Supplementary Materials

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Low-cost automated biochemical phenotyping for optimised nutrient quality components in ryegrass breeding

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Supplementary File 1a

WSC quantification protocol (WSC^{HT}) development

WSC extraction

Prior to extraction, plant material was incubated at 90^{o}C for 1 h (or microwaved for 1 min on a high setting) to degrade any glycolytic enzymes. In order to facilitate high-throughput and parallel extraction for multiple samples, reaction volumes were scaled-down. The majority of prior studies have used volumes between 2-80 ml. However, in order to automate the process in 96-well plate format, a volume of 600 µl was selected. In addition, sample mass of dried plant material was reduced to 20-60 mg. Previous studies have described extraction of WSCs from plant material using ethanol (at various concentrations), or water, or a combination of both. In the present study, the optimal extraction procedure for dried ryegrass leaf and stem tissue was found to be based on a combination, with heating to 60^{o}C prior to addition to the leaf material. This procedure increased the quantity of extracted WSC, presumably due to enhanced disruption of cell walls and consequent solubilisation.

Due to the effect of small extraction volume, a 3-fold extraction (first with water, followed by two rounds of ethanol) was performed. Extractions were performed in 1.4 ml 96-well cluster tube plates (Micronic, Netherlands) in which, after addition of each of the extraction volumes, samples were ground with 3 mm tungsten carbide beads (QIAGEN, Germany) in a GenoGrinder 2010 (SPEX SamplePrep, USA) at 1700 rpm for 10 minutes, 5 minutes and 5 minutes, respectively. After each of the 3 extraction steps, fluid surrounding the plant material could not be completely captured, and consequently a standardised volume was retained and permitted for carry-over into the following step. However, this process influenced the proportion of total WSC

captured in each extraction step, implying that extracts from each step cannot be pooled on the basis of equal volumes. Determination of the volume derived from each extraction that is required for pooling must consider the proportions of the previous extracts (due the carry-over volume). Consequently, equations were developed to calculate the volume from each extraction that is required to ensure equal pooling of true extract proportions.

$$Z_{1,2} = \frac{c}{v + c} \qquad Z_{1,3} = \frac{Z_{1,2} \times c}{v + c} \qquad Z_{2,3} = \frac{c - (Z_{1,2} \times c)}{v + c}$$

$$t = f - \left(f \times (Z_{1,3} + Z_{2,3})\right)$$

$$ext_3 = f$$

$$ext_2 = \frac{t - (Z_{2,3} \times f)}{1 - Z_{1,2}}$$

$$ext_1 = t - (Z_{1,3} \times f) - (Z_{1,2} \times ext_2)$$

Where;

c = carry-over volumes

v = Initial extraction volume

f = volume to retain and pool from the 4th extraction

$Z_{i,j}$ = proportion of extract from extraction *i* in extraction *j*

t = true volume of each extract that is represented in the final pooled extracts

ext_i = volume to retain from extraction *i*

Using these equations, and with initial extraction volume and carry-over volume set at 600 µl and 300 µl, respectively, the volumes pooled from the first, second and third rounds of extraction were 53.33 µl, 106.67 µl and 160 µl, respectively. Although 100% (v/v) ethanol was added as the extraction agent in the second and third rounds of extraction, the 300 µl carry-over volume ensured that the concentrations within the sample tube during extraction were actually 66.7% and 88.9%, respectively. Aliquots were collected after each extraction and pooled in 1 ml 96-well plates containing 60 mg of activated charcoal (per well). After the final extraction volume was collected, the charcoal-containing plates were vortexed for 30 seconds and incubated at room temperature for 5 minutes. Activated charcoal was required for removal of pigments and minerals that may interfere with subsequent quantification steps. The extracts were then centrifuged for 5 minutes at 4000 g, to separate the activated charcoal from purified WSC extract, which was then transferred to a fresh 200 µl 96-well plate. All liquid transfer and addition steps were automated on a Biomek® FX Automation Workstation (Beckman Coulter, USA).

