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A rapid and multi-element method for the analysis of major nutrients in grass (*Lolium perenne*) using energy-dispersive X-ray fluorescence spectroscopy

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Abstract

Elemental analysis of grass (*Lolium perenne*) is essential in agriculture to ensure grass quality and animal health. Energy-dispersive X-ray fluorescence (EDXRF) spectroscopy is a rapid, multi-element alternative to current methods using acid digestion and inductively coupled plasma optical emission spectrometry (ICP-OES). Percentage phosphorus (P), potassium (K), magnesium (Mg) and calcium (Ca), determined from grass samples using EDXRF, were within 0.035, 0.319, 0.025 and 0.061, respectively, of ICP-OES values. Concordance correlation coefficients computed using agreement statistics ranged from 0.4379 to 0.9669 (values close to one indicate excellent agreement); however, the level of agreement for each element depended on the calibrations used in EDXRF. Empirical calibrations gave excellent agreement for percentage P, K and Ca, but moderate agreement for percentage Mg due to a weaker correlation between standards and intensities. Standardless calibration using the fundamental parameters (FP) approach exhibited bias, with consistently lower values reported for percentage P and Mg, when compared with ICP-OES methods. The relationship between the methods was plotted as scatter plots with the line of equality included, and although correlation coefficients indicated strong relationships, these statistics masked the effects of consistent bias in the data for percentage P and Mg. These results highlight the importance of distinguishing agreement from correlation when using statistical methods to compare methods of analysis. Agreement estimates improved when a matching library of grass samples was added to the FP method. EDXRF is a comparable alternative to conventional methods for grass analysis when samples of similar matrix type are used as empirical standards or as a matching library.

Keywords

calcium • calibration • grass • magnesium • nutrients • phosphorus • potassium • X-ray fluorescence

Introduction

Agriculture in Ireland is predominantly grass based, with 90% of utilisable agricultural land devoted to grassland for livestock production under dairy and beef systems (O'Mara, 2008). In contrast to international systems in which animals are fed a total mixed ration diet, dairy farms in Ireland rely on grass as their primary source of feed, lending themselves to a complex system of ensuring that animals meet all of their dietary requirements from grass produced on the farm. Plants require up to 19 elements and animals require 25 elements for healthy growth (McGrath *et al.,* 2007). Major nutrients such as phosphorus (P), potassium (K), magnesium (Mg) and calcium (Ca) are removed in products (beef and milk) and silage or hay and need to be replaced in soils with fertiliser applications to avoid nutrient deficiencies in plants. If plant nutrient concentrations fall below the minimum requirements for animal health, the risk of nutrient deficiency in animals is increased, leading to poor animal performance and low live-weight gain (Fleming, 1977).

Dairy cows have a high dietary requirement for P, approximately 0.36% dry matter (DM) and P deficiencies such as aphosphorosis are likely if P values in feed fall below 0.20% DM (Coulter and Lalor, 2008; Ferris *et al*., 2010; Reid *et al*., 2015). As an essential nutrient for plant nutrition, Mg levels in grass can be indicative of animal intake as poor absorption of Mg by cows (only 20–25% of intake is ingested) requires a minimum requirement of 0.20% DM to prevent Mg deficiency known as grass tetany (NRC, 2001). Plants are generally regarded as efficient at taking up Ca, and average values in Irish herbage range from 0.5 to 1.4% DM, with an animal requirement of 0.45–0.65% DM for this element (NRC, 2001). The amount of K required for good-quality grass in pasture-based systems is estimated as 2.5% DM, although K deficiencies in animals are rarely reported. Given the highly variable nature of nutrients in Irish grass depending on the stage of growth (Fleming and Murphy, 1968) and soil type (Fleming *et al*., 1963), elemental analysis of grass is a useful

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measure of both plant nutrition and the ability to meet the minimum requirement of essential elements for animal health in pasture-based systems.

Conventional methods of crop analysis typically involve strong acid or alkaline digestion, followed by analysis of the filtrate by colourimetric analysis, atomic absorption or inductively coupled plasma (ICP) analysis. In routine analytical laboratories, delays are common due to high sample throughput, which can hold up the transfer of important results back to the farmer. Extending the grazing season in pasture-based agricultural systems requires rapid and reliable grass analysis to ensure grass quality throughout the growing season, so that grazing animals can meet their dietary needs at all times. Energy-dispersive X-ray fluorescence (EDXRF) spectroscopy can provide higher sample throughput with better reliable results than our current methods allow.

EDXRF spectroscopy offers an alternative to digestive methods, in the determination of total elements such as key nutrients, trace elements and heavy metals across a range of sample types. This technique allows simultaneous analysis of all elements from ^{11}Na to ^{92}U non-destructively in minutes, eliminating the time spent using different digestive reagents for different elements. Samples presented for XRF measurement are treated with an X-ray radiation source to excite the inner orbital electrons within the sample, to an excited state. When electrons relax to the ground state, fluorescent energy is emitted and the process results in measurable intensities and spectral lines, specific to each element. This technique has been more widely used in mining and geochemistry to determine the elemental composition of rocks and minerals (Al-Merey et al., 2005) and for ensuring quality control in the production of cement and other industrial materials (Bouchard et al., 2011). For environmental samples such as soils and plants, with large elemental compositions, the presence and predominance of other elements can interfere with the values for elements of interest. These are known as matrix effects and are often overcome by calibrating using simple matrices or synthetic materials spiked with a range of element concentrations (Paltridge et al., 2012; Reidinger et al., 2012). Alternatively, calibration of complex matrices often uses the fundamental parameters (FP) method, first developed in the mid-50s by Sherman (1955). This approach to calibration calls on a theoretical relationship between intensities and element concentrations and thereby creates ratios of measured intensities to that of pure elements to calculate the elemental composition of a sample. The Rigaku FP software used in this study also assumes that intensities can be affected by the absorbance and enhancement effects of other elements in the sample and provides coefficients to correct for these matrix effects (Kataoka, 1989; Kataoka et al., 2006). The application of EDXRF in plant science and agronomy has been limited to the monitoring of heavy metal concentrations during the remediation of contaminated soils; however, more recently, the use of EDXRF has provided comparable results with conventional methods for the determination of various elements in medicinal plants (Queralt et al., 2005; Elzain et al., 2016), as well as some trace elements in wholegrain wheat (Paltridge et al., 2012) and fodder (Necemer et al., 2003), using synthetic calibrators and certified reference materials (CRMs) as calibration standards.

