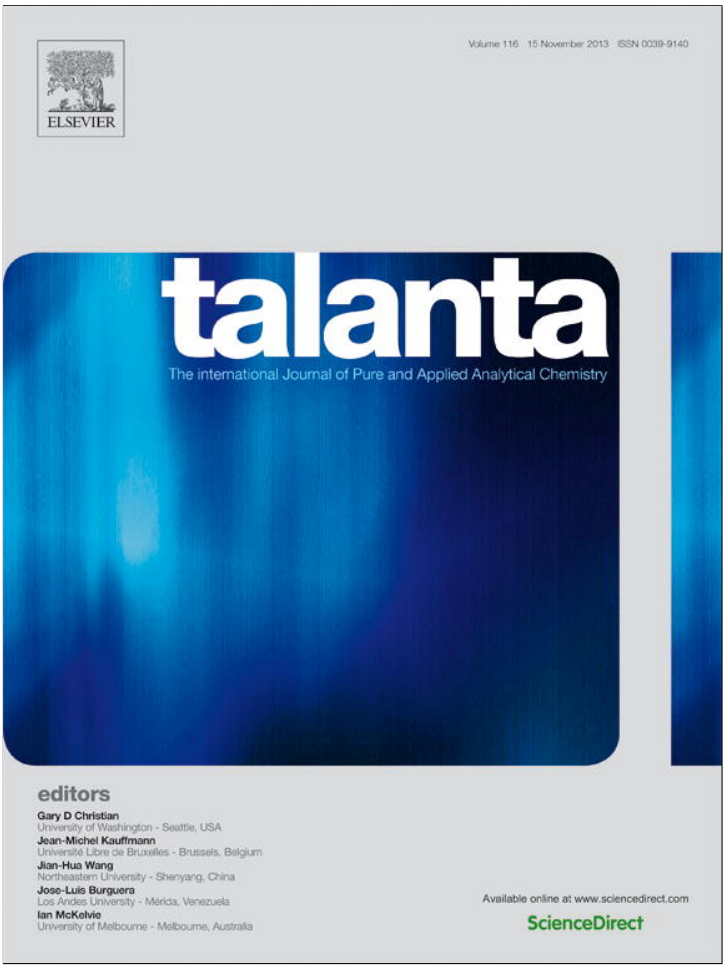

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Discrimination of grassland species and their classification in botanical families by laboratory scale NIR hyperspectral imaging: Preliminary results

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ABSTRACT

The objective of this study was to discriminate by a NIR line scan hyperspectral imaging, taxonomic plant families comprised of different grassland species. Plants were collected from semi-natural meadows of the National Apuseni Park, Apuseni Mountains, Gârda area (Romania) according to botanical families. Chemometric tools such as PLS-DA were used to discriminate distinct grassland species, and assign the different species to botanical families. Species within the *Poacea* family and other Botanical families could be distinguished (R^2=0.91 and 0.90, respectively) with greater accuracy than those species in the *Fabacea* family (R^2=0.60). A correct classification rate of 99% was obtained in the assignment of the various species to the proper family. Moreover a complete study based on wavelength selection has been performed in order to identify the chemical compound related to each botanical family and therefore to the possible toxicity of the plant. This work could be considered as a first step for the development of a complete procedure for the detection and quantification of possible toxic species in semi-natural meadows used by grazing animals.

1. Introduction

Forages are valuable for providing excellent, generally dilute sources of nutrients for ruminants, and, in general, providing forages is more sustainable and economical than other feeds. Feeding a mixture of hay forages rather than grass silage alone will generally give higher dry matter intakes resulting in higher milk production and growth, or allowing savings by reducing the amount of concentrate required to be supplemented. However, a number of plants that grow in pastures can be toxic to livestock, including cattle. In some cases, the components that make these plants toxic are still at toxic levels after being baled into hay. The best way to assure that forage is safe is to keep these plants out of the fields and pastures [1].

