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USING NIR AND NIR-HSI SPECTROMETRY TO STUDY FORAGE CHEMICAL AND BOTANICAL COMPOSITION

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Laura Monica Dale (2014). Using NIR spectrometry and NIR-HSI to study forage chemical and botanical composition (PhD Dissertation in English). Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liège, 134p., 15 tables, 17 figures.

Abstract

Forage quality or nutritive value is related to chemical composition, which can be determined by laboratory methods. The NIR technique in comparison with classical methods is non-destructive, non-polluting, fast and relatively inexpensive per analysis. Investigations on nutritional quality of Carpathians Apuseni Mountains (Romania) grasslands are rarely performed with NIR technique. Therefore, the objective of the thesis was to develop non-destructive methods for evaluating the quality of feed originating from the Gârda area of the Carpathians Apuseni Mountains (Romania) potentially and to similar grassland around the world. The first task was to study the potential of NIR spectroscopy for building a spectral database for forage quality based on a large collection of semi-natural grassland samples, using a 'local' calibration model built by the Walloon Agricultural Research Centre (CRA-W), in Belgium, to determine various parameters (e.g., protein, dry matter, ash, fibre, fat, aNDFom, ADF, lignin, digestibility, crude energy) from samples collected worldwide, outside Romania. The second task was to develop calibration models for an NIR-HSI system, which involved larger spectral data registration as an image. Until now, analyses to determine plant species were based on botanical composition evaluation, including visual observation, which is a subjective method involving identifying plants directly in the field. Distinguishing samples of pure grassland species can be time consuming, and it was therefore decided to build a spectral database of pure samples and then discriminate these samples into binary and ternary artificial sample mixtures. The main objective of these tasks was to identify the botanical families to which the samples belonged (Poaceae, Fabaceae and Other Botanical Families [OBF]). The focus was not on quantity monitoring, but rather on determining forage quality from stationary experiments in the grasslands. To conclude, this research has shown that it is possible to develop calibration models not only for quality assessment, but also for sample discrimination in dry powder samples. It was intended, that the mathematical models constructed and the database obtained, would be used for future research.

Laura Monica Dale. (2014). L'utilisation de la spectrométrie proche infrarouge (NIR) et NIR-HIS pour étudier la composition chimique et botanique des fourrages (Thèse de doctorat en anglais). Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège, 134p., 15 tables, 17 figures.

Résumé

La qualité du fourrage ou la valeur nutritionnelle dépend de la composition chimique, qui peut être déterminée par des méthodes de laboratoire. La technique NIR en comparaison avec les méthodes classiques est non destructive, non polluante, rapide et relativement peu coûteuse par analyse. Les études sur la qualité nutritionnelle des Carpates Monts Apuseni (Roumanie) Grasslands sont rarement réalisées avec la technique NIR. Par conséquent, l'objectif de la thèse est de développer des méthodes non destructives pour l'évaluation de la qualité des aliments provenant de la région de Garde des monts Apuseni Carpates (Roumanie) potentiellement et similaire à la prairie dans le monde. La première tâche a consisté à étudier le potentiel de la spectroscopie NIR pour la construction d'une base de données spectrale pour la qualité du fourrage basée sur une large collection d'échantillons de prairies semi-naturelles, en utilisant un modèle d'étalonnage «local» construit par le Centre wallon de Recherches agronomiques (CRA-W), en Belgique, pour déterminer différents paramètres (par exemple, teneurs en protéines, matière sèche, cendres, fibres, matières grasses, aNDFom, ADF, lignine, digestibilité, énergie brute) d'échantillons prélevés partout dans le monde, en dehors de la Roumanie. La deuxième tâche, qui impliquait l'enregistrement de larges données spectrales comme une image, était de développer des modèles d'étalonnage pour le système NIR - HSI. Jusqu'à présent, les analyses pour déterminer les espèces de plantes ont été fondées sur une évaluation de la composition botanique, y compris l'observation visuelle, qui est une méthode subjective impliquant une identification directe des plantes sur le terrain. La création d'échantillons purs de chaque espèce des prairies peut prendre beaucoup de temps, et il a donc été décidé de construire une base de données spectrales d'échantillons purs et puis de discriminer ces échantillons dans des mélanges artificiels binaires et ternaires. L'objectif principal de ces tâches était d'identifier les familles botaniques à laquelle les échantillons appartenaient (Poaceae, Fabaceae et d'autres familles botaniques [ABF]). L'accent a été mis non pas sur la quantification, mais plutôt sur la détermination de la qualité du fourrage à partir d'expériences stationnaires dans les prairies. Pour conclure, cette étude a montré qu'il est possible de développer des modèles d'étalonnage pour l'évaluation de la qualité, mais aussi pour la discrimination des échantillons de poudre sèche. Il était prévu que les modèles mathématiques construits et la base de données obtenue soient utilisés pour de futures recherches.

