Technical Note

Use of Fluorometry to Differentiate Among Clipped Species in the Genera *Astragalus*, *Oxytropis*, and *Pleuraphis*

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Abstract

A rapid and reproducible method to determine botanical composition of forage is an ecological and economic goal for range animal ecologists. Multidimensional fluorometry previously demonstrated the possibility of a unique optical approach for accurately determining species composition of clipped and digested plant materials. Fluorometry may be used to detect toxic plants in standing crop as well as diets by using electronic transitions in chemical structures at wavelengths between 370 and 580 nm. Grass hay (genus *Pleuraphis*) and 6 clipped forbs (4 species of *Astragalus* and 2 species of *Oxytropis*) were examined. The resulting spectral signatures were evaluated for differences in the blue and green regions of the visible spectrum using Principal Component Analysis (PCA). This represents the first published data using chemometrics to differentiate among fluorophores from these plant extracts. It was possible to distinguish between the grass and forbs and among forbs. Further research will be required to evaluate these same plant species in mixed diets and fecal samples.

Resumen

Una meta ecológica y económica de los ecológos de animales del pastizal es encntrar un método rápido y reproducible para determinar la composición botánica del forraje. La fluoroscopia multidimensional previamente demostró la posibilidad de ser un método óptico único para determinar certeramente la composición de especies de materiales vegetales cortados y digeridos. La fluorometría puede ser usada para detectar plantas tóxicas en la biomasa y en las dietas usando transiciones electrónicas en las estructuras químicas de longitud de onda entre 370 y 580 nm. Se examinaron henos del zacate (del género *Pleuraphis*) y de 6 hierbas (4 especies del género *Astragalus* y 2 del género *Oxytropis*). Las marcas espectrales resultantes fueron evaluadas por diferencia en las regiones azul y verde del espectro visible usando un Análisis de Componentes Principales (PCA). Este reporte representa los primeros datos publicados de uso de quimiometría para diferenciar entre fluoroforos de los extractos de estas plantas. Fue posible distinguir entre el zacate y la hierba y entre las hierbas. Se requerirá más investigación para evaluar estas mismas especies en dietas mezcladas y muestras fecales.

Key Words: botanical composition, fluorescence spectroscopy, poisonous plants, Principal Component Analysis (PCA)

INTRODUCTION

Determining herbivore diets in a rapid and accurate manner remains an unfulfilled goal for 21st century range animal ecologists. Poisonous plants pose a particular challenge to free-ranging animal production. Species in *Astragalus* and *Oxytropis* (locoweeds) can cause widespread poisoning of livestock in the western United States (Kingsbury 1964). In the last century, cattle and sheep mortalities due to toxic plants were estimated for the 17 western states at 1% and 3.5% of total livestock numbers, respectively (Nielsen et al. 1988; Nielsen and James 1992). Adding to the challenge in estimating animal losses from toxic plants is the fact that toxic and nontoxic plants are often found growing together, and some species may or may not be toxic depending on soils or growing conditions. The exact percentage of death losses varies by region and year to year, but recent estimates of the direct and indirect economic losses due to toxic plants are $340 million annually (James et al. 1992).

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Table 1. Names, harvest information, growing conditions and swainsonine content for 7 plant species.

Names				Harvest information		Growing conditions		Swainsonine
Plant type	Common	Scientific	Abbreviations	Date	Location	Soils	Plant community	content
Forb	Spotted milkvetch	*Astragalus lentiginosus* var. *diphysus*, (Gray) Jones	Asle	May 1998	27 km SE of St. Johns, AZ 34°24′N, 109°13′W	Sandy	Pinyon Juniper	0.14%
Forb	Drummonds milkvetch	*Astragalus drumondii* Dougl. ex Hook.	Asdr	July 1999	64 km NW of Fort Collins, CO 40°56′N, 105°15′W	Gravely loam	Foothill mixed grass prairie	Not detected
Forb	Laxmans milkvetch	*Astragalus laxmanii* Jack Quin	Asla	July 1999	64 km NW of Fort Collins, CO 40°56′N, 105°15′W	Gravely loam	Foothill mixed grass prairie	Not detected
Forb	Ft. Wingate milkvetch	*Astragalus wingatanus* Wats.	Aswi	July 1999	64 km NW of Fort Collins, CO 40°56′N, 105°15′W	Gravely loam	Foothill mixed grass prairie	Not detected
Forb	Lambert loco	*Oxytropis lambertii* var. *bigelovii* A. Gray	Oxla	July 1999	64 km NW of Fort Collins, CO 40°56′N, 105°15′W	Gravely loam	Foothill mixed grass prairie	Not detected
Forb	White locoweed	*Oxytropis sericea* Nutt. ex Torrey & Gray	Oxse	July 1999	64 km NW of Fort Collins, CO 40°56′N, 105°15′W	Gravely loam	Foothill mixed grass prairie	0.05%
Grass	Tobosa	*Pleuraphis mutica* Buckley	Plmu	August 1991	37 km north of Las Cruces, NM in Pasture 6, Jornada Experimental Range 32°29′N, 106°44′W	Clay loam	Foothill mixed grass prairie	Not analyzed

