





RESEARCH ARTICLE

Leveraging Natural History Collections to Understand the Impacts of Global Change

Reflectance spectroscopy allows rapid, accurate and non-destructive estimates of functional traits from pressed leaves

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Abstract

1. More than ever, ecologists seek to employ herbarium collections to estimate plant functional traits from the past and across biomes. However, many trait measurements are destructive, which may preclude their use on valuable specimens. Researchers increasingly use reflectance spectroscopy to estimate traits from fresh or ground leaves, and to delimit or identify taxa. Here, we extend this body of work to non-destructive measurements on pressed, intact leaves, like those in herbarium collections.
2. Using 618 samples from 68 species, we used partial least-squares regression to build models linking pressed-leaf reflectance spectra to a broad suite of traits, including leaf mass per area (LMA), leaf dry matter content (LDMC), equivalent water thickness, carbon fractions, pigments, and twelve elements. We compared these models to those trained on fresh- or ground-leaf spectra of the same samples.
3. The traits our pressed-leaf models could estimate best were LMA ($R^2 = 0.932$; %RMSE = 6.56), C ($R^2 = 0.855$; %RMSE = 9.03), and cellulose ($R^2 = 0.803$; %RMSE = 12.2), followed by water-related traits, certain nutrients (Ca, Mg, N, and P), other carbon fractions, and pigments (all $R^2 = 0.514$ – 0.790 ; %RMSE = 12.8–19.6). Remaining elements were predicted poorly ($R^2 < 0.5$, %RMSE > 20). For most chemical traits, pressed-leaf models performed better than fresh-leaf models, but worse than ground-leaf models. Pressed-leaf models were worse than fresh-leaf models for estimating LMA and LDMC, but better than ground-leaf models for LMA. Finally, in a subset of samples, we used partial least-squares discriminant analysis to classify specimens among 10 species with near-perfect accuracy (>97%) from pressed- and ground-leaf spectra, and slightly lower accuracy (>93%) from fresh-leaf spectra.
4. These results show that applying spectroscopy to pressed leaves is a promising way to estimate leaf functional traits and identify species without destructive analysis. Pressed-leaf spectra might combine advantages of fresh and ground leaves: like fresh leaves, they retain some of the spectral expression of leaf structure; but like ground leaves, they circumvent the masking effect of water

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absorption. Our study has far-reaching implications for capturing the wide range of functional and taxonomic information in the world's preserved plant collections.

KEYWORDS

functional traits, herbarium collections, leaf chemistry, partial least-squares regression (PLSR), reflectance spectroscopy, species identification

1 | INTRODUCTION

The world's herbaria together contain more than 390 million specimens (Thiers, 2021) which are an incomparably rich source of information about global plant diversity. Herbarium specimens are collected for many reasons—often to document where a species is present or to serve as vouchers for taxonomic studies. But these specimens are often repurposed for new ends, unforeseen by their collectors (Meineke et al., 2018). More than ever, ecologists and evolutionary biologists seek to use herbarium specimens to measure functional traits (Heberling, 2022): for example, to evaluate the long-term imprint of human activity on plant communities (Lang et al., 2019; Meineke et al., 2018); to fill in gaps in sparse trait databases (Perez et al., 2020); or to conduct comparative studies of clades (Jardine et al., 2020). Measuring functional traits on herbarium specimens carries the promise of letting us reach the inaccessible, including the past or distant parts of the world. Using herbarium specimens also allows researchers to benefit from the expertise of taxonomists and refer back to the same specimens for further use—for example, as sources of genetic data, or as references for species identification from new collections (Heberling, 2022). Using specimens, researchers can address ecological and evolutionary questions that require merging functional, genetic and distributional data at global scales.

Many functional trait measurements require destructive sampling—for example, by grinding up tissue for chemical analyses. Such measurements include most protocols to determine the elemental or molecular composition of a sample (Pérez-Harguindeguy et al., 2013). Because herbarium specimens are irreplaceable—especially those from historical collections—curators may hesitate to let them be destroyed, even in part, for ecological research. Using specimens in functional ecology might be more feasible with new, non-destructive techniques to estimate their traits.

Reflectance spectroscopy is a technique often used to estimate foliar functional traits non-destructively (Curran, 1989; Jacquemoud & Ustin, 2019). Spectroscopy is the study of matter's interactions with electromagnetic radiation across wavelengths (Jacquemoud & Ustin, 2019); spectroscopic studies of leaves often target reflectance—the proportion of incident radiation that is reflected—as a particularly revealing and easy-to-measure property. A typical leaf reflectance spectrum comprises reflectance at many narrow wavelength bands between about 350 and 2,500nm, which includes over 97% of energy from solar radiation reaching Earth's

surface (American Society for Testing and Materials, 2020). Because the leaf's chemical and structural makeup determines how it reflects, absorbs and transmits light, reflectance within this range carries information about many plant traits (Cavender-Bares et al., 2017).

Two main approaches exist to estimate traits using the full information in reflectance spectra. First, physics-based radiative transfer models like PROSPECT can be inverted to estimate a handful of traits with well-defined optical properties (Féret et al., 2017). Second, statistical models, often created using machine learning techniques like partial least-squares regression (PLSR), can estimate an even wider range of traits, albeit in a less mechanistic (and perhaps less general) way (Serbin & Townsend, 2020). This multivariate empirical approach gives researchers the flexibility to predict complex traits whose absorption features might not be as strong or well defined (Curran, 1989). Likewise, multivariate classification techniques like partial least-squares discriminant analysis (PLS-DA) use the full spectrum to discriminate species, lineages or other kinds of biological classes (Meireles, Cavender-Bares, et al., 2020).

Empirical approaches like PLSR are widely used to estimate plant traits from spectroscopic data measured on fresh or ground leaves. These traits include leaf N and leaf mass per area (LMA; Kothari, Beauchamp-Rioux, Blanchard, et al., 2022; Serbin et al., 2014, 2019; Streher et al., 2020), pigments (Kothari, Beauchamp-Rioux, Blanchard, et al., 2022; Yang et al., 2016), defence compounds (Couture et al., 2016; Nakaji et al., 2019), non-structural carbohydrates (Ely et al., 2019) and even photosynthetic capacity (Yan et al., 2021). Leaf-level PLSR models have been used to address such varied ecological topics as defence responses to herbivory (Kula et al., 2020) and the role of biodiversity in ecosystem function (Schweiger et al., 2018). Although this multivariate statistical approach is flexible, it is sensitive to the kind of leaf tissue used to train the model. Existing PLSR models have mostly been trained on reflectance spectra of fresh leaves (e.g. Serbin et al., 2019) or dried, ground leaves (e.g. Serbin et al., 2014). Such models are not expected to transfer to dried, intact leaves like herbarium specimens because both drying and grinding cause major changes in reflectance.

We built PLSR models to estimate traits from the reflectance spectra of pressed leaves, like herbarium specimens, and compared their accuracy with models built from fresh or dried, ground leaves. Our pressed leaf samples were prepared like herbarium specimens but not yet mounted on paper, and our analyses might thus serve as a proof of concept for the technique by setting aside the

methodological challenges related to working with older mounted specimens. Previously, Costa et al. (2018) had shown that a related spectroscopic technique, Fourier transform-near infrared (FT-NIR) spectroscopy, could predict several leaf structural traits from pressed leaves of tropical trees. Here, we explicitly compare the accuracy of trait estimation from the spectra of fresh, pressed and ground leaves for many leaf chemical and structural traits from multiple biomes and growth forms.

For most chemical traits, such as elemental composition or carbon fractions, we conjectured that ground-leaf spectral models would be the most accurate, as others have found (Couture et al., 2016; Serbin et al., 2014; Wang et al., 2020). Both drying and grinding might be important in achieving this accuracy. Drying may reveal minor absorption features of compounds in dry matter within the short-wave infrared (SWIR) range that, in fresh leaves, are obscured by the dominant effect of water absorption (Peterson et al., 1988). Grinding homogenizes the variation in structure and composition throughout the leaf lamina, which may allow us to capture a more even and representative sample of tissue (Richardson et al., 2021). Since pressed leaves are dried but not ground, we predicted they would yield intermediate accuracy for chemical traits.

For structural traits like LMA, we instead expected fresh and pressed leaves to outperform ground leaves because grinding disrupts the leaf structure. For water-related traits like leaf dry matter content (LDMC; dried mass divided by fresh mass), we expected fresh leaves to outperform both pressed and ground leaves because they retain the water absorption features that allow direct prediction of water content (Carter, 1991). Likewise, because pigments tend to degrade after collection, we expected to estimate their concentration best from fresh leaves. We also assessed sample discoloration and considered whether it reduces the accuracy of trait estimates, which may indicate whether spectroscopy is useful on old or degraded specimens.

