Bradford quantification of Glomalin-Related Soil Protein in coloured extracts of forest soils

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Abstract

Glomalin-related soil protein (GRSP) is thought to represent a fraction of recalcitrant organic matter in soil. But it is recognized that the autoclaved-citrate extraction procedure causes the co-extraction of humic substances which interfere (directly and indirectly) with the Bradford colorimetric assay. The aim of this work was to propose a reliable quantification method of GRSP from forest soil, very rich in organic matter and therefore in colour. We estimated the quantities of GRSP in the topsoil (0-10 cm) of 102 French forests using five methods: i) direct calibration, reasoned dilution with colour correction, ii) direct calibration, reasoned dilution but no colour correction, iii) direct calibration, 1:2 dilution, no colour correction, iv) indirect calibration and v) dilution method. Our results concur that the interference caused by the co-extracted compounds is not related simply to either the colour of the extracts or total soil organic matter content. These findings suggest that for improved accuracy of GRSP estimates using the Bradford method, extracts should be diluted, and the pH-specific absorbance of coloured extracts should be subtracted.

Keywords: Quantification of GRSP; Humic substances; Coloured extracts; Forest soils, Autoclaved-citrate extractable protein

Glomalin-Related Soil Protein (GRSP) is an operationally defined fraction of soil organic matter obtained by autoclaving soil in neutral or alkaline sodium citrate solution. It has been the object of many studies and some controversy since it was first described by Wright and Upadhyaya (1996). Reputed to be produced by arbuscular mycorrhizal fungi, it is increasingly used as a tracer of fungal activity. It is also said to confer particular physical stability to soils and to be stable and hence long-lived in soil (Treseder and Turner, 2007). However it is now recognized that the extract contains a mixture of proteins and that the co-extracted non-protein components interfere with the colorimetric Bradford assay usually employed to quantify extracted proteins (Whiffen et al., 2007; Gillespie et al., 2011). This results in negative interference (underestimation of protein content) and apparent protein content that depends on dilution (Jorge-Araújo et al., 2015; Reyna and Wall, 2014). Redmile-Gordon et al. (2013) showed that the Bradford assay consistently underestimated protein additions to citrate-extracts of soil, a problem that can be avoided by using a modified Lowry method. However, many researchers still prefer to use the Bradford method, which justifies investigation into the possibility that extract-dilution and colour correction might help to overcome this limitation. Moragues-Saitua et al. (2019) investigated several variants of protein quantification using the Bradford assay for five contrasting soils. They concluded that it is essential to subtract sample colour from measured absorbance and recommended dilution of the sample so that the intensity of absorbance at 465 nm did not exceed about 0.1. The most problematic soil among those tested by Moragues-Saitua et al. (2019) was a shrubland soil that gave highly coloured extracts. This observation led us to postulate that protein quantification in extracts from highly coloured forest soils, rich in organic matter and polyphenolic humic substances would be particularly subject to interferences. Our aim is to propose data analysis for the reliable quantification of proteins in GRSP extracts of organic-rich forest soils.

The soils were obtained from each of the 102 sites of the French national network for long-term forest ecosystem monitoring (Renecofor), which is part of the ICP Forests intensive (level II) monitoring network. The samples used were collected during the second field campaign between 2007 and 2012 from a depth of 0-10 cm in the mineral topsoil. Detailed information on the sites, sampling, storage and analysis are available ((http://www1.onf.fr/renecofor; Jonard et al., 2017; Ponette et al., 1997). They were provided as air-dried archived samples, that were further crushed and sieved (<200µm). Information on soil composition, climatic conditions and forest cover were supplied by the ONF. Air-drying has been reported to decrease the extractability of GRSP (Woignier et al., 2014), albeit in andosols, but no information is available on the effect of duration of subsequent storage. GRSP was extracted by autoclaving triplicate samples of soil in 20 mM sodium citrate solution (pH 7, soil:solution ratio of 1:8 g ml^{-1}) for 30 minutes at 121°C. After cooling suspensions were centrifuged at 15 000 g, 1.5 ml of supernatant was withdrawn and centrifuged again in a 1.5-ml Eppendorf tube at 15 000 g to ensure complete phase separation. Solutions were refrigerated and analysed within 3 days. Solutions were diluted (1:2, 1:3 or 1:10) in extraction solution prior to Bradford assay (Bio-Rad Quick-Start™), with 20 µl of sample and 250 µl of Bradford reagent in microplates. Absorbances were read at 465 and 595 nm (denoted A_{465} and A_{595}) with a ThermoScientific Multiskan GO spectrometer (Waltham, MA, USA). Calibration was performed using BSA as a standard. Sample blanks were also prepared with 20 µl of (diluted) sample added to 250 µl 1 M HCl (to obtain a final pH of about 1, as in the presence of Bradford reagent, since the colour spectra of soil extracts are pH-dependent). Absorbance was corrected for colour by subtraction of absorbance of the sample at the same dilution and pH. Since microplate spectrometers are single beam, the absorbance of wells filled with water was subtracted from the sample blank absorbance prior to colour correction, to avoid duplicate subtraction of the well absorbance. Five calculation