Quantification of individual WSC components

A sub-sample from each purified extract was diluted five-fold, and 4 µl was transferred to a 384-well UVstar® plate (Greiner bio-one, Germany), with 3 technical replicates, and incubated at 30^{o}C until all ethanol and water was evaporated. WSCs were resuspended in 4 µl of ddH_2O. Eight glucose standards (ranging from 1 mg/ml to 0.0156 mg/ml, including a blank) were also added to a 384-well plate, with 3 replicates. An aliquot of 20 µl of glucose assay reagent (G3293, Sigma-Aldrich, USA), containing hexokinase and glucose-6-phosphatase, was added to each

sample followed by incubation at 30ºC for 20 minutes, and absorbance of NADH was quantified at 340 nm with a Synergy 2 microplate plate reader (Biotek, USA). After the absorbance was recorded, 0.025 units of phosphoglucose isomerase (P9544, Sigma-Aldrich, USA) in 0.2M HEPES buffer, pH 7.8, was added to each sample. After incubation of the plates at 30ºC for an additional 15 minutes, absorbance at 340 nm was again quantified with a plate reader. Subsequently, 8.3 units of invertase (I4504, Sigma-Aldrich, USA) in 0.1 M citrate sodium citrate buffer, pH 6.0, was added to each sample and incubated at 30ºC for a final 60 minutes, after which absorbance at 340 nm was again measured. A standard curve relating absorbance to glucose concentration was established. By subtraction of the first and second absorbance readings from the third, the number of glucose-equivalent units of sucrose present in the samples could be determined. Subtraction of the first absorbance reading from the second permitted quantification of the amount of glucose equivalent units of fructose present in the sample.

For quantification of fructans, 20 µl of WSC extract was dried in an oven at 30ºC for 2 hrs in order to remove any ethanol and water, and the resulting pellet was resuspended in 50 µl of 0.2 M acetic acid. Plates were sealed and incubated in a water bath at 90ºC for 1 hour (with vortexing after 30 minutes) to allow degradation of fructans into sucrose and fructose units. To neutralise the acetic acid, 10 µl of 1 M sodium hydroxide (NaOH) was added and vortexed. Samples were then diluted up to 100 µl with ddH_2O. Aliquots of 4 µl from each sample were transferred to 384-well plates, to which 20 µl of glucose assay reagent, 0.025 units of phospho-glucose isomerase and 8.3 units of invertase were added. Samples were incubated at 30ºC for 1 hour, and absorbance at 340 nm was quantified on a plate reader. The concentration of glucose-equivalent units of fructans was determined by subtraction

of the concentrations of glucose, fructose and sucrose that were previously measured for each sample. As before, all liquid transfer and addition steps were automated on a Biomek® FX Automation Workstation (Beckman Coulter, USA). The high-throughput individual WSC extraction and quantification protocol described is henceforth designated as WSC^{HT}.

Supplementary File 1b

WSC and protein quantification protocol (FQ^{HT}) development

Protein and WSC extraction

The Kjeldahl procedure was unsuitable for rapid high-throughput extraction and quantification of protein content. True plant protein was consequently chosen as a proxy for CP. Numerous protein extraction and stabilisation buffers were tested, and use of a 1 M NaOH solution at 60^{o}C was found to consistently yield the highest proportion of CP (data not shown). The procedure for protein extraction was combined with the equivalent for WSC, so that sampled plant tissue was used for both forage quality components. Extraction involved a four-step process: an initial extraction of protein in the NaOH solution (involving sample grinding for 10 minutes at 1700 rpm and a 30 minute room temperature incubation), followed by the three-step WSC extraction method as previously described, with a single alteration. Instead of extraction in ddH_2O, acetic acid was used to neutralise the remaining NaOH in the 300 µl carry-over volume. As the procedure involved four steps, the previous equations for calculation of residual extraction liquid to combine from each extraction step (that were developed for three steps) were re-formulated.