There are over 2 000 species of *Astragalus* currently identified, with many of these species known to contain various toxins (Williams 1994). Adding to the challenge of identifying which plants are toxic is the fact that some animal species appear to be more susceptible to locoweed intoxication (Marsh 1909) than others. More recently, a plant fungal endophyte association in *Oxytropis lambertii* (Lambert loco) has been found that may be responsible for the high variability in toxicity found in certain populations of this species making identification of toxic from nontoxic plants impossible simply using taxonomic criteria since toxic and nontoxic plants can be found growing together (Ralphs et al. 2002). Therefore, a rapid and reliable method is needed to identify toxic from nontoxic plants.

The major toxin of concern in *Astragalus* and *Oxytropis* genera has been identified as a trihydroxy indolizidine alkaloid, or swainsonine (Molyneux and James 1982). This alkaloid acts as a reversible inhibitor of lysosomal alpha-mannosidase and Golgi complex alpha-mannosidase II. Swainsonine is weakly basic and mimics the shape of a cation intermediate involved in mannose hydrolysis, making it a potent enzyme inhibitor (Dorling et al. 1984). However, the lack of conjugate π-bonding (Ingle and Crouch 1988) in swainsonine eliminates its ability to fluoresce.

Preliminary data using a multidimensional optical technique termed fluorometry (Guilbault 1990) shows promise as a rapid, accurate, and repeatable method for identifying clipped digested plant materials (Anderson et al. 1996, 1998; Parker et al. 2000c). In the 1996 and 1998 studies, chloroform was used as the extracting solvent. This solvent produced fluorophores in the blue, green, and red regions of the visible spectrum. The red fluorescence was attributed to chlorophyll a (Lichtenthaler and Stober 1990). Goulas et al. (1990) suggest blue fluorescence from leaf material of higher plants is a complex signal involving at least 3 components. Lichtenthaler et al. (1991) indicated that the phenolic epidermal compounds in leaves including cafferic, ferulic, and sinapic acids as well as chlorogenic acid and quinic acid can contribute to fluorescence in the blue region of the visible spectrum. Green fluorescence is attributed to correspond with cell wall components berberine and quercetin (Lang et al. 1991), epidermal tissue (Broglia 1993), and mesophyll tissue (Lang et al. 1992). Though blue and green fluorescence apparently originate from more than 1 component (Lichtenthaler et al. 1991), whose chemical origin and precise location is yet unknown (Stober and Lichtenthaler 1993), it is possible to differentiate among plant materials without actually knowing which compounds are responsible for the fluorescence (Anderson et al. 1996; Anderson et al. 1998).

Recent research by T. L. Danielson (unpublished data, 2002) suggests that physiological buffered saline (PBS) may be a suitable solvent for extracting non-chlorophyll fluorophores from plant material, thus reducing the masking of blue and green fluorescence that can occur when chlorophyll is present. The objective of this research was to determine if fluorescence spectroscopy using Principal Component Analysis (PCA) could differentiate among data representing a composite of several plants within each of 7 species that had been ground and then extracted with PBS at pH 12.5.

MATERIALS AND METHODS

Plants

Six forbs and 1 grass hay were evaluated for spectral fluorescence using a fluorometer. Prior to the fluorometric evaluation of the forbs, a test, accurate to $< 0.001\%$, was used to determine that swainsonine was present in only 2 of the species (Table 1) while the grass, assumed not to contain swainsonine, was not tested for this alkaloid. The 6 forbs were obtained from the USDA-ARS Poisonous Plant Research Laboratory (Logan, UT) and had been collected from 2 locations, 1 in Arizona and the other in Colorado, while the grass hay was clipped on the USDA-ARS Jornada Experimental Range (Las Cruces, NM).

