Differentiating Among Plant Spectra by Combining pH Dependent Photoluminescence Spectroscopy with Multi-Way Principal Component Analysis (MPCA)

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Abstract: Photoluminescence spectroscopic probes offer the potential for differentiation among plant species in real-time. Spectral emission signatures (excitation at 365 nm) from three different pH (2.2, 7.5 and 12.5) phosphate buffered saline (PBS) extracts from two grasses, *Sporobolus flexuosus* (Thurb. ex Vasey) Rydb., [mesa dropseed], and *Pleuraphis mutica* Buckley [tobosa], two forbs, *Dimorphocarpa wislizenii* (Engelm.) Rollins [spectacle pod], and *Sphaeralcea incana* Torrey [pale globemallow], and leaves and twigs from two shrubs *Flourensia cernua* DC. [tarbush], and *Atriplex canescens* (Pursh) Nutt., [fourwing saltbush] were examined. Since pH has been shown to be pivotal in affecting extraction efficiency of other plant compounds pH seemed appropriate as an additional dimension within our multi-way principal component analysis (MPCA) to differentiate among six different plant species. In particular, MPCA allowed differentiation between *Sporobolus* and *Pleuraphis* that was not possible using only principal component analysis (PCA). This research suggests MPCA may be a more appropriate tool than PCA when attempting to discriminate among plant species.

Keywords: Botanical composition, chemometrics, fluorescence, luminescence, phosphate buffered saline (PBS).

1. INTRODUCTION

Determining standing crop composition and botanical composition of herbivore diets are the first two steps to optimize free-ranging herbivore nutrition [1]. Knowing if the standing crop contains toxic plants [2-4] or species pivotal to maintaining a desired landscape use [5] are key to managing the plant-animal interface. Using plant spectral signatures to distinguish plant life forms [6] and even genotypes [7] may provide a valuable tool for botanical analysis.

Previous fluorometry research has demonstrated the usefulness of spectroscopic methods for differentiating among pre- [8-10] and post-digested [6, 11] species of rangeland vegetation. Though Near Infrared Spectroscopy (NIRS) [12, 13] has been the automated optical method of choice in range animal ecology research, fluorometry [14] appears promising because of its practical differences (Table **1**). NIRS [19] as well as fluorometry have the potential to differentiate among plant species. However, within plant life forms, i.e., grasses, forbs and shrubs; it may be challenging to fluorometrically identify individual species without employing appropriate analytical tools. This challenge can be addressed efficiently using mathematical algorithms that take advantage of fluorometric data's multidimensional characteristics. Chemometric methods enable the analysis of complex data into sample constituents [20-22]. Distinguishing among species may require data sets with higher ordered dimensionality and use of chemometric procedures involving multi-way principal component analysis (MPCA) developed by Nomikos and MacGregor [23] rather than the simpler mathematical approach involving principal component analysis (PCA) [24-26], applicable to two-dimensional measurements.

These data were collected as part of a previous study to determine the effectiveness of PCA to discriminate among plant species. However, in the study by Danielson *et al.* [9] PCA was unable to adequately discriminate among visually similar spectral signatures, specifically, differentiation between two grass species was problematic. The objective of this research was to reevaluate a subset of Danielson's data using MPCA to determine if it is superior to PCA in differentiating among visually similar spectral signatures.

2. MATERIALS AND METHODS

Plants species potentially consumed by sheep and beef cattle [27-29] were harvested between 6 August and 2 October 1996 on the United States Department of Agriculture's Jornada Experimental Range (USDA-ARS-JER) located in south-central New Mexico, near Las Cruces. The harvested plants represented specimens having similar physiological

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Table 1. Six Practical Considerations when Comparing Near Infrared Reflectance (NIRS)[a] to Fluorometry[b]

