar. With increasing tiller height, sucrose concentration increased and fructan and total WSC concentrations decreased. While irrigation treatment increased the dry matter yield of all nine summer forage crops analysed, there was no consistent effect of irrigation on WSC concentration.

Abbreviations used

CP: crude protein; DM: dry matter; DP_{avg} : average degree of polymerization; ELSD: evaporative light scattering detector; HPLC: high performance liquid chromatography, RFO: raffinose family oligosaccharides; RO: reverse osmosis; UPLC: ultra performance liquid chromatography; WSC: water soluble carbohydrate

1. Introduction

Water soluble carbohydrates (WSC), which consist of simple sugars and complex storage carbohydrates such as fructans, are important nutrients that affect the nutritional value of pastures. The concentration of WSC in forages is associated with grazing preference by ruminants (Horadagoda, Fulkerson, Nandra, & Barchia, 2009; Smit, Tamminga, & Elgersma, 2006) and a high forage WSC concentration is considered essential for ensiling, as WSC provide the primary source of fermentable carbohydrates (Pettersson & Lindgren, 1990). It has been shown that there is a positive relationship between WSC concentration of post-defoliation residual and subsequent perennial ryegrass regrowth (J. M. Lee, Donaghy, Sathish, & Roche, 2009). In addition fructan, a carbohydrate reserve of plants, has an important role in protection of plants under conditions of drought and low-temperature stress (Liu, Mouradov, Smith, & Spangenberg, 2011). The potential of utilizing forages high in WSC to improve animal productivity through more efficient use of nitrogen and metabolisable energy has been demonstrated (M. R. F. Lee et al., 2003) and in response, plant breeding programs have selected for higher WSC concentrations (Easton, Stewart, Lyons, Parris, & Charrier, 2009).

Pasture management systems are often employed to increase dry matter (DM) yield and ensure optimal pasture nutritive value in order to maximize livestock production. This can be achieved through strategies such as irrigation, grazing management or fertilizer application. Pasture sward height has significant effect on forage nutritional characteristics which in turn influence ingestive behaviour (e.g. bite size and bite rate) of cattle (Fonseca et al., 2013; Ketelaar-De Lauwere et al., 2000) and lambs (Armstrong, Robertson, & Hunter, 1995). A significant vertical gradient in nutritional components of perennial ryegrass has been observed (Delagarde, Peyraud, Delaby, & Faverdin, 2000), and these variations were often greater than the variation observed in whole plants at different growth stages. Hence, one of the objectives of this study was to determine total WSC concentration and its composition in different tiller height fractions of temperate grasses.

Irrigation is an important farm management practice used to increase forage DM production (Eckard, Salardini, Hannah, & Franks, 2001; Jacobs, Ward, McKenzie, & Kearney, 2006). However, both Eckard et al. (2001) and Jacobs et al. (2006) reported that irrigation treatment had an inconsistent effect on the nutritional characteristics of different forage species. For example, irrigation treatment significantly reduced WSC concentration of millet but had no significant effect on WSC concentration of a hybrid brassica species (Jacobs et al., 2006). Water soluble carbohydrates have been associated with tolerance to environmental stress, including

drought (Moraes, Chatterton, Harrison, Filgueiras, & Figueiredo-Ribeiro, 2013). A further objective of this paper was to investigate the effect of irrigation on the total WSC and individual sugar concentrations of nine different summer forage crops.

The two most common methods of analysing WSC concentration in forages are colorimetry (Avigad, 1990) and liquid chromatography (Liu et al., 2011). There are a number of colorimetric assays for analysing WSC, based on various reagents. Examples include the anthrone assay (Trethewey & Rolston, 2009), reducing sugar assay and phenol-sulphuric acid assay (Hall, 2013). The colorimetric method based on anthrone, is a rapid, low cost and convenient method for the determination of carbohydrates, however, it does not distinguish between simple sugars and other complex carbohydrates and uses a single glucose calibration to quantify all WSC. Hall (2013) has shown that the results of colorimetric assays (reducing sugar and phenol-sulfuric acid assays) varied with the sugar standards chosen. Hence quantifying the WSC concentration using just glucose standard could lead to inaccurate estimation of WSC concentration. Conversely WSC analysis using liquid chromatography not only provides a total WSC concentration, but also individual WSC components. The individual WSC components are quantified using various sugar standards, thereby producing more accurate results (Moraes et al., 2013). Currently there are a number of methods using High Performance Liquid Chromatography (HPLC) for analysing WSC in forages (Chatterton, Harrison, Thornley, & Draper, 1990; Kagan, Kirch, Thatcher, Teutsch, & Pleasant, 2014). While HPLC methods provide accurate results, sample throughput is limited by long run times (e.g. 40 min). The introduction of Ultra Performance Liquid Chromatography (UPLC) offers a high throughput alternative to HPLC. This paper describes a high-throughput UPLC method to analyse total WSC and individual sugar concentrations of various forage samples.

