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Laser-induced fluorescence (LIF) spectra of herbaceous and woody pre- and post-digested plant material [1]

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Abstract

Filtrate from pre- and post-digested plant material was exposed to 355-nm pulsed laser light and the subsequent laser-induced fluorescence (LIF) was recorded. Similarities and differences among spectra from 20 materials are discussed. Each material was replicated once, dried, ground, and exposed to chloroform ($CHCl_3$) for 24 h. The material represented aged (1 to 18 years old) plants from different herbaceous (grasses and forbs) and woody plant life forms. Mean peak fluorescence recorded among materials differed ($P < 0.0001$) in both wavelength and peak amplitude (counts) across the spectral range (387 to 788 nm). Peak fluorescence was evaluated within each of three arbitrary color categories, blue near 455 nm and red near 674 nm, while only 16 of the materials produced a green peak near 528 nm. In general, the blue and green fluorescence peaks were broad while the red peak was narrow. Mean peak counts were largest in the red range. Varying amounts of laser beam absorption occurred among the materials evaluated

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due to different concentrations of filtrate and different absorption efficiencies; therefore, amplitude data (counts) were not used to determine statistical differences among materials. To overcome difficulties attributed to the raw count data, red/blue, red/green and blue/green count ratios within replicates were calculated. Using all three count ratios in a multivariate analysis of variance, the 16 materials could be separated into nine different ($P < 0.05$) material groupings. The LIF technique may provide a reliable means to separate ground pre- and post-digested plant materials following further research into determining what fluorophores are producing the spectral signatures and how sample preparation affect peak wavelengths. © 1998 Elsevier Science B.V.

Keywords: Laser-induced fluorescence; Botanical composition; Fecal analysis; Plants

1. Introduction

Techniques to provide high accuracy and precision when assessing rangeland vegetation quality (Cook and Stubbendieck, 1986) and utilization (Anderson, 1981) still await discovery. Beginning in the 1980s, laser-induced fluorescence (LIF) was used to investigate the effect of stressors on plant material including those due to disease, chemical pollutants or nutrient deficiencies and drought (Nilsson, 1995). Recent research by Anderson et al. (1996) indicates LIF may have potential as an alternative to visually based techniques for evaluating the botanical composition of plant material both pre- and post-digestion.

Fluorescence occurs when a photon of light with sufficiently high energy (equivalently: short wavelengths) is absorbed by a molecule, exciting an electron within the molecule to a higher energy state. The subsequent return of the electron to its ground state is often accompanied by fluorescence, the emission of a longer wavelength photon (Guilbault, 1990).

A plant's pigments determine its LIF yield and spectrum (Hoge et al., 1983; Lichtenthaler et al., 1986). In living plants, their LIF signature is not static but changes seasonally (Krajicek and Vrbova, 1994) and throughout the day (Rosema et al., 1991). Fluorescence intensity in soybean leaves appears greater from the abaxial (back) compared to the adaxial (front) side (Chappelle et al., 1984a). In growing Glenlea wheat, Brach et al. (1982) demonstrated leaf age and site on the leaf caused minor fluorescence differences.

To differentiate among plants, quantitative (amplitude) and/or qualitative (wavelength) differences in spectral signatures will be required. In vascular plants, red fluorescence is apparently governed primarily by chlorophyll concentration (Chappelle et al., 1984a), specifically chlorophyll *a* (Lichtenthaler and Stober, 1990). Blue fluorescence emission in leaves of higher plants is a complex signal involving at least three elementary components (Goulas et al., 1990). However, specific compounds responsible for blue fluorescence in green plants are yet unidentified (Lichtenthaler and Stober, 1990). The source of green fluorescence, at least in intact *Vicia faba* leaves, may originate from epidermal tissue (Broglia, 1993). Lang et al. (1992) found that green fluorescence (525 nm) in leaves originated from mesophyll tissue. The alkaloid berberine and quercetin, a widespread flavonol, are cell wall components of some plants and

Table 1
Excitation laser wavelengths (nm) and the resulting laser-induced fluorescence (LIF) produced in growing and dead vascular plant tissue

Laser			Specimen		LIF emission spectrum			Source
Excitation wavelength (nm)	Type, wave mode	Power	Plant	Part [age if given]	Spectral range (nm)	Peak wavelength ratios	Peaks (nm)	
A = 337.1 B = 441	A = N_2, pulsed B = He–Cd, continuous C = A + B	A = 25 mW B = 18 mW	A = *Allium cepa* (onion), *Pisum sativum* (pea), *Raphanus sativus* (radish), B = *Lactuca sativa* (lettuce) and radish C = onion and radish	Leaf canopy growing in situ	A = 340–650 B = 650–750 C = 340–750	None given	A = 450 B = 683 C = 440, 680, 725	Brach and Molnar, 1977
A = 337.1 B = 441.0	A = N_2, pulsed B = Cd–He, continous C = A + B	A = 25 mW B = 18 mW	A = Lettuce (7 varieties) B = A turf grass and lettuce	Leaves from greenhouse grown plants	A = 350–750 B = 650–800 C = 340–750	A = 556.6/743.4	A = 457, 556.6, 679.8, 743.4 B = 683.3, 741.6 779.14, C = 365, 725	Brach et al., 1977
A = 337.1 B = 410 C = 441	A = N_2, pulsed B = He–Cd, continuous C = He–Cd, continuous	A = 25 mW B = 18 mW C = 18 mW	A = Lettuce cultivars B = lettuce cultivars C = A turf grass and lettuce	Leaves from greenhouse grown plants	A = 350–650 B = 650–800 C = 650–800	A = None given B = 750/650, 714/750 C = B	A = 457 B = 686.3 C = 686.3 Grass: 741.6, 779.14	Brach et al., 1978
325	He–Cd, continuous	2.5 mV	*Hordeum vulgare* (barley), *Secale cereale* (rye), *Triticum aestivum* and *durum* (wheat), and Triticale T.	Leaves from plants growing in pots	350–850	None given	359.90, 391.59, 393.56, 418 (Triticale), 445.04, 474.74, 506.42, 538.10	Brach et al., 1982
A = 422 B = 532	A = excimer/dye B = frequency-doubled Nd:YAG	A = 300 kW B = 1.0 mW	*Ammophila spp.* (dune grass), *Myrica spp.* (wax myrtle) *Pinus taeda* (loblolly pine), and *Prunis spp.* (black cherry)	Excised leaf samples	400–800	685/730	685, 730	Hoge et al., 1983

Table 1 (continued)