The main objective of this work was to evaluate EDXRF as a non-destructive method for determining P, K, Mg and Ca in grass samples obtained from Irish dairy farms. This includes a comparison of calibration approaches for each element, whereby standard-based empirical calibration is compared with standardless calibration using the FP approach. Furthermore, we assess instrument repeatability and robustness of methods of sample preparation in EDXRF. Finally, we identify calibrations for each element in EDXRF that demonstrate the highest level of agreement with values determined using digestive techniques. This work represents the first study to evaluate the application of EDXRF for the determination of major nutrients in Irish grass samples.

Materials and methods

Overview

This study used an existing archive of 600 grass samples with accompanying database of known values of percentage P, K, Mg and Ca determined using acid digestion and ICP-optical emission spectrometry (OES) analysis. Archived samples of perennial ryegrass (Lolium perenne) were sampled during the stages of early and late grass growth from intensive dairy farms in the south and south-east of Ireland during 2015. From this archive and database, 21 samples were selected as empirical standards, 8 samples were selected to provide a matching library and 50 samples were selected as a validation set. These subsets of samples were selected using the following criteria. Empirical standards were selected to cover the range of values for each element in the database. Matching library samples were chosen to represent the minimum and maximum values for each element. Fifty grass samples with known values of percentages of P, K, Mg and Ca were randomly selected from the full archive (excluding standards) using the statistical function in MS Excel. Summary statistics for P, K, Mg and Ca values from the entire archive, empirical standards, matching library and validation dataset are presented in Table 1 and these values are in line with previously reported values for Irish grass (Parle et al. (2008). Using EDXRF, values of P, K, Mg and Ca were determined from the validation set using standards-based and standardless calibrations, and the level of agreement between EDXRF-determined values and ICP-

OES results was determined. CRMs were used to assess instrument stability and method robustness for grass analysis using EDXRF.

Chemical digestion and ICP-OES analysis

Grass samples returned to laboratories were dried at 70°C. All samples were ground before analysis. Grass samples were weighed out (0.5 g) and digested in 20% nitric acid using microwave heating. Digested samples were filtered prior to analysis by ICP-OES using an Agilent 5100 ICP-OES spectrometer. Calibrations for ICP-OES were prepared using multi-element certified standards and verified using an independent certified standard, and two CRMs for ICP-OES were included as quality control samples in each batch of grass samples analysed during 2015.

EDXRF measurements

EDXRF analysis was performed using a Rigaku NEX CG EDXRF spectrometer equipped with a nine-place sample changer with spin function using slow and steady spinning mode. This instrument uses secondary targets in a Cartesian geometry to produce indirect excitation of the sample. Analysis was performed under helium (He) atmosphere using a palladium (Pd) X-ray tube, with 14 mm beam spot size, and silicon (Si) drift detector with Peltier electronic circuit cooling system.

Samples were prepared in 32 mm sample cups with a polypropylene X-ray film of 4 μm thickness. Three grams of dried grass sample was weighed into sample cups and 250 inch-pound of pressure was applied using a hand press. Sample heights were measured in millimeters and sample cups were capped. All consumables were sourced from instrument supplier Dublin Analytical Instruments. EDXRF measurements were carried out according to the conditions set out in Table 2. Empirical standard samples (n = 21) selected from the archive with known percentage P, K, Mg and Ca values derived from ICP-OES methods were presented for EDXRF measurement, and the values of each element were entered into the instrument as reference concentrations. Samples were scanned and the relationship between fluorescence intensity and concentration was expressed by the linear model using the calibration function. Standardless calibration of grass samples using EDXRF used the FP method using Rigaku RPF-SQX software

Repeatability and robustness testing

Two CRMs, hay powder and Polish Virginia tobacco leaves, were used to determine instrument stability as well as method reproducibility and robustness. These materials represent EDXRF CRMs, not previously used for quality control of the ICP-OES methods mentioned earlier. Certified mean values

Table 1. Summary statistics of percentage phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg) in the grass archive, selected standards, matching library and validation dataset

Element	Full database (n = 637)	Standards (n = 21)	Validation (n = 50)	Matching library (n = 8)
	Percentage minimum–maximum (mean)			
P	0.134–0.506	0.162–0.506	0.213–0.433	0.162–506
	(0.312)	(0.305)	(0.322)	(0.321)
K	0.727–3.963	0.727–3.963	1.117–3.807	1.301–3.963
	(2.320)	(2.286)	(2.414)	(2.446)
Mg	0.072–0.303	0.072–0.3	0.104–0.216	0.072–0.30
	(0.163)	(0.186)	(0.161)	(0.199)
Ca	0.253–2.56	0.313–1.393	0.259–0.837	0.33–1.393
	(0.487)	(0.677)	(0.439)	(0.804)

Table 2. Energy-dispersive X-ray fluorescence measurement conditions for analysis of phosphorus (P), potassium (K), calcium (Ca) and magnesium (Mg)