Toxicity or poisonings can often be avoided by proper management of animals, pastures, and hay. Regular analyses of forage plants are required in order to determine the risk of poisoning grazing livestock, or to determine if harvested forage is likely to poison animals. The risk of intoxication will change depending on the phenological stage of growth of the plant [2], if forage quantity is limited, and when animals are very thirsty or hungry [3]. Thus, invasive and toxic plants can cause economic losses to the livestock industry; often these toxic plant species are aggressive invaders and reduce optimum utilization of pasture land [4].

Up to now the analyses regarding determination of invasive and toxic plants consisted of visual observations, indicating if the toxic plants were identified directly in the field. Toxic plant determination is very important not only for pasture quality, but also for animal intake [2]. The toxicity of plants varies can vary greatly depending on the species or variety, the concentration of the toxin in different plant parts, and also the state of maturity. Animals react differently depending on the plant species, age and health status of the animal before poisoning. The sensitivity to the toxic plant and the amount ingested are important points for animal health [5].

As practical strategy for preventing animal poisoning, some general guidelines are suggested: know which plants are toxic and when they are potentially dangerous; inspect pastures to identify

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and destroy poisonous plants before initiating grazing; be sure animals are neither starved nor thirsty when put on pasture; avoid hay that might contain toxic plants; provide supplemental feed and water to animals during periods of low pasture availability.

The grassland species can contain undesirable compounds from different chemical classes: alkaloids, phenolic compounds (some toxic phenol derivatives), as well as terpenes and sterols (terpenoids, sesquiterpenes, sesquiterpene lactone, saponins) [6]. Alkaloids are any chemical compound which contains group of heterocyclic nitrogenous substances of vegetable origin [7]. The toxicity of alkaloids may be variable, from low toxicity to fatal levels, as they are sometimes lethal for animals and humans [8]. The phenolic compounds are not uniformly distributed in plants at the tissue, cellular or subcellular levels [9]. The fundamental structural elements which characterize the phenolic compounds are important determinants in the sensory and nutritional quality of plants. Phenolic compounds have beneficial effects, but they may cause some allergic reactions, or can form some mutagenic and carcinogenic metabolites [10]. Urbas [11] states that terpene compounds represent the largest and functionally most diversified natural group; they are also known as plant essential oils.

Table 1
Grassland species used in the study to test the NIR-HSI technique. These species were harvested from a semi-native meadow in Romania, Gârda area, GPS coordinates of Gheţari Research Centre: latitude: 46.500, longitude: 22.816, elevation: 1212 m.

Poa	Faba	OBF
Agrostis capillaries L.	*Lathyrus pratensis* L.	*Adonis verinalis* L.
Anthoxanthum odoratum L.	*Lotus corniculatus* L.	*Arnica montana* L.
Briza media L.	*Trifolium montanum* L.	*Clematis integriforia* L.
Cynosurus cristatus L.	*Trifolium pratense* L.	*Clematis recta* L.
Festuca pratensis L.	*Trifolium repens* L.	*Conium maculatum* L.
Festuca rubra L.		*Datura stramonium* L.
Trisetum flavescens L.		*Equisetum palustre* L.
Nardus stricta L.		*Euphorbia cyparissias* L.
Deschampsia flexuosa L.		*Hieracium aurantiacum* L.
		Hypericum perforatum L.
		Pteridium aquilinum L.
		Stellaria media L.
		Veratrum album L.

Legend: Poa, *Poaceae* family, Faba, *Fabaceae* family, OBF, other botanical families.

The toxicity of terpene compounds consist of allergic reactions at the skin or mucus levels [6]. The sterols derived from plant sources are named phytosterols [13]. They can be identified by the integral constituents in the lipid layers; the sterols are involved in the regulation of membranes [12]. The most often found phytosterols are sigmasterol, sitosterol, brassinolide, clerosterol, cycloartenol and diosgenin [13].

Near Infrared Hyperspectral Imaging (NIR-HSI) was developed for a high level and frequency of product control leading to improved food safety and quality control systems [14,15]. NIR-HSI brings a new dimension in spectroscopy: the spatial resolution. Hyperspectral images allow identification and quantification of chemical constituents within samples [16]. With this, both the spectral and the spatial information will be available.