$$Z_{1,2} = \frac{c}{v+c} \qquad Z_{1,3} = \frac{Z_{1,2} \times c}{v+c} \qquad Z_{1,4} = \frac{Z_{1,3} \times c}{v+c}$$

$$Z_{2,3} = \frac{c - (Z_{1,2} \times c)}{v+c} \qquad Z_{2,4} = \frac{Z_{2,3} \times c}{v+c}$$

$$Z_{3,4} = \frac{c - (Z_{1,3} \times c) - (Z_{2,3} \times c)}{v+c}$$

$$t = f - \left(f \times \left(Z_{1,4} + Z_{2,4} + Z_{3,4}\right)\right)$$

$$ext_4 = f$$

$$ext_3 = \frac{t - (Z_{3,4} \times f)}{1 - \left(Z_{1,3} + Z_{2,3}\right)}$$

$$ext_2 = \frac{t - \left(Z_{2,4} \times f\right) - (Z_{2,3} \times ext_3)}{1 - Z_{1,2}}$$

$$ext_1 = t - \left(Z_{1,4} \times f\right) - \left(Z_{1,3} \times ext_2\right) - (Z_{1,2} \times ext_2)$$

Where;

c = carry-over volumes

v = Initial extraction volume

f = volume to retain and pool from the 4th extraction

$Z_{i,j}$ = proportion of extract from extraction *i* in extraction *j*

t = true volume of each extract that is represented in the final pooled extracts

ext_i = volume to retain from extraction *i*

The carry-over volume of 300 µl, and the volume to retain from the final extraction (160 µl) remained as before, so the volumes for pooling obtained from the first, second, third and fourth steps for WSC quantification were calculated as 53.33

µl, 106.67 µl, 106.67 µl and 160 µl, respectively. In addition, 10 µl from the first extraction was transferred to a separate plate for protein quantification, in which it was diluted 10-fold in order to obtain a final NaOH concentration of 0.1 M. After the first extract was collected, acetic acid was also added to neutralise NaOH. The extractions were again pooled in a plate containing activated charcoal and vortexed, incubated and centrifuged, the purified WSC extract being transferred to a fresh plate.

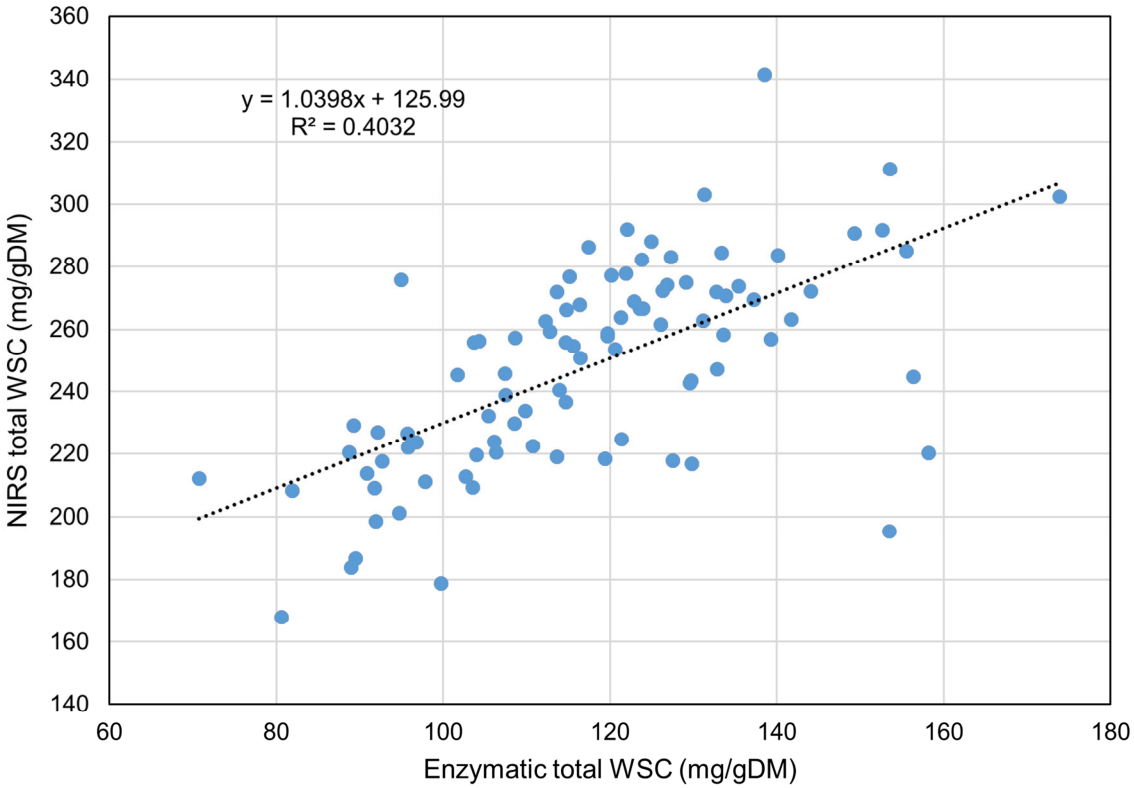
A higher concentration of NaOH (1 M) was observed to obtain extraction of c. 75% of CP (close to the expected level of true protein [Bryant *et al*. 2012,; Pacheco and Waghorn 2008]). However, use of concentrated NaOH solution was observed to lower the level of measured WSC, presumably due to degradation of simple sugars such as glucose and fructose (MacLaurin and Green 1969). Although use of a lower concentration of NaOH only obtains 55-60% of CP, WSC quantification was not affected, and so the 0.1 M NaOH concentration was adopted for the combined protein and WSC extraction protocol. As before, all liquid transfer and addition steps were automated on a Biomek® FX Automation Workstation (Beckman Coulter, USA).