74 methods were compared: (i) direct calibration (A_{595}), recommended dilution (to obtain sample
75 absorbance, $A_{465}\approx0.1$), colour correction; (ii) direct calibration (A_{595}), recommended dilution,
76 no colour correction; (iii) indirect calibration (A_{595}/A_{465}), recommended dilution, colour
77 correction; (iv) direct calibration, 1:2 dilution, no colour correction; (v) dilution method. For
78 the dilution method the colour corrected absorbance (A_{595}) was plotted against the dilution
79 (0.1, 0.33 or 0.5) and protein concentration of the least diluted solution calculated as the ratio
80 of the gradient of the dilution curve and the gradient of the standard calibration curve (Zor
81 and Selinger, 1996). The methods are compared by regression analysis of the calculated
82 values of GRSP (Figure 1), comparison of mean values (Table 1) and the experimental
83 variability (coefficient of variation calculated for each sample and each method with the mean
84 given in Table 1). Data were analysed separately for broadleaf and conifer forest soils.
85 Statistical analysis was carried out using R (R Core Team, 2018).

86 The soils tended to be acid, with average pH of 4.77 and only 16 samples (of 102) with pH
87 above 5.5 and only 5 with pH above 7. The organic carbon (OC) contents were in the range 8-
88 412 g kg^{-1}, with an average of 57 g kg^{-1}, with soils of conifer forests having larger C contents
89 than those of broadleaf forests, 71 and 43 g kg^{-1} respectively. All soils textures were found,
90 but most of the soils were silty loams. Significant positive correlations were observed
91 between organic carbon content and clay content, for the full data set and for deciduous and
92 conifer forest soils separately, with about 3 times more OC with respect to clay content in
93 conifer forest soils than broadleaf forest soils. Soils came from a range of altitudes from sea
94 level to 1850 m a.s.l., with an average of 500 and a median of 330 m a.s.l., with conifers
95 found at all altitudes, but in general at higher altitude than deciduous forests. The most
96 common deciduous species were oak (*Quercus petraea* and *Q. robur*) and European beech
97 (*Fagus sylvatica*), while the most common conifer species were spruce (*Picea abies*), silver

98 fir (*Abies alba*), Douglas fir (*Pseudotsuga menziesii*) and pines (*P. pinaster*, *P. sylvestris* and
99 *P. nigra ssp. laricio*). Most of the soils were Cambisols, Luvisols or Podzols.

100 Table 1 shows the average values of GRSP calculated for the soils by each of the five
101 calculation methods, with data for deciduous and conifer forest soils shown separately. It is
102 clear that the colour correction introduced a small decrease in the value of GRSP calculated.
103 This is largely compensated by the use of a greater dilution than may often be the case. In
104 most studies no mention is made of dilution, samples are probably diluted enough for the
105 absorbance to fall within the working range of the Bradford assay and dilution effect either
106 not noticed or ignored. Also shown are the average values of the coefficients of variability for
107 each method obtained from replicate measurements for each soil. The variability of the
108 dilution method is greatest, with c.v. values of more than 10%.