Laser			Specimen		LIF emission spectrum			Source
Excitation wavelength (nm)	Type, wave mode	Power	Plant	Part [age if given]	Spectral range (nm)	Peak wavelength ratios	Peaks (nm)	
337	N_2, pulsed	450 mW (9 mJ)	*Zea mays* (corn)	Plants growing in greenhouse [6–7 wk-old]	400–800	690/440	440, 690, 740	Chappelle et al., 1984a
337	N_2, pulsed	9 mJ	*Glycine max* (soybeans) and corn	Intact attached leaves growing in pots in greenhouse and fresh soybean leaves ground in distilled water [6 wk-old]	400–800	None given	440, 525 (soybean), 690, 740	Chappelle et al., 1984b
337	N_2, pulsed	9 mJ	*Acer rubrum* (red maple), *Beta vulgaris* (sugar beets), *Carya ovata* (shagbark hickory), soybeans, *Gossypium hirsutum* (cotton), barley, *Juniperus virginiana* (eastern red cedar), *Nicotiana tabacum* (tobacco), *Oryza sativa* (rice) *Picea glauca* (white spruce), *Pinus strobus* (eastern white pine), loblolly pine, *Querus ruba* (northern red oak), wheat, and corn	Excised mature conifer and hardwood leaves and greenhouse grown plant leaves [5–10 wk-old]	400–800	$(440)^2/685$	440, 525, (trees only) 685, 740	Chappelle et al., 1985
632.8	He/Ne	5 mW 30 mW	*Abies alba* (fir), *Acer plantanoides* (maple), *Fagus sylvatica* (beach), *Ginkgo biloba*, *Picea abies* (spruce), *Quercus robur* (oak), *Ulmus* spp. (elm)	Needles, leaves	600–800	690/740	690, 740	Lichtenthaler et al., 1986

337	N_2, pulsed	9 mJ	Red maple, sugar beet, shagbark hickory, soybean, cotton, barley, red cedar, tobacco, rice, *Picea glauca* (white spruce), white pine, loblolly pine, red oak, wheat, corn	Excised or attached outermost leaves and needles	400–800	440/685, 440/740	440, 525, 685, 740	Chappelle and Williams, 1987
337	N_2, pulsed	9 mJ	Soybeans and *Trifolium pratense* (clover)	Greenhouse grown intact leaves and water or acetone extracts	400–800	440/600	440, 525, 680, 740	Chappelle et al., 1991
337	N_2, pulsed	2.5 mJ	Tobacco, *Pseudotsuga menziesii* (douglas fir) and *Pinus sylvestris* (pine)	Leaves, needles	400–800	450/530, 450/690 690/735	450, 530, 690, 730, 735	Lichtenthaler et al., 1991
A = 308 B = 632.8	A = XeCl excimer B = He–Ne	A = 15 mJ/pulse B = 1 mW	*Medicage arborea* (medica), *Olea europea* (olive), *Vicia faba* (bean) and an evergreen	Intact and attached leaves	400–800	None given	A = 440, 530, 680, 740	Broglia, 1993
A = 355 B = 337	N_2, pulsed	A = Not given B = 2.5 mJ	A = Tobacco B = *Fagus slyvatica* (beech), wheat	Excised beech leaves, wheat seedlings [8–14 days old]	400–800	450/690 450/530 690/735	450, 530, 550, 680, 684, 690, 735	Lichtenthaler et al., 1993
337	N_2, pulsed	2.5 mJ	Wheat	Leaves: green, etiolated, white [8 days old]	400–800	450/690 450/530 450/735 690/735	450, 460, 530, 690, 735	Stober and Lichtenthaler, 1993
355	Nd:Yag	35 mJ	*Quercus pubescens* (oak)	Leaves	400–800	685/730 440/685	440, 520, 685, 730	Günther et al., 1994
Not given	XeCl	80 mJ to give 1.5 mJ/cm^2	Chestnut, dandelion, elder, grass, larch, lilac, pine, spruce, and walnut	Not given	350–800	None given	440, 525, 685, 736, 740	Krajicek and Vrbova, 1994

Table 1 (continued)

Laser			Specimen		LIF emission spectrum			Source
Excitation wavelength (nm)	Type, wave mode	Power	Plant	Part [age if given]	Spectral range (nm)	Peak wavelength ratios	Peaks (nm)	
A = 337 B = 632.8	A = N_2, pulsed B = He/Ne, continuous	A = 2.5 mJ B = 5 mW	Wheat B = soybeans	Leaves from greenhouse grown plants [14 days old] and field grown plants	400–800	450/690 690/735 450/530	450, 530, 685, 690, 695, 735	Stober et al., 1994
355	Nd:YAG	1 mJ	*Flourensia cernua* (tarbush leaves) and *Pleuraphis mutica* (tobosa hay), and feces from sheep fed diets containing varying amounts of the two species	Non-ground plant material and intact and crushed fecal pellets	400–800	Red/Blue	450 to 480, 675 to 676	Anderson et al., 1996

produce green fluorescence (Lang et al., 1991). The chemical origin and precise location in plant cells of blue and green fluorescence is yet unknown (Stober and Lichtenthaler, 1993), but obviously result from separate fluorophores (Lichtenthaler et al., 1991). Many compounds when present in concentrations or oxidation states specific to a particular plant type may provide the means for species identification because of their potential to produce LIF (Chappelle et al., 1989; Lichtenthaler et al., 1992). However, fluorophores in post-digested diets remain to be identified (Anderson et al., 1996).

Chappelle and Williams (1987) found LIF was related to plant species based on either number of fluorescent bands or relative band intensity. Brach et al. (1978), using a 441-nm laser beam, discriminated between a turf grass and lettuce by observing peak wavelength differences and quantitatively by fluorescence yield at 686.3 nm. Lichtenthaler et al. (1993) concluded that the best excitation wavelength for evaluating plant material was in the range between 370 and 395 nm from a pulsed laser, since longer wavelengths would overlap with blue fluorescence emission. Chappelle et al. (1989) concluded a pulsed laser emitting at 337 nm was preferred for evaluating plant material because pulsed monochromatic light overcame interference from ambient light and produced a multiband LIF.

Sample preparation methods can cause spectral shifts, absorption and quenching of LIF. Chappelle et al. (1991) found solvents, used to suspend extracted samples, can affect fluorescence. Sample pH, dissolved oxygen, temperature, filtrate concentration, and impurities in the extract also can affect fluorescence (Guilbault, 1990). The quartz cuvette, used to hold liquid samples in the path of the laser beam, should be cleaned with highly purified water (reverse osmosis) between samples; detergents, acetone and trace metal ions (chromium ions have a strong affinity for quartz) can quench fluorescence (Froehlich and Guilbault, 1990). Furthermore, cuvettes should be free of scratches to prevent entry of stray light (Froehlich and Guilbault, 1990).

No literature exists regarding the potential of LIF to differentiate among pre- and post-digested ground plant materials. Previously published LIF studies involving vascular plants focus on fresh or recently harvested intact leaves from horticultural and ornamental vegetation (Table 1). The objective of this research was to evaluate the potential of LIF for differentiating among 20 different ground pre- and post-digested plant materials found on arid rangeland. No attempt was made to identify the fluorophores (fluorescing compounds) responsible for LIF.