Laura Monica Dale. (2014). Utilizarea spectrofotometriei infraroșu apropiat (NIR) și a imagierii hiperspectrale infraroșu apropiat (NIR-HIS) pentru studierea compoziției chimice și botanice a furajelor (Teza de doctorat în limba engleză). Gembloux, Belgique, Gembloux Agro-Bio Tech, Universitatea din Liège, 134p., 15 tabele, 17 figuri.

Rezumat

Calitatea furajului sau valoarea nutritivă depinde de compoziția chimică, care pot fi determinate prin metode de laborator. Prin comparația tehnicii NIR cu metodele convenționale, aceasta este nedestructivă, nepoluantă, rapidă și relativ ieftină per analiză efectuată. Studii privind calitatea nutritivă a pajiștilor Carpații–Occidentali, Munții Apuseni (România), sunt rareori efectuate cu tehnica NIR. Prin urmare, obiectivul acestei lucrări este de a dezvolta metode nedestructive pentru evaluarea calității alimentelor din zona Garda de Sus, Munții Apuseni. Prima activitate a fost de a investiga potențialul tehnicii NIR pentru construirea unei baze de date spectrale privind calitatea furajului, bazându-se pe o colecție mare de probe de pajiști semi-naturale și folosind un model de calibrare „local“, construit de către Centrul Valon de Cercetări Agricole (CRA-W), Belgia. cu ajutorul acestui model se poate determina diverși parametri (de exemplu, proteina, substanța uscată, cenușă, grasimi, fibre, aNDFom, ADF, lignina, digestibilitatea sau energia brută). Modelul „local“ conține probe colectate din întreaga lume, mai puțin din România. A doua activitate, a fost de a dezvolta modele de calibrare pentru NIR-HSI, aparat care a implicat înregistrarea datelor spectrale sub forma de imagine. Până în prezent, analizele pentru determinarea speciilor de plante s-au bazat pe o evaluare a compoziției botanice, inclusiv observarea vizuală, o metodă subiectivă care implică identificarea directă a plantelor pe teren. Crearea de probe pure din fiecare specie de furaj de pe pajiștile semi-naturale poate dura o lungă perioadă de timp. Prin urmare, într-o prima etapă s-a decis să se construiască o bază de date spectrală pentru probe pure de furaj, iar apoi evidențierea acestor probe în amestecuri artificiale binare și ternare pentru validarea acestei tehnici. Obiectivul principal al acestei lucrări a fost de a identifica familia botanică din care fac parte probele (de exemplu: Poaceae, Fabaceae și alte familii botanice [ABF]). Accentul nu a fost pe cuantificarea probelor, ci mai degrabă pe determinarea calității furajului din experimente staționare a pajiștilor montane semi-naturale. În concluzie, acest studiu arată că este posibil să se dezvolte modele de calibrare pentru evaluarea calității, dar și pentru evidențierea probelor pure sub forma de pulbere. Este de așteptat că modelele matematice construite și rezultate să fie utilizate în cercetările viitoare.

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The thesis presents the results of my research, but from my point of view the most important result of this work is that the NIR spectroscopy technique is now being increasingly used in Romania. I hope that in the future it will become widely used in both industry and scientific research.