The HSI system has been previously used in agronomy, for example Suzuki et al. [17] used a ground-based HSI system to map the grass chemical components directly in the field. Similar studies were made by Okamoto et al. [18] for weed detection and plant classification in grasslands. Initially, the plant species were classified before segmentation, and then the plant leaves and background soil were separated. For plant discrimination, the Euclidean distance achieved with segmentation achieved a 75–80% classification rate, whereas discriminant analysis gave a rate of 90% of correct classification.

Recently, the discrimination technique, linear discriminant analysis (LDA) has been used on the same ground-based HSI systems for mapping botanical composition and herbage mass in pastures [19]. The herbage mass was first measured and the plant species were then classified as perennial ryegrass, white clover, other plants and dead material, with a classification accuracy of 91.6%. This study was one of the first studies regarding botanical composition discrimination directly in the field. Dale et al. [20] performed, with a laboratory scale NIR-HSI system, the discrimination of five distinct grassland species (*Arnica montana* L., *Agrostis capillaries* L., *Hieracium aurantiacum* L., *Festuca rubra* L. and *Trifolium repens* L.). The presence in binary and ternary artificial mixtures of the various grassland species was predicted by PLS-DA model, with a correct classification rate higher than 99%.

The research objective of this paper is to use laboratory scale NIR-HSI system to discriminate different grassland species according to botanical family membership: *Poacea* family (Poa), *Fabaceae* family (Faba) and other botanical families (OBF).

Table 2
List of toxic grassland species, their identified toxic compounds and some reactions that are caused by each toxic compound group [19].

Toxic compounds	Grassland species
Alkaloids	*C. maculatum* L. produces two types of intoxication (a) CNS depression, and (b) teratogenicity. The plant contains volatile pyridine alkaloids, primarily coniine, *N*-methylconiine, and gamma-coniceine. Other pyridines as well, but of much less importance *D. stramonium* L. is a neurotoxin, causing reduced GI mobility and appetite. Contains potent anticholinergic tropane alkaloids L-hyoscyamine and scopolamine. Similar toxicity to *Atropa belladonna* L. May also contain calystegin B2 in low concentrations *V. album* L. has toxic and medicinal effects. Causes nausea, vomiting, abdominal pain, hypotension, and cardiac effects also. Contains a series of nitrogenous steroidal alkaloids termed azasteroids, veratranine, cevanines, jervanines. Cyclopamine is probably most important of the jervanines, as it is most abundant and produces fetal deformities
Phenol derivates	*H. perforatum* L. can typically cause photosensitization but it can be used medicinally also. Glands in plant surface contain hypericine (a naphthodianthrone) and pseudohypericin (all highly condensed quinones)
Terpenoids	*E. palustre* L. is neurotoxic and can cause muscle weakness, trembling and collapse. Toxins are primarily a thiaminase (inactivate thiamine). Does contain small amounts of pyridine alkaloids that are probably not involved in the toxicity *C. integriforia* L. and *C. recta* L. can produce some digestive disturbances and gastrointestinal (GI) irritation. Toxin may be protoanemonin; may also contain glycosidic derivatives of oleanolic acid terpenoids, similar to Ranunculus *P. aquilinum* L. provokes toxicity in ruminants as bone marrow depression and/or neoplasia; in horses as thiamine deficiency. The other toxicity problems (*i.e.*, carcinogenesis and hematologic effects) result from ptaquiloside, an unstable norsesquiterpene glycoside with an illudane skeleton *A. verinalis* L.—causes digestive disturbances. Contains a series of potent 23-C cardenolides: adonitoxin (a mannoside of adonitoxigenin); cymarin; and K-strophanthin. Concentrations of these glycosides are highest in leaves and flowers, may also contain other toxins such as protoanemonin *E. cyparissias* L. have irritant properties in sap. Irritation of skin, mucous membranes, conjunctiva and GI tract. Contain complex diterpenoid euphorbol esters (*e.g.*, esulones A–C, esulatins A–C); ungenols (*e.g.* miliamines). Great variation in which diterpenoids are present in these plants *S. media* L.—annual weed, is not known to be toxic but is an invasive plant for meadows. Related genera (*e.g.*, *Saponaria*) contain steroidal saponins (often githagenin) that are glycosides of pentacyclic oleananes

2. Material and methods

For this study, species used for each group are species growing in the Apuseni Mountains, more precisely in a semi-natural grassland of National Apuseni Park, Gârda area (Romania) (GPS coordinates of Gheţari Research Centre: Latitude: 46.500–Longitude: 22.816): Poa, Faba and OBF collected samples are presented in Table 1.