Consideration	NIRS	Fluorometry	Practical Implication for Using Fluorometry Instead of NIRS	References
Sample properties	Many materials absorb, linear dynamic range of < 1.5 orders of magnitude.	Fewer materials fluoresce, linear dynamic range as great as six orders of magnitude.	Aids in specificity and accuracy of identification at trace to percentage levels of concentration.	[16]
Equipment	Limited to laboratory.	Potential for field portability.	Provide real-time data for making management decisions.	[17]
Measurement	Vibrational energies (λ > 700 nm).	Electron transitions resulting from excitation energies between 190 to 800 nm.	Greater discernment specificity.	[18]
Extraction solvent (for solution analyses)	Must be transparent.	Polar and non-polar solvents.	Solvent used to prepare samples can become the first step in discrimination.	[9]
Identification algorithms	Specificity important for accuracy, i.e., calibration samples and unknowns must show similar variability.	Less specificity required for equal accuracy, i.e., calibration samples and unknowns do not have to have the same variability.	Allows unknown samples to be accurately discernable even when calibration algorithms do not include the extent of variability shown in the unknowns making the methodology robust for prediction.	[10]
Data dimensionality	Only two dimensions. Signal intensity results only from reflection vs. wavelength allowing many possibilities coming from blended chemical structures.	Up to five dimensions. Intensity - function of three parameters involving specific chemical structures affecting: excitation wavelength (λ), emission (λ), and time expressed as polarization anisotropy.	The greater the number of dimensions the greater the techniques power to discriminate among specie differences for accurate discernment.	[16]

[a][15].
[b][14].

maturity (phenology) and were harvested from several different locations on the landscape to provide as much soil and topographic variability as possible. Nine plants from each of six different species were harvested: two grasses, *Sporobolus flexuosus* (Thurb. ex Vasey) Rydb., [mesa dropseed], and *Pleuraphis mutica* Buckley [tobosa]; two forbs, *Dimorphocarpa wislizenii* (Engelm.) Rollins [spectacle pod], and *Sphaeralcea incana* Torrey [pale globemallow]; and two shrubs consisting of leaf and stem material, *Flourensia cernua* DC. [tarbush] leaves and *Atriplex canescens* (Pursh) Nutt., [fourwing saltbush] leaves and current years stem growth (twigs) were obtained. The plant nomenclature follows that of Allred [30].

The 36 herbaceous plants, nine from each of four species, were clipped at ground-level while leaves from nine tarbush plants and leaves and twigs from nine fourwing saltbush plants were stripped from branches giving a total of 54 unique plant samples to be analyzed. Detailed sample collection and preparation protocol can be found in Danielson *et al.* [9]. Harvested plant materials were oven-dried to a constant mass at 60^{o} C before the contents of each of the 54 bags were ground to pass a 1-mm micro-Wiley mill screen. The ground plant material was collected in labeled plastic vials and stored at room temperature. Immediately prior to fluorometric analysis, the vial contents were re-dried at 60^{o} C and triplicate 0.15 g samples of the dried material from each of the 54 vials were weighed into labeled glass culture tubes, sealed with Parafilm®, and stored at room temperature. A 10 mL volume of phosphate buffered saline (PBS), containing sodium azide (to inhibit microbial growth), at one of three pH values (2.2, 7.5, or 12.5) was added to each of the 162 tubes. These pH values were chosen to ensure that the highest buffering capacity of the solvent would be maintained following the extraction procedure thus preventing changing of the extract's pH. Details of how the PBS and three pH solutions were made have previously been published [9]. The filled tubes were shaken for one hour at 900 RPM followed by filtration through Whatman No. 4 paper into clean, labeled tubes, sealed with Parafilm®, and stored at 3^{o} C until analysis.

Besides being environmentally safe, PBS was used to minimize chlorophyll extraction [9] and to minimize pre-and post- spectral filtering effects. Danielson *et al.* [9] reported the need to dilute the resulting plant extract solutions (a pH 2.2, 7.5, and 12.5) by factors of 12.50%, 6.25%, and 3.13%, eg. 1 ml of extract was diluted to a total of 8, 16, and 32 ml of the appropriate pH buffered solutions, respectively. Research has revealed that extraction solvents having different pH values can be useful in extracting various compounds including pectin [31] and glutaric, malic and maleic acids found in plants [32]. Therefore, extracting solution pH was included as a variable in the present study.

Excitation radiation of 365 nm was selected from a 500 W Xe/Hg-arc lamp using a 0.25 m monochromator (Melles Griot, Model HR-20, [9]). The emitted radiation was collected and transferred using F-matching transfer optics [33] to the entrance slit of a 1.0 m focal length monochromator

equipped with a 1200 mm holographic grating with a 1.7 nm bandpass (2.0 mm entrance and exit slit widths). Use of the holographic grating resulted in the Wood's grating anomaly [34-36] being observed and it was left uncorrected. It manifested itself as an apparent local minimum in all spectra at ~515 nm. This study was not designed to determine the molecular species responsible for the various fluorometric signatures; therefore, the uncorrected spectra reported using this system are not directly transferable to other spectroscopic configurations. Fluorometric instrumentation details have been previously described by Danielson *et al.* [9].

