SILICON AND OTHER ESSENTIAL ELEMENT COMPOSITION IN ROOTS USING X-RAY FLUORESCENCE SPECTROSCOPY: A HIGH THROUGHPUT APPROACH

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Introduction

Silicon (Si) is the second most abundant element in soil, just after oxygen, making up 30% of the upper continental crust (w/w, ca. 146 times more abundant than carbon) (Hans Wedepohl, 1995). On a day-to-day basis, Si is used in countless households as building materials, in all electronic devices and even cosmetics (Vasanthi et al., 2012). This element is not essential only to human societies, but is also used by a large range of living organisms, including plants (Cooke and Leishman, 2011).

Whereas Si concentrations vary considerably among plants, trace amounts of this element have been found in all tested species (Hodson et al., 2005). Intraspecific variation between cultivars has also been documented (Deren, 2001). Poaceae have been shown to accumulate relatively large amounts of Si (Hodson et al., 2005), but rice, *Oryza sativa* L., has the highest Si concentrations, representing up to 10% of its dry mass (Epstein, 1999). Silicon contributes to plant strength, providing grazing resistance to stems and leaves (Ando et al., 2002; Osborne, 2008). Below-ground, silicon aids *Distichlis spicata* L. root penetration in the soil matrix (Hansen et al., 1976).

Accumulation of Si in plant tissue is energetically inexpensive compared to carbon fixation (energy costs of Si vs. carbon 1:10-1:20) (Raven, 1983). This could have been at the origin of the diversification of Poales during the Miocene, when atmospheric CO_2 levels were low (Craine, 2009) therefore favoring plants able to accumulate high levels of Si to substitute carbon in some essential functions, such as mitigation of abiotic stresses (e.g. Epstein, 1999; Currie and Perry, 2007) and defenses against herbivores (e.g. Reynolds et al., 2009).

There is increasing evidence that Si accumulated in plants plays a role in physical and chemical defenses against herbivores. Massey and Hartley (2009) demonstrated that the performance of the herbivore *Spodoptera exempta* Walker was reduced when the insect was offered grass species grown under Si-rich, compared to low Si conditions. The authors proposed that Si reduces the digestibility of the consumed plant tissues and that Si phytoliths cause wear to the insect mandibles, eventually impacting its performance (Massey and Hartley, 2009). Solubilized Si appears to be linked to the biochemistry of several plant secondary metabolites involved in plant defenses (Datnoff et al., 2007). Additionally, grass leaf siliceousness (i.e. silica content) impacts the behavior of certain insect herbivores. For example, the white fly *Bemisia tabaci* Gennadius preferentially lays eggs on low-Si leaves, probably to ensure reliable food resources to its progeny (Correa et al., 2005). In plant roots, Si has also been found to mitigate the effects of abiotic stresses such as salinity and heavy metals (e.g. Kim et al., 2014; Vaculíková et al., 2014; Fialová et al., 2016) and improves resistance to pathogens (e.g. Cherif et al., 1994; Safari et al., 2012; Fortunato et al., 2014). Furthermore, as is seen with insect herbivores, Si accumulation in roots can affect rodent populations, via reduced digestibility and increased wearing) (Wieczorek et al., 2015a; Wieczorek et al., 2015b). To date, there is only one example of a demonstrated impact of Si on insect root herbivores (Frew et al., 2016).

Typically, elemental analyses in plants are based on digestion protocols, that are both time consuming and potentially hazardous. Reidinger et al. (2012) reported a rapid, accurate and relatively cheap method for analyzing Si and phosphorus (P) in plant material using an X-ray fluorescence spectrometer (XRF). Here, we follow up this earlier work, presenting a protocol for analyzing the elemental composition of roots using XRF techniques. Because root material can be very scarce and laborious to collect, we have also tested various sample preparation methods to reduce the quantity of plant material required for the analysis.