Fluorometric Procedure

Approximately 1 g of each of the 7 different ground plant species was placed in aluminum weigh boats and dried at 60°C for 24 hours. From each weigh boat triplicate samples, approximately 0.15 g (mean = 0.1501 ± 0.00002 g, $n = 21$), of plant material was weighed into each of 3 labeled 16 × 25 mm borosilicate culture tubes (Kimble Kontes, Vineland, NJ). The culture tubes were covered with Parafilm® (Pechiney, Menasha, WI) and placed in a test tube rack. The rack was covered with aluminum foil, and held at room temperature until the extraction solution (stored at ~2°C) was added.

Culture tubes containing each sample were randomized prior to addition of autoclaved (35 minutes at 121°C, 125 kPa) PBS extraction solution. During preparation of the PBS solution the solution was adjusted to a pH of 12.5 using a 1 M solution of NaOH (Mallinckrodt, St. Louis, MO) while monitored using an Orion, Model 210 A, (Beverly, MA) pH electrode. The extraction solution contained 0.263 g NaN_3 (sodium azide, a microbial growth inhibitor), 1.422 g $NaHPO_4$, 3.801 g Na_2HPO_4 (all from Alfa Aesar, Ward Hill, MA), 0.408 g KCl (Sigma) and 13.567 g NaCl (J. T. Baker, Phillipsburg, NJ) dissolved in 2 L of HPLC grade water.

Each replicate consisted of 7 treatments (plant species), 3 extraction solvent blanks, and a single scattering suspension (a TiO_2 suspension) used within and among all 3 replicates. The experimental protocol involved exposing the solvent blanks of PBS drawn from the stock solution at the beginning, middle, and end of each replicate followed immediately by exposing the single TiO_2 suspension solution. Data from these 2 materials were used to remove any solvent related spectral information and correct for instrument fluctuations both within and among days, respectively. Incorporation of the light scattering suspension of TiO_2 provided a signal indicative of the wavelength-dependent intensity of the incident radiation, which enabled compensation for significant drifts in the output of the Xe-arc lamp excitation light source.

A Lab Industries Repipet II (Barnstead/ Thermolyne, Dubuque, IA) calibrated to deliver 10 mL of extraction solvent was used to fill tubes containing ground plant material and the PBS blanks. The 10 tubes per replicate were then sealed with Parafilm® and shaken in an attempt to saturate the "plug" of ground plant material that floated on the extraction solvent. The sealed tubes were then placed on an orbital shaker (VWR Model 98001, Albuquerque, NM) for 1 hour at 100 RPM. While on the shaker the tubes were held in a foam rubber holder adjusted at an angle to minimize contact of the contents with the Parafilm® and rotated 180° after 30 minutes of shaking to ensure maximum contact of the plant material with the extraction solvent.

Tubes were centrifuged immediately after shaking at 925 × g for 25 minutes using a Beckman Model TJ-6 Centrifuge (Labx, Midland, ON, Canada). Following centrifugation, supernatent from each culture tube was decanted into a non-sterile 10 mL syringe (Allometrics Inc., Franklin Lakes, NJ) with a 0.2 μm non-sterile nylon filter (Millex, Bedford, MA) attached. Care was taken not to disturb the solid pellet of material in each culture tube. A plunger was then inserted into the syringe barrel and approximately 3 mL of filtrate was collected in a 3.5 mL non-sterile acrylate cell with a 10 mm light path (Spectrocell, Oreland, PA). The acrylate cell was then closed with a Spectrocell Teflon® (LDPE) cell cap and placed in the fluorometer and exposed for 0.5 seconds per excitation wavelength. The samples were scanned while being excited between 200 and 400 nm in 5 nm increments (i.e., 41 excitation wavelengths), resulting in a fluorescence spectrum acquired at each excitation wavelength with 7 nm resolution. This produced 1 026 data pairs consisting of fluorescence wavelengths and intensity counts.