Data acquisition was accomplished using in-house software developed using LabView® 5.0 (National Instruments). Data analysis utilized algorithms found in the PLS-Toolbox (Eigenvector Research, Wenatchee, WA) operated within MATLAB (Mathworks, Lowell, MA).

2.1. Multi-Way Principal Component Analysis (MPCA)

The fluorescence emission data consisted of broad and superimposed spectral features from yet to be identified fluorophores within the 375-620 nm region of the spectrum. This technique neither enables a comprehensive molecular component level understanding of the spectral response curve (fingerprint) nor relies on the identification of the specific molecular species. However, this spectral approach has been found useful in differentiating among plants.

Earlier use of PCA by Danielson *et al.* [9] involved decomposition of the two-dimensional data matrix (i.e., emission intensities at each of 247 wavelengths for each of the six plant species) into a collection of principal components and an error matrix. PCA can only deal with two-dimensional data matrices. Conversely, MPCA enables analysis of three dimension data matrices.

Though statistically and mathematically similar to PCA, MPCA (also called unfolded PCA or U-PCA) involves the generation of a representation of the eigenvectors for the covariance or correlation matrix of the original measured variable data matrix [37]. Simply stated, MPCA enables the discernment of principal components within an entire data set that account for the variance present in those data and an effective reduction of the effective variables describing each sample. It should be noted that for both PCA and MPCA, the emission response curve or surface, respectively, is used in the analysis of the collected measurements thereby eliminating the need for any subjective selection of specific variables (e.g., wavelength or wavelength and pH). For a more detailed discussion of the MPCA procedure, see Obeidat *et al.* [10] and cited references therein. Although there are other data analysis tools that can be applied to data sets with higher dimensionality (e.g., measured intensities as a function of more than one variable), previous use of PCA suggested that an expanded form of the same approach would enable segregation of plants using emission intensities as a function of both wavelength and extracting solution pH.

Using MPCA makes it possible to differentiate, identify, and classify plant material without any *a priori* information [37-39]. In our data, pH served as the third dimension because of its usefulness in extracting different plant compounds. The extracts from the six plant species used in this research were run independently at three pHs (2.2, 7.5, and 12.5). This allowed us to build a two way data matrix at each pH with dimensions of 247 x 6 for total dimensionality of 247 x 6 x 3.

3. RESULTS AND DISCUSSION

3.1. Observed Fluorescence

Spectra were normalized to their respective intensity maxima to enhance both the observed differences and similarities among the six plant species. The 420 nm peak observed at all three pHs (Fig. **1A, B** and **C**) can be attributed to a Raman scattering band arising from the water based solvent [9]. Efforts to eliminate this artifact through background correction yielded either sample-dependent under or over correction. This suggests the presence of a sample-dependent attenuation from pre- or post-filter effects by absorbing concomitant molecular species. Correction for this feature was not undertaken eliminating the ability to compare these data with similar fluorometric studies.

3.2. Effect of Solvent pH on Fluorescence Spectra

The PBS extracts at the acidic pH (2.2) appeared to produce visually similar fluorescence spectra among the six plant species (Fig. **1A**). In contrast, the solvents at a neutral pH of 7.5; (Fig. **1B**) and an alkaline pH of 12.5; (Fig. **1C**) produced spectral signatures with greater visual variation among the six plant species. Overall, fluorescence was observed in two regions of the visible spectrum. One region exhibited a maximum between 440-470 nm (blue) with a shift to longer wavelengths (red shift) as pH was increased from 2.2 to 12.2 (Table **2**). The other visually distinguishable region occurred at ~523 nm (green) but it did not exhibit a red shift with pH (Table **2**). This suggests that blue-emitting fluorophore(s) exhibited greater pH dependence among the six different plant species than those emitting in the green.

The two grasses, mesa dropseed and tobosa, showed very similar fluorescence responses at pH 2.2 and pH 7.5 (Figs. **2A** and **2B**) making them visually indistinguishable between 375 and ~414 nm. This similarity thwarted PCA from being able to discern between these two plants [9].