Parameter	P	K	Mg	Ca
	Conditions			
Voltage	25 kV	50 kV	25 kV	50 kV
Secondary target	RX9 Graphite	Copper	Silicon	Copper
Peak detected	Kα, 2.015 keV	Kα, 3.313 keV	Kα, 1.254 keV	Kα, 3.691 keV
Acquisition time	100 s	100 s	300 s	100 s

and uncertainty values of percentage P, K, Mg and Ca for the CRMs used are outlined in Table 3. Instrument repeatability was examined by preparing each CRM sample and obtaining 11 EDXRF measurements of percentage P, K, Mg and Ca, determined using the empirical calibrations derived for each element. This process was repeated using standardless calibrations, using FP and FP with a matching library (FPML). In order to evaluate the repeatability of EDXRF measurements for each calibration method, EDXRF measurements were carried out under identical experimental conditions. The robustness of the method of sample preparation for EDXRF measurement was tested using both CRMs over an 11-day period using a different analyst each day. Instrument limits of detection (LODs) were determined using 3 g of clean microcrystalline cellulose prepared and pressed in a sample cup and scanned 10 times. The LOD values for each element were expressed as three times the computed standard deviation (s.d.) from 10 scans using each calibration.

Results

Developing empirical calibrations for EDXRF
Quality control of ICP-OES analyses was determined as percentage recovery of each element using two ICP-OES CRMs during all analyses carried out with the instrument in 2015. Average recovery values over this time period for CRM 1 (NCS DC 73349) were 97%, 110%, 102% and 104% for percentage P, K, Mg and Ca in grass, respectively, while CRM 2 (Polish tobacco) gave recoveries of 111%, 107%, 110% and 109% for percentage P, K, Mg and Ca, respectively.

Pearson product–moment correlation coefficients between EDXRF element intensities and ICP-OES concentrations were high for all elements ($r > 0.80$) and the computed coefficients of determination (R^2) varied from 0.79 to 0.94. These statistics represent the proportion of variation in element values explained by intensity, with R^2 values of 90% and 94% for P and K, respectively. These relationships were weaker for Mg and Ca, and the intensities explained 79% and 85% of the variation in Mg and Ca values determined by ICP-

OES. Using the Rigaku software, alpha corrections were used to correct for matrix effects or X-ray absorption/enhancement in the samples using the modified Lachance–Traill technique (Lachance and Traill, 1966), within the calibration function on the instrument. This function was used to improve the R^2 values for Mg and Ca only as no improvement to R^2 values for P and K were observed. Magnesium was corrected for the effects of copper (Cu) and Ca, and the R^2 value increased to 0.90 when the corrected concentrations were regressed against intensities. Matrix corrections for Ca values used alpha corrections for the effects of manganese (Mn), which increased the R^2 value to 0.96. Values determined from validation samples using empirical calibration are denoted by the term EDXRF_EMP.

Standardless calibration methods in EDXRF
In this study, percentage P, K, Mg and Ca determined using the FP calibration method is denoted by the term EDXRF_FP. In addition, a third calibration method was included using the FP approach in which a matching library containing eight grass samples of known concentrations was included in the method to examine how matching libraries influence values determined using FP scattering. In this study, eight samples with known concentrations of P, K, Mg and Ca were selected and these reflected the range of values described in the archive (Table 1). The results from this calibration method are denoted with the term EDXRF_FPML.

Validation of methods
EDXRF measurements were carried out on 50 samples randomly selected for validation, and concentrations of P, K, Mg and Ca were determined using the empirical standard and standardless (FP) calibration methods described earlier. Values of each element determined using each EDXRF calibration method were paired with values obtained from acid digestion and ICP-OES analysis from the same sample to compare the measurement techniques. ICP-OES measurements are treated herein as the gold standard or target values when agreement statistics were applied to the data. Measurements of agreement were derived using a SAS (SAS, 2003) macro program to compute the estimates and

Table 3. Certified reference materials (CRMs) used to test the repeatability and robustness of energy-dispersive X-ray fluorescence measurements for phosphorus (P), potassium, (K), magnesium (Mg) and calcium (Ca) (± standard deviation)

Reference material	P (%)	K (%)	Mg (%)	Ca (%)	Source
BCR–129 (Hay powder)	0.236 ± 0.007	3.38± 0.08	0.145±0.004	0.6± 0.01	Institute for Reference Materials and Measurements
INCT-PVTL-6 (Polish Virginia tobacco leaves)	0.242± 0.015	2.64± 0.09	0.241± 0.015	2.297± 0.078	Institute of Nuclear Chemistry and Technology

BCR = Community Bureau of Reference. INCT = Institute of Nuclear Chemistry and Technology.

confidence limits described in Table 4. Agreement between values obtained by XRF methods (EDXRF_EMP, EDXRF_FP and EDXRF_FPML) and values measured using ICP-OES is described using agreement statistics derived by Lin (1989) and Lin *et al*. (2002). These agreement statistics describe the proportion of data that lies within an acceptable boundary from the ICP-OES value. This boundary is the maximum acceptable difference between measurements using EDXRF and ICP-OES and it is estimated here as the total deviation index (TDI). The proportion of data lying within that boundary is defined here as the coverage probability (CP). Agreement statistics require that a boundary value and CP are set to

Table 4. Agreement estimates of concordance correlation coefficient (CCC), total deviation index (TDI) and coverage probability (CP) with 95% confidence limits (CLs) for each element determined by energy-dispersive X-ray fluorescence (EDXRF) using empirical (EMP), fundamental parameters (FP) and FP with a matching library (FPML)