Quantification of total WSC and true plant protein

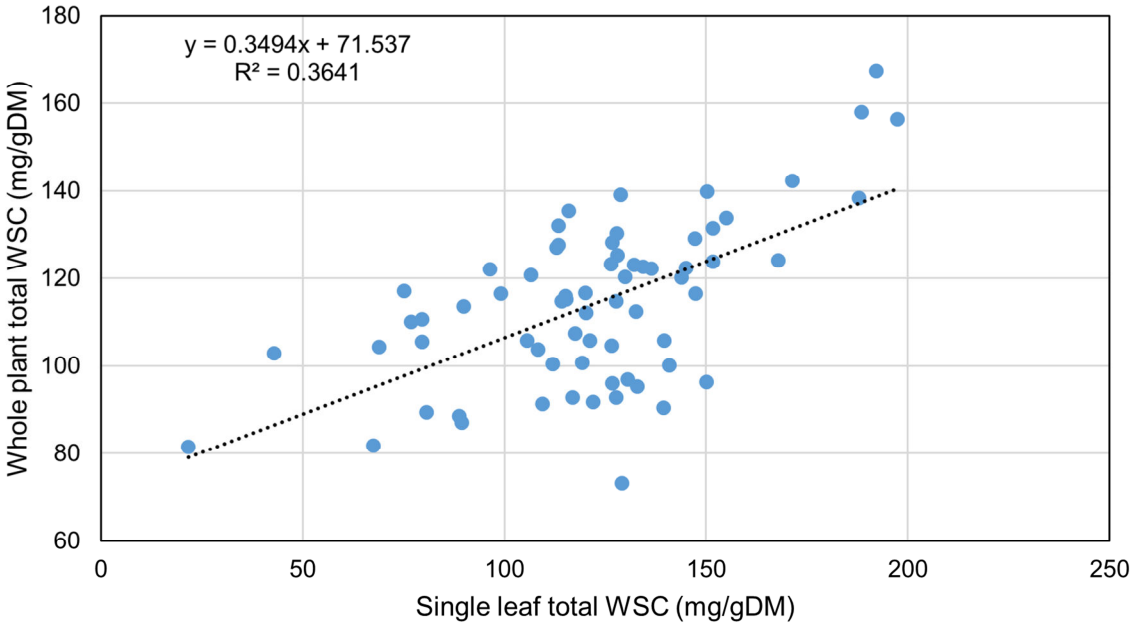
As previously described, plant tissue samples required incubation at 90^{o}C prior to extraction in order to denature any plant enzyme that could degrade individual WSCs. However this denaturation step would also reduce the level of measured true plant protein, due to the common structural character of proteins. Therefore, as enzyme activity did not affect total WSC, samples were not incubated prior to extraction for the combined protein and WSC quantification assay, and WSCs were hence quantified as a whole rather than individually.