Finally, we asked whether pressed-leaf spectra can be used to identify samples to species. Reflectance spectra often show phylogenetic

signal in certain wavelength ranges because of phylogenetic conservatism in their underlying traits (Diniz et al., 2020; McManus et al., 2016; Meireles, O'Meara, & Cavender-Bares, 2020). This signal is what often makes it possible to classify species or higher-level taxa from fresh-leaf spectra (Cavender-Bares et al., 2016; Meireles, Cavender-Bares, et al., 2020). Studies with tropical forest species have also shown that FT-NIR absorbance spectra of pressed leaves can be used to classify species or higher-level taxa (Durgante et al., 2013; Lang et al., 2015; Prata et al., 2018). Based on these studies, Draper et al. (2020) proposed using spectra of herbarium specimens as part of an integrative process of species delimitation and identification. However, it remains uncertain whether fresh- or pressed-leaf spectra are better suited to the task of classifying species. Here, we compared the accuracy of supervised classification from fresh-, pressed- and ground-leaf spectra among the common species in our dataset. Because they preserve some of the leaf structure (unlike ground leaves) and reveal the distinctive SWIR absorption features of macromolecules and other compounds (unlike fresh leaves), pressed leaves may represent the best of both worlds for distinguishing species using spectroscopy.

2 | MATERIALS AND METHODS

2.1 | Spectral and leaf trait measurements

We trained PLSR models on leaf reflectance spectra and traits measured as part of four projects conducted by the Canadian Airborne Biodiversity Observatory (CABO). We validated the models both internally and on an independent dataset of pressed tree and herb samples collected at Cedar Creek Ecosystem Science Reserve (East Bethel, MN, USA). Table 1 describes the projects and lists how many samples and species they include for each growth form or functional group. The leaf sampling procedure is described in Supporting Information.

TABLE 1 A summary of CABO projects used for model building and internal validation (above the thick black line) and the Cedar Creek dataset used for external validation (below the thick black line). The column heading 'broadleaf' refers to broadleaf trees only

Project	Description	Samples (species) per functional group					Total
		Broadleaf	Conifer	Herb	Shrub	Vine	
Beauchamp-Rioux	Deciduous forest trees sampled throughout the growing season in 2018 at several sites in southern Québec and Ontario (Beauchamp-Rioux, 2022)	405 (9)					405 (9)
Dessain	Forbs, grasses, shrubs and broadleaf trees from forests and open areas sampled throughout the growing season in 2017 at four sites in southern Québec	31 (24)		15 (13)	26 (22)		72 (59)
Boucherville	Forbs, grasses, shrubs and vines sampled in August 2018 from the Parc national des Îles-de-Boucherville in southern Québec			45 (8)	22 (3)	6 (1)	73 (12)
Warren	A single broadleaf evergreen tree species (<i>Agonis flexuosa</i> [Willd.] Sweet) collected in November 2018 across a soil chronosequence (Turner et al., 2018) in southwestern Australia	68 (1)					68 (1)
Total		504 (25)		60 (19)	48 (23)	6 (1)	618 (68)
Cedar Creek	Forbs, grasses and broadleaf and coniferous trees sampled at Cedar Creek LTER in Minnesota in late summer and fall 2018	177 (10)	101 (4)	55 (14)			333 (28)

For each CABO sample, we measured full-range reflectance spectra (350–2,500 nm) of the leaves at three stages: (a) freshly sampled, (b) pressed and (c) oven-dried and ground into a fine powder. We measured directional-hemispherical reflectance spectra of fresh leaves on the adaxial surface of multiple leaves or leaf arrays from each sample using spectroradiometers equipped with integrating spheres. We pressed a portion of the sample and measured reflectance spectra on the adaxial surface of pressed leaves between 6 months and 3 years later using a spectroradiometer with a leaf clip (Figure S1). Lastly, we measured ground-leaf spectra using a spectroradiometer with a benchtop reflectance probe that pressed loose leaf powder into an even pellet with very low transmittance. We trimmed all spectra to 400–2,400 nm. Detailed notes on measurement and post-measurement processing of reflectance spectra are found in Supporting Information.

While measuring pressed-leaf spectra, we inspected each pressed specimen by eye to note signs of discoloration in preparation or storage. While all leaves have some changes in their appearance as they dry, we were particularly interested in the loss of green colour, such as blackening, browning or the development of a silvery or whitish finish on the leaf surface. We scored each leaf on a discrete scale from 0 to 4 (see examples in Figures S2–S5). A score of 0 indicates no noticeable discoloration. Scores 1 through 4 indicate increasing discoloration, from 1 (either <10% blackening/browning or development of a slight silvery finish to the leaf) to 4 (>75% blackening/browning).

We measured the following leaf structural and chemical traits on each CABO sample: LMA (kg/m^2), LDMC (mg/g), equivalent water thickness (EWT; mm), carbon fractions (soluble cell contents, hemicellulose, cellulose and lignin; %), pigments (chlorophyll *a*, chlorophyll *b* and total carotenoids; mg/g) and concentrations of a variety of elements (Al, C, Ca, Cu, Fe, K, Mg, Mn, N, Na, P, Zn; % or mg/g). Protocol summaries are in Supporting Information.

2.2 | Partial least-squares regression modelling for trait estimation

We used a PLSR modelling framework to predict each trait from each of fresh-, pressed- and ground-leaf spectra across the full range (400–2,400 nm). PLSR is suited to handle spectral datasets, which have many collinear predictors, because it projects the spectral matrix onto a smaller number of orthogonal latent components in a way that maximizes the ability to predict the response variable. We simply used reflectance values as predictors, since calculating various common transformations of reflectance (see Serbin et al., 2014) did not increase predictive accuracy in preliminary tests. We also did not transform any trait values to reduce skewness, again finding in preliminary tests that it did not improve predictive accuracy enough to warrant the added complexity. Some studies restrict the wavelengths used in prediction, often to ranges known or assumed to contain features relevant to a given trait (e.g. Serbin et al., 2014). We considered that the 400–1,300 nm range might be most liable to change in storage due to degradation of

photosynthetic pigments and accumulation of brown pigments that absorb in the near-infrared range (NIR; Fourty et al., 1996). Thus, we also built pressed-leaf models restricted to 1,300–2,400 nm ('restricted-range models'), which might be expected to generalize better to datasets that include older or more discoloured leaves. We present these results mainly in Supporting Information.

Our methods for model calibration and validation largely follow Burnett et al. (2021). First, we randomly divided the data into calibration (75%) and validation (25%) datasets, stratified by growth form. We began by fitting a model for each trait on the calibration dataset. We selected the smallest number of components for which the root mean squared error of prediction (RMSEP) from 10-fold cross-validation fell within one standard deviation of the global minimum. We used this number of components—a different number for each trait—in further analyses to predict traits on the internal validation dataset. We calculated the variable importance in projection (VIP) metric for calibration models to see which parts of the spectrum were most important for predicting each trait (Wold, 1994).

To test how well we could predict traits on the internal validation subset, we first did a jackknife analysis by iteratively (100 \times) dividing the 75% calibration data further into random 70% training and 30% testing subsets. For each trait, we trained models on the 70% using the previously determined optimal number of components and predicted the remaining 30%. This analysis gave us a distribution of model performance statistics across the 100 iterates (R^2 , %RMSE), which reveals the sensitivity of model performance to randomly varying sets of training and testing data.

Next, we applied the 100 jackknife models for each trait to the 25% internal validation subset, yielding a distribution of 100 predictions for each validation sample. We quantified model performance using R^2 and root mean squared error (RMSE) between measurements and mean predictions. We also report the RMSE as a percentage of the 2.5% trimmed range of measured values (%RMSE), which we used rather than the entire range (as in e.g. Burnett et al., 2021) for robustness to outliers. For each trait, we also tested whether the magnitude of residuals (observed minus predicted) in the validation dataset varied among leaves with different discoloration scores. We performed all statistical analyses in R v. 3.6.3 (R Core Team, 2020) and used package *pls* v. 2.7.1 (Mevik et al., 2019) for PLSR modelling.

In our main set of models, we kept chemical traits on a mass basis for consistency with the usual basis on which such traits (except pigments) are measured and reported. However, some traits are most often distributed proportionally to area and some users may have reasons to prefer area-based estimates (Kattenborn et al., 2019), so have we also made area-based models available (see Table S1 for performance summary statistics). All models (mass- and area-based) are available to download (see *Data Availability Statement*).