Calibration	CCC	Precision coefficient	Accuracy coefficient	$TDI_{0.95}$	CP	RBS[1]
Phosphorus[2]				$TDI_{0.95}$	$CP_{0.05}$	
EDXRF_EMP	**0.9313**	**0.9428**	**0.9878**	**0.035**	**0.9942**	0.03
95% CL	0.8957	0.9091	0.9666	0.041	0.9760	
EDXRF_FP	**0.4379**	**0.9018**	**0.4857**	**0.136**	**0.2578**	7.85
95% CL	0.3398	0.8459	0.3992	0.148	0.1820	
EDXRF_FPML	**0.8542**	**0.8647**	**0.9878**	**0.051**	**0.9413**	0.02
95% CL	0.7791	0.7901	0.9453	0.060	0.8807	
Potassium[3]				$TDI_{0.95}$	$CP_{0.40}$	
EDXRF_EMP	**0.9557**	**0.9788**	**0.9764**	**0.384**	**0.9646**	0.72
95% CL	0.9333	0.9660	0.9597	0.447	0.9177	
EDXRF_FP	**0.9662**	**0.9768**	**0.9851**	**0.319**	**0.9862**	0.29
95% CL	0.9427	0.9628	0.9714	0.375	0.9570	
EDXRF_FPML	**0.9626**	**0.9774**	**0.9849**	**0.353**	**0.9734**	0.32
95% CL	0.9433	0.9637	0.9713	0.416	0.9321	
Magnesium[2]				$TDI_{0.95}$	$CP_{0.05}$	
EDXRF_EMP	**0.6008**	**0.6537**	**0.9190**	**0.043**	**0.9766**	0.15
95% CL	0.4450	0.4944	0.8128	0.050	0.9378	
EDXRF_FP	**0.6791**	**0.8768**	**0.7745**	**0.048**	**0.9843**	2.19
95% CL	0.5720	0.8082	0.6883	0.054	0.9537	
EDXRF_FPML	**0.8994**	**0.9081**	**0.9905**	**0.025**	**0.9999**	0.02
95% CL	0.8460	0.8555	0.9604	0.029	0.9984	
Calcium[2]				$TDI_{0.95}$	$CP_{0.05}$	
EDXRF_EMP	**0.8440**	**0.9313**	**0.9063**	**0.126**	**0.5187**	1.04
95% CL	0.7762	0.8913	0.8538	0.145	0.4311	
EDXRF_FP	**0.9230**	**0.9705**	**0.9510**	**0.099**	**0.6397**	1.20
95% CL	0.8868	0.9528	0.9227	0.114	0.5449	
EDXRF_FPML	**0.9669**	**0.9692**	**0.9976**	**0.061**	**0.8822**	0.03
95% CL	0.9480	0.9508	0.9869	0.073	0.8044	

[1]The relative bias squared (RBS) must be less than 1.0 or 8.0 for CP of 0.9 or 0.8, respectively, in order for the total deviation index (TDI) to be valid.
[2]For P, Mg and Ca, $TDI_{0.95}$ is the TDI when CP is set at 0.95; $CP_{0.05}$ is the CP estimate when TDI is set at 0.5.
[3]For K, $TDI_{0.95}$ is the TDI estimate when CP is set at 0.95; $CP_{0.40}$ is the CP estimate when TDI is set at 0.40. Agreement estimates are highlighted in bold.

fixed values before running the agreement statistics macro. For our data, we set the criterion that at least 95% (CP = 0.95) of the EDXRF measurements must be within a defined boundary (TDI) of the ICP-OES values for each element. For percentage P, Mg and Ca, this boundary value was fixed at 0.05, and based on the scale of observations in the percentage K data, this value was set at 0.40.

For fixed TDI (0.05 for percentage P, Mg and Ca; and 0.40 for percentage K) values, the agreement macro computed a value of CP that represents the proportion of data lying within 0.05 or 0.40 of the target (ICP-OES) value. This CP value is denoted as $CP_{0.05}$ and $CP_{0.40}$ in Table 4 and elsewhere. Correspondingly, when CP values were fixed to a predetermined value of 0.95, the analysis computed a TDI value that represented the boundary values captured in 95% of the data. This value is denoted in Table 4 and elsewhere as $TDI_{0.95}$. Estimates of $CP_{0.40}$, $CP_{0.05}$ and $TDI_{0.95}$ and their confidence limits are presented in Table 4 for each element determined using each EDXRF calibration method. Coverage probability computed from the predetermined boundary condition (0.05 and 0.40) were compared with the CP criterion of 0.95, and TDI values computed when CP was set to 0.95 were compared with TDI criterion values set to 0.05 (percentage P, Mg and Ca) and 0.40 (percentage K).

In addition, the SAS program generated the agreement plot (scatter plot) describing the linear relationship between the results from EDXRF methods and the ICP-OES values for each element, as shown in Figures 1–4, with a line of equality included in each. In addition to the TDI and CP estimates, the concordance correlation coefficient (CCC) is presented as an additional measurement of agreement between the methods (Lin, 1989). Derived from the mean squared deviation (MSD), the CCC is a product of accuracy and precision, and it measures the agreement along the line of equality when methods are presented as a scatter plot. The CCC values are presented as coefficients in Table 4, for which a value of 1.0 represents perfect agreement (EDXRF = ICP) and a value of zero represents no agreement. This value ranged from 0.4379 to 0.9669 in our analysis, indicating moderate-to-excellent agreement between the values from EDXRF methods and ICP-OES values across all elements. In Table 4, the Pearson correlation coefficient computed between EDXRF and ICP-OES values is represented by the precision coefficient. As the CCC value contains components of precision and accuracy, the coefficients of both are presented in Table 4 for each analysis. Where lack of agreement between the methods is indicated by low CCC values, the precision and accuracy coefficients provide information on whether the source of disagreement originates from within the sample variation (precision) or a shift from the true value (accuracy).