For quantification of total WSC, 20 µl of WSC extract was transferred to a 96-well microtitre plate. The samples were then dried in an oven at 30^{o}C to remove any ethanol and water. The dried WSC extracts were resuspended in 50 µl of 0.2 M acetic acid. Plates were incubated in a water bath at 90^{o}C for 1 h (with vortexing after 30 minutes) to degrade fructans into sucrose and fructose units. To neutralise the acetic acid, 10 µl of 1 M NaOH was added and vortexed. Sample volumes were increased to 100 µl with ddH_2O. Aliquots of 4µl of each digested WSC sample were transferred to a 384-well plate, to which 20 µl of glucose assay reagent, 0.025 unit phosphoglucose isomerase and 8.3 units of invertase were added to each sample. Samples were incubated at 30^{o}C for 1 h, and the absorbance at 340 nm was quantified on a plate reader. The concentration of glucose-equivalent units of WSC was determined by using a standard curve based on known glucose concentration samples.

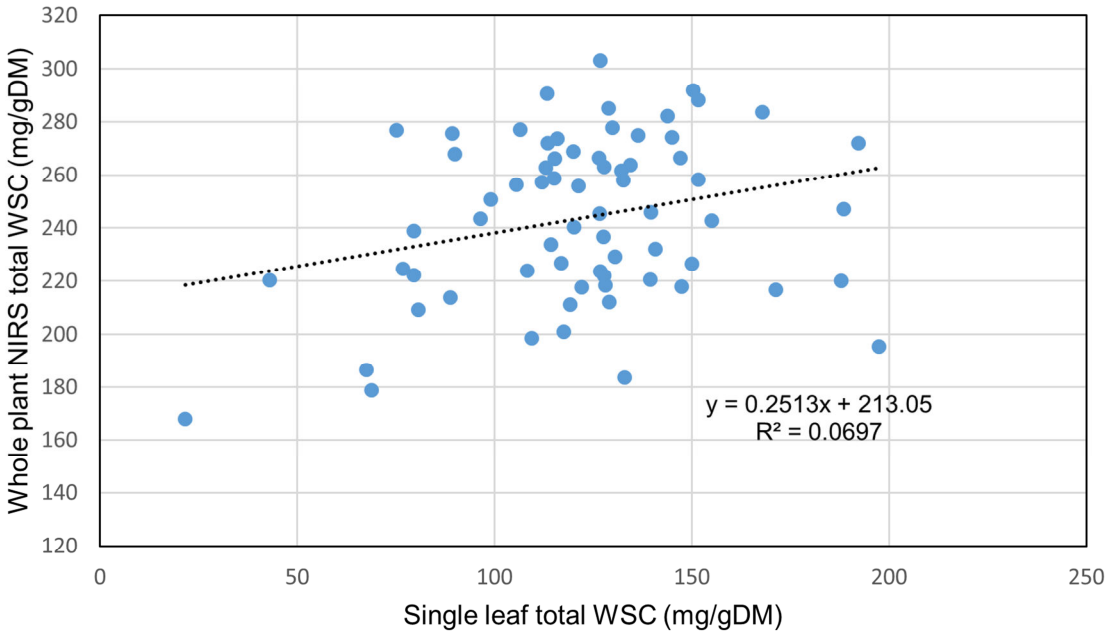
For quantification of true plant protein, the Coomassie Brilliant Blue (Bradford) assay (Bradford 1976) was used. From the 10-fold diluted protein extracts, 5 µl aliquots were transferred to flat-bottomed colorimetric quantification plates with three technical replicates. An aliquot of 250 µl Coomassie protein assay reagent (Thermo Scientific, USA) was added, and manufacturer's instructions were followed for quantification. As before, all liquid transfer and addition steps were automated on a Biomek® FX Automation Workstation (Beckman Coulter, USA). The high-throughput WSC and protein extraction and quantification protocol described is henceforth designated as FQ^{HT}.