2.3 | External validation

To test how well our PLSR models would transfer to a fully independent dataset, we applied the ensemble of pressed-leaf models

for five traits (LMA, LDMC, EWT, N and C) to pressed-leaf spectra from Cedar Creek, then compared the model-derived trait estimates with measured values. Like most of the CABO dataset, the Cedar Creek dataset comprises trees and herbs from northeastern temperate North America, but it includes an entire functional group (needleleaf conifers) absent among the CABO projects in this study. We collected the spectra with the same instrument and foreoptic as the pressed-leaf spectra in the CABO dataset, but used different sampling, preparation and measurement protocols. We aimed to see whether the inclusion of a new functional group and the various subtle differences in protocols would affect the models' performance. Full details on this dataset are provided in Supporting Information.

2.4 | Partial least-squares discriminant analysis modelling for species classification

We tested the potential to classify species with fresh-, pressed- and ground-leaf spectra using partial least-squares discriminant analysis (PLS-DA; Barker & Rayens, 2003). We took spectra from the 10 most common species in our dataset—all of which are deciduous trees except *A. flexuosa*, which is evergreen. Each species was represented by at least 20 specimens (~480 total). For each tissue type, we divided the full dataset into 60% calibration and 40% validation subsets, stratified by species. In the R library *caret* v. 6.0.84 (Kuhn, 2020), we trained models on the calibration subset using 10-fold cross-validation repeated 10 times. We chose the number of PLS components during cross-validation by maximizing Cohen's kappa (κ), which describes the agreement between the true and predicted species identities while accounting for the probability of agreement by chance. Imbalanced training data can bias classification algorithms (Sun et al., 2009), so we used a two-step procedure to balance classes while maintaining enough training data and avoiding overfitting. First, we downsampled within better-represented species classes at random so that all classes had equal size, then chose the number of components (n) that maximized κ . Second, we upsampled from less-represented classes at random with replacement so that classes had equal size—again maximizing κ , but restricting the range of components evaluated to no more than the n chosen during the downsampling step. We applied the cross-validated PLS-DA model from the upsampling step to the validation subset and summarized its performance using raw classification accuracy and κ .

3 | RESULTS

3.1 | Patterns in traits and reflectance spectra

We saw large variation among samples in each of our target traits within the CABO data, ranging from 1.4-fold variation in C to more than 20-fold variation in traits like lignin, P, K and Zn (Table 2). The ranges of most traits in our dataset covered a large portion of the global distributions in the TRY dataset, but tended to be narrower

at both extremes (Kattge et al., 2020). Many traits—including LMA, LDMC, EWT, cellulose and many elements—had distributions with a pronounced skew (most often positive). Broadleaf trees tended to have higher LDMC, C and lignin than other growth forms. Among the herbs, grasses had very high hemicellulose and cellulose and low lignin content, while forbs often had high N. Some of the trait variation was driven by specific projects; for example, *A. flexuosa* in the Warren project tended to have particularly high LMA, Na and C and low N.

Both pressed and ground leaves had higher median reflectance across nearly the entire spectrum (Figure 1), as expected based on changes in water content and structure (Carter, 1991). Indeed, water absorption features (the largest of which are centered around 1,450 and 1,930nm) largely disappeared in pressed and ground leaves. The red edge between the visible and NIR regions was also blunted by both pressing and grinding, causing the global maximum of median reflectance to shift from 872nm (fresh) to 954nm (pressed) to 1,313nm (ground).

Within each tissue type, the coefficient of variation (CV) of reflectance was generally highest where average reflectance was lowest. Across tissue types, pressed-leaf spectra tended to show greater absolute variation in reflectance throughout much of the spectrum, particularly towards the tails of the distribution (e.g. the middle 95% in Figure 1b). The species that have the most exceptionally reflective pressed leaves across the spectrum (mainly *Phragmites australis* [Cav.] Trin. ex Steud., *Phalaris arundinacea* L. and *Asclepias syriaca* L.) do not have particularly reflective fresh leaves, leaving it uncertain why their pressed leaves are so reflective. In contrast, discoloured leaves tended to have lower reflectance throughout the visible and NIR ranges (Figure S5). Unlike pressed leaves, ground leaves showed very low absolute variation in reflectance throughout the SWIR, likely because grinding eliminates variation in leaf structure. However, they showed high variation from 700 to 1,100nm, which may also result from varying degrees of discoloration.

3.2 | Partial least-squares regression modelling for trait estimation

Tested on the internal validation dataset, pressed-leaf models performed best at predicting LMA ($R^2 = 0.932$; %RMSE = 6.56), C ($R^2 = 0.855$; %RMSE = 9.03) and cellulose ($R^2 = 0.803$; %RMSE = 12.2). These traits were followed by a mixture of water-related traits, carbon fractions and pigments, as well as N and Ca (all $R^2 = 0.582$ – 0.790 ; %RMSE = 12.8–17.0). Although some other elements (Mg, P) could be estimated with $R^2 > 0.5$ and %RMSE < 20, most models for other elements showed lower accuracy (Table 2; Figures 2 and 3; Figures S6–S12). In most cases, restricted-range (1,300–2,400nm) models had similar predictive accuracy to full-range models (Table S2).

We compared pressed-leaf models with both fresh-leaf and ground-leaf models. The optimal number of components selected to

TABLE 2 Summary statistics of PLSR model internal validations from full-range fresh-, pressed- and ground-leaf models. %RMSE is calculated as RMSE divided by the 2.5% trimmed range of measured values within the validation subset. Global ranges are derived from the TRY database (Kattge et al., 2020; accessed 13 April 2021), omitting data with error risk greater than 3. Carbon fractions (solubles, hemicellulose, cellulose and lignin) and carotenoids are each represented by <1,250 points in TRY, which may limit the estimates of the global range. EWT is not available on TRY

Trait	Dataset range	Global range	Fresh				Pressed				Ground			
			No. comps	R ²	RMSE	%RMSE	No. comps	R ²	RMSE	%RMSE	No. comps	R ²	RMSE	%RMSE
LMA (kg/m ²)	0.0233–0.217	0.00866–0.354	15	0.961	0.00740	5.02	9	0.932	0.00970	6.56	18	0.858	0.0139	9.44
LDMC (mg/g)	155–573	68.2–857	16	0.830	27.5	9.25	13	0.671	38.5	12.9	19	0.800	29.9	10.1
EWT (mm)	0.0488–0.282	NA	7	0.868	0.0153	10.4	10	0.790	0.0193	13.2	18	0.733	0.0213	14.5
Solubles (%)	38.5–88.9	20.0–74.6	10	0.591	5.03	16.3	13	0.743	3.97	12.8	22	0.810	3.54	11.5
Hemicellulose (%)	2.77–34.1	8.71–45.0	10	0.577	3.15	17.6	12	0.730	2.49	13.8	19	0.707	2.73	15.3
Cellulose (%)	5.19–25.8	7.65–42.6	22	0.645	1.85	16.5	16	0.803	1.38	12.2	24	0.934	0.791	7.05
Lignin (%)	0.918–21.5	2.27–32.4	8	0.551	2.10	18.4	13	0.675	1.81	15.8	22	0.818	1.35	11.8
C (%)	39.5–53.6	25.5–60.3	11	0.751	1.09	11.7	17	0.855	0.839	9.03	22	0.932	0.562	6.06
N (%)	0.883–5.62	0.14–7.27	12	0.692	0.329	15.1	15	0.754	0.297	13.6	21	0.961	0.117	5.38
Chl a (mg/g)	1.24–15.5	1.38–22.1	5	0.724	1.09	14.2	8	0.675	1.18	15.4	16	0.744	1.04	13.6
Chl b (mg/g)	0.486–5.29	(total Chl)	5	0.693	0.402	14.5	4	0.582	0.469	16.8	7	0.671	0.413	14.9
Carotenoids (mg/g)	0.193–3.05	0.00102–5.1	5	0.672	0.235	15.9	8	0.632	0.251	17.0	20	0.790	0.189	12.8
Al (mg/g)	0–0.379	0.004–19.7	6	0.326	0.0423	23.0	8	0.350	0.0418	22.7	10	0.504	0.0362	19.7
Ca (mg/g)	1.64–36.9	0.68–69	14	0.436	4.83	19.7	17	0.752	3.23	13.1	26	0.877	2.22	9.02
Cu (mg/g)	0–0.052	0.00154–0.064	6	0.428	0.00471	21.6	20	0.314	0.00508	23.1	9	0.500	0.00443	20.3
Fe (mg/g)	0.0150–0.266	0.0128–1.1	3	0.165	0.0399	21.7	8	0.209	0.0390	21.2	8	0.394	0.0341	18.6
K (mg/g)	1.06–38.2	0.3–78.1	7	0.553	3.23	19.3	16	0.488	3.33	21.4	14	0.710	2.61	15.6
Mg (mg/g)	0.482–7.86	0.277–15.6	14	0.243	0.890	24.5	10	0.514	0.714	19.6	18	0.603	0.659	18.2
Mn (mg/g)	0–1.02	0.00560–5.37	4	0.242	0.182	22.1	13	0.386	0.165	20.0	17	0.487	0.148	18.0
Na (mg/g)	0–4.87	0.00499–48.7	9	0.480	0.508	21.8	10	0.494	0.503	21.5	8	0.353	0.565	24.3
P (mg/g)	0.222–7.18	0.1–9.9	10	0.449	0.859	17.3	20	0.621	0.714	14.3	12	0.550	0.776	15.6
Zn (mg/g)	0.00142–0.647	0.00221–0.242	8	0.361	0.0658	25.1	11	0.444	0.0615	23.4	11	0.517	0.0571	21.8