2. Methods and materials

2.1 Chemicals

Thiourea was obtained from Ajax Chemical (Auburn, VIC, Australia). Calcium chloride dehydrate, maleic acid and trisodium citrate dehydrate were obtained from BDH Merck Ltd (Poole Dorset, UK). Glacial acetic acid and sodium hydroxide pellet were obtained from Chem Supply (Gillman, SA, Australia). Acetonitrile was obtained from Merck (Darmstadt, Germany). Hydrochloric acid (32%) and sulphuric acid (97%) were obtained from Scharlau Chemie s.a (Sentmenat, Spain). Anthrone, fructose, glucose, p-hydroxybenzoic acid hydrazide, maltose,

raffinose, stachyose and sucrose were obtained from Sigma Aldrich (St. Louis, MO, USA). All reagents used were of analytical grade, the solvents were of HPLC grade and MilliQ water was used for preparing solvents for all UPLC runs.

2.2 Sugars and fructans analysis

2.2.1 WSC - anthrone method

The colorimetric determination of WSC concentration in the forage samples in the method development study were carried out using Australian Fodder Industry Association (AFIA) Method 1.11R (AFIA, 2014). Briefly, forage samples (0.1 g) were extracted into 50 mL reverse osmosis (RO) water by mixing at room temperature, using a rotary mixer for 1 h. A 1 mL aliquot of the extract and 5 mL WSC reagent (consisting of 25 N (c. 70%) sulphuric acid with 0.9 mg/L thiourea and 0.9 mg/L anthrone) were placed in boiling water for 20 min. The absorbances of the resultant mixtures were measured at 620 nm and WSC concentration in the samples were calculated using a glucose standard calibration curve.

2.2.2 Fructan – Megazyme method

The colorimetric determination of fructans in the WSC extract was undertaken using Megazyme Method (K-FRUC 11/12; Megazyme, Bray, County Wicklow, Ireland) (McCleary et al., 2000)).

2.2.3 WSC and Fructan – UPLC method

A method based on UPLC was developed which was adapted from an existing HPLC method by Liu et al. (2011). Ground plant material (0.2 g) was extracted in RO water (12 mL) and heated to 90°C for 60 min with vortexing at 10 min intervals. The mixture was cooled to room temperature and centrifuged at 3,000 rcf for 10 min (Eppendorf Centrifuge 5810, Hamburg, Germany). The resultant supernatant (termed WSC extract) was used for subsequent analyses.

Liquid chromatographic analyses were undertaken using UPLC system (Waters Corporation, Milford, MA, USA) which consisted of UPLC Binary Solvent Manager (BSM) (H10UPB941A), UPLC Sample Manager (G10UPA77M) and UPLC Evaporative Light Scattering Detector (ELSD) (K09UPE660M). The column used for sugar and fructan analysis was an ACQUITY Amide column 1.7µm, 2.1×100 mm (Waters Corporation, USA). The chromatographic data were collected and analysed using the Empower 3 Software (Waters Corporation, USA).

The Waters UPLC ELSD settings were as follow;

Nebuliser: Cooling; Gain: 100

Drift tube: 50°C; Gas: 35 psi

The Waters UPLC BSM settings were as follow;

Solvent A1: 80% Acetonitrile with 0.05% ammonia

Solvent B1: 30% Acetonitrile with 0.05% ammonia

Gradient: 90% A1 to 70% B1 over 10 min at 0.13 mL/min with subsequent 7 column volume re-equilibration with 90% A1

For individual sugar analysis, the WSC extract (0.75 mL) was mixed with acetonitrile (0.75 mL) and the mixture was centrifuged at 10,000 rcf for 10 min (Eppendorf Centrifuge 5430R, Hamburg, Germany). The supernatant was filtered through a 0.2 µm PTFE syringe filter (Grace Davison Discovery Sciences, Illinois, USA). The filtrate (2 µL) was injected into the UPLC to determine sugar concentrations in the sample.