Agreement statistics for percentage P are presented in Table 4. Values determined by EDXRF using empirical calibration
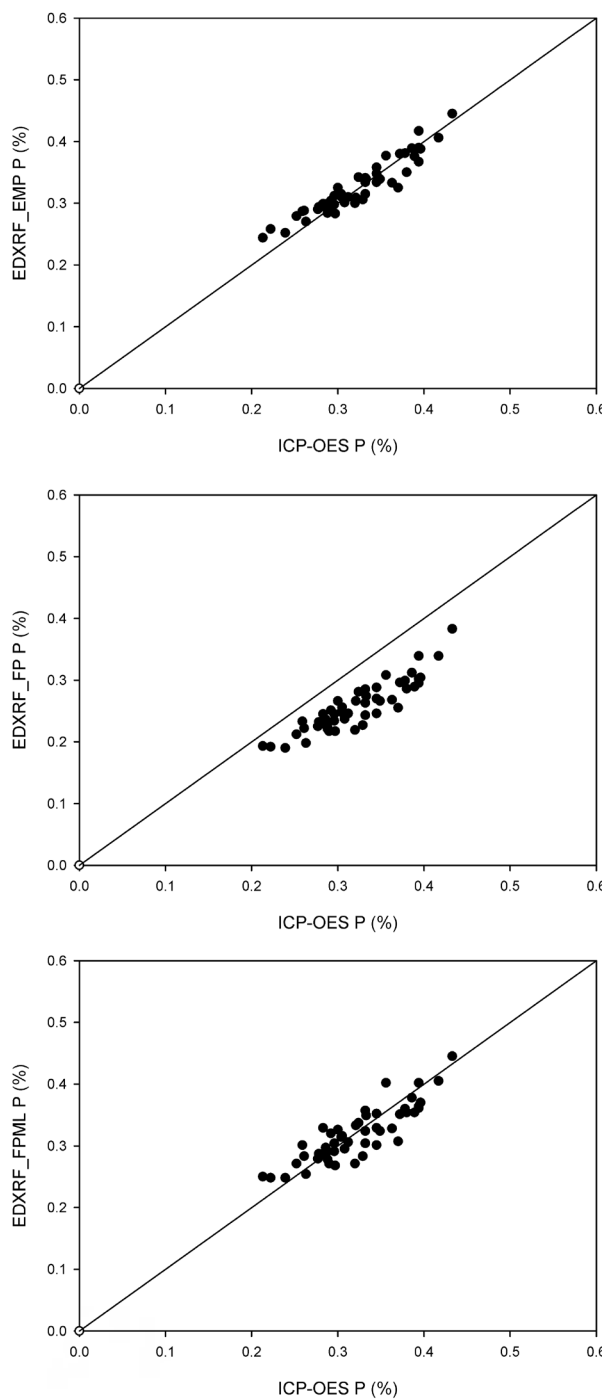
Figure 1. Scatter plots of percentage phosphorus (P) determined by ICP-OES and EDXRF using empirical method (EDXRF_EMP), fundamental parameters method (EDXRF_FP) and FP method with a matching library (EDXRF_FPML), with the line of equality included. ICP-OES = inductively coupled plasma optical emission spectrometry; EDXRF = energy-dispersive X-ray fluorescence.
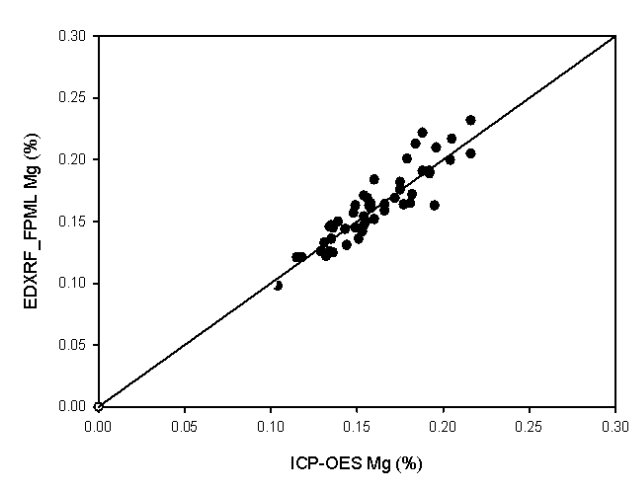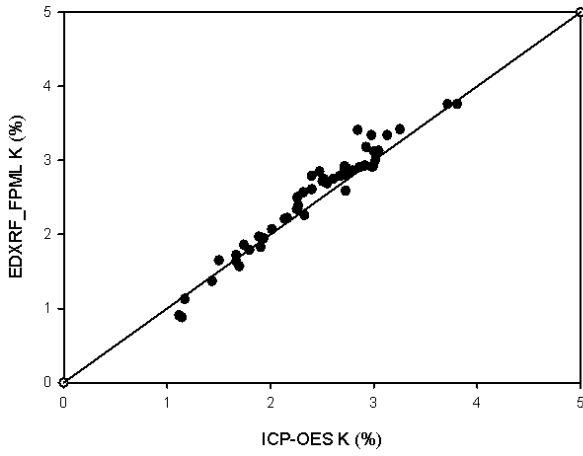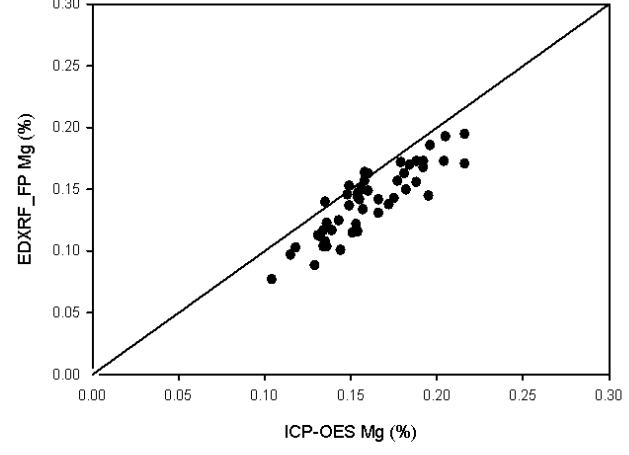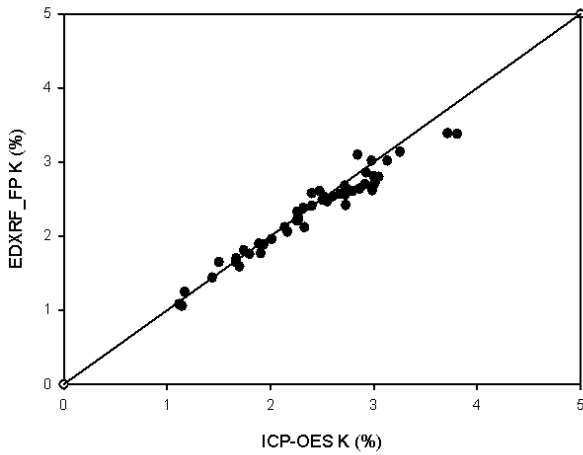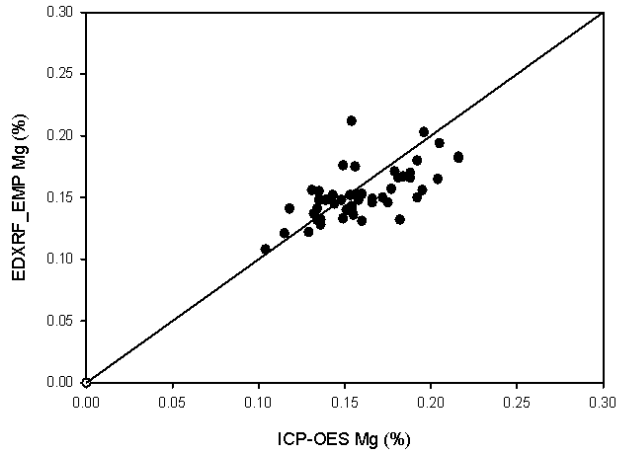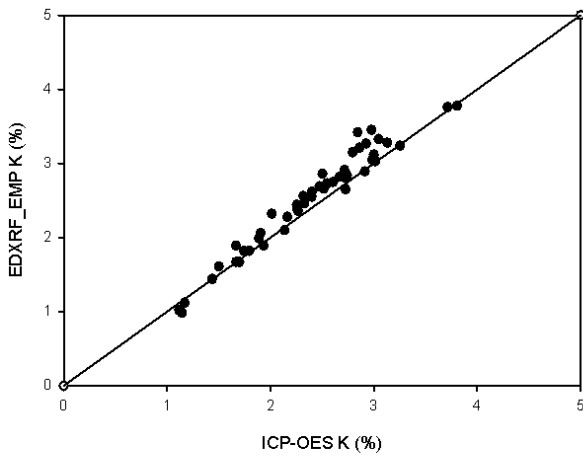