2. Materials and methods

2.1. Materials

The 20 different pre- and post-digested plant parts, mixed diets, and post-digested diets (goat feces) from eight different plant species were dried and ground to pass a 1-mm Wiley screen before being evaluated for LIF (see Table 2). The materials included are four woody species consisting of leaves and/or leaves and twigs of creosotebush (*Larrea tridentata* [DC.] Cov.), winterfat (*Ceratoides lanata* [Pursh] J.T. Howell), fourwing saltbush (*Atriplex canescens* [Pursh] Nutt.) and tarbush (*Flourensia cernua*

Table 2
Means ($n = 2$ replicates per material) of laser-induced fluorescence (LIF) wavelengths corresponding to peak counts for 20 red, 20 blue and 16 green pre- and post-digested plant materials exposed to 10 pulses of 355-nm laser light each having ~4 mJ of energy and lasting ~10 ns

Materials	Wavelengths			Peak counts		
	Blue	Green	Red	Blue	Green	Red
Woody species						
Creosotebush *Larrea tridentata*	477.4^{A}	552.6^{A}	676.1^{C}	1419^{I}	3159^{E}	$120{,}732^{GH}$
Winterfat *Ceratoides lanata*	447.5^{HIJ}	527.3^{BCD}	673.8^{EF}	7315^{A}	6917^{B}	$76{,}242^{GHI}$
Fourwing saltbush *Atriplex canescens*[1]	460.6^{CDE}		675.2^{D}	2604^{EFG}		$676{,}107^{B}$
Tarbush *Flourensia cernua*						
August 1991 harvest	472.5^{AB}	552.9^{A}	678.4^{B}	7473^{A}	$11{,}699^{A}$	$840{,}469^{A}$
October 1995 harvest	448.8^{GHIJ}	552.6^{A}	680.3^{A}	2996^{E}	7218^{B}	$647{,}460^{B}$
Forb species						
Leatherweed croton *Croton corymbulosus*	452.1^{FGHI}	533.0^{B}	676.1^{C}	4861^{B}	5859^{C}	$816{,}411^{A}$
Grass species						
Barley straw *Hordeum spp.*	454.3^{EFGH}	531.6^{B}	673.7^{F}	1325^{I}	1488^{GH}	$68{,}467^{HI}$
Tobosa hay *Pleuraphis mutica*						
August 1991 harvest[1]	447.4^{HIJ}		674.4^{E}	2963^{E}		$265{,}962^{DE}$
October 1995 harvest[2]	449.8^{GHIJ}		672.6^{HI}	2163^{GH}		$374{,}486^{C}$
Mesa dropseed *Sporobolus flexuosus*						
July 1977 stems	445.8^{IJ}	527.6^{BCD}	673.7^{F}	1312^{I}	1140^{HI}	$91{,}437^{GHI}$
December 1977 stems	443.5^{J}	511.0^{DE}	672.6^{HI}	1209^{I}	946^{HI}	720^{I}
July 1977 leaves	455.9^{DEFG}	543.3^{AB}	674.4^{E}	2258^{FG}	2413^{F}	$299{,}659^{CD}$
December 1977 leaves	448.9^{GHIJ}	515.1^{CD}	671.0^{J}	4226^{C}	3620^{DE}	3049^{I}
December 1977 inflorescence	449.1^{GHIJ}	501.1^{F}	672.8^{GHI}	1018^{I}	844^{I}	633^{I}
December 1977 dead material	449.4^{GHIJ}	509.5^{DE}	671.6^{J}	2696^{EFG}	2268^{F}	3328^{I}
October 1995 whole plant[1]	462.9^{CD}		672.3^{I}	1597^{HI}		$171{,}353^{EFG}$
Angora goat diets						
25% winterfat + 75% barley straw	450.0^{GHIJ}	528.5^{BC}	673.7^{F}	3098^{DE}	3180^{E}	$83{,}820^{GHI}$
23% fourwing saltbush + 77% barley straw	450.0^{GHIJ}	530.8^{BC}	674.4^{E}	2114^{GH}	2036^{FG}	$276{,}294^{CD}$
Angora goat feces						
25% winterfat + 75% barley straw	460.3^{CDEF}	526.4^{BCD}	673.3^{FG}	3641^{CD}	3981^{D}	$242{,}379^{DEF}$
23% fourwing saltbush + 77% barley straw	466.2^{BC}	532.5^{B}	673.0^{GH}	2838^{EF}	3417^{DE}	$157{,}097^{FGH}$
Standard error	±2.81	±5.79	±0.22	±200	±194	±34,772
Overall P value for testing material differences	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
High value	477.4	552.9	680.3	7473	11,699	840,469
Low value	443.5	492.5	671.0	1018	845	633

^{ABCDEFGHIJ}Means in the same column with different superscripts differ ($\alpha = 0.05$).
[1]No green fluorescence in either replicate.
[2]Green fluorescence in only one replicate.

DC.); one forb, leatherweed croton (*Croton corymbulosus* Engelm.); three grass species, barley (*Hordeum spp.*) straw, tobosa (*Pleuraphis mutica* Buckley) (formally *Hilaria mutica* [Buckl.] Benth.) hay and mesa dropseed (*Sporobolus flexuosus* [Thurb.] Rydb.).

Winterfat leaves and twigs were harvested near Taos, New Mexico during the winter of 1985 and fourwing saltbush leaves and twigs were harvested near Las Cruces, New Mexico during June 1986 (Nunez-Hernandez, 1987). Barley straw was purchased in Las Cruces during the mid to late 1980s. Croton plants and creosotebush leaves and twigs were hand-harvested in Doña Ana County, New Mexico during the mid to late 1980s (Jerry Holechek, personal communication).

Tarbush leaves and tobosa hay were collected from pasture 6 on the Jornada Experimental Range (JER) near Las Cruces during August 1991 (King et al., 1996) and October 1995. Five tarbush plants and five tobosa plants were harvested in 1995, placed in separate paper bags, brought to the laboratory and air-dried. Samples of the entire tobosa plant and leaves only from tarbush were ground and analyzed for LIF. Air-dried and ground material (0.2 g) was composited by species and thoroughly mixed to make 1.0 g samples.

Twice in 1977 and once in 1995, five different mesa dropseed plants were harvested at ground level and placed in separate paper bags. The plants harvested in 1977 were immediately dried at 60°C while the material harvested in 1995 was air-dried before being ground. The July and December 1977 harvests were from similar areas in JER pastures 2N and 2S, respectively. The five mesa dropseed plants harvested in October 1995 were collected from JER pasture 10B. Plants harvested in 1977 were separated into plant parts (stems, leaves, floral appendages and dead gray material) prior to grinding and storage at room temperature in amber colored plastic medicine-type vials with snap on lids. Inflorescence and dead material was unavailable from mesa dropseed harvested in July 1977. Each of the plants harvested in 1995 were individually ground whole and stored in five separate plastic vials. Prior to determining LIF on the mesa dropseed parts, contents in each plastic vial were dried at 60°C followed by weighing out 1.0 g composite samples for each plant part by combining 0.2 g of material from each of the five vials.

Material representing the 'as fed' diets were reconstructed by weighing appropriate ratios of the ground (1 mm), air-dried plant materials into 1.0 g composite samples. Fecal material representing these diets was obtained from castrated Angora goats fed diets containing either 25% winterfat + 75% barley straw or 23% fourwing saltbush + 77% barley straw that had been ground to pass a 2.5-cm hammer mill screen. The composited Angora goat rectal-grab fecal samples (4 goats $\times$ 4 days $\times$ 2 pellets goat^{-1} day^{-1} = 32 pellets diet^{-1}) were obtained following a 10-day period of adaptation in a digestion trial conducted during the late fall and early winter of 1987 at New Mexico State University (Vernet, 1989).