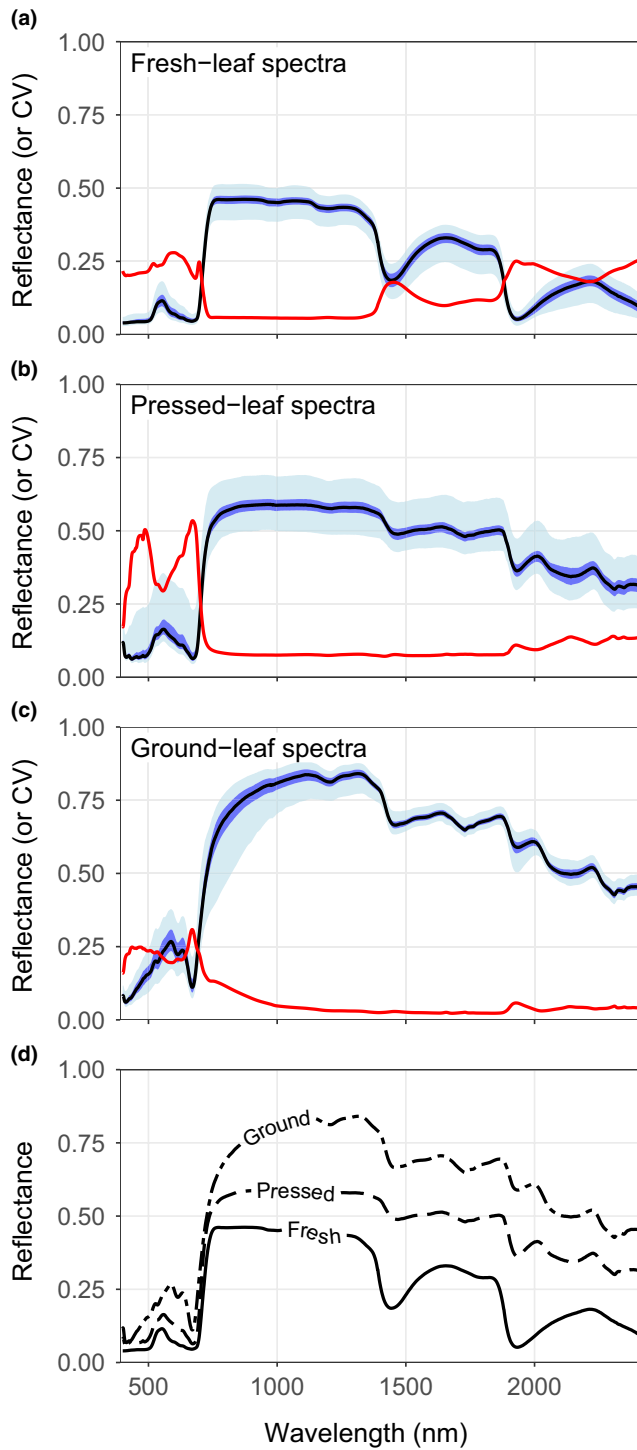


FIGURE 1 Distributions of spectral reflectance and its coefficient of variation (CV) among fresh (a), pressed (b) and ground (c) leaf samples. A solid black line connects the median reflectance at each wavelength. Dark blue and light blue ribbons denote the middle 50% and 95%. The solid red line shows the coefficient of variation at each wavelength. In (d), the medians of all three are shown for comparison.

predict each trait was between 3 and 26. For any given trait, ground-leaf models usually had the most components, often followed by pressed-leaf models (Table 2). Fresh-leaf models were best for

predicting the structural and water-related traits—LMA, LDMC and EWT (Figures 2 and 3; Figures S6–S12). Ground-leaf models were best for predicting chemical traits, like carbon fractions and most elements. For most traits, pressed-leaf models had intermediate performance, although for some (e.g. pigments, LDMC) both fresh- and ground-leaf models performed better. Statistics from jackknife analyses showed that model performance was more variable for traits that were predicted less accurately (Figures S13–S15). There was no correlation between the magnitude of residuals from pressed-leaf models and our discoloration index for any trait ($p > 0.05$).

For all traits except LMA and Fe, the VIP metric for fresh-leaf spectra showed a global maximum between 710 and 720 nm (Figure 4; Figures S16–S18)—wavelengths slightly longer than the typical inflection point of the red edge (Richardson et al., 2002). Many traits also show high VIP across the green hump at ~530–570 nm. Bands in the NIR range were less important for predicting most traits than much of the visible range. The SWIR range was generally important for predicting LMA, EWT, Na and pigments, and many other traits showed several local peaks of importance, most prominently at about 1,880 nm, but also near 1,480 and 1,720 nm.

For predicting traits from pressed-leaf spectra, the general trend held that visible reflectance and certain ranges in the SWIR were important for predicting most traits, while the NIR and much of the shorter SWIR (800–1,750 nm) were less important (Figure 4; Figures S16–S18). The red edge peak of importance for most traits was near 705 nm. Other prominent local maxima for many traits lay close to 1,440, 1,720, 1,920, 2,130 and 2,300 nm. We saw broadly similar patterns in ground-leaf spectra, except that VIP for most traits was lower at longer SWIR wavelengths (2,000–2,400 nm).

3.3 | External validation

For most traits, pressed-leaf model performance on the external validation dataset from Cedar Creek was not quite as strong as the internal validation (Table 3; Figure 5). For C, the models performed very poorly ($R^2 < 0.05$). Among the remaining traits, R^2 ranged from 0.350 (LDMC) to 0.876 (LMA). For N in particular, %RMSE was high (37.8%) due to bias—N concentrations were underestimated for conifers but slightly overestimated for remaining samples.

Since conifers were absent from the CABO training dataset, we considered whether the models we built would extend to this new functional group. For LDMC, models performed better when excluding conifers ($R^2 = 0.406$, %RMSE = 22.8) than when retaining them ($R^2 = 0.350$; %RMSE = 24.7). In contrast, for LMA and EWT, models performed better when retaining conifers. For LMA in particular, estimates for conifers were quite good, and their extension of the trait range raised R^2 (from 0.752 to 0.876) and reduced %RMSE (from 16.4 to 10.3). Restricted-range models yielded better external validation R^2 for N and LDMC both including and excluding conifers (Table S3; Figure S19). For LDMC in particular, this improvement resulted from improved estimates for extremely discoloured samples of *Populus tremuloides* Michx.