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Abbreviations

ADF, acid detergent fibre	OMD: in vitro organic matter digestibility
aNDFom: neutral detergent fibre	PCA: principal component analysis
ANN: artificial neural networks	PCR: principal component regression
AVIRIS: airborne visible/infrared imaging spectrometer	PHyTIS: portable hyperspectral tunable imaging system
Bias: errors derived from predicted-actual component values	PLS: partial least squares regression
CF: crude fibre	PLSDA: partial least squares discriminant analysis
CP: crude protein	Poa: Poaceae family
EE: crude fat	R^2 : R^2 Cal: coefficient of determinations for calibration model
Faba: Fabaceae family	R^2 CV: coefficient of determinations for cross validation model
FPA: focal plane array;	R^2 Pred: coefficient of determinations for external validation model
FT-NIR: Fourier transform near infrared	RMSEC: root mean square error of calibration
HPLC: high performance liquid chromatography	RMSECV: root mean square error of cross validation
HSI: hyperspectral imaging	RMSEP: root mean square error of the prediction
IR-HSI: infrared hyperspectral imaging spectroscopy	RPD: residual predictive deviation
IR: infrared spectroscopy	SD: standard deviation
ISL-HSI: instrumentation and sensing lab hyperspectral imaging system	SEC: standard error of calibration
LD: lorentzian distribution function	SECV: standard error of cross validation
LDA: linear discriminant analysis	SEM: standard error of the mean of ANOVA test
Lignin(sa): acid detergent lignin	SEP: SEP(C): standard error of prediction, standard error of prediction corrected for bias
M1: control	SID: spectral information and divergence
M2: 50N 25P ₂ O ₅ 25K ₂ O	SVM: support vector machines
M3: 100N 50 P ₂ O ₅ 50K ₂ O	T1: semi-natural meadow (control)
M4: 150N 75 P ₂ O ₅ 75K ₂ O	T2: 20 t/ha cow and horse manure
MLR: multi-linear regression	T3: 10 t/ha cow and horse manure + 50N 25 P ₂ O ₅ 25K ₂ O
MS: mass spectroscopy	T4: 100N 50 P ₂ O ₅ 50K ₂ O
MSI: multispectral imaging	T5: 10 t/ha cow and horse manure + 100N 50 P ₂ O ₅ 50K ₂ O
NIR spectroscopy: near infrared spectroscopy	USI: ultraspectral imaging
NIR-HSI: near infrared hyperspectral imaging system	VIS: visible
O1: control	WBSF: warner bratzler shear force
O2: 10 t/ha cow and horse manure	WIR-HSI: short wavelength infrared hyperspectral imaging system
O3: 20 t/ha cow and horse manure	
O4: 30 t/ha cow and horse manure	
OBF: other botanical families	

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Introduction



... to the research

Context

Grassland utilization has undergone considerable change in Europe in the past two decades. Some grasslands have been completely abandoned in terms of agricultural use and are now exposed to various changes. Forage production from temporary rather than permanent grassland has become an integral part of agricultural land management. This has contributed to the economic and social viability of farming and to the sustainable development of rural areas in an increasingly urbanized world.

Animal breeders are aware of the importance of forage quality because poor nutrition can limit the productivity of ruminants selected for genetic purposes (Ulyatt and Waghorn 1993). Until 1990, obtaining quantitative information from large numbers of samples was often difficult and unaffordable, and numerous studies conducted on forage quality under various production systems and using various methods highlighted the need for new technologies. With the advent of near infrared (NIR) spectrometry, a fast and non-destructive method that can be used for forage quality determination, many of the difficulties were overcome. The technology has proved able to provide reliable and affordable assessments of feed composition (crude protein, lipids, ash, structural fibre, soluble carbohydrates), digestibility and metabolisable energy (Joffre et al., 1992; De Boever et al., 1995).

NIR spectrometry was first used in Romania in 2000 when Vidican et al. (2000) presented their study on using it to determine forage dry-matter digestibility. Although the technology has been used in some European Union (EU) countries since 1980, in most EU countries it is still at an early stage of development. Research on forage composition and quality has been initiated by several laboratories with the aim of:

- *developing non-destructive methods*
- *assessing the potential of using NIR spectrometry to build a spectral database*
- *building NIR spectrometry calibration and validation models for various parameters, such as protein, dry matter, ash, fibre, fat, neutral detergent fibre (aNDFom) acid detergent fibre (ADF), lignin, digestibility and crude energy*
- *developing NIR spectrometry instruments to an advanced level, by using them in combination with hyperspectral imaging (NIR-HSI) to handle up to 76,800 spectra/5min (InGaAs camera), as image collection*
- *developing NIR-HSI methods for grassland species discrimination and forage quality calibration and validation*

NIR spectrometry is a subjective method that requires different types of classical analysis for calibration (Carlier et al., 1998). A permanent validation of the calibration models is necessary to ensure their flexibility and robustness (Shenk et al., 1989).