The Fluorometer

The fluorometer, built at Sandia National Laboratories (Wagner et al. 1996; Parker et al. 2000a and 2000b), has previously been described by Mukherjee et al. (2001). It contained a 150 watt high-pressure Xenon arc lamp (Oriel Model 66002, Newport Oriel Instruments, Stratford, CT). White light from the Xenon lamp was focused into an F/4, 1/8 m double monochrometer (CVI Model CM 110, Albuquerque, NM) for scanning wavelengths of excitation in the 200 to 400 nm range in 5 nm increments with a bandwidth of 7 nm. Light coming from the monochrometer was then imaged into the center of the acrylate sample cell. Scattered light and fluorescence from the PBS filtrate was detected at 90° to the incident excitation source. The emitted radiation was imaged onto the entrance slit of an F/4, 1/8 m imaging spectrometer with a 200 groove mm^{-1} holographic grating (ISA Jobin Yvon, Edison, NJ). A 1024-element intensified Reticon array (Model 1420, EG&G Princeton Applied Research, Trenton, NJ) detected the light at the image plane. The detection spectrometer had 5 nm resolution.

Data

Data were obtained using LabVIEW™ 7 software (National Instruments, Austin, TX). Appropriate programs developed in-house in addition to PLS Toolbox 3.5 software (Eigenvector Research, Inc., Wenatchee, WA) were used for further processing of the data using MATLAB™ 7.0.4 (The MathWorks, Natick, MA) software that operates as an interactive programming environment. The spectrum of raw data for each of the 7 plants (Fig. 1) consisted of 1 026 data points representing variations in fluorescence intensity (i.e., emitted radiation) between 370 nm and 580 nm. The spectra are not reported on an absolute intensity scale because there was no calibration of the fluorometer/camera system's responsivity vs. wavelength. The shapes of the spectra in Figure 1 depend in part on the instrumental responses of our fluorometry system and a different system would provide spectra that look somewhat different. The spectra have been corrected for PBS fluorescence (blanks) from the spectrum of each sample. The arbitrary spectral regions considered "blue" lie between 424.0 nm to 491.1 nm while "green" includes the region between 491.2 nm and 575.0 nm (See West 1967, pages E–133).

Statistical Analysis

Multivariate statistics have successfully been used to differentiate botanical composition of digested diets using fluorometric data (Mukherjee et al. 2001). However, for this paper Principle Component Analysis (PCA, Jolliffe 1986; Wise et al. 2003), was used to investigate differences among the spectra of the 7 plant samples.

Mathematically, PCA represents the eigenvectors for the covariance or correlation matrix of the original data matrix