Regarding the grassland species chosen for OBF group, they are of special interest because many of them are toxic. In Table 2, the grassland species were grouped according to the toxic compounds they may contain: alkaloids, phenolic compounds, terpenes and sterols; further some reactions that can be caused by the toxic compounds are shown [20].

The grassland plants species were collected directly in the field after the grassland botanical composition determination. The samples were air-dried and then prepared using the protocol for NIRS analysis adapted on the scanning linear system [21].

The NIR-HSI system used was a push-broom system (SWIR Hyperspectral ImSpector N25E Burgermetrics, Riga, Latvia); the NIR-HSI analyses were performed at the Walloon Agricultural Research Centre in (CRA-W Gembloux). The wavelength range used was 1100–2400 nm with a spectral resolution of 10 nm. The images consist in lines of 209 channels of wavelength and each image is a mean of 32 scans. For image acquisition, the Hyper See program (Burgermetrics, Riga, Latvia) was used.

Once the images were random acquired on a species by species basis (over two consecutive days), a total of 750 spectra (250 spectra in 3 repetition images) were selected in order to build and validate PLS-DA (Partial Least Squares Discriminant Analysis) discrimination models under MatLab R2010a program (Mathworks, Natick, MA) to discriminate different grassland species. The 1st and 3rd repetition images (500 spectra per sample) were used to construct the calibration models (training set) and the 2nd repetition images (250 remaining spectra) as external validation (test) set.

The calibration algorithm was carried out on absorbance spectra pre-treated by normalization and 1st Savitzky–Golay derivative (window=11, polynomial=2). The values were normalized to (divide each variable by) the sum of the absolute value of all variables for the given sample. The normalization of samples returns a vector with unit area (area=1) "under the curve" [22]. The PLS-DA calibration model created was validated using "leave one out" cross validation and 12 latent variables (LVs) were chosen to build the discriminant equation. The optimal LVs depend on the specific objectives of the modelling project, and the number chosen, as addition of other LVs does not greatly improve the performance of the model [22]. Outliers were detected by using score, residual, and leverage plots, and outliers were eliminated to achieve robustness and to increase the accuracy of the estimation [23].

3. Results and discussion

In order to discriminate between Poa *vs.* Faba *vs.* OBF the PLS-DA algorithm was applied. The PLS-DA algorithm is based on the classical PLS regression algorithm and is performed to construct models to discriminate nonviable and viable classes [24].

The PLS-DA results are expressed in terms of correct classification rates for each class. These rates are grouped into the so-called confusion table as shown in Table 3 for the training set, cross validation and test set., This table shows that, for the test set, 100% of Poa, 96.2% of Faba and 99.9% of OBF samples have been respectively correctly detected as such.

The outlier elimination plays an important role in the NIR-HSI discrimination model developments [25]. The results indicated

Table 3
Confusion matrix for the *Poaceae* family *vs. Fabaceae* family *vs.* other botanical families PLS-DA model.

Calibration results (%)	Poa	Faba	OBF
Predicted Poa	100	0	0
Predicted Faba	0	100	0
Predicted OBF	0	0	100
Cross validation results (%)			
Predicted Poa	100	0.09	0
Predicted Faba	0	99.91	0
Predicted OBF	0	0	100
External validation results (%)			
Predicted Poa	100	1.20	0
Predicted Faba	0	96.20	0.09
Predicted OBF	0	2.60	99.91

Legend: Poa, *Poaceae family*, Faba, *Fabaceae* family, OBF, other botanical families.

Table 4
Calibration performance for the *Poaceae* family *vs. Fabaceae* family *vs.* other botanical families PLS-DA model.