In recent years, NIR spectrometry has been used to evaluate plant selection, plant nutritive value, silage and crop information, feed ration balance and quality (Decruyenaere et al., 2009). Whatever its NIR applications, this technology is only as good as the calibration data derived from wet chemistry analysis that are available. Once adequate calibration models are in place,

NIR spectrometry can provide rapid, inexpensive and accurate assessments of feed composition in a large number of samples (Weiss and Pell, 2007).

Over the past 30 years, research at the Walloon Agricultural Research Centre (CRA-W) in Gembloux, Belgium in wet chemistry and on calibration models has led to the development of ‘global’ and ‘local’ calibration models (Sinnaeve et al., 1994). The ‘global’ models make predictions based on an entire population of samples (Tran et al., 2010), whereas the ‘local’ models make predictions based on a small homogenous group of samples selected from a larger group of samples (Fernández Pierna and Dardenne, 2006). In both cases, wet chemistry analyses need to be conducted on the samples. ‘Local’ NIR models could be developed using different mountain grassland samples from throughout Europe or even worldwide.

There has long been much research interest in the semi-natural grasslands of Romania’s Apuseni Mountains in terms of fertilization regimes, botanical composition, biodiversity and medicinal plants. One of the most important endemic species of this area, because of its medicinal properties, is *Arnica montana* L. To date, only classical methods have been used to determine the rich botanical composition of these grasslands because NIR spectrometry has not been developed for application in the area.

Objective

The objective of this thesis was to develop innovative methods for characterizing: (i) the forage quality of grassland samples from the Gârda area of the Apuseni Mountains, using NIR spectrometry for an external validation of CRA-W’s ‘local’ calibration model; and (ii) the endemic species and grassland botanical composition, using NIR-HSI techniques. This required the development of new mathematical models to calibrate the NIR spectrometry and NIR-HSI systems for these semi-natural grasslands. The focus was not on quantity monitoring, but rather on determining forage quality from stationary experiments in the grasslands. It was intended that the mathematical models constructed and the database obtained would be used for future research.

Outline

A compilation of published and accepted articles, the manuscript is divided into eight chapters. They open with a description of the importance of grassland and forage quality (Chapter I), two reviews of the literature on the use of chemometric tools in NIR spectroscopy and NIR-HSI (Chapter II) and on HSI applications in agriculture and agro-food product quality and safety control (Chapter III), and a description of the research strategy (Chapter IV). Chapter V (Article 1) describes the fertilization effects on the chemical composition and *in vitro* organic matter digestibility of the Apuseni grasslands studied, as predicted by NIR spectrometry. Two studies were carried out on plant species discrimination. The first study (Chapter VI, Article 2)

focused on the discrimination of some species (*Festuca rubra* L., *Trifolium repens* L., *Agrostis capillaris* L., *Hieracium aurantiacum* L. and *Arnica montana* L.); the model consisted of 1000 spectra of each species. The second study (Chapter VII, Article 3) focused on the discrimination of botanical families (*Poaceae*, *Fabaceae* and other botanical families) and also looked a species' toxicity discrimination. The final chapter (Chapter VIII) presents the general conclusions drawn from the research and looks at future prospects in the use of NIR-HSI systems in agriculture.