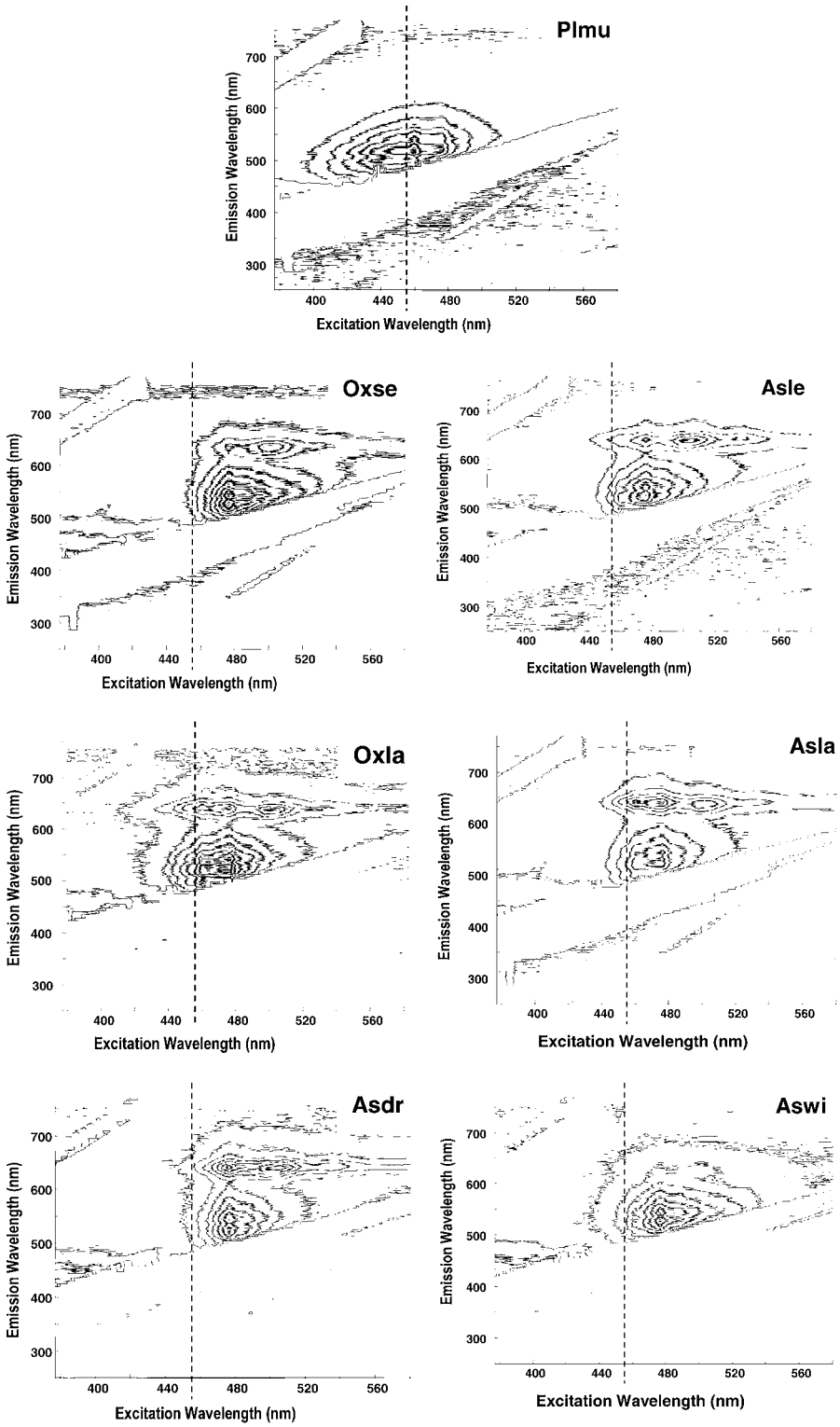

Figure 1. Data from *Astragalus lentiginosus* var. *diphysus* (Asle), *Astragalus drumondii* (Asdr), *Astragalus laxmanii* (Asla), *Astragalus wingatanus* (Aswi), *Oxytropis lambertii* var. *bigelovii* (Oxla), *Oxytropis sericea* (Oxse), and *Pleuraphis mutica* (Plmu) plant materials extracted with a pH 12.5 phosphate buffered saline solvent containing sodium azide to inhibit microbial growth, excited with a fluorometer between 370 and 580 nm in 5 nm increments. Labview™ 7 software was used to produce the emission intensity contour showing increasing intensity between blue and red regions, the dashed line (455 nm) represents the studied slice of each spectra using principal component analysis.

containing the measured variables. There may be as many eigenvectors as there are variables. The eigenvector associated with the largest eigenvalue has the same direction as the first principal component. The eigenvector associated with the second largest eigenvalue determines the direction of the second principal component. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible.

Because PCA is capable of simultaneously investigating measurements dependent on several variables to determine differences from among treatments, it has proven to be a useful tool for analyzing spectroscopic data such as is obtained from fluorometry (Geladi et al. 2003). In this study, the first 3 components are identified as PC1, PC2, and PC3. They are mutually orthogonal and can be represented in 3 dimensions as shown in Figure 2. The greater the separation in this 3-dimensional principal component space among the 7 treatments, the greater is the statistical difference between sample spectra.