Calibration performance	Poa	Faba	OBF
R^2 Cal	0.909	0.601	0.900
R^2 CV	0.908	0.598	0.899
R^2 Pred	0.889	0.463	0.859
RMSEC	0.148	0.210	0.157
RMSECV	0.149	0.211	0.157
RMSEP	0.158	0.241	0.186

Legend: Poa, *Poaceae* family, Faba, *Fabaceae* family, OBF, other botanical families, R^2 Cal, coefficient of determinations for calibration model, R^2 CV, coefficient of determinations for cross validation model, R^2 Pred coefficient of determinations for external validation model, RMSEC, root mean square error of calibration, RMSECV, root mean square error of cross validation, RMSEP, root mean squared error of the prediction.

that after the outliers were eliminated (2% of all spectra), the determination coefficients (R^2) of the discrimination models of Poa, Faba and OBF were 0.91, 0.60 and 0.90, respectively (Table 4); the root mean square error of calibration (RMSEC) of the three models were 0.148, 0.210 and 0.157, respectively; and root mean square error of cross validation (RMSECV) of the three models were 0.149, 0.211 and 0.157, respectively. The R^2 found for Faba was lower ($P=0.05$) in comparison with Poa and OBF, while the RMSEC and RMSECV were higher. The poor results in Faba discrimination could not be fully explained, but may be linked to the lower number of samples taken in the study (five distinct grassland species).

For the estimation of the predictive capacity of the discrimination model the external validation procedure was applied. The coefficients of determination for the calibration and cross validation compared to the prediction set were similar: R^2 Pred=0.89 for Poa, 0.46 for Faba and 0.86 for OBF. The statistics obtained for prediction efficiency of the models could be expressed by the value of the root mean squared error of the prediction (RMSEP=0.158 for Poa, 0.241 for Faba, and 0.186 for OBF).

The differences among the botanical families were assessed using PLS-DA scores plots; complete and significant ($P=0.05$) separation of Poa, Faba and OBF groups was shown (Fig. 1). When the PLS-DA model is complete, the score plot can be visualised for qualitative results of discrimination model, each data point representing one sample. In the corresponding PLS-DA score plot the

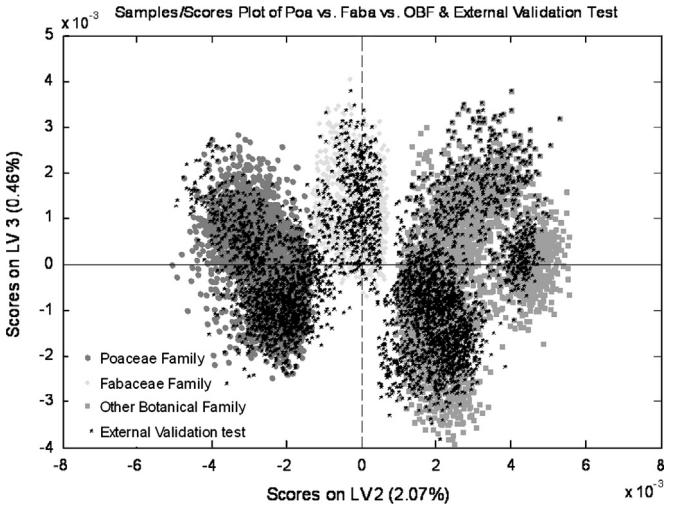

Fig. 1. PLS-DA score plot—LV2 (2.07%) *vs.* LV3 (0.46%).

Table 5
Specific bands and related vibration of *Poaceae* family and other botanical families registered in LV 2 (2.07%) loading.