2.2. Laser procedure

The 20 ground materials were oven-dried at 60°C for 24 h, and two 0.15 g samples (replicates) were weighed into 40 glass test tubes and stoppered with cork and left at room temperature until 10 ml of HPLC grade chloroform ($CHCl_3$) was added. Following 12 to 14 h in $CHCl_3$, corks were removed and the contents in the 40 test tubes were filtered through Whatman No. 4 paper (retained particles > 20 μm in size) into clean glass test tubes and re-stoppered. To reduce $CHCl_3$ evaporation, all corks were wrapped with Parafilm®. The 40 test tubes containing $CHCl_3$ filtrate and four test tubes

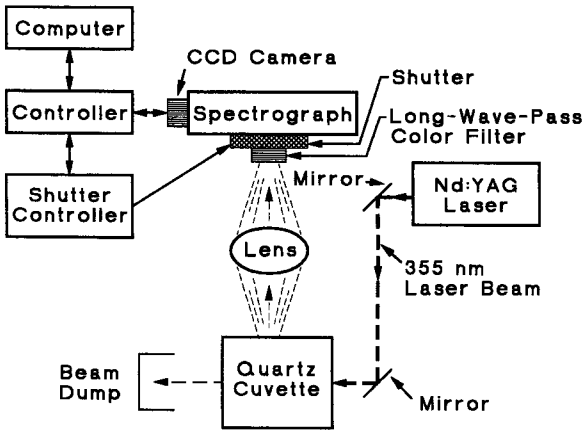

Fig. 1. Schematic (top view) of laboratory setup for determining laser-induced fluorescence (LIF).

containing only $CHCl_3$ were placed in a freezer at $-20°C$. Following 33 days in the freezer, $CHCl_3$ was added when necessary to compensate for any solvent evaporation prior to exposing the 40 samples and four $CHCl_3$ blanks to the laser's energy. The replicates were tested in random order for LIF by decanting approximately 3 ml aliquots from each test tube into a 3.5-ml quartz fluorometer cell (cuvette) (Catalog Number 3-Q-10 manufactured by Starna Cell; interior dimensions, 10×10 mm for use in the 170 to 2700 nm range). The cuvette was then placed in a holder and exposed to 10 pulses of 355 nm laser light each having ~ 4 mJ of energy and lasting ~ 10 ns (Fig. 1). A single $CHCl_3$ blank was run following the first four exposures, and thereafter, single blanks were excited following each twelfth sample with the last blank excited following exposure of all 40 samples. Once exposed, filtrate in the cuvette was discarded, the cuvette was rinsed with acetone and allowed to air-dry before reuse. Each sample required ~ 2 min to evaluate.

The 40 samples were each excited at 355 nm by a frequency-tripled neodymium:yttrium aluminum garnet (Nd:YAG) laser (Model DCR-2A Quanta-Ray, manufactured by Spectra-Physics). The power was measured periodically using a power meter (Model 380101 sensor and Model 365 Power Energy Meter, both manufactured by Scientech, Boulder, CO). Laser light not absorbed in the sample was captured by a cardboard shield ('beam dump'; Fig. 1). Some of the resulting broadband fluorescence was captured by a 5-cm diameter lens (10 cm focal length) and imaged to the entry slit of the spectrograph (Model UFS 200, manufactured by Instruments S.A., with 300 groove/mm holographic grating) located 20 cm from the lens. The spectrograph entry slit width of 200 μm yielded spectral resolution of about 2.5 nm. A long-wave-pass color filter (385 nm) was used at the spectrograph input to prevent scattered laser light from entering the instrument and possibly distorting the fluorescence spectrum.

The dispersed spectra at the spectrograph's output were detected using a cooled, charged-coupled-device (CCD) camera (Model CH250A, manufactured by Photometrics, Tucson, AZ). A computer operated the camera and recorded its output. The computer also controlled the mechanical shutter (Uniblitz Model D122, manufactured by

Vincent Associates, Rochester, NY) used to limit exposure time. Connections between computer, camera, and shutter were made via the camera controller (Model CE200A, manufactured by Photometrics, Tucson, AZ).

The laser operated at a steady rate of 10 pulses/s. All exposures were 1 s to reduce the possibility of inducing chemical changes in the samples as a result of longer periods of laser exposure. (The camera's CCD chip accumulated the fluorescence spectrum resulting from the ten excitation pulses during each 1 s exposure.) In a previous trial, we observed filtrate in the cuvette to lose its color following ~ 90 s of laser exposure.

2.3. Data

Each spectrum of raw data (Figs. 2–4) consists of 1152 data points representing variation in fluorescence intensity between wavelengths 387 nm and 788 nm. Data in our spectra beyond 770 nm should be ignored, since they may also contain wavelengths that are half those shown. For example, if there is fluorescence at 390 nm it will also appear at the 780 nm position. The 385-nm longwave-transmission filter prevented this problem from occurring for positions less than 770 nm.

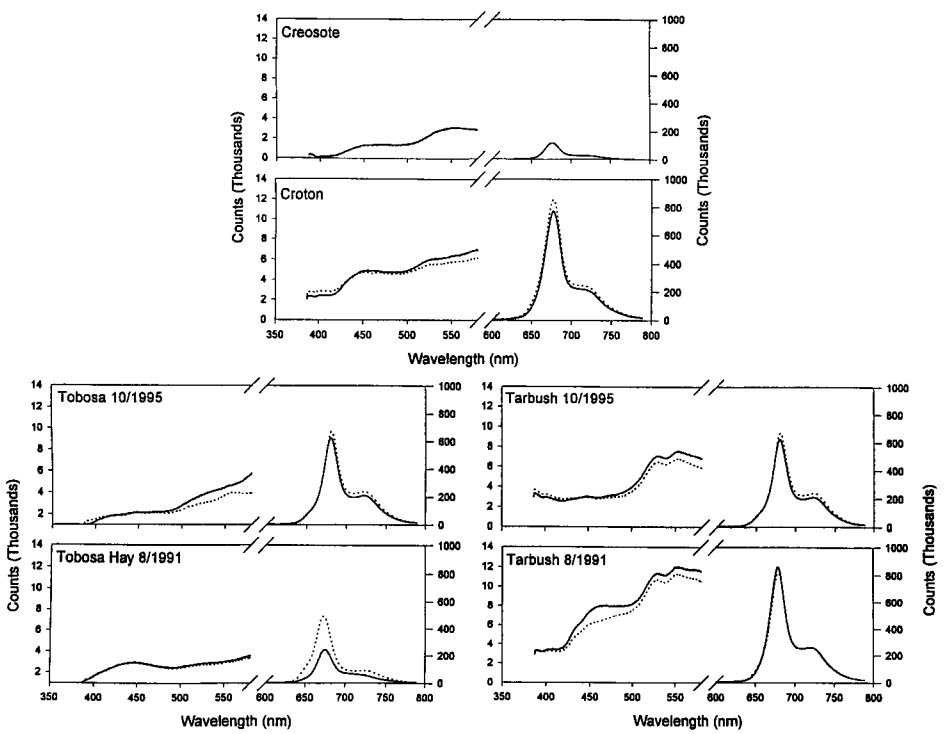

Fig. 2. Laser-induced fluorescence (LIF) of two replicates (.... and ____) of leaves from two shrubs (creosotebush and tarbush), one forb (leatherweed croton) and one grass (tobosa).