For fructan analysis, the WSC extract (0.8 mL) was mixed with 0.2 N sulphuric acid (0.2 mL) and incubated at 80°C for 120 min. Acetonitrile (0.6 mL) was added to the resultant acid digest and the mixture was vortexed followed by centrifugation at 10,000 rcf for 10 min. The supernatant was filtered through 0.2 µm syringe filter and the filtrate (2 µL) injected into the UPLC to determine glucose and fructose concentrations in the samples. Fructan concentration in the samples was calculated using following equations (Liu et al., 2011):

$$M_f = k (G_f + F_f) \text{ where,}$$

$$G_f = G_{\text{total}} - G_{\text{free}} - 0.53S_{\text{free}},$$

$$F_f = F_{\text{total}} - F_{\text{free}} - 0.53S_{\text{free}},$$

$$k = [180 + 162(n-1)] / 180n, \text{ and}$$

$$n = DP_{\text{avg}} = (F_f / G_f) + 1$$

(M_f = mass of fructans; G_f = glucose released from fructans; F_f = fructose released from fructans; G_{total} = total glucose in H_2SO_4 digest; G_{free} = glucose in WSC extract; F_{total} = total fructose in H_2SO_4 digest; F_{free} = fructose in WSC extract; S_{free} = sucrose in WSC extract; k = correction factor for water uptake during hydrolysis and DP_{avg} = average degree of polymerization)

In the Megazyme fructan assay, the procedural-step to preclude sucrose and reducing sugars does not affect raffinose family oligosaccharides (RFO) and hence these WSC would be measured as fructans (Jensen, Harrison, Jerry Chatterton, Shaun Bushman, & Earl Creech, 2014).

Similarly in UPLC method for the fructan analysis, the acid hydrolysis step does not differentiate between fructans and RFO and sucrosyloligosaccharides and thus the measured fructan value would include all of these carbohydrates.

2.3 Forage samples

In the method development study, five diverse forage samples consisting of oaten hay, timothy hay, chicory, lucerne and perennial ryegrass were used. The samples were dried at 60°C for 72 h and ground to pass through a 1 mm sieve, and stored in an airtight container until further analysis.

Samples used in the tiller height study were obtained from a replicated experiment (n=3) grown at Ellinbank, Victoria, Australia (38° 14' S, 145° 55' E). The experimental plots were grown on acidic, mesotrophic, red ferrosol top soil. The site has a temperate climate, with an average annual rainfall of ~1000 mm. Four different temperate grass varieties perennial ryegrass (*Lolium perenne* L. cv. Bealey); prairie grass (*Bromus willdenowii* Kunth cv. Atom); tall fescue (*Festuca arundinacea* Schreb cv. Jethro); and cocksfoot (*Dactylis glomerata* L cv. Savvy) were grown as monocultures at the same field site. For WSC analysis, grass tillers (in the vegetative state) were collected from each plot in late winter (21 July to 20 August 2015) by cutting 80 individual tillers to ground level and these were subsequently dissected into three height fractions: 0 – 5 cm, 5 – 10 cm and 10 + cm. Sampling occurred between 10:00 and 13:00 hours on each day. The minimum and maximum air temperature during the sampling period were -2.6°C and 16.5°C respectively. The samples were dried at 60°C for 72 h, ground to pass through a 1 mm sieve, and stored in an airtight container until further analysis.