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Chapter 1



*Importance of
Grassland and Forage Quality*

Importance of Grassland and Forage Quality

Context

Natural grasslands are an important factor in the existence and balance of the global ecosystem, particularly in terms of sustainable development. There are numerous definitions of the word 'grassland', many with specific connotations. The United Nations Education, Scientific and Cultural Organization (UNESCO) gives a fairly narrow definition: "land covered with herbaceous plants with less than 10% tree and shrub cover" (Suttie et al., 2005). Globally, agriculture accounts for 37.59% of utilizable land, and this area is divided into arable land, permanent grassland and permanent cropland. Permanent grassland occupies the highest percentage (68.64%) of the agricultural area (FAO STAT, 2012) and is among the largest ecosystems in the world. In Europe, permanent grassland accounts for 16.15% of the used land area and 35.85% of the agricultural area (FAO STAT, 2012). Grasslands are an important element in the European landscape; some of the semi-natural grasslands are rich in flora and fauna, often sustain much loved iconic species that put on spectacular floral displays and offer a refuge to disappearing species (Knowles, 2011). They are part of a rural landscape that is much appreciated for its aesthetic qualities by visitors and locals alike. This landscape is now threatened to some extent by mechanization and agricultural intensification, and some species no longer occur widely (Knowles, 2011).

From an agricultural perspective, grasslands make an important contribution and perform important functions, their main role being a source of feed for herbivores and ruminants, although this means that some grassland area face the threat of intensification. From an ecological perspective, their main role is to maintain biodiversity (Rotar et al., 2010). Grassland systems contribute to soil conservation, provide medicinal plants and attractive landscapes, mitigate greenhouse gas emission and play an important role in soil carbon storage (Păcurar et al., 2012). They are net sinks for atmospheric CO₂, storing 500-1,200 kg C/ha/year, depending on the prevailing management practices (e.g., stocking rate, utilization mode, fertilization) (Dollé et al., 2011).

The study area

Our studies were conducted in Romania in central Europe. The country lies halfway between the North Pole and Equator, and halfway between the Atlantic Ocean and Ural Mountains (Figure 1). Its total area is 23,839,100 ha. Permanent grasslands account for 19.72% of this area, and for 33.46% of Romania's agricultural area (FAO STAT, 2012). As in much of central Europe, most grasslands in Romania are in the mountainous areas, with only a low percentage of grassland in the lowland areas used for agriculture. There is a great diversity of species and ecosystems, especially in the Carpathian Mountains, where the natural grasslands comprise a wide variety of plant species.



Figure 1. Map of Europe, showing Romania



Figure 2. The Garda de Sus area in Alba County, Romania

The Apuseni Mountains grasslands that were the subject of this study lie at an altitude of 1,130m asl. They are on a glacial plateau, Poiana Călineasa, near the village of Gârda de Sus in the north-west of Alba County (Figure 2).

Grasslands have a variety of functions, including providing good quality forage for livestock, creating habitats for flora and fauna, contributing to the attractiveness of the landscape (Vîntu et al., 2004), providing livelihoods for livestock rearers and herders (Suitte et al., 2005) and being important reserves of biodiversity. Tourism has increased their importance in recent years, attracting people to the designated hiking trails in some grassland areas (Gârda, 2010), to sites of religious significance in others (Suitte et al., 2005) and to mountain resorts such as Stâna de Vale and Arieșeni.

Romania is the only European country with five biogeographically distinct regions: continental, pannonic, steppic, pontic and alpine (Page et al., 2012). The village of Gârda de Sus has a typical mountain climate, generally wet and cold on the high peaks with a gradual change in temperature the lower down one goes. In 2010, the average annual temperature in Gârda de Sus was 4°C, with frosty winters (-5°C), warm summers (15.6°C) and an average precipitation of 997.4 mm (Gârda, 2010).

Romania's soil consists of the most of the soil types common in Europe, with underlying volcanic, sedimentary and metamorphic rocks. The Apuseni Mountains are rich in minerals (including gold and copper) and caves. There are more than 400 caves in the area and some of the most spectacular limestone cave systems in the world (Gligan, 2006). These caves contain remains of the extinct bears (*Ursus speleus*) and prehistoric humans, huge colonies of bats, subterranean lakes, striking calcareous formations and giant earthworms living in the guano-covered cave floors. There are also ice caves in the mountains, among the largest being the Scărișoara, Focul Viu and Vârtopeice caves (Gligan, 2006).

The relief in the studied area consists of a set of peaks with different orientations, where grasslands are currently the most used land, as well as some arable land near the houses. The region's morphological relief type is a plateau, with advanced flattening a major feature in some areas, and closed basins developing, especially in the southern half of the territory.