109 Figure 1 shows the comparison of values of GRSP for each soil calculated using the
110 recommended method on the x-axis and each of the other methods. In the absence of colour
111 correction (Fig 1a) GRSP is overestimated as non protein chromophores contribute to the
112 calculation. There is a similar overestimation for both conifer and broadleaf forest soils
113 (gradients of 1.37 and 1.32 respectively). Figure 1b shows the combined effect of colour
114 correction and dilution. Solid regression lines are for the full data set, for soils of either
115 deciduous or conifer forests, whereas dotted lines are also shown for the soils where the
116 recommended dilution was not 1:2, the more intensely coloured soils, and often soils with
117 larger GRSP contents. Despite the positive interference of colour, the overall effect of less
118 than the recommended dilution is to underestimate GRSP due to the strong inhibition of co-
119 extracted compounds. Figure 1c shows excellent correlations with gradients of nearly unity
120 when the direct (A_{595}) and indirect methods (A_{595}/A_{465}) are compared (both after colour
121 correction and recommended dilution). Both correlations are equally recommended. Figure 1d
122 shows that the Dilution method overestimates GRSP for deciduous forest soils, but

underestimates it for conifer soils, particularly those with larger GRSP contents. This observation, along with the large experimental variability (more than 10% on average) indicates that this method is not to be recommended.

To conclude, we strongly recommend that soil extracts be diluted and pH-dependent colour absorbance subtracted before quantification of protein using the Bradford assay. This is an alternative to the use of the modified Lowry assay recommended by Redmile-Gordon (2013) to avoid the negative and positive interferences of co-extracted soil phenolic compounds in the Bradford assay of citrate extracted soil proteins. The nature and extent of interferences caused by co-extracted compounds are not simple functions of colour, protein or organic matter content, as previously reported (Redmile-Gordon et al., 2013; Woignier et al., 2014); Jorge-Araújo et al., 2015).

Acknowledgements

GC received a travel grant from the Ministry of Higher Education and Research, Côte d'Ivoire.

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181 **Figure and Table Captions**

182 **Figure 1**

183 Regression analysis between GRSP quantification methods plotted against data obtained with
184 the reference method (direct calibration (A_{595}), recommended dilution and colour correction).
185 **a**) direct calibration (A_{595}), recommended dilution no colour correction; **b**) direct calibration,
186 1:2 dilution, no colour correction; **c**) indirect calibration (A_{595} / A_{465}), recommended dilution
187 and colour correction and **d**) dilution method. The ○ and ● symbols represent broadleaf and
188 conifer respectively. In Fig 1b dotted lines are regressions for data where the recommended
189 dilution differed from 1:2 for soils of either broadleaf or conifer forests, and in both cases the
190 number of points (N) is indicated.

191 **Table 1**

192 Mean and standard deviation (s.d.) of data calculated by each of the methods and the mean of
193 coefficient of variation (c.v.) of the values of GRSP calculated from replicates for each soil.
194 The different letters indicate significant differences between the mean values of GRSP
195 content.