Figure 2. Scatter plots of percentage potassium (K) determined by ICP-OES and EDXRF using empirical method (EDXRF_EMP), fundamental parameters method (EDXRF_FP) and FP method with a matching library (EDXRF_FPML), with the line of equality included. ICP-OES = inductively coupled plasma optical emission spectrometry; EDXRF = energy-dispersive X-ray fluorescence.
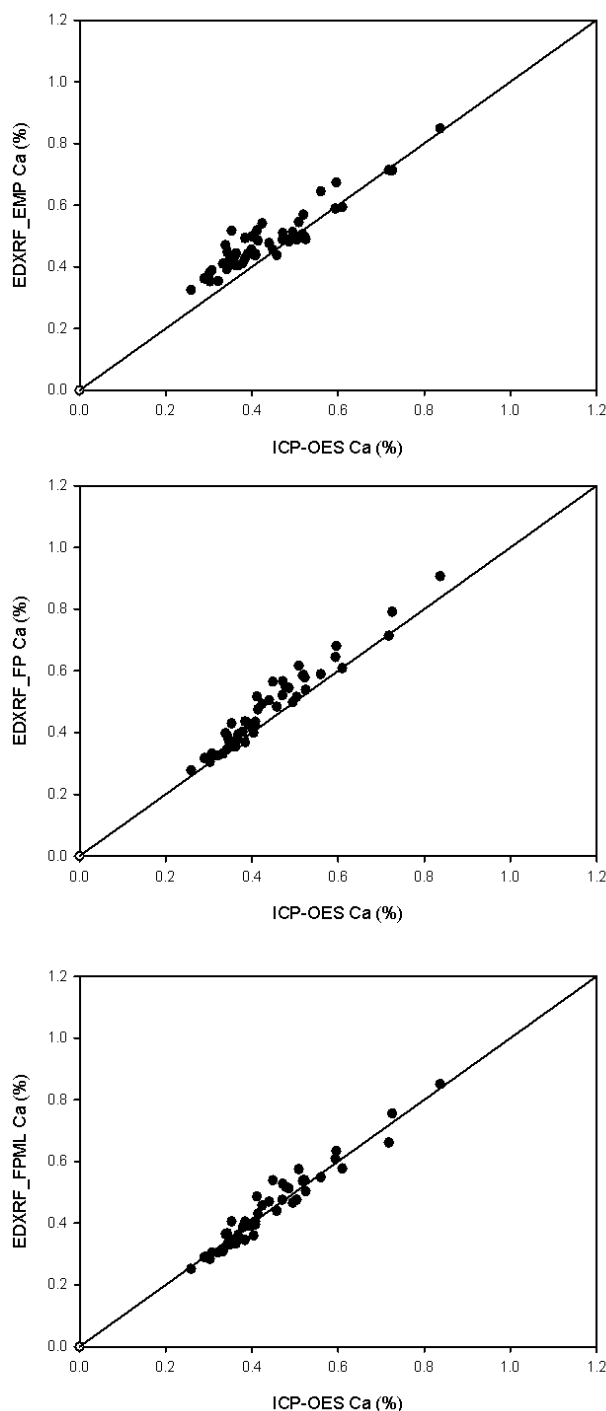
Figure 3. Scatter plots of percentage magnesium (Mg) determined by ICP-OES and EDXRF using empirical method (EDXRF_EMP), fundamental parameters method (EDXRF_FP) and FP method with a matching library (EDXRF_FPML), with the line of equality included. ICP-OES = inductively coupled plasma optical emission spectrometry; EDXRF = energy-dispersive X-ray fluorescence.