Materials and Methods

All measurements were carried out on a PANalytical Epsilon 3 EDXRF spectrometer. Silicon was measured at a tube voltage of 5 kV, and a current of 60 µA in the presence of helium. Silicon standards were prepared by mixing SiO_2 with methyl cellulose (oven dried at 60°C overnight) at a range of Si concentrations (0.5%, 1%, 2%, 4%, 6%, 8%, 10%) and milling for 2 minutes at 30 Hz. Standards were measured as either big pellets (2 g of material, 33 mm diameter), small pellets (300 mg material, 13 mm diameter) or as a loose powder in a small mass holder (100 mg), and a standard curve was produced.

To validate this standard curve, Si standards were run alongside several certified plant samples, to produce a multi-element calibration allowing for simultaneous measurement of a range of elements (Table 2). Of these standards, three included Si values (NJV 94-4 Energy grass 2.1%, IPE 132 Broccoli 0.2%, NCS ZC73018 Citrus leaves 0.41%). Calibration ranges and correlation coefficients are given in Table 3.

Tests with root material

To evaluate if the XRF protocols described above can accurately measure the elemental composition of root material, we randomly selected seven root samples of long rotation Italian ryegrass (*Lolium multiflorum* Lam.) from amongst a larger number of samples harvested during a field experiment in 2016. Briefly, ryegrass plants were grown in pots for 80 days (September to December) before soil was carefully washed from the root balls. Roots were oven-dried at 40°C for three days and ground in a mixer mill (Retsch MM400) for 3 min (30 Hz) and stored at -20°C until processed. The large quantity of root material allowed for four replicates of each measurement per sample.

Root material can be laborious to collect and, once dried, only minute amounts could remain. Therefore, in an attempt to reduce the quantity of plant material used for this analysis, three sample preparation methods were assessed: 1) big pellets were pressed (10 t for 2 min, Manual Hydraulic

Press 15 t, Specac Limited, UK) using 2 g of root material (pellet size: 33 mm diameter, 2 mm thick); 2) using the same device, small pellets were pressed using 300 mg of root material (pellet size: 13 mm diameter, 2 mm thick); 3) finally, small-mass holders (PANalytical, B.V., The Netherlands) were tested with 100 mg of root material.

Statistical analyses

The overall effect of the sample preparation on measured root Si concentrations was tested with an ANOVA (*lm()* in R 3.2.3 (R Development Core Team, 2015)) and plotted with *boxplot().* Differences between sample preparation methods within samples were evaluated using the function *kruskal.test()* (One-way ANOVA on ranks) as the data were not normally distributed.

To evaluate whether elemental concentrations were similar between replicates, we examined the distribution of the residuals and identified outliers using the function *boxplot.stats().* To offer a visual assessment, the residuals were plotted with *boxplot().*

Results and Discussion

Overall, the sample preparation approach did not affect mean root Si concentrations (Figure 1, $F_{2\text{-}81}$ = 0.038, p = 0.923). Within samples, significant differences between sample preparation technics were measured in only one of the 15 samples (Table 1.). This is very encouraging for researchers looking at measuring elemental composition of roots. Indeed, collection of root material is laborious and often the quantity of dried material is not sufficient to prepare big (or even small) pellets as described above. Small-mass holders have a 20-fold smaller requirement for analytical material compared to large pellets, and do not appear to compromise the accuracy of the measurement.

Given the results discussed in the previous paragraph, the second analysis of Si, along with Mg, Al, P, K, Ca, Mn, Fe, Cu, Zn, S and Cl was performed with small-mass holders. Despite some variations within samples (Figure 2), only three outliers were detected (Si: within sample 64, Al: within sample 183, Fe: within sample 134) out of a total of 84 measurement. This makes this approach a true high throughput technique as it is virtually not necessary to replicate measurements for individual samples.

Figure 1.
Boxplot representing the total average Silicon concentration measured with three different sample preparation techniques (details on the sample preparation in the text).