Spectra obtained from spectacle pod extracts at pH 7.5 demonstrated a significant increase in one or more compounds that fluoresced in the green region (515-525 nm) of the spectrum compared to those components responsible for blue (375-498 nm) emission. Furthermore, spectacle pod at pH 7.5 did not exhibit the same red shift as did tarbush. Tarbush demonstrated an interesting characteristic in having the largest relative green wavelength (523 nm) emission among all the six plant species evaluated. The red shift of emission wavelength with increasing extraction pH can be seen in Table **2**. These observations can be explained by extraction of a longer wavelength emitting fluorophore resulting in the measured red shift. In similar studies, Billa *et al.* [21] in wheat straw, sorghum fiber and sweet sorghum stalks demonstrated pH dependent fluorescence spectra could be used to distinguish types of pulps used in the manufacture of various papers. The alkaline treatment of grass and legumes with NaOH has been shown to release the phenolic compounds, ferulic acid and *p*-coumaric acid, from cell walls and these phenolics have therefore been proposed to be major contributors to plant fluorescence in the blue-green region of the

Table 2. Maximum Blue (424.0-491.2 nm)[a] and Green (491.3-575.0 nm)[a] Emission Wavelengths (λ) and Blue to Green Emission Wavelength Ratios along with an Intensity Ratio of the Maximum Blue Emission Intensity to the Maximum Green Intensity for Six Ground Arid Rangeland Forage Species Extracted with Buffered Saline at Three pH Values. The Grasses, Forbs and Shrubs were Exposed to a 365 nm Excitation Wavelength from a 500 W Xe/Hg-Arc Lamp

Grassses	***Sporobolus Flexuosus*** **(Mesa Dropseed)**				***Pleuraphis mutica*** **(Tobosa)**			
pH	**λ**		**λ Ratio**	**Intensity Ratio**	**λ**		**λ Ratio**	**Intensity Ratio**
2.2	434	522	.83	3.27	439	518	.85	3.17
7.5	438	520	.84	2.96	443	520	.85	2.70
12.5	458	521	.88	2.34	458	521	.88	2.22
Forbs	***Dimorphocarpa Wislizenii*** **(Spectacle Pod)**				***Sphaeralcea Incana*** **(Pale Globemallow)**			
pH	**λ**		**λ Ratio**	**Intensity Ratio**	**λ**		**λ Ratio**	**Intensity Ratio**
2.2	442	522	.85	2.86	446	520	.86	3.23
7.5	448	521	.86	1.37	453	519	.87	2.99
12.5	469	521	.90	1.48	459	520	.88	2.16
Shrubs	***Flourensia Cernua*** **(Tarbush Leaves)**				***Atriplex Canescens*** **(Fourwing Saltbush Leaves Plus Current Years Twigs)**			
pH	**λ**		**λ Ratio**	**Intensity Ratio**	**λ**		**λ Ratio**	**Intensity Ratio**
2.2	444	521	.85	2.63	436	520	.84	2.87
7.5	453	523	.87	1.67	446	521	.85	3.03
12.5	466	522	.89	1.57	456	521	.88	2.91

[a][40].

visible spectrum [41]. Furthermore, these researchers concluded that the relative concentrations of these molecules vary among various grass species, although this conclusion suggests genetic differences based on plant species. Other explanations of these variations await investigation, (e.g., differences related to changes in plant physiological state or a combination of other biotic or abiotic factors acting either independently or synergistically). Although plant chemical composition can vary due to many factors [42], it is unknown how these chemical changes affect fluorometry. Visual evaluation of mean fluorescence spectra from the six different plant species at each pH (Fig. **1A** through **C**) indicated both similarities and differences.

The forbs (spectacle pod and pale globemallow) and shrubs (tarbush and fourwing saltbush) appear to exhibit higher extraction efficiency for green-emitting fluorophores regardless of pH (i.e., the ability to transfer one or more chemical compounds from the plant sample into the PBS solution). Additionally, shifts in wavelengths of maximum emission between each of the forbs and two shrubs towards the red (i.e., longer wavelengths) were observed. At neutral and high pH, the fluorescence responses in both forbs and shrubs appear to be visually distinguishable. Two distinctive peaks were observed from the data; one centered at ~450 nm, the other at ~525 nm.