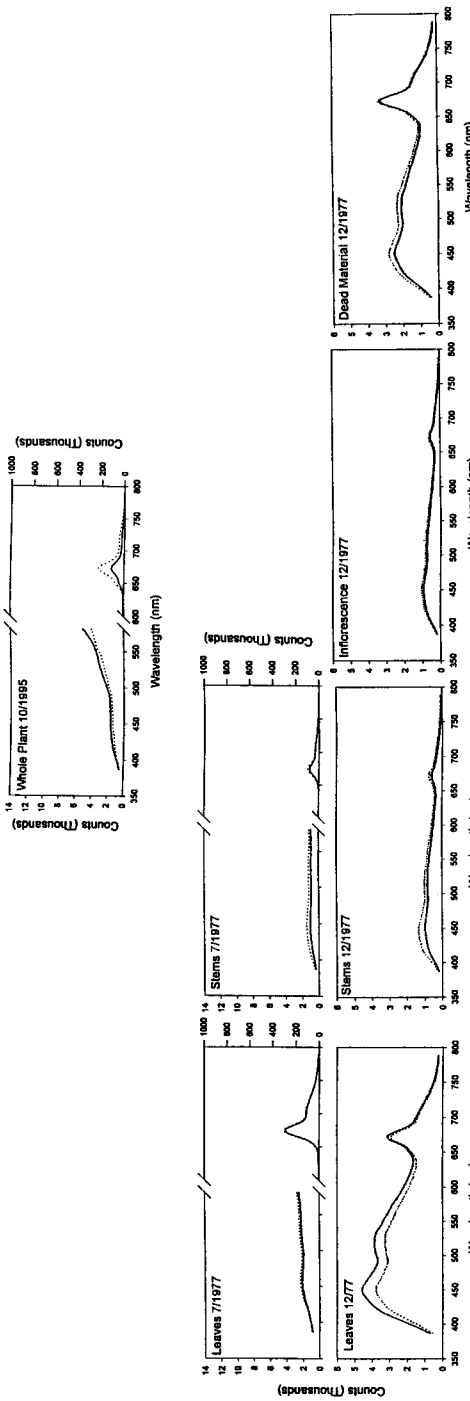

Fig. 3. Laser-induced fluorescence (LIF) of two replicates (.... and ____) of mesa dropseed plant parts harvested in 1977 and 1995.

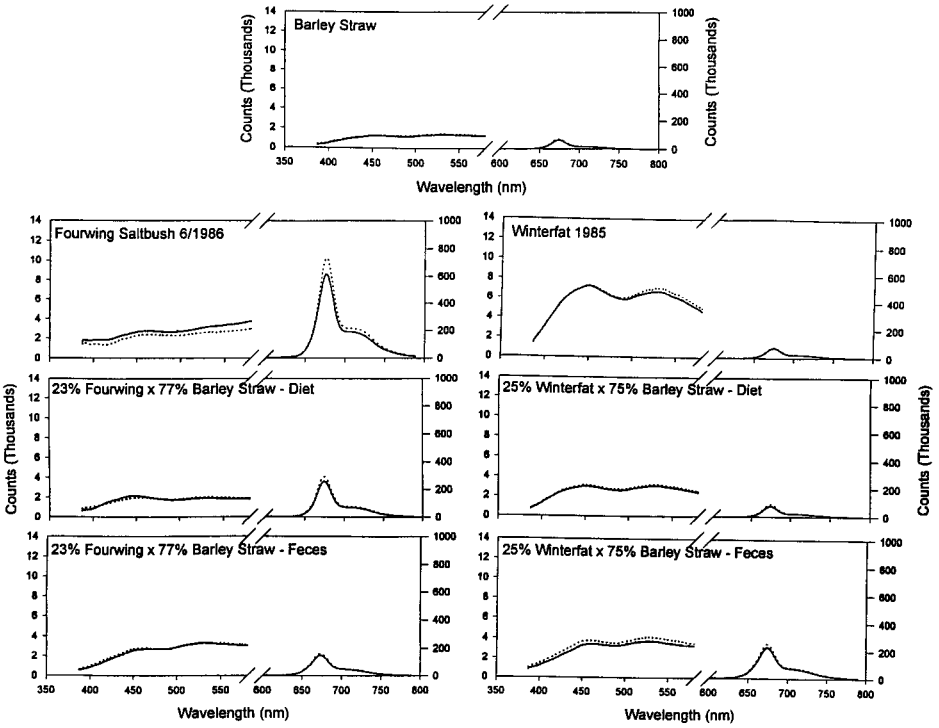

Fig. 4. Laser-induced fluorescence (LIF) of two replicates (.... and ____) of barley straw, fourwing saltbush leaves and winterfat leaves as components and as mixtures in diets, and feces from Angora goats fed these two diets.

A single data point gives the detected 'counts' (proportional to fluorescence intensity) in the corresponding 0.35-nm wide wavelength interval. We are not reporting spectra on an absolute intensity scale since there was no calibration of the spectrograph/camera system's responsivity vs. wavelength. The shapes of these spectra depend in part on the instrumental responses of our spectrograph and camera, and a different spectrograph/camera pair would obtain spectra that look somewhat different. In contrast, count ratios accurately differentiate among materials when laser energy entering and exiting the fluorescing sample is unknown. Furthermore, by using ratios, standards are not required to calibrate the spectrograph since all LIF spectra are taken with the same instrumentation. Ratios do depend on the spectral response of the spectrograph/detector equipment used. However, ratios should be independent of fluctuations in the laser's power (Chappelle et al., 1984a).

The spectra have been corrected for $CHCl_3$ fluorescence by subtracting wavelength-by-wavelength the mean $CHCl_3$ fluorescence from the spectrum of each sample. Each 'corrected' spectrum was characterized by six numbers: the peak count, and wavelength of the peak count in each of three arbitrary spectral regions (see Weast, 1967, page E-133): 424.0–491.1 nm ('blue'), 491.2–575.0 nm ('green'), and 647.0–700.0 nm ('red'). No distinct spectral peaks were detected outside the blue, green and red regions.

2.4. Statistical analysis

All 40 samples had two or more fluorescence peaks. Both replicates in the 20 materials evaluated had at least one peak wavelength in the blue and one in the red. The highest peak wavelengths in the blue and in the red, and the counts corresponding to these peaks were statistically analyzed. In contrast, both replicates in only 80% of the materials exhibited a peak wavelength in the green. Wavelength of the peak in the green and the counts corresponding to this peak were statistically analyzed for 16 materials.