The experiment

Our experimental fields are located on red clay soil. The processes involved in the soil's formation account for this red colour. The main characteristics of the soil are: soil pH 5.34, total N 0.212%, mobile P 3 ppm and K 25 ppm.

Grasslands in the Gârda de Sus area are rich in species of many colours, which adds to the beauty of the landscape (Figure 3). The colour combinations in the natural grasslands on the plains and higher up in the mountains are symptomatic of a healthy environment (Rotar et al., 2010). The biodiversity hot spots in the Apuseni Natural Park are protected as special conservation areas (Figure 4).

The communal pastures tend to be typical *Arnica* habitats, the dominant species being *Nardus stricta* L., *Agrostis capillaris* L. and *Festuca rubra* L., along with other herbaceous species. Fertilization in these *Arnica* habitats has reduced species richness and increased grassland productivity. The composition of these grasslands will undergo further change if farmers give

up mowing but maintain moderate grazing, which will encourage woody plants and invasive species to spread into the area (Păcurar et al., 2005; Păcurar, 2005).



Figure 3. Mountain grassland landscape in the Gârda de Sus area



Figure 4. Plant biodiversity in the Gârda de Sus area

The semi-natural grasslands ecosystems typical of the Apuseni Mountains are now very rare in much of Europe, but in Romania they are still common in mountainous areas. The flagship species of the grasslands in the Apuseni Natural Park is *Arnica montana* L., an important medicinal plant, its inflorescence used for both pharmaceutical and cosmetic purposes. Endemic to Europe, this species occurs in Austria, Belgium, Denmark, Germany, Hungary, Italy, Luxemburg, the Netherlands, Norway, Poland, Romania, Russia and Sweden (Michler et al., 2004), with half of these endemic populations dependant on grasslands for their development and survival. Only in Romania and southern Spain, however, is this species still

harvested (Michler et al., 2004). In the Apuseni Natural Park, *Arnica montana* L. is now being threatened by land abandonment, system intensification and inappropriate harvesting.

Romania's human population has declined since 1989, the sharpest fall occurring in the rural areas, including the Apuseni Mountains. Most of the population in this area live at the base of the mountains in villages, hamlets or isolated households. The semi-natural grasslands of the mountains provide summer grazing and winter fodder (hay, silage) for cattle, sheep, goats, horses and other livestock. People build small huts in the grasslands to live in during the hay-making period in summer (Gârda, 2010). Extensive farming is practised up to an altitude of 1,200m asl. Mechanized agriculture was impossible before 2005 because of the altitude and cold climate (Păcurar, 2005), but some farmers are now using it; many others are prevented from doing so, however, because of cost and still use manual and animal management practices. In the mountainous area, only potatoes can be cultivated with confidence; barley and wheat are grown, but farmers run the risk of these cereals not ripening before the autumn hoarfrost.

The spruce woods of the Apuseni Mountains are a rich source of timber for the local people known as the Moți, who are skilled in timber processing and wood carving. This craft began with the selection of wood for staves and has been passed down the generations. Among the traditional handicrafts associated with the Moti are tulnics, a type of alpine horn instrument (Gârda, 2010).

Several studies of the botanical composition of the semi-natural grasslands of the Apuseni Mountains, and the effect of fertilization on them, have been conducted (Reif et al., 2005). The rich biodiversity of the Platoul Ghețari–Poiana Calineasă area was studied by Reif et al. (2005), who identified 491 plant species. In 2005, Păcurar et al. (2005), as part of an overall study of the biodiversity of the mountain grasslands, initiated a series of studies on forage quality in the area, one the few such studies to have been done here.

Forage quality

Forage quality is important in determining nutritionally balanced feed rations, developing forage inventories, evaluating forage management practices (growing, harvesting and storage) and pricing and marketing forage (Dale et al., 2012). The term 'quality' can be defined in terms of whether or not something is 'suitable for use' (Juran J. M.) or as the 'set of characteristics of an entity that gives it the ability to meet the needs expressed or implied' (ISO 8402:1995). 'Suitable for use' is probably the most comprehensive definition, emphasizing both product use and the needs of product users. For users, quality is determined by desires and expectations.