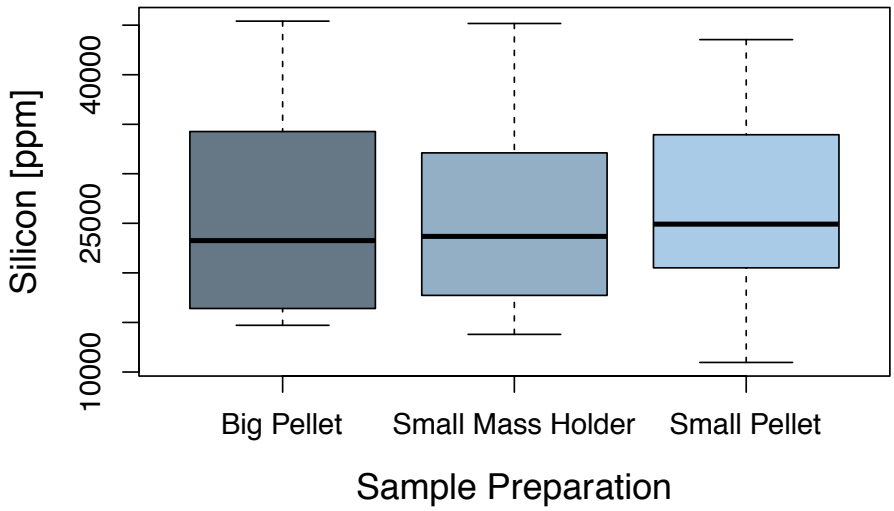
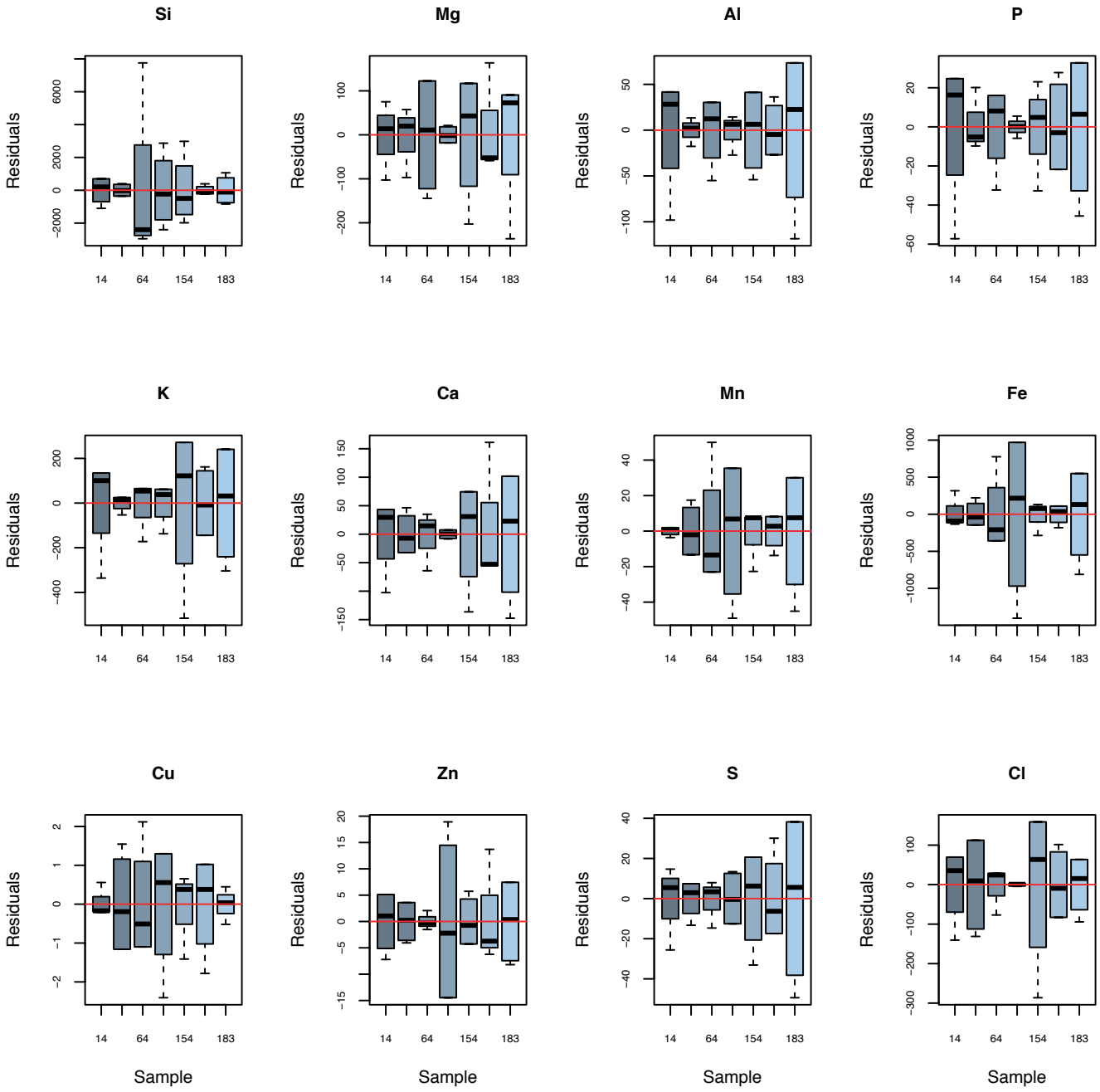