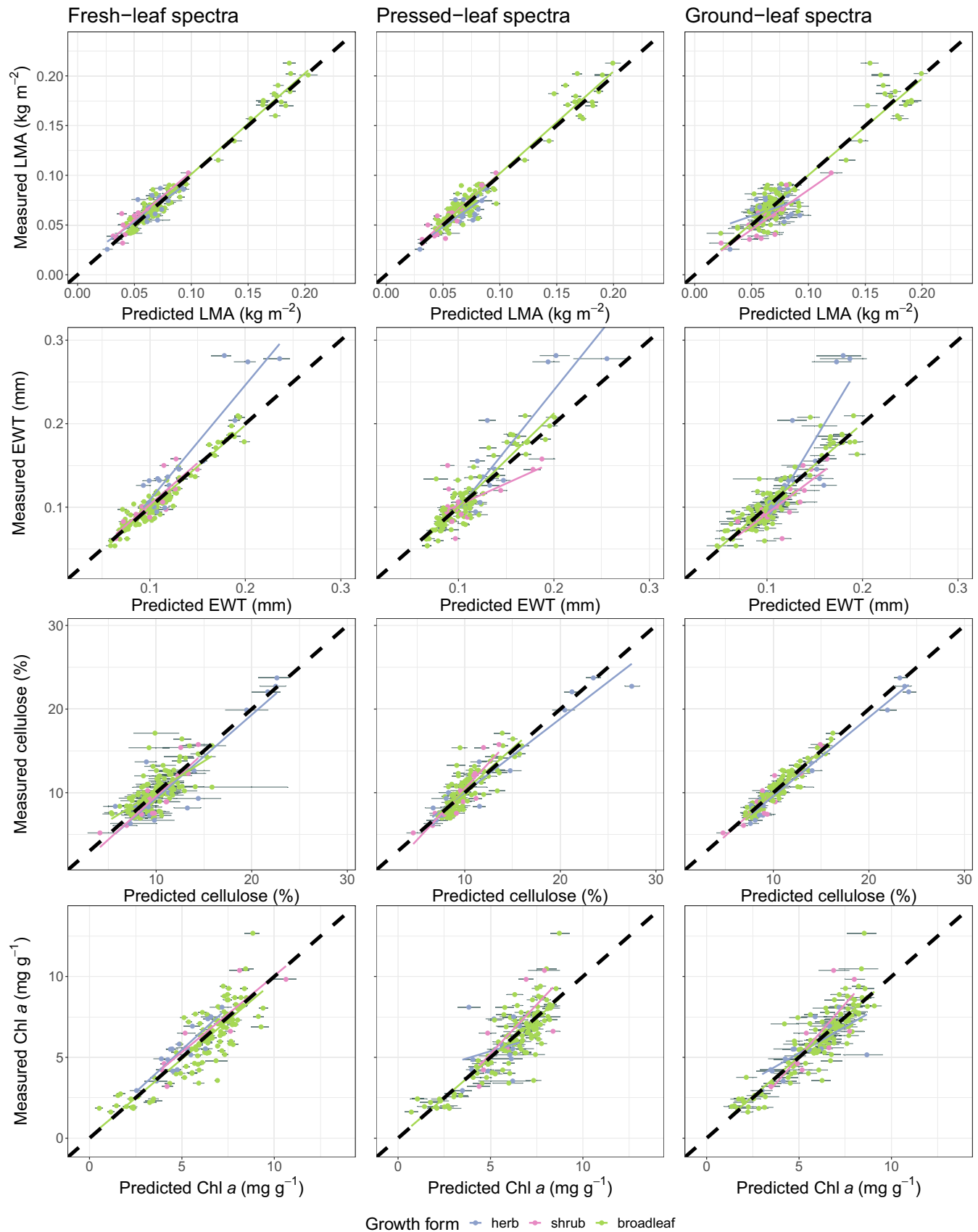


FIGURE 2 Internal validation results for LMA, EWT, cellulose and chlorophyll *a* predicted from fresh- (left), pressed- (middle) and ground-leaf (right) spectra. In each panel, each growth form has a separate ordinary least-squares regression line overlaid on top of the thick dashed 1:1 line. The error bars for each data point are 95% confidence intervals calculated from the distribution of predictions based on the ensemble of 100 models produced in jackknife analyses. **Figures 2** and **3** display a subset of traits that represent various functions and span the range of model performance. Plots for remaining traits are in Supporting Information.

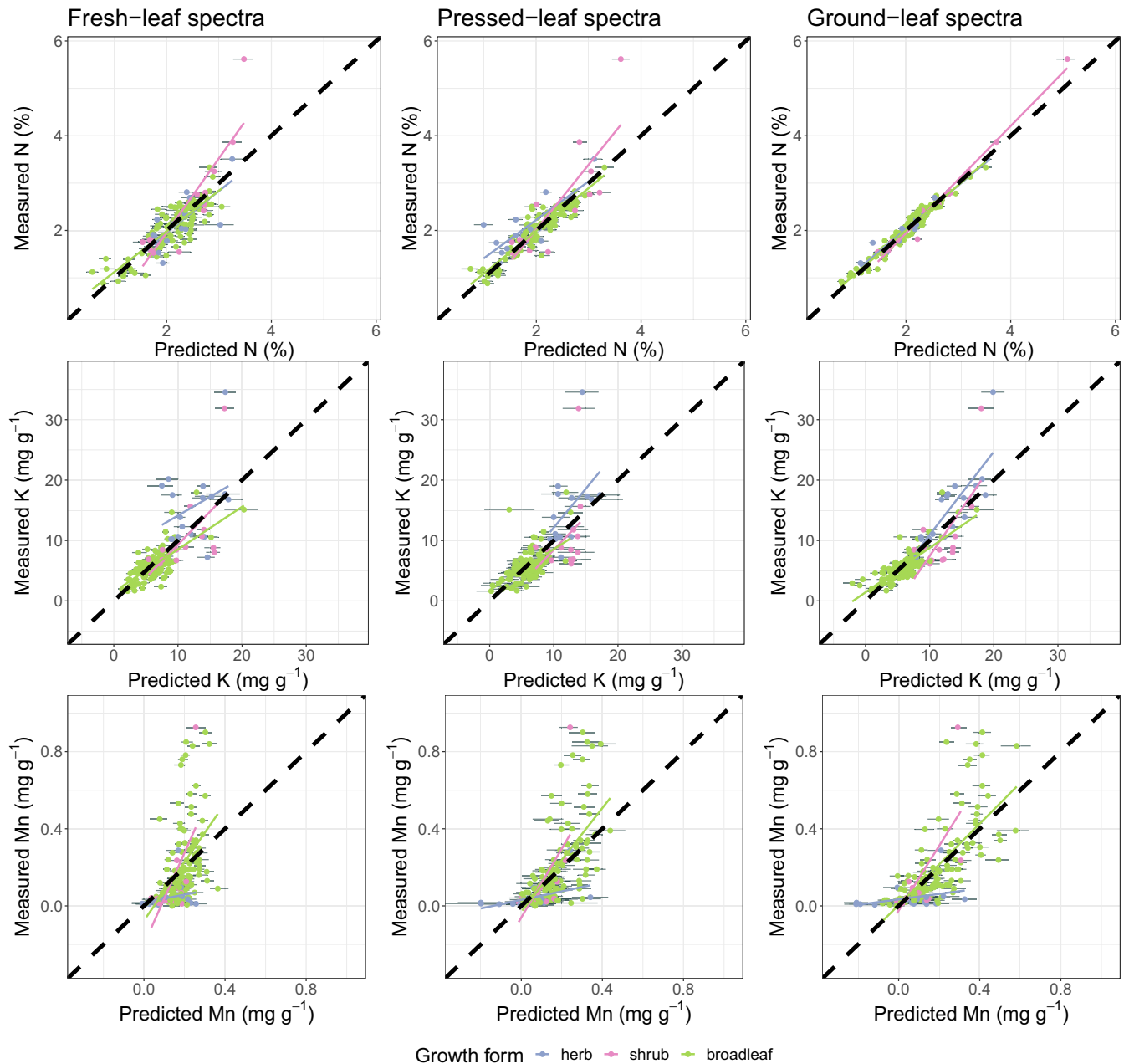


FIGURE 3 Internal validation results for three elements (N, K and Mn) predicted from fresh- (left), pressed- (middle) and ground-leaf (right) spectra, displayed as in Figure 2.

3.4 | Partial least-squares discriminant analysis modelling for species classification

PLS-DA models using pressed- and ground-leaf spectra showed near-perfect performance at classifying species (Figure 6). Models using fresh-leaf spectra were slightly worse but still showed strong performance. The optimal fresh-leaf model, which had 28 PLS components, correctly predicted the taxonomic identity of 175 of the 188 samples in the training dataset ($\kappa = 0.920$; $p < 0.0001$). The best pressed-leaf model, which had 37 PLS components, correctly predicted 184 of the 188 samples ($\kappa = 0.975$; $p < 0.0001$). The best ground-leaf model, which had 48 PLS components, correctly

predicted all 189 samples ($\kappa = 1$; $p < 0.0001$). The majority (>70%) of misclassifications were between congenics.