Samples used in the irrigation treatment study were obtained from a replicated forage experiment (n=3) grown at Panmure, Victoria, Australia (38°33'S, 142°63'E). A diverse set of summer forage crops were used in this experiment. These included two turnip varieties (*Brassica rapa* L. cvs. Appin and Australian Purple Turnip), two hybrid Brassica varieties (cvs. Graza (a complex hybrid of *Raphanus sativus*) and Hunter (*Brassica campestris* L. × *Brassica napus* L.)), two forage rapes (*Brassica napus* L. cvs. Rangi and Winfred), chicory (*Cichorium intybus* L. cv. Grouse), millet (*Echinochloa utilis* cvs. Ohwi and Yabuno) and plantain (*Plantago lanceolata* L. cv. Tonic). All forage crops were grown under dryland (rain-fed, 76 mm precipitation over the experimental period – December 2012 to February 2013) and full irrigation with irrigation based on 100% of estimated water requirements. For all forage, samples were collected by cutting to ground level. The samples were washed to remove soil contamination and then dried at 60°C for 72 h and ground to pass through a 1 mm sieve, and stored in an airtight container until further analysis.

2.4 Statistical analysis

Statistical analyses were based on average values of the three replicates. Minitab 17 (Minitab Inc., PA, USA) was used to undertake ANOVA and Tukey's comparison tests. Significant difference between treatments were reported with different capital letters when $P < 0.01$ and with different small letters when $P < 0.05$.

3. Results and Discussion

3.1 Development of UPLC WSC and Fructans/RFOs method

The calibration curve for the individual sugars was obtained by injecting five sets of mixed sugar standards consisting of fructose, glucose, sucrose, maltose, raffinose and stachyose (Figure 1; Appendix I), with concentrations ranging from 0.1 mg/mL to 0.8 mg/mL for each component. There was clear baseline separation between the peaks of various sugar standards. As the ELSD response to sugar concentration is non-linear, a power-equation model was fitted to the calibration curve for quantifying sugar concentrations in the samples analysed. The sugar standards were run at the beginning, middle and end of each batch of samples analysed. The sugar peaks of the samples were identified by retention time and spiking the samples with sugar standards.

< insert Figure 1 near here >

The five samples (oaten hay, timothy hay, chicory, lucerne and perennial ryegrass) analysed had very different sugar profiles (Table 1). For oaten hay and timothy hay, fructose was the most abundant sugar (c. 45% and c. 52% of total sugars respectively) followed by glucose. For chicory and lucerne, sucrose was the most abundant sugar (c. 40% and c. 60% of total sugars respectively) followed by fructose and glucose (c. 15 to 25% of total sugars). Maltose was only detected in chicory and consisted of 16% of the total sugars. For perennial ryegrass the only mono- or disaccharides measured was fructose. Raffinose and verbacose were not detected in any of the five samples analysed.

< insert Table 1 near here >

In the WSC extracts, fructan/RFO concentrations were comparable when determined with the Megazyme and UPLC methods, but some differences were observed (Table 2). For the summer forage crop (oaten hay and chicory) and Lucerne, fructans have not been reported and hence the oligosaccharides in these forages are most likely the RFO. On the other hand, for cool-season grasses (perennial ryegrass and timothy hay), fructan is one of the major component of WSC.

For oaten hay, chicory and lucerne the Megazyme assay gave higher ($P < 0.01$) fructan/RFO values whereas for perennial ryegrass the UPLC method gave a higher ($P < 0.01$) fructan/RFO value. Furthermore, for timothy hay, there was no significant difference in fructan/RFO values obtained using the two different methods. The discrepancies in oligosaccharide concentration observed may be due to the difference in the hydrolysis method (acid hydrolysis in UPLC method and enzyme hydrolysis in Megazyme method). The fructans found in various plant species are structurally diverse and are broadly classified as inulin, levan, mixed levan, inulin neoseries, and levan neoseries (Vijn & Smeekens, 1999). The effectiveness of the acid hydrolysis and/or enzyme hydrolysis could be dependent on the fructan composition, which varies significantly with plant species, for example acid-hydrolysis is more effective than inulinase hydrolysis for 2-6-linked fructans i.e. levan and levan neoseries (Jensen et al., 2014). This variation in fructan composition could, in turn, lead to variation in results obtained from different analytical methods.