Three count ratios—red/blue, red/green and blue/green—were also analyzed for just 16 materials. Each variable was analyzed separately by analysis of variance in a completely randomized design using the GLM procedure in SAS (SAS Institute, 1989). Observed significance levels (p) are reported for the AOV F-tests. Means and standard errors were also calculated and the least significant difference (LSD) test was used for mean separation at $\alpha = 0.05$ when the F for materials was significant. In addition, a multivariate analysis of variance was performed on the red/blue, red/green and blue/green count ratios and a multivariate means separation technique was used to group similar materials (David W. Smith, personal communication).

3. Results and discussion

3.1. Spectral signatures

The 20 materials excited at 355 nm produced different LIF spectral signatures between 387 nm and 788 nm in the regions of the spectrum arbitrarily designated blue, green and red (Figs. 2–4). Most replicates had similar spectral profiles. Neither the carrier solvent $CHCl_3$ nor the acetone wash used to clean the cuvettes produced fluorescence peaks.

It was not the intent of this research to determine the compounds responsible for producing fluorescence but rather to determine similarities and/or differences in spectral signatures among the 20 pre- and post-digested plant materials. Determining which compounds produce a particular spectral signature will be complicated because emission wavelength of a fluorophore can shift depending on its local (i.e., molecular) environment (Goulas et al., 1990). Van Soest (1994) points out the difficulty in distinguishing endogenous matter from matter contributed by the digestion and fermentation processes in ruminant feces. Therefore, determining the fluorophores producing spectral signatures from pre- and post-digested heterogeneous diets will be challenging, especially for feces, since it contains undigested plant residues, microbial debris and endogenous matter arising from digestion.

3.2. Counts

At least one of the peak amplitudes (counts), associated with the highest peak in each of the blue and red ($n = 20$ materials) and green ($n = 16$ materials) wavelengths, differed among materials as shown by an overall significant ($P < 0.0001$) F-test (Table 2). The largest numerical count differences among samples occurred in the red region of

the spectrum making it necessary to depict wavelengths of 16 materials on different intensity scales (Figs. 2–4). Only in mesa dropseed leaves, stems and floral appendages harvested in December 1977 did peak blue counts exceed red counts (Fig. 3). We experienced different absorption of the laser beam among the 40 samples preventing a constant amount of laser energy from passing through the cuvette. Therefore, differences among materials based on raw count data do not appear meaningful and will not be discussed further.

3.3. Wavelengths

Replicates of all 20 materials had at least one peak feature in both the blue (mean = 455 ± 0.63 nm) and red (mean = 674 ± 0.05 nm) region, while only replicates of 16 materials had a green (mean = 528 ± 1.45 nm) peak (Figs. 2–4). Fluorescence in the red region of the spectrum produced a prominent, and relatively narrow feature for most materials. The LSDs showed only three materials—tarbush leaves harvested in August 1991 (678.4 nm) and October 1995 (680.3 nm) and fourwing saltbush (675.2 nm)—had red wavelengths different from the rest while none of the materials had uniquely different wavelengths in the blue ($n = 20$) or green ($n = 16$) spectra (Table 2).

In pre-digested vascular plant material, red emissions have been attributed to chlorophyll *a* fluorescence (Chappelle et al., 1984a; Lichtenthaler and Stober, 1990) with chlorophyll fluorescence maxima occurring between 650 and 800 nm (Stober et al., 1994; Nilsson, 1995; see Table 1). Even though the red emission in our pre- and post-digested samples was in this spectral range (671 to 680 nm), it is uncertain what compounds are responsible for the red emissions. Furthermore, it would be premature to speculate that products of chlorophyll degradation in goat feces are responsible for the red spectral signature we observed, even though the relatively indigestible chromogens originating from chlorophyll have been used as internal indicators (Kotb and Luckey, 1972). Further research will be required to determine if $CHCl_3$ has an effect on chlorophyll fluorescence since Chappelle et al. (1991) and Wehry (1990) report type of solvent and pigment dilution can affect fluorescence.

At least one of the highest peak wavelengths, in each of the three arbitrary color categories (blue, green and red), differed among the 20 materials as shown by an overall significant ($P < 0.0001$) F-test (Table 2). A fluorescence spectrum is fundamentally a quantitative relationship between emitted intensity and wavelength: $I(\lambda)$. The quantitative differences among some of the LIF spectra (Figs. 2–4) are gross enough to obviously look very different, i.e., they have different shapes and require no statistical analysis to distinguish them. The spectral shape actually characterizes just the relative values $I(\lambda_1)$ and $I(\lambda_2)$ at different wavelengths, λ_1 and λ_2, within a spectrum. Thus, if two spectra have intensities $I_1(\lambda)$ and $I_2(\lambda)$ such that $I_2(\lambda) = C \cdot I_1(\lambda)$ for some constant $C \neq 1$, the two spectra still have the same shape. The qualitative shape differences in our LIF spectra allow us to evaluate variations among the spectra arising from the 20 materials and discuss the 'characteristic wavelengths'. For example, in Fig. 3 the fluorescence spectrum for mesa dropseed leaves harvested in December 1977 is present at all wavelengths beyond about 390 nm. However, one's eye notes obvious local maxima in $I(\lambda)$ at wavelengths ~ 445 nm ('blue'), ~ 520 nm ('green'), and ~ 670 nm

('red'). Thus, identification of such 'blue', 'green', and 'red' peaks is one way we characterize this material's spectrum for further discussion and comparison with others. Phenolic compounds including the cinnamic acids such as caffeic, ferulic and sinapic acid as well as chlorogenic acid and quinic acid preferentially localize in the leaf epidermis and can contribute to fluorescence in the blue region (Lichtenthaler et al., 1991). Recent research by Bongi et al. (1994) indicated blue-green fluorescence arises mainly from outer epidermal leaf layers. Post-digested samples (feces) contain substantial epidermal fragments, given that microhistological procedures (Alipayo et al., 1992) use epidermal fragment identification to identify the botanical composition of animal diets. Therefore, it is not surprising all our samples fluoresced in the blue spectral region.

Tarbush leaves, tobosa hay, mesa dropseed stems and mesa dropseed leaves harvested at different dates had different red fluorescence wavelengths (Table 2). The wavelength for peak green fluorescence was similar for tarbush leaves, and for mesa dropseed stems harvested in July and December 1977, while July and December mesa dropseed leaves differed in peak green fluorescence. The blue fluorescence wavelength was similar for tobosa harvested in August 1991 and October 1995 and for mesa dropseed stems and leaves harvested in July and December 1977. However, the blue fluorescence wavelength from tarbush leaves differed; a shorter wavelength was associated with leaves harvested in 1995 compared to those harvested in 1991 (Table 2).

Mesa dropseed harvested in December 1977 may have contained less chlorophyll and carotenoids than July materials due to senescence; therefore, pigment dilution may partially explain the lower red fluorescence counts (Stober and Lichtenthaler, 1993). However, Brach et al. (1978) found fluorescence amplitude to be highest in the oldest growing lettuce plants. An identical red (673.7 nm) fluorescence was observed for barley straw, mesa dropseed stems harvested in July 1977, and Angora goat diet containing 25% winterfat and 75% barley straw. A slightly longer (674.4 nm) and identical red wavelength was found for tobosa hay harvested in 1991, mesa dropseed leaves harvested in July 1977 and Angora goat diet containing 23% fourwing saltbush and 77% barley straw. Leaves of creosotebush and croton both fluoresced in the red at 676.1 nm. The only materials fluorescing at identical green (552.6 nm) and blue (450.0 nm) wavelengths were creosotebush and tarbush harvested in October 1995, and the two Angora goat diets, respectively (Table 2).