4 | DISCUSSION

We show that we can estimate a wide range of leaf functional traits among 68 woody and herbaceous species from reflectance spectra of pressed leaves (Table 2; Figures 2 and 3; Figures S6–S12). Model performance was highest for LMA, C and N, followed by a mixture of water-related traits, carbon fractions, pigments and a few important elements (Ca, Mg and P). Other elements could only be estimated with fairly low

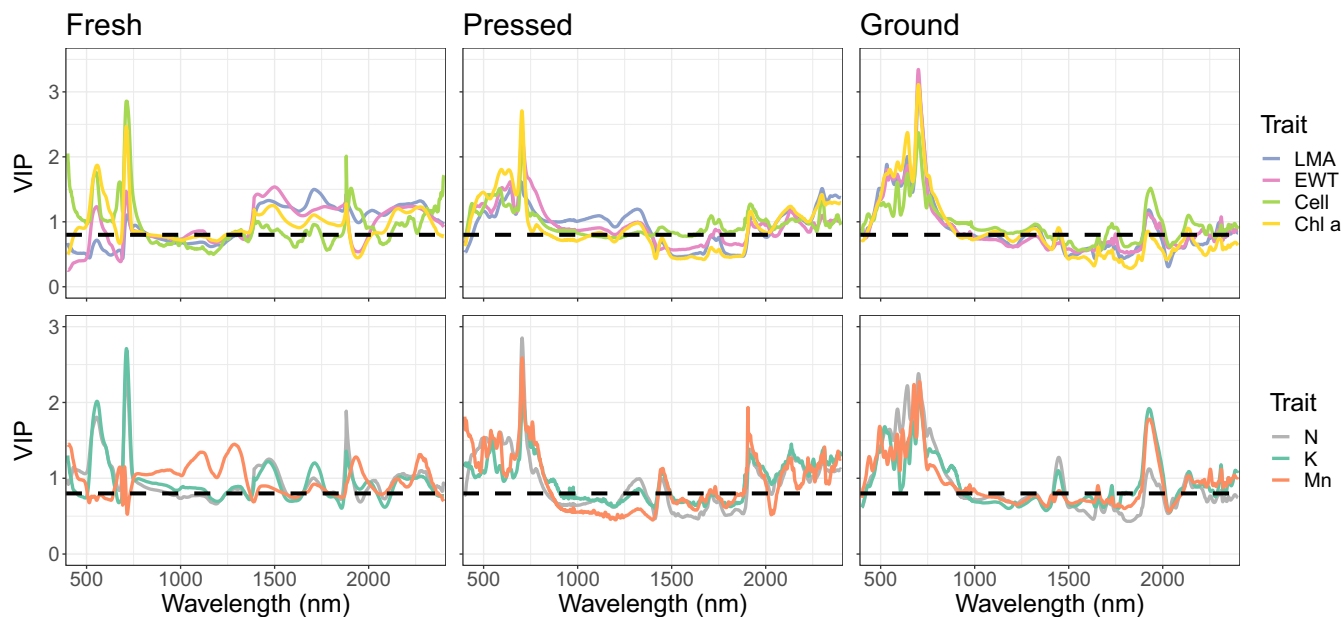


FIGURE 4 The variable importance in projection (VIP) metric calculated based on fresh- (left), pressed- (middle) and ground-leaf (right) models. As in [Figures 2 and 3](#), we selected seven traits that represent a range of patterns in VIP across the tissue types (cell = cellulose). The dashed horizontal line at 0.8 represents a heuristic threshold for importance suggested by [Burnett et al. \(2021\)](#). VIP plots for remaining traits are in Supporting Information.

TABLE 3 Summary statistics for external validation of pressed-leaf PLSR models. The models were trained on CABO data (see [Table 2](#)) and applied to a dataset collected at Cedar Creek

Trait	Including conifers				Excluding conifers			
	Range	R^2	RMSE	%RMSE	Range	R^2	RMSE	%RMSE
LMA (kg/m^2)	0.0179–0.229	0.876	0.0186	10.3	0.0179–0.136	0.752	0.0132	16.4
LDMC (mg/g)	133–525	0.350	69.8	24.7	133–504	0.406	65.6	22.8
EWT (mm)	0.0602–0.372	0.666	0.0422	17.1	0.0602–0.363	0.586	0.0337	18.6
C (%)	35.7–59.8	0.027	4.58	25.6	35.7–54.1	0.025	4.40	27.2
N (%)	0.75–4.68	0.544	0.723	37.8	0.75–4.68	0.548	0.797	31.5

accuracy. These results show that pressed-leaf spectra provide an integrative measure of leaf phenotypes, much like fresh-leaf spectra ([Cavender-Bares et al., 2017](#)), but with stronger potential to characterize variation in chemical traits. Perhaps as a result, we could use pressed-leaf spectra to classify species as accurately as ground-leaf spectra and better than fresh-leaf spectra. Our results underscore the potential that using reflectance spectroscopy on herbarium specimens could yield rapid and non-destructive estimates of many functional traits, enabling more expansive studies of trait variation across space and time.

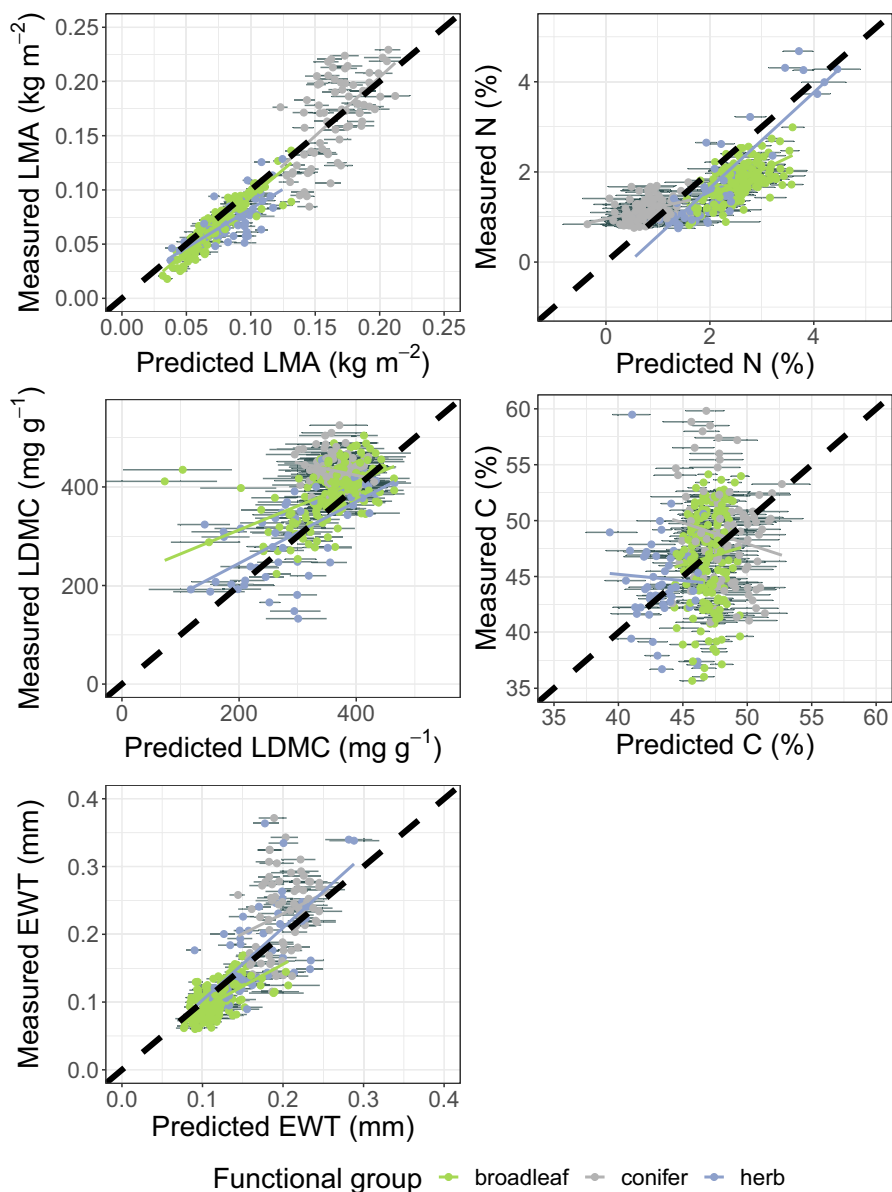
4.1 | Comparing partial least-squares regression analysis model performance

We compared pressed-leaf models to fresh-leaf and ground-leaf models from the same samples. Our findings about which kind of tissue was best for predicting each trait mostly supported our hypotheses. Ground-leaf spectra showed the strongest performance for most chemical traits, likely because grinding homogenizes the lamina

and removes the potentially confounding influence of leaf structure ([Table 2](#)). Pressed-leaf spectra showed intermediate performance for most chemical traits, perhaps because, like ground leaves, they lack the major water absorption features that mask the smaller features of other compounds in the SWIR range ([Peterson et al., 1988](#)). Contrary to our predictions, ground-leaf spectra performed about as well as fresh-leaf spectra, and better than pressed-leaf spectra, for estimating pigment concentrations. The same factors that provided an advantage to ground-leaf spectra in estimating chemical traits also perhaps explain why pressed- and (especially for LMA) ground-leaf spectra performed worse for estimating water-related and structural traits (LMA, LDMC and EWT). Pressed-leaf models may represent a good compromise in allowing many traits to be estimated with mostly intermediate but nonetheless quite high accuracy.