< insert Table 2 near here >

The total WSC (sum of sugars and oligosaccharides (fructans & RFOs)) concentration obtained from UPLC method were compared to those obtained with the anthrone method, and were found to be lower ($P < 0.01$; by 0.6 to 2.8 g/100 g DM; Table 2). The lower WSC concentrations quantified by UPLC compared to the anthrone colorimetric method were similar to the findings of Kagan et al. (2014) and Hall (2014), where these investigators compared WSC concentration using HPLC and colorimetric methods. The anthrone method involves a more extreme hydrolysis (i.e. with 25 N (c. 70%) sulphuric acid at 100°C) and this color-complex assay is not specific to sugars and oligosaccharides. Soluble non-cellulosic non-starch polysaccharides which would not be detected by HPLC or by UPLC in this study are included in the colorimetric assays (Hall, 2014). Furthermore, with the colorimetric WSC assay, color development varies with the different sugars (Hall, 2013) and different forages contain different individual sugar concentrations (Table 1). This variability in sugar composition in turn could lead to inaccurate estimation of sugars when an empirical colorimetric method with one sugar (i.e. glucose) standard calibration is used to quantify total WSC concentration.

The UPLC method provides a more descriptive, specific and precise analysis of WSC concentration compared to the traditional colorimetric method used. Furthermore, it also eliminates the need to use thio-urea and allows the use of lower concentrations of strong acids. For this reason, in subsequent analysis of samples from tiller height and irrigation treatment experiments, the total and individual WSC concentration were analysed using UPLC method.

3.2 Effect of tiller height on sugar concentrations in perennial grasses

The four perennial grasses analysed in this study had diverse sugar concentrations at all three tiller height fractions (Table 3; Figure 2). The most substantial changes with increasing tiller height fraction were an increase ($P < 0.01$) in sucrose concentration and a decrease ($P < 0.01$) in fructan concentration. With increasing tiller height fraction from 0-5 cm to +10 cm, the sucrose concentration increased by 118%, 71%, 85% and 153% for perennial ryegrass, prairie grass, tall fescue and cocksfoot respectively. The corresponding percentage decrease in fructan concentrations was 72%, 77%, 64% and 32% for perennial ryegrass, prairie grass, tall fescue and cocksfoot respectively. However, a decrease in total WSC concentration with increasing tiller height fraction was observed only for perennial ryegrass ($P < 0.01$), prairie grass ($P < 0.01$) and tall fescue ($P < 0.05$). For cocksfoot there was no significant change in total WSC concentration because the decrease ($P < 0.01$) in fructose, glucose and fructan concentrations was counterbalanced by an increase ($P < 0.01$) in sucrose concentration.

< insert Table 3 and Figure 2 near here >

The decrease in total WSC concentration with increasing tiller height fraction was negatively correlated with an increase in crude protein (CP) concentration (Table 4). The WSC and CP were negatively correlated for perennial ryegrass, prairie grass and tall fescue (R^2 of 0.83, 0.97 and 0.82 respectively). In contrast to total WSC concentration, sucrose concentration was positively correlated with increasing CP concentration with tiller height fraction. The R^2 values for the relationship between sucrose and CP for perennial ryegrass, prairie grass, tall fescue and cocksfoot were 0.97, 0.95, 0.59 and 0.92 respectively.

< insert Table 4 near here >

The above data obtained for four cool-season perennial grasses at the three tiller heights suggest that tiller height fraction has a significant effect on total WSC concentration and composition. Unlike starch synthesis, fructan synthesis is less sensitive to low temperatures and hence plants active in winter and early spring accumulate fructan as a carbohydrate reserve (Vijn & Smeekens, 1999). In all four grasses, there was a significant concentration of fructans in all samples but the values varied depending upon the species and tiller height fraction. The fructan concentration ranged from as high as 70% of total WSC (0 – 5 cm perennial ryegrass) to 20% of total WSC (5 – 10 cm cocksfoot). The average degree of fructan polymerization (DP_{avg}) were significantly different between the tiller height fractions for perennial ryegrass and cocksfoot (Table 3). For these two species, the DP_{avg} were greatest for the lower tiller fraction (0 – 5 cm) and smallest for

the highest tiller fractions (10+ cm). Grotelueschen and Smith (1968) also observed fructans with higher degree of polymerization in the stem bases of Timothy and Bromegrass.