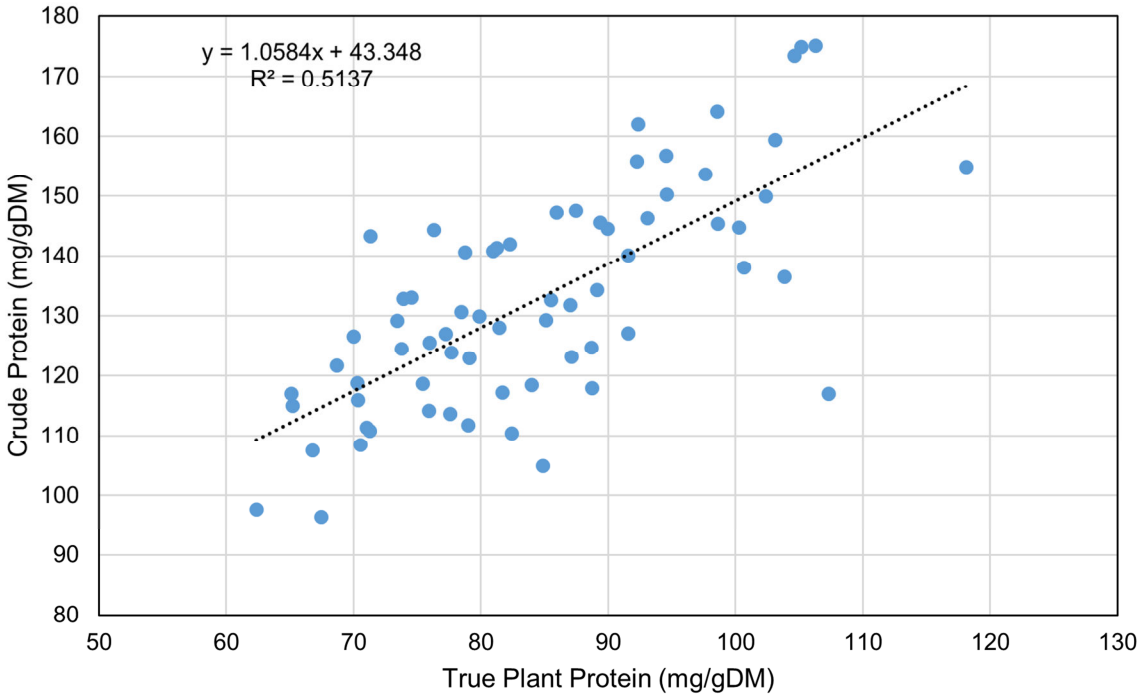
Supplementary Figure 1a. Significant (p-value <0.001) correlation between total WSC quantified enzymatically, after protein extraction and total WSC estimated using NIRS.