An assessment of 'forage quality' on the basis of whether or not it is suitable for use needs to take into account of nutritive value. The main factors determining nutritive value are nutrient concentration and digestibility (Collins and Fritz, 2003). Forage contains a mixture of chemical, physical and structural characteristics that determine the quality of semi-natural grasslands and the nutrients that are accessible to the target animals (Fales and Fritz, 2007). Forage nutritional chemistry focuses on the composition and structure of forage in animal feed. Ruminant productivity is affected by forage quality: the higher the quality of the forage, the greater the productivity and economic return (Fales and Fritz, 2007).

As far as animals are concerned, the most important forage components are carbohydrates, proteins, lipids, vitamins and minerals. There are also other significant components in forage cell walls that are important in ruminant nutrition. Forage quality changes with plant maturity and is affected by a wide range of factors, including genotypic variation, plant species, morphology, chemical and physical composition, climate, environment, soils and management practices (Fales and Fritz, 2007). Regular analyses of forage are needed in order to determine whether or not it meets the daily nutritional requirements of the target animals. Although considerable advances have been made in understanding forage quality, more work is needed on breeding forage species with improved digestibility and a reduction in characteristics that are not important in animal nutrition. Improved quality based on an appropriate nutrient balance leads to animal weight gain and an increase in milk production.

Forage quality analyses in the laboratory

In order to assess the forage quality of semi-natural grasslands, a set of general analyses need to be conducted on botanical composition, palatability, nutrients and digestibility. The botanical composition of forage is an important factor in forage quality (Bush et al., 2007). Sensory evaluation can be used as a method of analysis in order to identify toxic plants in the field and eliminate unsuitable forage.

The nutritional value of forage is a function of its chemical composition, which can be determined in the laboratory. In order to assess most of the quality parameters, such as nutrients and digestibility, wet chemistry methods are applied. Several techniques based on direct analyses using solvents, acidic or basic solutions, or other chemicals, can be applied to assess the various parameters of feed samples (Dale et al., 2012). In a typical feed analysis, measurements of quality parameters such as crude protein and cellulose can be used to assess the nutritive value. Wet chemistry methods are the most accurate ones for determining the nutrient value of forage and feed, which is needed for quality assurance purposes and for development of new techniques. Wet chemistry is also the reference method for all spectrometric methods currently being used as discussed later.

Laboratory analyses based on wet chemistry are the official analytical methods endorsed by the Association of Official Agricultural Chemists (AOAC) and used to assess nutrient concentration in forage. The main characteristics measured are: dry matter, pH, titratable acidity, organic acids, soluble N, ammonia, carbohydrate fractions (aNDFom, ADF, acid detergent lignin [lignin (sa)], hemicelluloses, cellulose, starch, sugars, non-structural carbohydrates), nutrient availability (enzymatic digestion, *in vitro* gas production) and nitrogen fractions (total nitrogen, soluble nitrogen, neutral detergent insoluble nitrogen [NDIN], acid detergent insoluble nitrogen [ADIN]). The wet chemistry analytical methods used are: the Kjeldhal method for crude protein (CP) (AOAC, 1990); method 942.05 for crude ash (AOAC, 1990); method 920.39 for ether extract (EE) (AOAC, 1990); method 73/46/CEE – Fibre Cap for crude fibre (CF) (FOSS, DK); the Van Soest–Fibre Cap method for aNDFom, ADF and lignin (sa) (FOSS, DK); and the De Boever method for *in vitro* organic matter digestibility (OMD) (De Boever et al., 1986).

In agriculture, the most common method used to analyse plant cell walls is the detergent fibre procedure (Van Soest, 1964), which determines the aNDFom as a proportion of plant-derived feeds of limited digestibility and the ADF and lignin (sa) using gravimetric measurements after

extraction (Hatfield et al., 2007). The *in vitro* OMD of forage is an important characteristic which indicates how well feed would potentially be absorbed by the animal when passing through the digestive tract (Dale et al., 2012).