Fructan concentration was inversely correlated with the sucrose concentration in all four perennial grasses analysed. The 10+ cm tiller fraction, which consists of fresh tissue and leaves, had the highest concentration of sucrose and lowest concentration of fructans. Conversely, the 0-5 cm fraction had the lowest concentration of sucrose and highest concentration of fructan. The observed reduction in fructan concentration in plant height is in line with the findings of Sprague and Sullivan (1950) and Silva et al. (2015). Sprague and Sullivan (1950) found that the fructan concentration in orchard grass (*Dactylis glomerata*) was highest in lower stem followed by upper stem and upper leaf blades had the least concentration of fructans. Sucrose is synthesized in the leaf tissue via photosynthesis. When the supply of carbon (sucrose) via photosynthesis exceeds the carbon utilized for the growth and respiration, the simple sugars are translocated to the root and stem and either consumed via cellular metabolism or converted to storage carbohydrate reserves (fructans). After defoliation of forages, the storage carbohydrate reserves act as the source of energy for growth and metabolism until the plant has sufficient leaf area to support its growth through photosynthesis (J. M. Lee et al., 2009). Thus, the high concentration of fructan found in the lower fraction of grass tillers (0-5 cm) is an adaptive mechanism for regrowth of the analysed cool-season grass species.

3.3 Effect of irrigation on the sugar concentration of various forage samples

There was substantial variation in sugar concentration between the summer forage species analysed (Table 5). For all forage types, fructose and glucose were the predominant sugars and when combined accounted for > 80% of total sugars (except for irrigated Hunter, c.70%). Sucrose was the next most abundant WSC component (up to 20% of total sugars). The irrigated Hunter hybrid brassica, and the irrigated forage rape cultivars Rangi and Winfred had a modest amount of the fructans/RFOs (c. 1 – 2% of DM) and for all remaining samples the measured fructan/RFO concentration was lower than 1% of DM.

< insert Table 5 near here >

There was no consistent effect of irrigation on total WSC concentration of the samples analyzed. Rangi and turnip cultivar Appin, had an increase ($P < 0.05$) in the total WSC concentration in response to irrigation whereas for millet and plantain there was a reduction ($P < 0.01$ and $P < 0.05$ respectively) in total WSC concentration. Furthermore, for all other species there was no effect ($P > 0.05$) of irrigation on the total WSC concentration.

The effect of irrigation on individual sugar concentrations was inconsistent (Table 5). For irrigated Appin and Rangit, there was more than a two-fold increase ($P < 0.01$) in glucose concentration. Conversely irrigated millet and plantain exhibited a decline ($P < 0.01$) in glucose concentration. Likewise, irrigation treatment reduced ($P < 0.01$) fructose concentration in Australian Purple-Top turnip and millet but increased ($P < 0.05$) fructose concentration in chicory and plantain.

Irrigation increased the average DM yield (Table 6) and this observation is similar to the findings of Jacobs et al. (2006). The greatest DM yield increase in response to irrigation was obtained for chicory (287%) and the smallest increase was obtained for the Graza hybrid brassica (37%). Similarly, irrigation was associated with an increase in total WSC yield, being the greatest for chicory (384%) and the smallest for millet (21%).

< insert Table 6 near here >

It has been reported that WSC concentration is associated with plant responses to environmental stress such as drought (Bohnert & Sheveleva, 1998; Subbarao, Nam, Chauhan, & Johansen, 2000). Hence it was anticipated that irrigation would lead to decrease in WSC concentration. However, the WSC concentration and composition of forages grown under dryland or irrigated conditions were diverse. For some varieties irrigation had a significant effect ($P < 0.05$) on WSC concentration whereas in others there was no effect (Table 4). However, due to increased DM yield, irrigation treatment increased average total yield of water soluble carbohydrates for all nine summer forages investigated.

In summary, this study demonstrates the applications of the UPLC WSC method in forage analysis by analysing the WSC concentration of samples from two different experiments (1) temperate forages at different tiller heights and (2) summer forage crops grown under rain-fed and irrigated conditions. It was observed that tiller height had significant effect on the WSC concentration and composition. Nevertheless, there was no consistent effect of irrigation on WSC concentration.

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Figure captions

Figure 1 Chromatogram of mixed (fructose, glucose, sucrose, maltose, raffinose and stachyose) sugar standards at different concentrations (0.1 mg/mL to 0.8 mg/mL)

Figure 2 Box plot of individual sugars and fructan concentrations in four grasses (A: perennial ryegrass, B: prairie grass, C: tall fescue & D: cocksfoot) at three different tiller height fractions (**1**: 0 – 5 cm, **2**: 5 – 10 cm & **3**: +10 cm). The boxes represent the spread of data with median value as the middle line in the boxes.