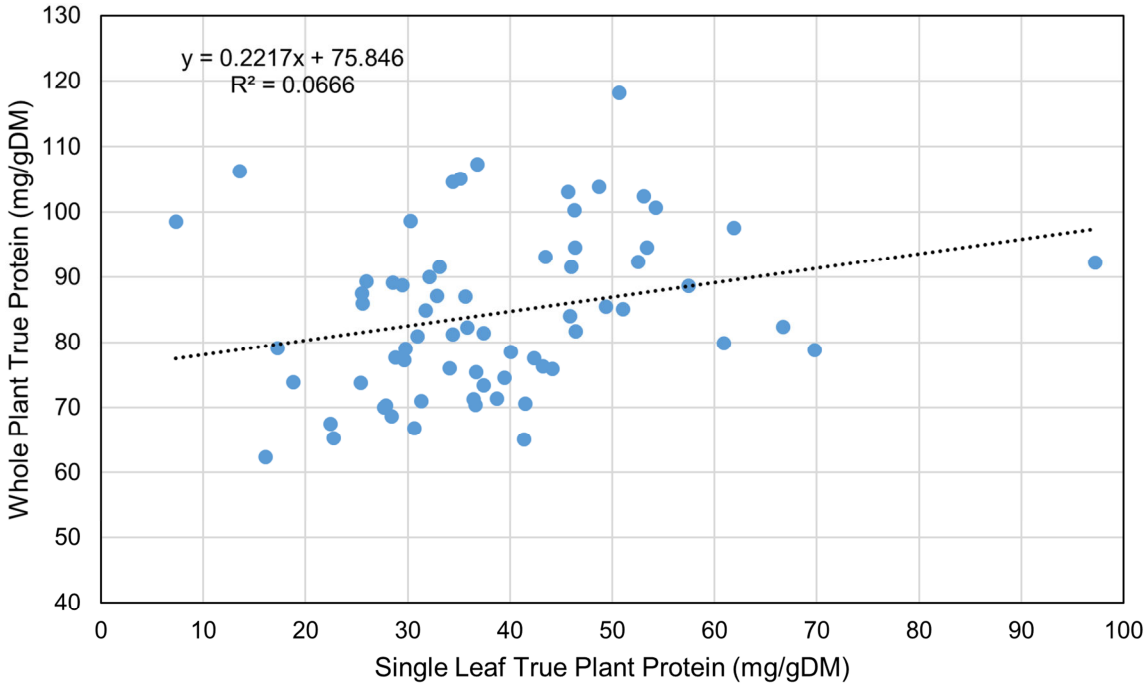
Supplementary Figure 1b Significant (p-value <0.001) correlation between WSC quantities measured from a single leaf base and from a whole plant.



Supplementary Figure 1c. Significant (p-value = 0.031) correlation between WSC quantities measured from a single leaf base and from NIRS analysis of a whole plant.



Supplementary Figure 1d. Significant (p-value <0.001) correlation between measurements of true plant protein from ground tissue of whole plant and CP quantified by NIRS.



Supplementary Figure 1e. Significant correlation (p-value = 0.035) between single leaf plant protein and whole plant protein, quantified using the Coomassie Brilliant Blue protein assay.

Supplementary Table 1. Breeding history and associated breeding company for each cultivar used during protocol development and validation

Cultivar name	Breeder	Species		Cultivar marketed as	Ploidy level	Parentage	Origin	Reference
		PBR status in Australia	PVR status in New Zealand					
Aberdart	IGER	*L. perenne*	*L. perenne*	*L. perenne*	Diploid	European	Europe	
Alto	NZ Agriseeds	*L. perenne*	*L. perenne*	*L. perenne*	Diploid	Bronsyn x Impact	(NZ x Spain)	PVJ, 20(3)
Arrow	NZ Agriseeds		*L. perenne*	*L. perenne*	Diploid	Yatsyn x SP470986	(NZ x Spain)	
LP423	NZ Agriseeds	*L. perenne*		*L. perenne*	Diploid	Tolosa x Bronsyn	(NZ x Spain)	
LP447	NZ Agriseeds			*L. perenne*	Diploid			
Matrix	Cropmark Seeds	*L. hybrid*	*L. x Festulolium*	*L. perenne*	Diploid	Impact, Aires HD, Meadow fescue	(NZ, Spain, Meadow Fescue)	PVJ, 14(3)
One50	PGG Wrightson Seeds	*L. perenne*	*L. perenne*	*L. perenne*	Diploid	Tolosa cross	(Spain x NZ)	PVJ, 23(2)
Trojan	NZ Agriseeds	*L.* x *boucheanum*	*L.* x *boucheanum*	*L. perenne*	Diploid	Tolosa re-selection	Spain	