Figure 2.
Boxplots of the residuals of each sample for each of the measured elements. Very few outliers were detected, supporting the current approach to measure these selected elements in root material.



Conclusion

The production of pellets involves more sample processing, yet they can be more easily stored or even shipped if necessary. By comparison with pressed pellets, small-mass holders offer the advantage of maintaining sample integrity and reducing post-handling of the samples.

Using small-mass holders to perform XRF elemental analyses is reliable, at least for the 12 elements tested here. This approach minimizes the quantity of material required to perform the measurements without compromising the quality of the analysis. Within samples, repeat measurements are sufficiently reproducible that only a single replicate is needed to obtain a confident estimate of sample concentrations, making this approach both effective and precise. The only current limitation is the availability of standards to validate additional calibration curves in order to expand the range of elements that can potentially be analysed.

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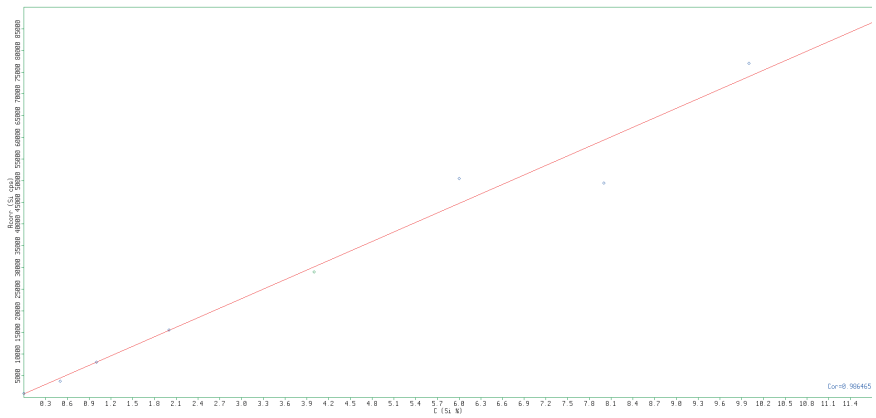