Table 1: Concentration (g/100 g dry matter) of individual sugars in the water soluble carbohydrate fractions of oat hay, timothy hay, chicory, lucerne and perennial ryegrass (n=3; Average $\pm$ Standard deviation)

	Oaten hay	Timothy hay	Chicory	Lucerne	Perennial ryegrass
Fructose	3.2 $\pm$ 0.1	2.0 $\pm$ 0.0	0.9 $\pm$ 0.0	0.9 $\pm$ 0.1	0.8 $\pm$ 0.1
Glucose	2.1 $\pm$ 0.1	1.9 $\pm$ 0.0	1.1 $\pm$ 0.1	0.6 $\pm$ 0.2	nd
Sucrose	1.7 $\pm$ 0.0	nd	2.0 $\pm$ 0.0	2.5 $\pm$ 0.0	nd
Maltose	nd	nd	0.9 $\pm$ 0.0	nd	nd
Fructans/RFOs	1.6 $\pm$ 0.2	5.8 $\pm$ 0.2	0.6 $\pm$ 0.1	1.4 $\pm$ 0.1	5.6 $\pm$ 0.1
Total WSC	8.6 $\pm$ 0.3	9.7 $\pm$ 0.2	5.5 $\pm$ 0.1	5.4 $\pm$ 0.2	6.4 $\pm$ 0.1

Table 2: Comparison of the fructans/RFO and total water soluble carbohydrate concentration (g/100 g dry matter) using the Ultra Performance Liquid Chromatography method and colorimetric methods (n=3)

	Fructans/RFO (g/100 g DM)		Total WSC (g/100 g DM)	
	UPLC method	Megazyme method	UPLC method	Anthrone method
Oaten hay	1.6 ^A	2.5 ^B	8.6 ^A	11.3 ^B
Timothy hay	5.8	5.7	9.7 ^A	12.5 ^B
Chicory	0.6 ^A	1.2 ^B	5.5 ^A	6.1 ^B
Lucerne	1.4 ^A	1.7 ^B	5.4 ^A	7.2 ^B
Perennial ryegrass	5.6 ^A	4.3 ^B	6.4 ^A	8.2 ^B

Values within the same column with different letters differ (capital letters = $P < 0.01$). **Table 3:** Water soluble carbohydrate concentrations (g/100 g dry matter) and average degree of fructan polymerization for four grasses at different tiller heights (n =3)

	Tiller fraction (cm)	Water soluble carbohydrate components (g/100 g DM)					Degree of polymerization of fructan
		Fructose	Glucose	Sucrose	Fructans	Total WSC	
Perennial ryegrass	0 – 5	3.4 ^A	3.2 ^A	4.9 ^A	26.0 ^A	37.4 ^A	12.0 ^A
	5 – 10	4.3 ^B	3.4 ^A	6.9 ^B	14.8 ^B	29.4 ^B	9.7 ^A
	10 +	2.8 ^A	1.8 ^B	10.7 ^C	7.2 ^C	24.4 ^C	6.4 ^B
Prairie grass	0 – 5	3.6 ^A	1.9 ^A	4.1 ^A	18.4 ^A	30.4 ^A	7.6
	5 – 10	2.3 ^B	1.1 ^B	5.1 ^B	11.9 ^B	22.5 ^B	6.9
	10 +	1.3 ^C	0.7 ^C	7.0 ^C	4.3 ^C	14.4 ^C	6.4
Tall fescue	0 – 5	3.2 ^A	1.4 ^{AB}	4.7 ^A	12.9 ^A	24.0 ^a	7.5
	5 – 10	3.2 ^A	1.7 ^A	7.1 ^B	9.4 ^A	23.3 ^{ab}	6.9
	10 +	1.7 ^B	0.9 ^B	8.7 ^C	4.6 ^B	17.3 ^b	6.5
Cocksfoot	0 – 5	3.1 ^A	1.3 ^A	3.4 ^A	4.1 ^A	12.9	22.8 ^A
	5 – 10	2.4 ^B	1.1 ^A	5.2 ^B	2.3 ^B	12.0	14.4 ^B
	10 +	0.0 ^C	0.0 ^B	8.6 ^C	2.8 ^B	12.4	3.7 ^C

Values within the same column and cultivar with different letters differ (lower case letters = $P < 0.05$; capital letters = $P < 0.01$).