(EDXRF_EMP) showed excellent agreement with ICP values (CCC = 0.9313). The computed $TDI_{0.95}$ and $CP_{0.05}$ indicated that 95% of the data were within 0.035 for percentage P of ICP-OES values and that 99.42% of the data were within the predetermined boundary value of 0.05 for percentage P, of the target, with confidence limits of both TDI and CP lower than the set of predetermined values. However, agreement statistics computed for the EDXRF_FP method indicated poor agreement (CCC = 0.4379) with ICP-OES values, derived from poor accuracy of the methods. While the precision coefficient (Pearson correlation) was high (0.9018), indicative of a strong correlation, the level of agreement is poor, and visual inspection of the relationship between the methods in Figure 1 shows points lying to the right of the line of equality, such that P values determined by the FP method using EDXRF were consistently lower than ICP-OES values. Agreement using FP calibration for P was improved when a matching library of samples of known percentage P values were included in the FP method and estimates of CCC improved to 0.8542. This was largely due to improvement in accuracy when a matching library of similar matrix type was included. As the computed $TDI_{0.95}$ value of 0.051 for percentage P approached the predetermined boundary value of 0.05, it is likely that improved agreement could be related to the influence of known values from the matching library.

For K, conditions set for TDI and CP were 0.40 for percentage K and 0.95, respectively, and estimates computed for each calibration method in EDXRF indicated excellent agreement with ICP-OES values, with CCC values > 0.95. Comparable CCC, TDI and CP values were computed for each calibration method, indicative of excellent precision and accuracy across calibration methods to determine EDXRF values. Scatter plots in Figure 2 confirm these estimates, with the majority of points lying very close to the line of equality for each calibration method.

Magnesium values determined by EDXRF_EMP and ICP-OES were weakly correlated, as indicated by the precision coefficient (0.6537) giving moderate agreement estimates (CCC = 0.6008), even though CP and TDI values met the conditions set, with 97.66% of data lying within 0.05 for percentage Mg of ICP-OES values and 95% of data lying within 0.043 for percentage Mg of target values. Poor precision in this method was the source of disagreement with ICP-OES values, illustrated by the scatter between EDXRF_EMP and ICP-OES values in Figure 3. It is likely that the source of disagreement originated from the correlation between the standards and intensities (R^2 = 0.79) even though alpha corrections were applied to the linear relationship to generate corrected values. Magnesium values determined from the FP method of calibration indicated moderate agreement with ICP-OES values (CCC = 0.6791). Although values of TDI and CP estimates indicated that 98.43% of the data were

Figure 4. Scatter plots of percentage calcium (Ca) concentrations determined by ICP-OES and EDXRF using empirical method (EDXRF_EMP), fundamental parameters method (EDXRF_FP) and FP method with a matching library (EDXRF_FPML), with the line of equality included. ICP-OES = inductively coupled plasma optical emission spectrometry; EDXRF = energy-dispersive X-ray fluorescence.

within 0.05 for percentage Mg of ICP-OES values, the high relative bias squared (RBS) indicated that the computed TDI (0.048) was invalid. Figure 3 illustrates poor accuracy using this method, denoted by a high number of points lying to the right of the line of equality, as this method produced lower Mg values compared to ICP-OES values. When a matching library of known Mg values with a similar matrix was added to the FP calibration, the computed $TDI_{0.95}$ value of 0.025 for percentage Mg was within the target boundary, and the CCC estimate improved to 0.8994, showing excellent agreement and improved accuracy. Figure 3 depicts points lying closest to the line of equality for this calibration method for the determination of percentage Mg using EDXRF.

Computed CCC values for percentage Ca indicated good-to-excellent agreement for all calibration methods, with values ranging from 0.8440 to 0.9669; however, none of the methods met the boundary conditions or were within 0.05 for percentage Ca of ICP-OES values. Computed CP values ranged from 0.5187 to 0.8822, indicating that the proportion of the data falling within 0.05 for percentage Ca was lower than the target of 95% (0.95). The FPML method provided the highest measurement of agreement (CCC = 0.9669); however, 95% of EDXRF_FPML determined values were within 0.061 for percentage Ca of ICP-OES values (greater than the 0.05 predetermined boundary) but could provide an acceptable alternative boundary value for percentage Ca, given that high precision and accuracy coefficients were computed for this calibration method. Figure 4 illustrates the improved agreement with ICP-OES values using FPML calibration as points lie closer of the line of equality, compared to the other calibration methods.