Winterfat and fourwing saltbush differed in peak blue and red wavelengths when comparing fluorescence (Table 2). Comparison between these two species for green fluorescence was not possible since fourwing saltbush did not exhibit a peak green wavelength while winterfat did. The peak blue wavelength of barley straw did not differ from either winterfat or fourwing saltbush while the peak wavelength responsible for red fluorescence of barley straw was shorter than for fourwing saltbush and similar to winterfat (Table 2). When winterfat and fourwing saltbush were each combined with barley straw, fluorescence at the peak red wavelength differed between the two diets, while the peak blue wavelength was similar between the two diets. Post-digestion peak blue wavelengths and peak red wavelengths from the goat feces were similar for the two diets (Table 2). Peak blue wavelength differed between diets and feces. However, peak red wavelength, though different between diets was similar among the diet containing

winterfat and the feces containing either winterfat and barley straw or fourwing saltbush and barley straw (Table 2).

Not finding unique peak wavelength differences among plants composing a diet and the feces obtained from animals fed the diets is not surprising since many fluorophores like chlorophyll *a* are common to vascular plants and any quantitative differences in similar fluorophores may have been lost due to a blending of the spectral signatures. However, with sophisticated computer software such as multivariate 'patch' algorithms and a genetic optimizer for the multivariate routine, it may be possible using computer-based analysis to estimate individual chemical concentrations in multicomponent mixtures by making reference to a base set of pure materials having calibrated spectral signatures (Wagner et al., 1996). In the case of winterfat and fourwing saltbush, the spectral signature of each may have become blended with that from the straw since barley composed $> 70\%$ of the material in both diets. However, if plant species were to contain a fluorescing compound unique only to that species, even if it were present in minute concentrations, LIF may be able to detect its presence since Hickman and Moore (1970) report detecting dyes in water using LIF when concentrations were as low as 0.1 ppb.

In contrast to red fluorescence, much broader features were observed in the blue and/or green portions of the spectrum among the 20 materials (Figs. 2–4). The wider fluorescence bands in the blue and green compared to the red region may indicate complex and less symmetrical fluorophores (Guilbault, 1990).

The tarbush and tobosa (Fig. 2) harvested in 1991 was identical to material previously reported to fluoresce when exposed to a 355-nm laser beam (Anderson et al., 1996). However, in the earlier study, neither plant material was ground before exposure to $CHCl_3$ and the period of extraction in $CHCl_3$ was substantially shorter (20 s) compared to the present study (24 h). In the earlier study, tarbush leaves exhibited only one peak between 450 nm and 463 nm (blue), while tobosa hay had two peaks, one between 451 nm and 466 nm (blue) and a second at ~ 675 nm (red). In this study, tarbush leaves harvested in 1991 and 1995 both produced peak fluorescence in the blue, green and red regions of the spectrum (Table 2). In contrast, ground tobosa hay harvested in 1991 and 1995 produced peak fluorescence in only the blue and red regions of the spectrum (Table 2).

Except for the CCD camera which replaced the photodiode array described by Anderson et al. (1996), the instrumentation was set up and operated in a similar manner for both studies. However, these equipment differences could have been responsible for the differences in the various color ratios "within" a spectrum without changes in the actual spectrum. As an example, the tobosa to tarbush blue count ratio ($10/23 = 0.43$) reported by Anderson et al. (1996) is approximately equal to the tobosa to tarbush count ratio in this study ($2963/7473 = 0.40$).

Preparation of samples differed between the two studies. Surface area for the $CHCl_3$ to act upon was greater in the current study compared to the earlier study because the material used in this study was ground, while in the initial study, the materials were not ground. Second, the ground material was in contact with the $CHCl_3$ for 24 h in this study, while in the past study, the intact material was in contact with the solvent for only 20 s. The 20 s exposure time probably removed only cutin (extracellular components)

Table 3
Means ($n = 2$ replicates per material) of red/blue, red/green and green/blue laser-induced fluorescence (LIF) peak count ratios for 16 pre- and post-digested plant materials exposed to 10 pulses of 355 nm laser light each having ~4 mJ of energy and lasting ~10 ns

Materials	Peak count ratios		
	Red/blue	Red/green	Green/blue
Woody species			
Creosotebush *Larrea tridentata*	85.1^{E}	38.2^{FG}	2.2^{B}
Winterfat *Ceratoides lanata*	10.4^{I}	11.0^{H}	1.0^{GH}
Fourwing saltbush *Atriplex canescens*[1]			
Tarbush *Flourensia cernua*			
August 1991 harvest	113.1^{D}	71.9^{DE}	1.6^{C}
October 1995 harvest	216.2^{A}	90.2^{C}	2.4^{A}
Forb species			
Leatherweed croton *Croton corymbulosus*	168.2^{B}	139.9^{A}	1.2^{D}
Grass species			
Barley straw *Hordeum spp.*	51.7^{G}	46.0^{F}	1.1^{DE}
Tobosa hay *Pleuraphis mutica*			
August 1991 harvest[1]			
October 1995 harvest[2]			
Mesa dropseed *Sporobolus flexuosus*			
July 1977 stems	70.6^{EF}	80.7^{CD}	0.9^{HI}
December 1977 stems	0.6^{I}	0.8^{H}	0.8^{I}
July 1977 leaves	132.9^{C}	124.3^{B}	1.1^{DEFG}
December 1977 leaves	0.7^{I}	0.9^{H}	0.9^{HI}
December 1977 inflorescence	0.6^{I}	0.8^{H}	0.8^{HI}
December 1977 dead material	1.2^{I}	1.5^{H}	0.8^{HI}
October 1995 whole plant[1]			
Angora goat diets			
25% winterfat + 75% barley straw	27.0^{H}	26.3^{G}	1.0^{EFG}
23% fourwing saltbush + 77% barley straw	131.3^{C}	135.6^{AB}	1.0^{FGH}
Angora goat feces			
25% winterfat + 75% barley straw	66.6^{FG}	60.9^{E}	1.1^{DEF}
23% fourwing saltbush + 77% barley straw	55.3^{FG}	46.0^{F}	1.2^{D}
Standard error	±5.47	±4.18	±0.05
Overall P value for testing material differences	0.0001	0.0001	0.0001
High value	216.2	139.9	2.4
Low value	0.6	0.8	0.8

^{ABCDEFGHIJ}Means in the same column with different superscripts differ ($\alpha = 0.05$).
[1]No green fluorescence in either replicate.
[2]Green fluorescence in only one replicate.

from the intact leaves. Chlorophyll does not exist in the cutin layer; therefore, the 20 s extraction may not have been adequate to extract chlorophyll (if chlorophyll fluorescence is responsible for the red peak).