Our pressed-leaf models often performed as well as fresh- and ground-leaf models published here and elsewhere. For example, our models for LMA had an RMSE ($0.00970 \text{ kg}/\text{m}^2$) lower than many fresh-leaf models from the literature, including [Serbin et al. \(2019; \$0.015 \text{ kg}/\text{m}^2\$ \)](#), [Nakaji et al. \(2019; \$0.015 \text{ kg}/\text{m}^2\$ \)](#) and

FIGURE 5 External validation results for various traits predicted from pressed-leaf spectra of Cedar Creek samples using models trained on CABO data. Panels are displayed as in Figures 2 and 3.



Streher et al. (2020; 0.051 kg/m²). The ground-leaf models in Serbin et al. (2014) had a validation RMSE of 1.4 and 2.4 for cellulose and lignin percentages, comparable with 1.38 and 1.81 for our pressed-leaf models (Table 2). On the other hand, Serbin et al. (2014)'s ground-leaf models for N performed better than our pressed-leaf models (2014; validation RMSE = 0.13 vs. 0.297). Our models' error could be within acceptable bounds for addressing many questions about large-scale ecological or evolutionary patterns that encompass a wide range of trait variation. For some traits (e.g. EWT, N, K, Mn) many of the samples with the greatest errors were at the poorly sampled tails of the measured trait distribution, which suggests that more thorough sampling may be needed to ensure models can make reliable predictions at these extremes (Figures 2 and 3). Nevertheless, our external validation analyses indicate that our models for some important traits—like LMA, LDMC, EWT and N, but not C—can transfer reliably to other datasets and even sometimes to new functional groups (Figure 5).

4.2 | Interpreting partial least-squares regression model performance

It may seem perplexing that we could succeed at all in predicting LMA from ground-leaf spectra or LDMC and EWT from pressed- and ground-leaf spectra. The ability to estimate these traits must not result from the optical expression of the traits themselves. We suggest that we instead sense these traits via their correlations with other traits that have a stronger optical expression. This kind of effect—a 'constellation effect' (sensu Chadwick & Asner, 2016; Nunes et al., 2017)—has been invoked to explain the ability to estimate traits like rock-derived nutrients (Nunes et al., 2017) and $\delta^{15}\text{N}$ (Serbin et al., 2014) that are not known to have strong absorption features in the measured range of wavelengths. However, models that rely on such constellation effects could break down when patterns of trait covariance vary (Kothari & Schweiger, 2022), which could make models for certain traits fail beyond the domain of the training data.

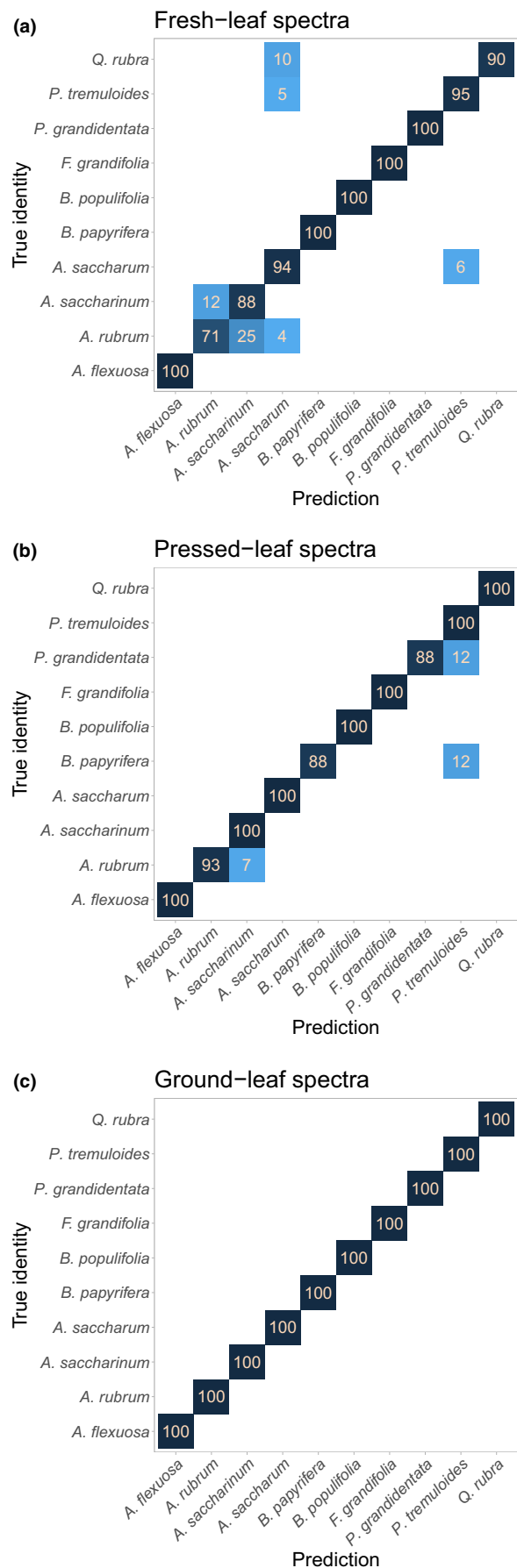


FIGURE 6 Confusion matrices for partial least-squares discriminant analysis from (a) fresh-, (b) pressed- and (c) ground-leaf spectra. Rows specify the true species identity, while columns specify the models' predictions. Each row sums to 100: Numbers on the diagonal represent the percentage of specimens of each species that were correctly classified, while off-diagonals represent the percentage misclassified as other species. Full binomials: *Agonis flexuosa*, *Acer rubrum* L., *Acer saccharinum* L., *Acer saccharum* Marshall, *Betula papyrifera* Marshall, *Betula populifolia* Marshall, *Fagus grandifolia* Ehrh., *Populus grandidentata* Michx., *Populus tremuloides* Michx. and *Quercus rubra* L.

The potential role of constellation effects makes it important to interpret how our models work. The VIP metric helps us understand what features are most important, but interpreting it can be challenging because reflectance at a given band is never driven by a single trait. Fresh-leaf VIP for most traits had peaks in the visible and SWIR ranges, with a global maximum at 705 nm (Figure 4 and Figures S16–S18). The pattern of high VIP along the green hump and red edge is common in PLSR models from fresh leaves (Ely et al., 2019; Streher et al., 2020; Yan et al., 2021; Yang et al., 2016). The red edge (particularly 700–725 nm) may be so important because of its sensitivity to both chlorophyll content and leaf structure (Richardson et al., 2002). Much of the visible range was proportionally even more important for pressed- and ground-leaf models, and most of the NIR was less important (except for LMA; Figure 4; Figures S16–S18). There were multiple small VIP peaks in the SWIR. Although some (e.g. at 1,440 and 1,920 nm) lie within major water absorption features, any causal link to the leaf's fresh water content is unlikely for pressed and ground leaves. Many of these peaks also lie near broad absorption features for many components of dry matter, including protein, cellulose, lignin and starch, which complicates their interpretation (Curran, 1989; Fourty et al., 1996).

With some exceptions, the VIP metric showed that the same bands are often important for predicting different traits. This pattern might be taken as an artefact of trait covariance: For example, the three pigment pools covaried strongly ($R^2 = 0.827\text{--}0.969$) and had nearly identical VIP across the spectrum (Figures S16–S18). One might take similarities in VIP further to imply that there are a small number of traits whose tight coordination with others underlies the performance of all models through constellation effects. Nevertheless, across the whole dataset, many traits covaried only weakly but still shared VIP patterns. For example, EWT, cellulose, N and K were not tightly coordinated ($R^2 = 0.003\text{--}0.152$) but shared similar patterns of pressed-leaf VIP across the spectrum (Figure 4), including peaks at 705 and 1,920 nm. While VIP is a useful heuristic, it does not show the direction in which a band's reflectance alters trait estimates; the same bands may matter for different traits in different ways. Here, similarities in VIP do not appear to result solely from strong networks of trait covariance. Nevertheless, the fact that we can estimate traits like LDMC and EWT from pressed-leaf spectra appears to imply some role for trait covariance, perhaps in a more diffuse way.

4.3 | Partial least-squares discriminant analysis modelling for species classification

PLS-DA models showed that fresh-, pressed- and ground-leaf spectra alike could be used to classify species with perfect accuracy for ground leaves, near-perfect accuracy (>97%) for pressed leaves and excellent accuracy (>93%) for fresh leaves (Figure 6). In contrast to prior work that deliberately selected many congeners (Lang et al., 2015), our most common species were often distantly related. Among the misidentified samples, most were mistaken for congeners, which implies that related species are more spectrally similar (Meireles, O'Meara, & Cavender-Bares, 2020; Schweiger et al., 2018). However, past studies using dry leaves have shown great success with closely related species (Lang et al., 2015; Prata et al., 2018) or even populations (Stasinski et al., 2021).