A similar study reported by Johnson *et al*. [43] indicated peak similarities and differences for seven different types of plants consisting of five species each of grasses/sedges, conifers, herbaceous dicotyledons, succulents, palms, woody deciduous dicotyledons, and woody evergreen dicotyledons. They observed for all 35 species a violet to blue emission peak with a maximum between 405-475 nm, while in about one third of their species they observed a predominant green emission peak between 510-550 nm. Although not seen in the present study, one species of plant (*Aloe barbadensis*, a succulent) was reported to exhibit an emission maximum at 568 nm [43]. It should be noted that samples in the Johnson study were prepared from the adaxial and abaxial surfaces of plant foliage while our samples were aqueous plant extracts from ground whole plants or plant parts.

There have been many suggestions as to what fluorophores are responsible for the observed fluorescence spectra (Table **3**). The UV-induced fluorescence of specific organic compounds known to be endogenous to plant leaves has also

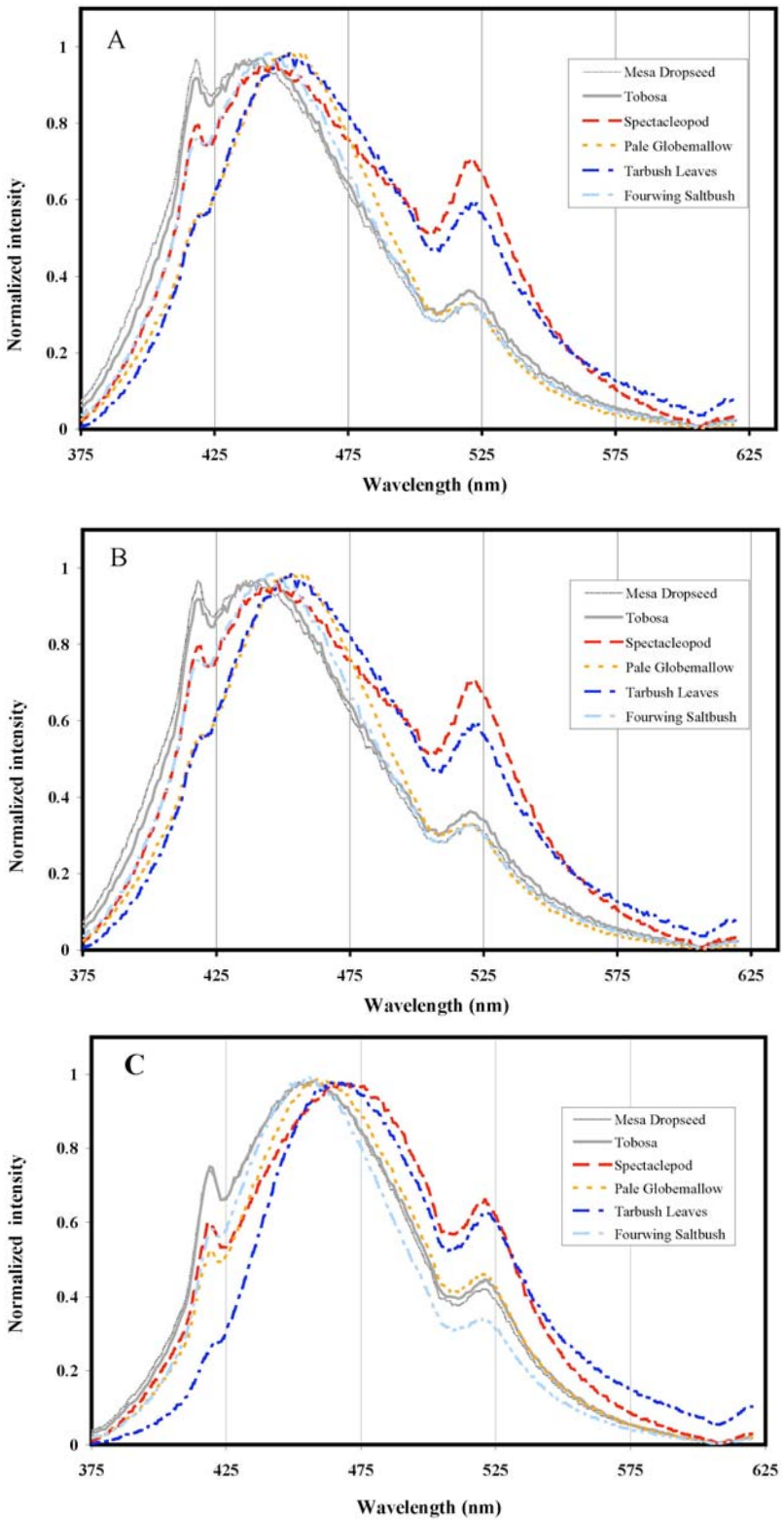

Fig. (1). Mean (n = 9) normalized emission spectra for phosphate buffer saline (PBS) extracts of two grasses, two forbs and two shrubs (leaves and current year's twigs only for shrubs) at three pHs (A = 2.2; B = 7.5 and C = 12.5).

been reported. It should be noted with respect to the present study that coumarins tend to fluoresce more intensely in alkaline solutions while not at all in acidic solutions. Hence, these types of compounds may have contributed to the red-shift observed in the present study.

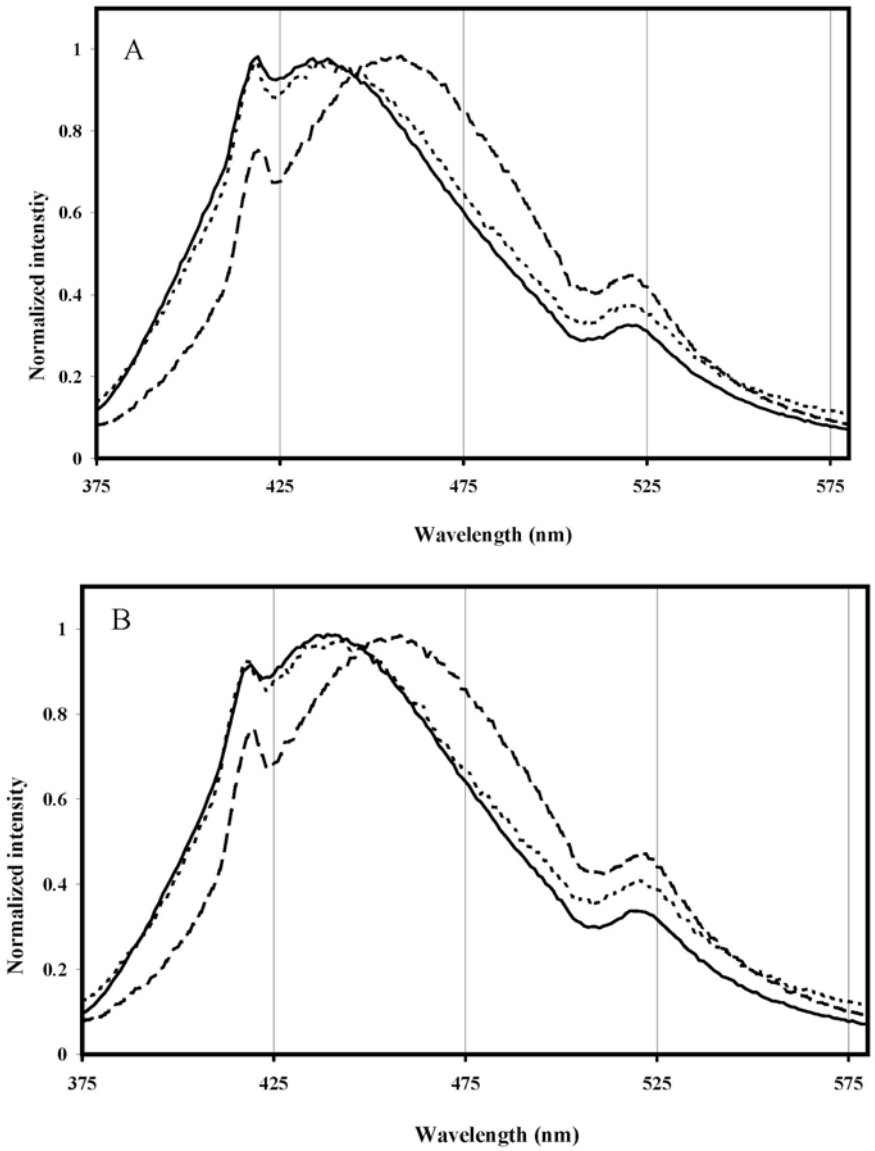

Fig. (2). Mean (n = 9) normalized emission spectra for phosphate buffered saline (PBS) extracts of **A**) *Sporobolus flexuosus* [Thurb.] Rydb., mesa dropseed, **B**) *Pleuraphis mutica* Buckley, tobosa at pH values of 2.2 (–––), 7.5 (•••), and 12.5 (- - -).