Repeatability and robustness testing

The robustness of the method of sample preparation for EDXRF measurement was also tested using both CRMs (Table 3) over an 11-day period using a different analyst each day, and these results are presented in Table 5. For each element determined using EDXRF, results exhibited low percentage relative standard deviation (RSD) regardless of calibration used, indicating good method robustness. The instrument LOD was <0.001 for P, K and Ca and <0.04 for Mg. For repeatability testing, values of P, K, Mg and Ca were determined using EDXRF for both CRMs (hay powder and Polish Virginia tobacco leaves) and the relative standard deviation (RSD) was expressed as a percentage. These estimates are presented in Table 5, with low percentage RSD values for all elements, regardless of calibration approach, indicating good instrument stability and repeatability.

Discussion and conclusion

EDXRF values were within 0.035, 0.319, 0.025 and 0.061 of ICP-OES values for percentage P, K, Mg and Ca, respectively, for 95% of the data, demonstrating excellent agreement between spectroscopic and digestive methods for these elements in grass samples. However, agreement statistics between EDXRF and ICP-OES values depended on the calibration methods used in EDXRF. Necemer *et al*. (2003) determined sulphur (S) and chlorine (Cl) in fodder using XRF calibrated with CRMs and found comparable results with values measured using ICP-atomic emission spectrometry (AES), with accuracy between 5 and 10% for S and Cl, respectively.

In this study, using the empirical calibration method to determine P, K, and Ca provided excellent agreement with ICP-OES values; however, agreement estimates for Mg were lower and the source of disagreement originated from the linear relationship between known standards and fluorescence intensities. The empirical approach to calibration in EDXRF is recommended, provided strong linear correlations between standard concentrations and intensities can be achieved.

Table 5. Percentage relative standard deviation (RSD) calculated for each element in the CRMs hay powder and Polish Virginia tobacco leaves, derived from each calibration approach, to assess instrument stability and repeatability

	Hay powder				Polish Virginia tobacco leaves			
	P	K	Mg	Ca	P	K	Mg	Ca
Instrument stability and repeatability, percentage RSD								
Calibration								
EMP	0.27	0.35	3.86	1.06	0.35	0.40	4.46	0.74
FP	0.52	0.31	6.87	071	0.47	0.30	3.32	0.26
FPML	0.28	0.35	6.83	0.63	0.38	0.46	3.32	0.26
Method robustness, percentage RSD range[1]								
Method robustness testing	0.88–4.58	0.40–4.48	5.5–8.2	1.1–3.9	0.98–1.55	0.84–1.1	3.2–5.7	0.79–1.07

[1]A range of percentage RSD values for each element across calibration approaches is presented for method robustness testing.

Standard samples based on the same matrix type can minimise the matrix effects of absorbance and enhancement caused by the presence of other elements in the sample, which could be especially relevant for environmental samples with complex matrices such as soils, sediments, grains and grass samples. The FP approach was used in this study as a standardless calibration method. Omote *et al*. (1995) concluded that while the FP method is effective as a screening tool in instances where estimates of concentrations across a broad range of elements are required, their research on moor vegetation recommended that standard samples with similar composition to unknowns may be necessary to improve predictions. Often, these standards are prepared using similar materials, and for plant analysis for instance, cellulose with spiked concentrations of elements is used (Reidinger *et al*., 2012)

However, in this study, percentage P and Mg determined using the FP method in EDXRF were consistently lower than the ICP-OES values, despite a high correlation ($r > 0.80$) between methods for both elements. The source of disagreement between EDXRF and ICP-OES methods when FP calibration was used was largely due to lack of accuracy, which reduced the estimates of CCC for both elements. While the correlation coefficients between EDXRF and ICP-OES indicated strong relationships between methods for P and Mg, these statistics masked the effects of consistent bias in the data. These results highlight the importance of distinguishing agreement from correlation when using statistical methods to compare methods of analysis. The scatter plots between methods indicate good association or correlation between methods for all elements, with points lying along a straight line describing the relationship between methods. This approach has been used to compare EDXRF measurements on wheat with ICP values for zinc, iron and selenium (Paltridge *et al*., 2012). However, relying on correlation and regression coefficients to describe agreement between methods can be misleading (Altman and Bland, 1983; Bland and Altman, 1986; Bland and Altman, 2003) as perfect agreement exists if points lie along the line of equality but perfect correlation exists if points lie along any straight line. In our study, the level of agreement between values determined by each method is illustrated by how close the points lie along the line of equality (Figures 1–4).

The FP calibration method was supplemented with a matching library of eight grass samples covering the range of values for percentage P, K, Mg and Ca captured in the archive. When the FP method was combined with a matching library of similar matrix type, agreement improved from adequate to excellent for P, Mg and Ca. Based on the results of this study, we recommend including samples with a range of values within a similar matrix in both empirical and FP calibration methods for EDXRF to ensure high level of accuracy and

overall agreement (CCC) between EDXRF and ICP-OES values for P, K, Mg and Ca in grass samples. Reidinger *et al*. (2012) found excellent repeatability (counting and instrument statistics) in EDXRF for the determination of Si and P using plant CRMs. The repeatability and robustness testing of EDXRF calibrations methods in our study indicated steady and consistent results from both instrument measurement and sample preparation, which – combined with excellent agreement with ICP-OES – supports our conclusion that EDXRF measurement of grass samples can replace digestive analysis and provide rapid analysis of high volumes of samples to capture a range of elements with excellent accuracy and precision.

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