Our analysis reinforces that that pressed- or ground-leaf models might be particularly suited to the task of classifying or delimiting species (Figure 6). This finding is notable because measuring spectra of pressed leaves in an herbarium is also much simpler than measuring spectra of fresh leaves through an intensive field campaign across the range of a clade. We conjecture that these models have an advantage because drying reveals the absorption features of multiple compounds in the SWIR range that might together allow finer discrimination of species than water content does. Indeed, ground-leaf spectra have greater intrinsic dimensionality than fresh-leaf spectra (Kothari & Schweiger, 2022), which suggests they have more independent axes of variation along which species may separate. Our results support the growing practice of using spectra of pressed herbarium specimens in species delimitation and identification (Draper et al., 2020; Prata et al., 2018). However, classification models from pressed or ground leaves have less relevance for research using remotely sensed imagery, which is typically dominated by fresh leaves.

4.4 | The future of spectroscopic trait estimation

Although trait predictions from spectral models are not perfect, they have a few advantages over conventional trait measurements: they (a) can be non-destructive, (b) are fast and require relatively little training and (c) have very low marginal cost, despite the high capital cost of buying a spectrometer (Costa et al., 2018). These advantages could make it easier to address questions that require large datasets of functional traits. But researchers may be deterred if they must each build their own models tailored to particular uses—and for herbarium specimens, it might not be possible to do the destructive trait measurements often needed to train the models. Ideally, spectral models would be general enough that researchers could confidently use them without further validation, but this aim is not easy to achieve: for several reasons, a model trained on any particular spectral dataset may make poor trait predictions on new data. As with any other technique, the goal for spectroscopic trait estimation is to improve model accuracy

and generality as much as they can be jointly improved. Below, we discuss some challenges one by one, particularly as they concern pressed leaves.

One concern is that the new data could be outside the range of traits or optical properties in the training dataset (Schweiger, 2020). A general model, if such a thing is possible, would need to represent the vast range of leaf functional traits and optical properties. Another kind of concern about model generality concerns sample preparation before spectral measurements. For example, particle size influences ground-leaf spectra (Foley et al., 1998). For pressed leaves, it may be particularly important to prepare samples in consistent ways that preserve the leaves' anatomical integrity. In our external validation analyses, we found that pressed-leaf models yielded reasonably accurate predictions of most traits, even though the validation dataset differed in sample preparation protocols and included conifers, which were absent from the training dataset. Nevertheless, even setting aside conifers, external validation for one trait (C) was very poor, and for another (N) showed noticeable bias—enough that researchers might need to develop their own correction factors to use the model in practice.

Another class of challenges concerns spectrometers and their foreoptics. Spectra of fresh leaves can be measured with different foreoptics, including integrating spheres, contact probes or leaf clips. We used a leaf clip with pressed specimens because mounting delicate pressed leaves in an integrating sphere could damage them. While leaf clips and contact probes often have a higher signal-to-noise ratio, they are less likely to produce consistent measurements among instruments or replicate samples due to variation in viewing geometry and anisotropic surface reflectance (Petibon et al., 2021). The logistical constraint of having to use them on pressed leaves could thus make it harder to compare data among instruments. In theory, the greater inconsistency of leaf clip measurements could have reduced the performance of our pressed-leaf models compared with our integrating sphere-based fresh-leaf models, but we still found that the former performed better for most chemical traits.

Another challenge is that while many herbarium specimens are glued to a paper backing, measuring reflectance with a leaf clip or probe usually requires placing a black absorbing background under the sample to keep transmitted light from being reflected back into the sensor. When unattached leaves are not available, using spectra from these specimens may require new methods to correct for reflectance from the mounting paper.

Spectrometers and their software also vary subtly in their sensors and techniques for processing spectra (Castro-Esau et al., 2006), and in some cases researchers must take steps to reconcile spectra measured from different instruments (Meireles, Cavender-Bares, et al., 2020). Assuming that these kinds of technical challenges can be overcome, our results and others encourage confidence about building general models to estimate traits from a wide variety of plants (Serbin et al., 2019). The creation of open libraries for spectral data (like EcoSIS, <https://ecosis.org/>; or the CABO data portal, <https://data.caboscience.org/leaf>) and spectral models (like EcoSML; <https://ecosml.org/>) will contribute to this goal. Lastly, we note that

many of the same concerns about discrepancies among sampling and measurement protocols could arise when using existing spectral libraries to aid in species identification (Draper et al., 2020).

4.5 | Implications for herbarium-based research

A particular challenge for herbarium specimens is that their optical and chemical properties (especially light-sensitive pigments) may degrade during preparation or storage. Such degradation could make it hard to distinguish changes in the traits of living plants over time from changes in storage. Even in this study, where no specimens were collected before 2017, many underwent visible changes in colour, including browning or blackening; ~12% were scored at 2 or higher, with large variation among species (e.g. 42% of *Populus grandidentata* specimens, but 0% of *Betula papyrifera* specimens). We found little evidence that such discoloration hinders trait estimation: Both the results of our discoloration analyses and the similar performance of full- and restricted-range models suggest that PLSR is flexible enough to predict traits despite the variable influences of discoloration in our specimens. This capacity likely depends on using samples for model calibration that show a similarly wide range of discoloration.

Our specimens were collected no more than 3 years before measurement, but ecologists may want to use specimens collected decades ago. While our findings give reason to be optimistic that properly calibrated models could return accurate estimates of many traits from old or discoloured specimens, it remains untested whether there are any limits to this potential. In general, not much is known about long-term changes in specimen chemical (aside from DNA) or optical properties (Lang et al., 2019). Colour changes are known to accelerate under certain preparation and storage conditions—including exposure to some chemical preservatives and high light, drying temperature or humidity (Bridson & Forman, 1999; Metsger & Byers, 1999)—which it may be worth avoiding when possible. Long-term studies of specimens—perhaps subjected to varying preparation and storage techniques—could establish how chemical and optical properties change over time and help refine these guidelines further.

Some of the challenges we describe pertain to projects that would measure spectra on samples already collected, but spectroscopy—like other novel uses for herbarium specimens—could also prompt changes in collection practice. For example, it underscores the potential value of gathering and storing extra leaf material (e.g. in fragment packets), which would circumvent the challenge of measuring mounted leaves and aid destructive analyses of herbarium specimens (Heberling, 2022). We propose that herbaria could also incorporate spectroscopy into their operations by measuring incoming specimens shortly after pressing, which could mitigate the challenges caused by mounting and degradation.

Linking spectral data measured on herbarium specimens to the digital record of the voucher could be a powerful tool to enable data synthesis, but it may require new informatic tools (Draper

et al., 2020). The hyperdimensionality of the reflectance spectrum could make it hard to accommodate within existing standards like the Darwin Core (Wieczorek et al., 2012), at least without extensions. One could link records to external spectral databases like EcoSIS or SPECCHIO (Hueni et al., 2020), which are also designed to store metadata about instrumentation and processing. We would advocate for coordination between herbarium managers and researchers who use reflectance spectroscopy, which could build agreement about best practices for spectral measurement and curation and allow data to be synthesized and compared across research groups.

We show that non-destructively measured pressed-leaf spectra retain much of the information about many leaf functional traits found in fresh-leaf spectra. While validating this technique on older specimens will require extensive further research, our findings suggest that reflectance spectroscopy could allow herbaria to take on a greater role in plant functional ecology and evolution. Our study has far-reaching implications for capturing the wide range of functional and phenotypic information in the world's preserved plant collections.

AUTHORS' CONTRIBUTIONS

Shan Kothari, Jeannine Cavender-Bares and Etienne Laliberté conceived the ideas and designed the methodology; Rosalie Beauchamp-Rioux and Shan Kothari collected the data; Shan Kothari analysed the data and wrote the first draft of the manuscript. All authors made substantial contributions to further drafts and gave final approval for publication.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/2041-210X.13958>.

DATA AVAILABILITY STATEMENT

We uploaded spectral and trait data to the Ecological Spectral Information System (EcoSIS) in separate packages for CABO fresh-leaf spectra (Kothari, Beauchamp-Rioux, Laliberté, & Cavender-Bares, 2022b), CABO pressed-leaf spectra (Kothari, Beauchamp-Rioux, Laliberté, & Cavender-Bares, 2022c), CABO ground-leaf spectra (Kothari, Beauchamp-Rioux, Laliberté, & Cavender-Bares, 2022d) and the Cedar Creek pressed-leaf spectra used in the external validation (Kothari, Erding, & Cavender-Bares, 2022). We uploaded all spectral models and a guide to using them to the Data Repository for the U of M (DRUM; Kothari, Beauchamp-Rioux, Laliberté, & Cavender-Bares, 2022f). Spectral processing and analysis scripts are available in a GitHub repository (<https://github.com/ShanKothari/pressed-leaf-models>) and the version used here is archived at Zenodo (Kothari, 2022).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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