Table 3. Plant Molecules Known to Fluoresce

Molecules	Emission Wavelength (λ)	References
Chlorophyll a	666	[44]
Chlorophyll b	646	
NAD(P)H	430 (bound to proteins) & 460 (free form)	[45-47]
FAD, FMN, Riboflavin	525	[45-47]
Caffeic acid, Ferulic acid, Chlorogenic acid, Sinapic acid, (+) Catechin and Phyllo-quinone (reduced form) Aesculetin, Scopoletin	440	[47]
t-stilbene, Rhaponticin	380-390	[47]
Berberine, Quercetin	520-530	[47]

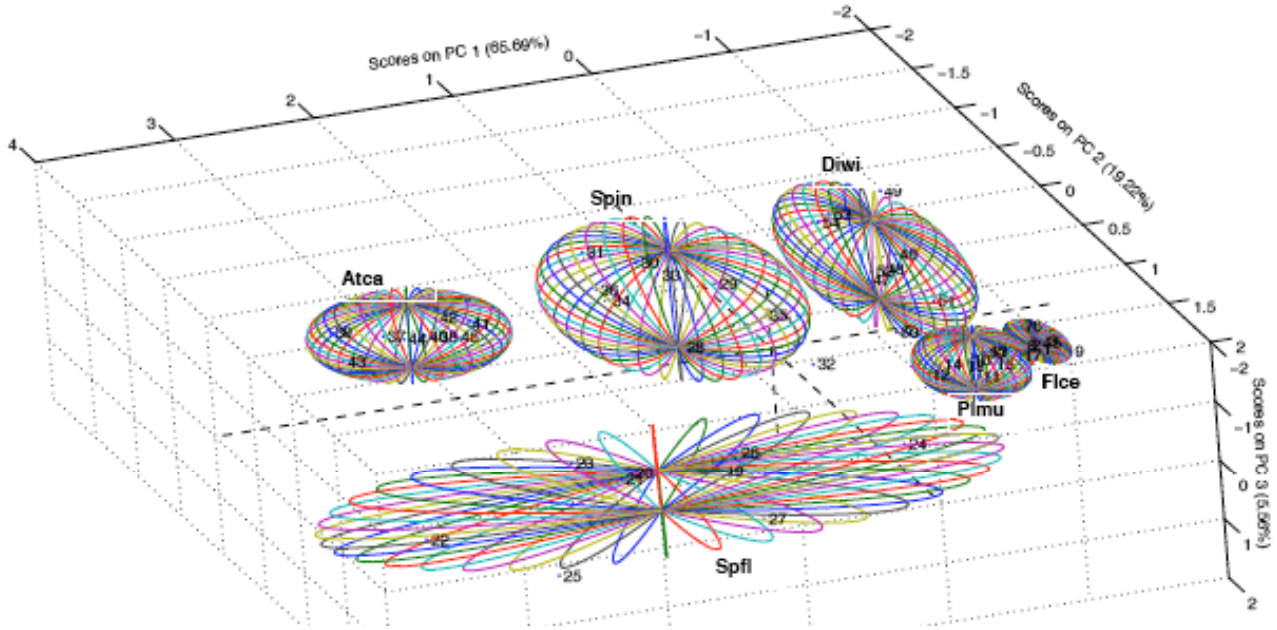

Fig. (3). Plot of PC scores showing six species (*Sporobolus flexuosus* (Spfl), *Pleuraphis mutica* (Plmu), *Dimorphocarpa wislizenii* (Diwi), *Sphaeralcea incana* (Spin), *Flourensia cernua* (Flce) and *Atriplex canescens* (Atca)) each with nine plants per species resulting from the application of a multi-way principal component analysis (MPCA) data analysis of the pH-dependent emission spectra from each phosphate buffered saline solution extract of plant species samples. The three dimensions of the data set were signal intensity as a function of emission wavelength and extracting solution pH. The numbers correspond to samples from individual plants. Ellipsoids depict 95% confidence volumes about the centroid projection of each plant species (see Table **4**).