196

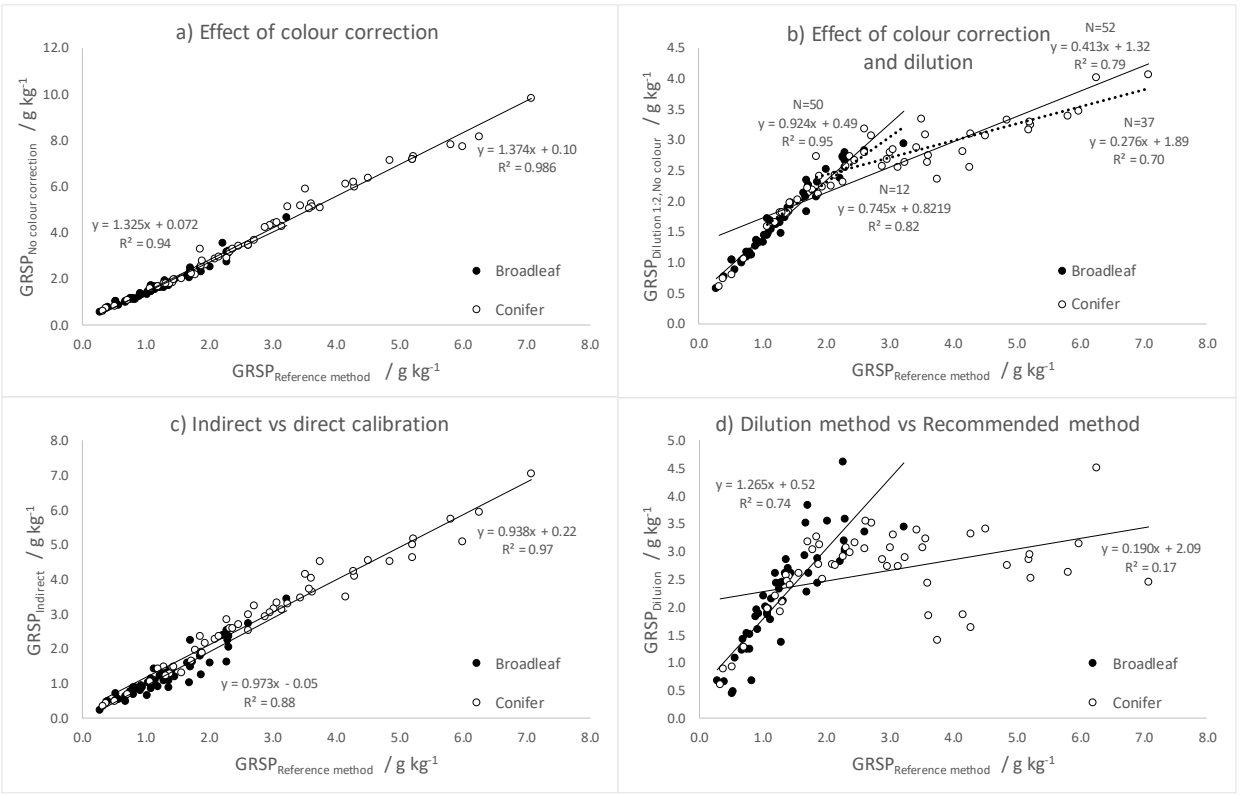
197 **Table 1**

198

Methods/Vegetation	Mean	s.d.	Mean c.v. (%)
Direct, Variable dilution, colour correction (Reference method; x-axis of Figure 1)			
Broadleaf	1.34[a]	0.63	2.80
Conifer	2.87[b]	1.61	3.62
All	2.11	1.45	3.22
Direct, Variable dilution, no colour correction			
Broadleaf	1.85[a]	0.87	4.09
Conifer	4.04[b]	2.23	3.13
All	4.05	2.02	3.60
Indirect, Variable dilution, colour correction			
Broadleaf	1.23[a]	0.68	2.49
Conifer	2.86[b]	1.59	3.35
All	2.06	1.47	2.93
Direct, 1:2 dilution, no Colour			
Broadleaf	1.71[a]	0.64	4.15
Conifer	2.50[b]	0.75	3.09
All	2.11	0.80	3.61
Dilution method			
Broadleaf	2.22[a]	0.93	10.50
Conifer	2.67[b]	0.74	12.65
All	2.43	0.86	11.60

199

200


a) Effect of colour correction
GRSP No colour correction / g kg-1
GRSP Reference method / g kg-1
y = 1.374x + 0.10
R² = 0.986
y = 1.325x + 0.072
R² = 0.94
Broadleaf
Conifer
b) Effect of colour correction and dilution
GRSP Dilution 1:2, No colour / g kg-1
GRSP Reference method / g kg-1
N=52
y = 0.413x + 1.32
R² = 0.79
N=50
y = 0.924x + 0.49
R² = 0.95
N=37
y = 0.276x + 1.89
R² = 0.70
N=12
y = 0.745x + 0.8219
R² = 0.82
Broadleaf
Conifer
c) Indirect vs direct calibration
GRSP Indirect / g kg-1
GRSP Reference method / g kg-1
y = 0.938x + 0.22
R² = 0.97
y = 0.973x - 0.05
R² = 0.88
Broadleaf
Conifer
d) Dilution method vs Recommended method
GRSP Dilution / g kg-1
GRSP Reference method / g kg-1
y = 1.265x + 0.52
R² = 0.74
y = 0.190x + 2.09
R² = 0.17
Broadleaf
Conifer