Figure 3.
Si calibration using large pellets

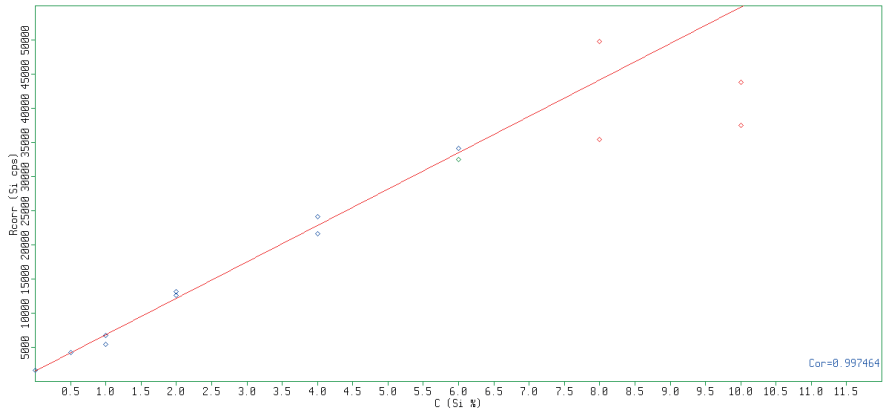

Figure 4.
Si Calibration using small pellets. Points highlighted red were excluded

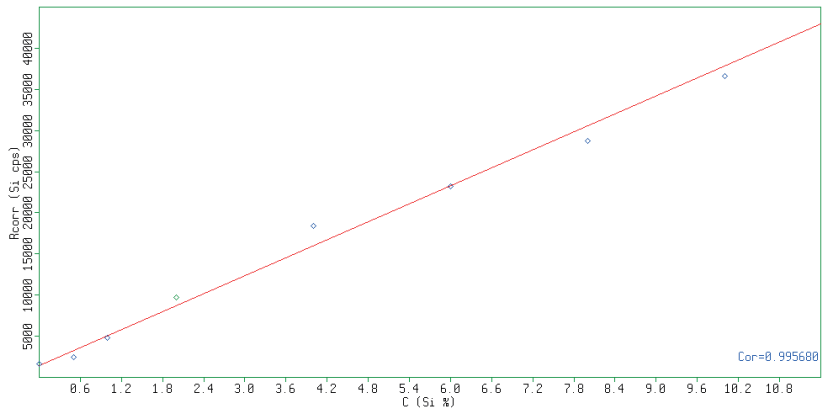

Figure 5.
Si Calibration using loose powder in small mass holders

Table 1.
Differences within samples between the sample preparation techniques.

Sample	X^2	Df	p-value	
14	7.446	2	0.024	*
31	2.470	2	0.291	ns
64	4.308	2	0.116	ns
134	3.846	2	0.146	ns
154	1.077	2	0.584	ns
181	2.007	2	0.367	ns
183	4.308	2	0.116	ns

Table 2.
Standards used to prepare calibration curves

Standard	Source
NCS ZC73018 Citrus leaves	China National Institute for Iron and Steel
IPE 132 Broccoli	Wageningen evaluating programs for analytical laboratories
IPE 682 Wheat Straw	Wageningen evaluating programs for analytical laboratories
IPE 114 Rosa (plant)	Wageningen evaluating programs for analytical laboratories
1515 Apple leaves	National Institute of Standards and Technology (NIST)
NJV 94-4 Energy grass	Swedish University of Agricultural Sciences

Table 3.
Calibrated concentration ranges and correlations *Si includes artificial standards

Element	Calibrated concentration range	Correlation (R^2)
Al	228 – 1800 (ppm)	0.90000
Ca	2100 – 42000 (ppm)	0.98596
Cl	320 – 6510 (ppm)	0.99938
Cu	2.33 – 6.6 (ppm)	0.86582
Fe	83 – 480 (ppm)	0.98852
K	2000 – 38300 (ppm)	0.99048
Mg	417 – 2710 (ppm)	0.97336
Mn	21.4 – 72.0 (ppm)	0.98466
Na	24.4 – 1530 (ppm)	0.73764
P	500 – 6310 (ppm)	0.99666
S	280 – 9850 (ppm)	0.98703
Si*	0 – 10 (%)	0.99131
Zn	10.6 – 75.00	0.91537