3.4. Univariate count ratios

In 16 materials, the overall F differed ($P < 0.0001$) for red/blue, red/green and green/blue LIF count ratios indicating differences among materials (Table 3). None of the 16 materials differed in the red/green count ratio. Tarbush leaves harvested in both 1991 and 1995, leatherweed croton and the 25% winterfat + 75% barley straw diet differed in red/blue count ratio while creosotebush and tarbush leaves harvested in 1991 and 1995 differed in the green/blue count ratio (Table 3).

Count ratios changed over time and plant part within a species. Smaller red/blue, red/green and green/blue count ratios were calculated for tarbush leaves harvested in August 1991 compared to those harvested in October 1995 (Table 3). Comparing count ratios for mesa dropseed leaves and stems, the red/blue, red/green and green/blue ratios in July and December indicate differences between plant parts only during growth (Table 3). For mesa dropseed leaves, red/blue, red/green and green/blue count ratios were larger in July than in December. However, for mesa dropseed stems, the green/blue ratio was similar in July and December while the red/blue and red/green count ratios for stems were significantly larger in July than in December (Table 3). Mesa dropseed leaves, stems, floral appendages and old dead gray material harvested in December 1977 all had similar red/blue, red/green and green/blue peak count ratios.

The red/green and green/blue count ratios of diet and feces arising from the mixture of fourwing saltbush and barley straw would not be due to fourwing saltbush since it did not produce peak fluorescence in the green (Table 2). The plant material, winterfat and

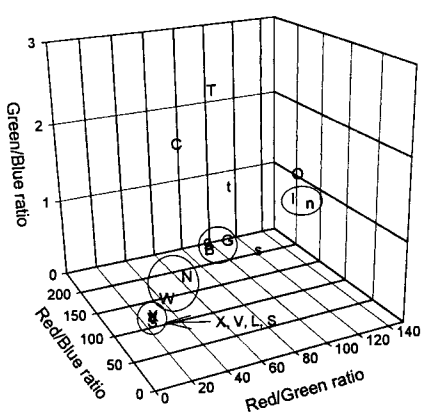

Fig. 5. A 3-D plot of mean laser-induced fluorescence (LIF) green/blue, red/blue and red/green count ratios from: barley straw = (B), creosotebush leaves = (C), croton leaves = (O), tarbush leaves harvested in 1991 and 1995, respectively = (t) and (T), goat feces composed of 23% fourwing saltbush leaves and 77% barley straw = (g), goat feces composed of 25% winterfat and 75% barley straw = (G), mesa dropseed leaf material harvested in July and December 1977, respectively = (l) and (L), mesa dropseed stem material harvested in July and December of 1977, respectively = (s) and (S), mesa dropseed floral appendages harvested in December 1977 = (V), mesa dropseed dead gray material harvested in December 1977 = (X), winterfat leaves = (W), diet composed of 23% fourwing saltbush leaves and 77% barley straw = (n), and diet composed of 25% winterfat leaves and 75% barley straw = (N). Letters not enclosed in the same circle differ ($\alpha = 0.05$).

barley straw, and the diet made by combining the two species had different red/blue, red/green and green/blue peak count ratios. Furthermore, the two plant species, the diet they composed and the feces obtained by feeding Angora goats the diet all differed in red/green peak count ratios. However, among the four materials, the red/blue and green/blue peak count ratios were not all statistically different (Table 3).

3.5. Multivariate count ratios

By combining the red/blue, red/green and green/blue peak count ratios into a multivariate analysis, means were separated on all three ratios at once (Fig. 5). Fluorescence spectra with a multiplicity of bands will be more useful for plant identification than a spectrum consisting of chlorophyll bands only (Chappelle et al., 1985). Lichtenthaler et al. (1993) found the 450/690, 450/530 and 690/735 fluorescence ratios to vary independently of one another and are complementary for determining the physiological state of terrestrial vegetation.

Using multivariate mean separation with all three count ratios, we statistically differentiated nine groupings among the 16 materials. Five materials (tarbush harvested in August 1991 and October 1995, leatherweed croton, creosotebush and mesa dropseed stems harvested in July 1977) differed from one another while the remaining 11 materials formed unique subgroups containing two or more materials (Fig. 5). The similarity among mesa dropseed material (stems, leaves, floral appendages and old dead gray material) harvested in December 1977 suggests fluorophores responsible for LIF were similar among plant parts, but differences between leaves and stems in July and September suggest fluorophores may be affected by maturity. Based on the univariate analysis, winterfat and barley straw differed in all three count ratios, while winterfat and the diet composed of 25% winterfat + 75% barley straw differed in only the red/blue and red/green count ratios (Table 3). However, in the multivariate analysis (Fig. 5), winterfat could not be distinguished from 25% winterfat + 75% barley straw probably due to a 'blending' of the spectra. With digestion of the two diets by Angora goats, the relative influence of winterfat may have been substantially reduced since winterfat and fourwing saltbush are more digestible than hay (blue grama; *Bouteloua gracilis*; Vernet, 1989). Though mesa dropseed leaves harvested in July and December of 1977 differed using all three count ratios, it is probably only a coincidence that mesa dropseed leaves harvested in July should be similar to a diet composed of 23% winterfat and 77% barley straw (Fig. 5).

4. Conclusions

Chloroform filtrate from ground pre- and post-digested plant materials exposed to 10 pulses of 355-nm laser light each having ~ 4 mJ of energy and lasting ~ 10 ns produced LIF between 387 and 788 nm. All 20 materials ranging in age from current year's growth to 18-year old mesa dropseed exhibited localized peak intensities in the blue (range 444 to 477 nm) and red (range 671 to 680 nm) regions of the spectrum while localized peak intensities occurred in the green (range 493 to 553 nm) in only 16

materials. Spectral plots were similar between replicates but visually different among materials.

Red fluorescence peaks were the most pronounced spectral feature across materials except in mesa dropseed stems, leaves and floral appendages harvested in December 1977, in which blue peak counts exceeded red peak counts. Though red peak fluorescence was narrow and distinct among most materials, amplitude of this spectral signature was quite variable. In contrast, most materials showed relatively broad blue and green peaks with fluorescence intensity (amplitude) less variable compared to that in the red.

Because laser beam intensity passing through the cuvette was not constant across materials evaluated, peak counts were not used to statistically differentiate among materials. Instead count "ratios" were used to separate LIF signatures among materials because they are statistically and spectroscopically robust. By simultaneously analyzing red/blue, red/green and green/blue count ratios in a multivariate analysis, we were able to separate 16 materials with fluorescence in the blue, green and red spectral regions into nine different groupings.

Using only variation in peak fluorescence values and calculated count ratios, it was possible to detect differences in LIF among pre- and post-digested plant materials. Detecting changes in LIF within a species over time and differences in LIF among plant parts within a species may be possible pending further research. Furthermore, one may assume additional information exists in the non-peak portion of the spectral signatures between 387 nm and 788 nm. Use of this information will require development of appropriate models and algorithms and a more complete understanding of the compounds responsible for producing the fluorescence obtained.

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