Table 4. Confidence Surface Values (95%) for Each Species, see Fig. (3)

Plant Species	Centroid of Each Elipsoid			95% Confidence Ellipsoidal Surfaces					
	PC1	PC2	PC3	Score 1		Score 2		Score 3	
				Upper	Lower	Upper	Lower	Upper	Lower
***Sporobolus flexuosus* (Mesa dropseed)**	-1.58	0.56	-0.27	-1.40	-1.76	0.82	0.30	-0.15	-0.39
***Pleuraphis mutica* (Tobosa)**	-1.17	0.45	0.08	-0.78	-1.56	0.78	0.13	0.53	-0.36
***Dimorphocarpa wislizenii* (Spectacle pod)**	1.53	1.17	0.30	3.97	-0.91	1.89	0.46	0.64	-0.05
***Sphaeralcea incana* (Pale globemallow)**	0.03	-1.13	0.65	0.88	-0.82	-0.32	-1.95	1.47	-0.17
***Flourensia cernua* (Tarbush leaves)**	2.31	-0.45	-0.48	3.01	1.61	-0.05	-0.86	0.05	-1.01
***Atriplex canescens* (Fourwing saltbush leaves plus current years twigs)**	-1.13	-0.60	-0.28	-0.60	-1.65	0.24	-1.45	0.39	-0.96

Our data suggest that using extraction solutions differing in pH may be a useful first step when attempting to distinguish among various plant species if MPCA procedures are used. Danielson *et al*. [9] determined that the scores of the first three PCs from a PCA model of emission spectra of solutions at each pH value enabled adequate separation among all plant species except for the two grasses while accounting for more than 95% of the total variation within the extract spectral signature. However, separation of the two grasses (tobosa and mesa dropseed) remained unrealized.

Three two-way data matrices corresponding to each extract solution pH were concatenated into a single data matrix with three dimensions of 247 x 3 x 6. Application of MPCA to this matrix yielded a separation enhancement for all six plant extracts. This approach increased the dimensionality of the data set by incorporating data from all three pH extracts simultaneously enhancing separation among the six plant species (Fig. **3**). This enabled the successful separation of all six species using scores from the first three PCs in an MPCA model in which 95% of the total variation was accounted for. This three dimensional data set was composed of the two–way MPCA models using the first versus the second and the first versus the third PCs. Fig. (**3**) shows that by increasing the dimensionality of the data using three pH values the technique's ability to separate fluorometric signatures improved. Spectra from each of the 54 plants show the variability around each of the six specie groupings (Fig. **3**) is less

than that among the six species (Table **4**). Ellipsoidal surfaces in Fig. (**3)** indicate volumes defined by 95% confidence intervals relative to the centroid location of score projections of combined spectra from plants of a specific species. Separation of ellipsoids then illustrates significant (i.e., >95% confidence) differences in processed spectra from different plant species while accounting for statistical differences between different plants of the same species. Specifically, the two grasses were spatially separated compared to PCA procedures in which the grasses were not clearly separated, as previously reported by Danielson *et al.* [9]. The three-dimensional PC-score space resulting from the MPCA analysis suggests that the greater separation of the score vectors in this three-dimensional principal component space among the six treatments is indicative of increased statistical differences among the corresponding sample spectra.

The next possible research step in evaluating the usefulness of MPCA would be an attempt to accurately different ate from among fluorometric data arising from known mixtures of plant species. If differentiation were possible and repeatable it would be logical to then investigate the usefulness of MPCA as a tool in differentiating among spectral fluorometric diet data obtained from free-ranging animals. Such information remains challenging to obtain except where highly skilled labor and money are both abundant. The utility of this approach has been suggested through preliminary results published elsewhere [17].

4. CONCLUSIONS

This research using MPCA as a tool to differentiate among normalized fluorometric spectral data obtained from six plant species provided positive results. As the dimensionality of the data set was increased to include solution pH as a third variable, it impacted the measured fluorescence signal by removing ambiguities in sample identification seen with PCA. Even without accounting for the source of variation among the individual plants within each of the six species evaluated, MPCA was able to visually separate the six species. This suggests MPCA may be a promising tool for analyzing future fluorometric data.

DISCLAIMER

Mention of a trade name does not constitute a guarantee, endorsement, or warranty of the product by the USDA-ARS or New Mexico State University over other products not mentioned.

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