Constituents	Wavelengths (nm)	Bond vibration
Poa LV 2 (−)		
Carbohydrate	1212	C–H str. second overtone
Protein	1432	N–H str. second overtone
	2050	N–H sym. str.+amide II
Fiber	1777	C–H str. first overtone
	1816	O–H str.+2 × C–O str.
Alcohols	2073	O–H str.+ O–H def.
Starch	2253	O–H str.+O–H def.
OBF LV 2(+)		
Carbohydrate	1193	C–H str. first overtone
	1356	2 × C–H str.+C–H def.
	1395	2 × C–H str.+C–H def.
	1482	O–H str. first overtone
	1614	C–H str. first overtone
	1652	C–H str. first overtone
	1696	C–H str. first overtone
	1953	C–O str. second overtone
Protein	1501	N–H str. first overtone
	1564	N–H str. first overtone
	2154	2 × amide I+amide III
	2292	N–H str.+C=O str.
Starch	1540	O–H str. first overtone (intermol. H-bond)

Legend: Poa, *Poaceae* family, OBF, other botanical families.

calibration (grey marks) and external validation (black marks) samples can be displayed together.

The score plot of the 2nd and the 3rd latent variable (LV 2 and LV 3) presented a good discrimination between the botanical groups. Because Faba contain similar crude fiber content as Poa and similar crude protein content as OBF it was not possible to characterize the Faba group. Interestingly, examining the score plot showed that the primary differences accounting for the separation between Poa and OBF was the 2nd latent variables. However, regarding the loadings of 2nd latent variables, the important bands for Poa and OBF (not shown), which correspond to chemical components are shown in Table 5.

According to the loading plot 2nd latent variables, the bands correlated with Poa group were located in the negative part (LV 2 [−]), while the bands correlated to OBF group were found in the positive part (LV 2 [+]). The important NIR absorption bands found in loading were presented and a tentative chemical interpretation was proposed on the basis of the information available in the literature [26].

The differences between Poa and OBF could be partly related to potential toxicity and secondary compounds contained within each botanical group. The specific bands registered in OBF belongs to vibration combination band of C–H stretching and deformation vibration in CH_3 (1356 nm), combination band of C–H stretching and deformation vibration in CH_2 (1395 nm) and following by combination band of O–H stretching and deformation vibration (1482 nm) have been associated to constituents as: polyphones, alkaloids, protein and volatile and non-volatile acid [27] (Fig. 2). The same combination bands have been correlated to constituents observed by Chan et al. [27] (2275 nm, 2320 nm and 2090 nm, respectively), because both present bands of combination and of hydroxyl groups. The band around 1193 nm (C–H stretching vibration) has been included in the discrimination model of both Poa and OBF groups. It is interesting to mention that Wu et al. [28] have correlated a band in the vicinity of 1197 nm (8351 cm^{-1}) to alpha-pinene alkaloid (Fig. 2).

Regarding the spectral regions close to 1564 nm LV 2 (+), 1777 nm LV 2 (−) and 2154 nm LV 2 (+), these regions provided an important contribution to the loading, and the spectral regions could be related to the combination band of N–H stretching and deformation vibration, combination band of C–H stretching, and deformation vibration and combination band of amide vibration [29] (Fig. 2). The combination bands can be attributed to the chemical structure of total tannins (2150[−] and 2318 [+]) and total phenols (2150[−], 1772[+] and 1560[−]) [27]. Roberts et al. [30] noted that the main interest in identification of tannins in *Lotus corniculatus* L. was in the vicinity of 2140 nm wavelength range, which in our case could be the 2154 nm.

Another similar study to Roberts et al. [30] was performed by Sinnaeve et al. [31] and the specific wavelengths for condensed tannins were reported at 1460 nm and 2144 nm for bands of O–H bonds and 1132, 1650, 2144, 2306 and 2350 for bands of C–H bonds. The 2154 nm, 1652 nm and 2293 nm wavelengths have been also highlighted in the discrimination PLS-DA model and are similar wavelengths to 2150 nm, 1604 nm and 2312 nm, respectively which were attributed by Goodchild et al. [29] below to condensed tannins (Fig. 2).

Phenolic compounds are omnipresent in most plant species and play an important role in resistance of plant disease [32]. The spectral bands between 8000–4000 cm^{-1} (1250–2500 nm) were mainly caused by the stretching or deformation vibration of C–H, O–H and N–H groups; these groups are abundant in phenolic acids [33]. The wavelengths close to 1100–1300 nm present an important contribution to the LV loading and are mainly related to the combination band of O–H symmetric and anti-symmetric stretching vibration, the combination band of C–H aromatic second overtones, and C–H third overtone vibration. These can be attributed to the chemical structure of phenolic compounds [34,35].