Table 4: Crude protein concentration (g/100 g dry matter) of four grasses at different tiller heights (n=3; Average $\pm$ Standard deviation)

Tiller fraction (cm)	Crude protein (g/100 g DM)			
	Perennial ryegrass	Prairie grass	Tall fescue	Cocksfoot
0 – 5	8.3 $\pm$ 0.4 ^A	14.5 $\pm$ 0.2 ^A	12.2 $\pm$ 0.9 ^A	14.9 $\pm$ 0.8 ^A
5 – 10	12.2 $\pm$ 0.7 ^B	20.0 $\pm$ 0.5 ^B	12.8 $\pm$ 1.2 ^A	19.8 $\pm$ 0.8 ^B
10 +	21.8 $\pm$ 0.7 ^C	29.4 $\pm$ 0.7 ^C	20.9 $\pm$ 0.5 ^B	28.9 $\pm$ 0.8 ^C

Values within the same column with different letters differ (capital letters = $P < 0.01$).

Table 5: Concentration of water soluble carbohydrate (g/100g dry matter) of nine forage varieties grown under dryland and irrigated conditions (n=3)

Forage variety		Treatment	WSC (g/100 g DM)				
			Fructose	Glucose	Sucrose	Fructan/RFO	Total WSC
Turnip	Appin	Dryland	4.7	4.0 ^A	0.5 ^A	0.1	9.3 ^a
		Irrigated	4.1	9.6 ^B	0.0 ^B	0.9	14.6 ^b
	Australian Purple Top	Dryland	4.0 ^A	1.8	0.2	0.0	6.0
		Irrigated	2.5 ^B	2.2	0.2	0.0	4.9
Hybrid Brassica cultivars	Graza	Dryland	2.8	2.0	0.1	0.1	5.0
		Irrigated	3.4	3.9	0.1	0.7	8.1
	Hunter	Dryland	4.1	3.2	0.9	0.5 ^a	8.7
		Irrigated	2.0	4.4	1.9	1.1 ^b	9.4
Forage rape	Rangi	Dryland	4.2	4.1 ^A	0.5	1.2	10.0 ^a
		Irrigated	3.9	8.6 ^B	0.3	2.0	14.8 ^b
	Winfred	Dryland	5.4	3.4	0.2	1.6	10.6
		Irrigated	4.7	5.4	0.0	1.6	11.7
Chicory		Dryland	3.1 ^a	3.3	0.8 ^a	0.4	7.6
		Irrigated	3.8 ^b	5.2	0.2 ^b	0.3	9.5
Millet		Dryland	4.8 ^A	4.6 ^A	2.1 ^A	0.0	11.5 ^A
		Irrigated	3.4 ^B	3.0 ^B	1.2 ^B	0.0	7.6 ^B
Plantain		Dryland	0.6 ^a	8.2 ^A	0.0	0.0	8.8 ^a
		Irrigated	1.0 ^b	5.3 ^B	0.0	0.0	6.3 ^b

Values within the same column with different letters differ (lower case letters = $P < 0.05$; capital letters = $P < 0.01$).

Table 6: Dry matter yield (kg dry matter/ha) and total yield water soluble carbohydrate (kg dry matter/ha) of nine summer forage varieties grown under dryland and irrigated conditions

Forage variety		Dryland (kg DM/ha)		Irrigated (kg DM/ha)		Total yield water soluble carbohydrates (kg DM/ha)	
		Average	Standard deviation	Average	Standard deviation	Dryland	Irrigated
Turnip	Appin	3031	745	5086	1170	282	743
	Australian Purple Top	2210	772	4925	794	133	241
Hybrid brassica cultivars	Graza	4222	1186	5784	1768	211	469
	Hunter	2713	487	4517	1111	236	425
Forage rape	Rangi	3139	674	6381	1499	314	944
	Winfred	3298	989	7525	896	350	880
Chicory		1270	659	4921	572	97	467
Millet		4680	455	8561	812	538	651
Plantain		1538	218	4773	774	135	301

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