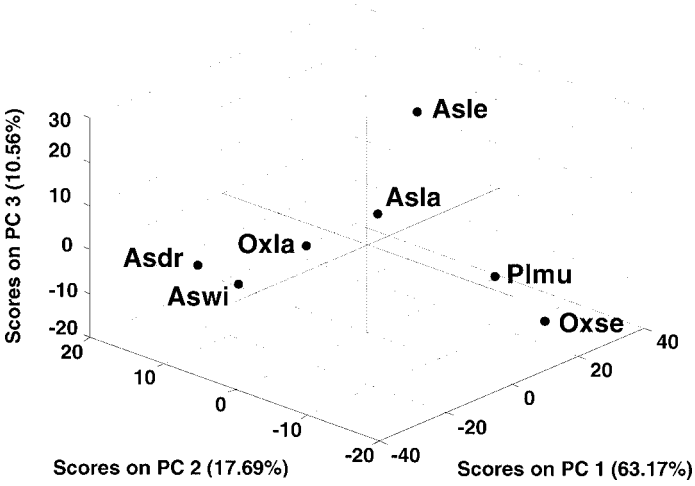

Figure 2. Principal component analysis applied to emission spectra from *Astragalus lentiginosus* var. *diphysus* (Asle), *Astragalus drumondii* (Asdr), *Astragalus laxmanii* (Asla), *Astragalus wingatanus* (Aswi), *Oxytropis lambertii* var. *bigelovii* (Oxla), *Oxytropis sericea* (Oxse), and *Pleuraphis mutica* (Plmu) plant materials extracted with a pH 12.5 phosphate buffered saline solution containing sodium azide to inhibit microbial growth when excited at 455 nm using a Xenon fluorometer. At this particular excitation wavelength 91.42% of the variability among plant emission spectra can be accounted for by applying three principal components: PC1, PC2, and PC3.

RESULTS AND DISCUSSION

The 9 PBS solvent blanks did not differ ($P = 0.916$); subsequently the mean effect of the PBS solution was subtracted from the emission spectrum from each of the 21 samples before PCA was performed. Emission spectra recorded from PBS extracts of the 7 plant species are depicted in Figure 1. These data represent a preliminary analysis of plants, 2 of which are known to contain toxins to cattle (i.e., swainsonine). There was no indication of photo-degradation of the samples over the 3 days during which data were collected. The 9 data sets obtained from the TiO_2 scattering solution had a mean intensity of 160 968 ± 3 022 at an excitation wavelength of 455 nm.

The contour lines in Figure 1 include regions of similar emission intensity with the center contour lines of Plmu and Asle having the highest emission among all 7 plant species. Diagonal features depicted as broad, white lines with slopes of approximately 1.0 and 2.0 correspond to the detection of the first and second order diffraction of the incident radiation ($\lambda_{(emission)} = \lambda_{(excitation)}$), respectively. It is, therefore, the region located above this Rayleigh scattered radiation that was of interest in this study (i.e., $\lambda_{(emission)} > \lambda_{(excitation)}$).

A visual comparison of the spectra shown in Figure 1 reveal both similarities, and, more important, spectral differences. Each spectrum shows broad spectral features adjacent to the first-order Rayleigh scattering feature. However, close examination of the spectra reveal differences regarding both the number of maxima present and their respective locations within the spectral window. While the spectrum corresponding to *Pleuraphis mutica* revealed a single large peak with a maximum at an excitation-emission (λ_{ex}-λ_{em}) coordinate of 450–522 nm, those from each of the other plant species showed red-shifted features relative to *Pleuraphis mutica*. Table 2 list the λ_{ex}-λ_{em} coordinates for the dominate maxima of each spectra.

Although the above method of visual spectral comparison is strongly suggestive of the ability of luminescence spectra to identify plant species, it is neither quantitative nor conclusive. In an effort to better quantify differences (or similarities) among such spectral signatures, PCA was applied to the emission spectra arising from a single excitation wavelength previously used by Anderson et al. (1996; 1998). These spectral "slices" are identified by the vertical dashed lines superimposed on each spectra shown in Figure 1.

Figure 2 is the projection of each of the 2-dimensional spectra in a 3-dimensional principal component coordinate space (PC-space). These 3 principal components were derived from the eigenvalues associated with a matrix of the emission intensities at each wavelength for each plant species ($\lambda_{excitation} = 455$ nm). These components (PC1, PC2, and PC3) accounted for 63.2%, 17.7%, and 10.6% of the variance, respectively, for a total of 91.5%. With PCA data a 2 PC model that captures more than 78% of the variance in a data set suggests the data are fairly well correlated (Wise et al. 2003). For these data the first 2 PC model captures more than 80% of the variance.

Readily apparent from Figure 2 are the relatively broad distributions among the 7 clipped plant species within the PC-space. Greater separation between the projected points is indicative of greater differences between the corresponding spectra. Notable among these results is the relatively large distance of separation in PC-space between the 2 species that contain swainsonine, *Oxytropis sericea* (Oxse) and *Astragalus lentiginosus* var. *diphysus* (Asle). Regardless how the PC-space is rotated these 2 plants appear to produce the extreme values on the PC3 axis. Both *Oxytropis sericea* (Gardner et al. 2001) and *Astragalus lentiginosus* var. *diphysus* (Gardner et al. 2003) have been reported to contain significant amounts of swainsonine. Even though pure swainsonine does not fluoresce these 2 species apparently contain 1 or more fluorophores that allow differences between them to appear different from other species within their respective genera and from the *Pleuraphis mutica*.