The terpenoid concentration in plants is also a component that helps the OBF and Poa separation. All terpenoids are derived by repetitive fusion of carbon units based on isoprene skeletons [36–39]. Isoprene spectra are combinations of C–H stretching and deformation vibrations from $–CH_3$ groups (1696 nm), C–H stretching and deformation vibrations from $=CH_2$ groups (1614 nm) [35] (Fig. 2). Also the combinations and overtones of more fundamental bands such as C–H stretching and deformation vibrations $–CH_3$ groups (1696 nm), C–H stretching and deformation vibrations $=CH_2$ groups (1614 nm) and C–O stretching and deformation vibrations CO_2R groups (1953 nm) can be considered as fatty acids [36] (Fig. 2).

On the basis of the relation existing between spectral fingerprint enhanced by discriminant models and chemical groups, it can be stated that the toxic constituents of plants provided a partial basis on which the discrimination between plant species and families was achieved. The OBF plants are both invasive and toxic plants, and could be discriminated from nontoxic plants.

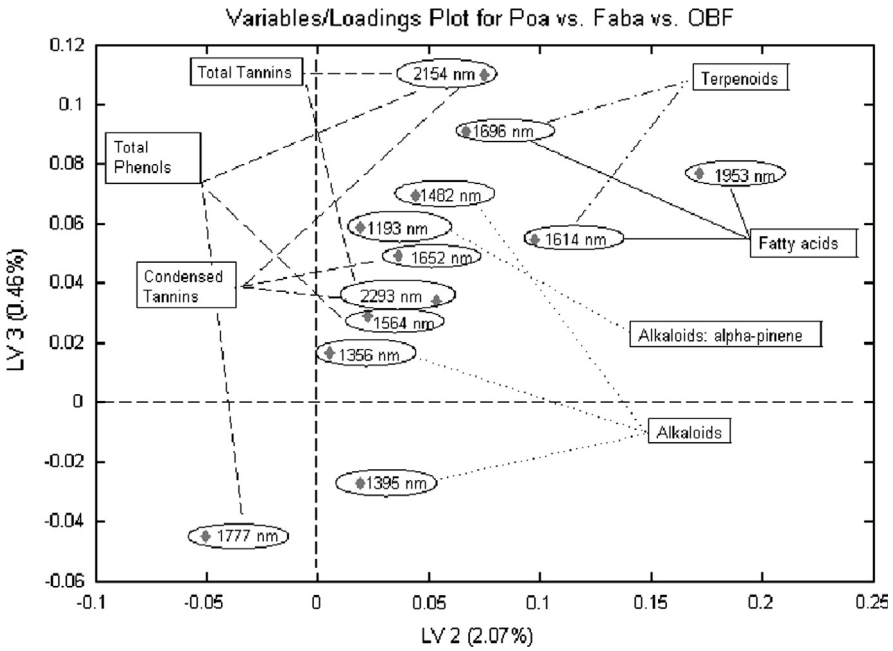

Fig. 2. Combination bands for alkaloids, terpenoids, phenolic derivates and fatty acids in the PLS-DA space determined by LV2 and LV3.

4. Conclusion

The PLS-DA model was successfully performed to discriminate the grassland species according to botanical families by NIR-HSI system. This work is a first step for the development of a procedure for detection and quantification of toxic species in forages harvested from semi-native grasslands. The NIR-HIS technique has the ability to discriminate the floristic composition of plants harvested from grazing lands. The technique could be used to determine if invasive and toxic plant species are present or not. To affirm this, the next step should chemical analysis of all the samples for all the targeted compounds, which would help to confirm the discrimination seen in the NIR images. Further studies are still necessary to refine the discrimination between Poa *vs.* Faba and Faba *vs.* OBF, respectively, as well as to demonstrate unequivocally the chemical compounds responsible for the discrimination. It is important to underline that the floristic composition of a meadow could be determined only if the spectra of each identified species are included in the databases used to calibrate the system.

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