Table 2. Number and location of dominant peak maxima intensities for *Astragalus lentiginosus* var. *diphysus*, *Astragalus drumondii*, *Astragalus laxmanii*, *Astragalus wingatanus*, *Oxytropis lambertii* var. *bigelovii*, *Oxytropis sericea*, and *Pleuraphis mutica* extracted with a pH 12.5 phosphate buffered saline solution containing sodium azide to inhibit microbial growth when excited between 370 and 580 nm using a Xenon fluorometer.

		Locations of dominant peak maxima intensities			
		Excitation (λ_{ex})		Emission (λ_{em})	
Species	No. peaks	370–474	475–580	500–624	625–750
Astragalus lentiginosus	6	457	—	—	648
var. *diphysus*		465	—	530	—
		465	—	550	—
		465	—	—	648
		—	500	—	648
		—	530	—	648
Astragalus drumondii	5	470	—	525	—
		470	—	540	—
		470	—	—	645
		—	500	—	645
		—	530	—	645
Astragalus laxmanii	5	452	—	—	649
		454	—	520	—
		470	—	525	—
		470	—	—	649
		—	495	—	649
Astragalus wingatanus	2	470	—	525	—
		470	—	550	—
Oxytropis lambertii	4	450	—	—	648
var. *bigelovii*		460	—	520	—
		465	—	—	648
		—	492	—	648
Oxytropis sericea	4	470	—	540	—
		470	—	500	—
		470	—	—	648
		—	495	—	648
Pleuraphis mutica	1	450	—	522	—

The 2 species, *Astragalus drumindii* (Asdr) and *Astragalus wingatanus* (Aswi), within the genus *Astragalus* appear to occupy a relatively similar PC-space (Fig. 2). These 2 species did not contain swainsonine, and are considered not very palatable and palatable, respectively (Mike Ralphs, personal communication, 2005). In contrast, the 2 species within the genus *Oxytropis* appeared quite different in PC-space (Fig. 2). Though *Oxytropis lambertii* var. *bigelovii* (Oxla) may be toxic and is considered very palatable (Ralphs et al. 2002) our material did not contain swainsonine and produced fluorescence quite distinctly different from *Oxytropis sericea* (Oxse) that is toxic and contained swainsonine.

Given the differences seen among the 3-dimensional spectra shown in Figure 1, it is envisioned that expansion of dimensionality of the data will enhance the discrimination capabilities of this technique. By using additional emission spectra besides those at 455 nm, that characterize the data described in this paper, there exists a high probability that meaningful spectral differences exist among species.

CONCLUSION

These preliminary results from *Pleuraphis mutica*, and plants within the genera *Astragalus* and *Oxytropis* suggest fluorometry has potential for identifying these species prior to ingestion by free-ranging herbivores. Though it is currently not known what particular fluorophores are present in these spectral signatures, the data obtained in this study suggests the fluorophores in each replicate were similar. These data suggest for the first time it is possible to discriminate differences among plant species (some of which are poisonous) using fluorometry. Furthermore, these data represent the first successful attempt at elucidating these differences using sophisticated computing capability to apply PCA methods to differentiate toxic from nontoxic plants. Developing robust statistical procedures to tease apart differences between vegetation samples both independently and in mixtures will be one of the initial requirements for routine use of fluorometry as a tool for determining botanical composition of free-ranging animal diets. Once digestion studies on these same plants are conducted it will be possible to determine if plant material containing swainsonine can be predicted from fluorophores associated with swainsonine.

MANAGEMENT IMPLICATIONS

How to rapidly, precisely, and accurately obtain botanical information on plant material to make near real-time management decisions does not exist at the present time. However, these data are urgently needed especially when poisonous plants are part of the forage matrix. Documenting the spectral fingerprint of fluorophores in plant material is a relatively new, rapid, and robust optical approach (Anderson et al. 1996) for providing high-resolution data on botanical composition of clipped as well as digested plant material. Fluorometry appears to be an interesting tool, especially if plants containing toxic properties can be identified with this relatively rapid and environmentally friendly technique. Further research will be required to investigate the spectral signature of clipped as well as digested plant materials on individual plants as well as composite mixtures of the same species and dietary mixtures that would accurately reflect a foraging animal's diet.

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