Forage quality analyses using animals

In order for ruminants to receive their daily nutrient requirement, it is important for farmers to ensure they are providing the optimum quantity and quality of feed. Numerous field methods using animals are available for assessing forage quality. These methods seek to define the ability of forage to support ruminant welfare and productivity (Bush et al., 2007) and are used mainly to determine nutritive or net energy value (Blaxter, 1989). The live weight gain (LWG) method, a simple gravimetric measurement, is used for developing standardize feeding systems. The LWG measurement focuses on the difference between an animal's starting and final weight and can show significant variation from one animal to another, as well as from pasture to pasture (Mott and Lucas, 1953 cited by Cochran et al., 2007). Another method used to assess forage quality is the body condition score, a subjective measurement of the amount of metabolisable energy stored in a live animal (Bastin, 2013). Widely used in beef and dairy cattle studies, this method is based on giving each animal a score (in dairy cattle the score scale is 1-5, and in beef cattle it is 1-9) (Bastin, 2013).

Animals are also used in *in vivo* experiments. Diet selection is used to measure nutrient digestion and balance in grazing animals, as well as the nutritional characteristics and botanical composition of the grazed forage (Cochran et al., 2007). Assessing the nutritive value the milk yield requires is determinate in conjunction with digestion trials or other evaluations based on intense sampling (Cochran et al., 2007). The most accurate method for determining diet selection is to collect samples from the oesophageal or ruminal fistulae (cannulas) of animals (Cochran and Galyean, 1994). Similar procedures are used to assess forage intake, a valuable index of forage quality that can also be affected by animal characteristics. Used to calculate forage consumption, this method is based on investigating faecal output and diet digestibility (Cochran et al., 2007).

In assessing total tract digestion, for example, faecal energy loss is calculated from total energy consumed, where digestible energy is the total dry matter intake multiplied by the gross energy concentration in the diet. The digestion of a given nutrient can be measured by calculating the difference (in kg) between nutrient consumed and nutrient excreted. Another method used to assess forage digestibility is based on mixing indigestible intestinal markers in animal feed; the markers are recovered in the faeces (Cochran et al., 2007). *In situ* studies are conducted to identify which dietary components are absent. One example is the method known as the 'mobile nylon bag technique' (Stern et al., 1997). It involves using porous bags of feed samples, which are introduced at the start of the gastrointestinal tract and removed at the distal end of the segment.

Several methods have been developed to assess animal's balance when feed a forage and nitrogen balance. Determining energy balance is based on quantifying total energy input and all sources of energy loss, and focuses on such factors as total intake energy (gross energy), faecal energy, urinary energy, heat energy and gaseous energy (Cochran and Galyean, 1994). Gaseous energy can be determined by measuring the methane gas emission that ruminants produce (Cochran et al., 2007). Establishing a good nitrogen balance is based on capturing as

much nitrogen as possible in an animal's tissue or products. In order to assess nitrogen balance, it is necessary to determine all the sources of nitrogen loss (e.g., faecal, urinary, scurf and eructated ammonia) and to subtract this loss from the nitrogen input in diets (Cochran et al., 2007). One of the metabolic indicators of high nitrogen concentration is the amount of urea in an animal's blood, milk and urine.

All these analyses using animals provide specific information that can be used to establish indicators of nutritive value and to monitor nutritive intake (Cochran et al., 2007).

Forage quality analysis using NIR spectrometry

There has been increasing emphasis in the past 25 years on using non-destructive methods for determining forage and feed quality. Many new analytical tools based on spectroscopic technologies have been developed since the pioneering work undertaken by Karl Norris (Hart et al., 1962). In the agricultural sector, near infrared (NIR) spectroscopy is a well-known technology for acquiring chemical information from samples. The discovery of NIR energy was made by Frederick William Herschel in the 19th century, but the first industrial application was in 1950 (Workman, 2005). In the 1980s, NIR spectroscopy began to focus more on chemical analysis and in the past 20 years it has become a powerful tool for scientific research in agriculture and the food industry (Dale et al., 2012).

An NIR spectrum can be generated when a sample is introduced into an NIR spectrometer and a reflectance or transmittance of light in the NIR region is measured (Weiss and Pell, 2007). NIR is a spectroscopic method based on a combination of fundamental overtones and molecular vibrations that are excited to higher energy levels (Romanach, 2011).

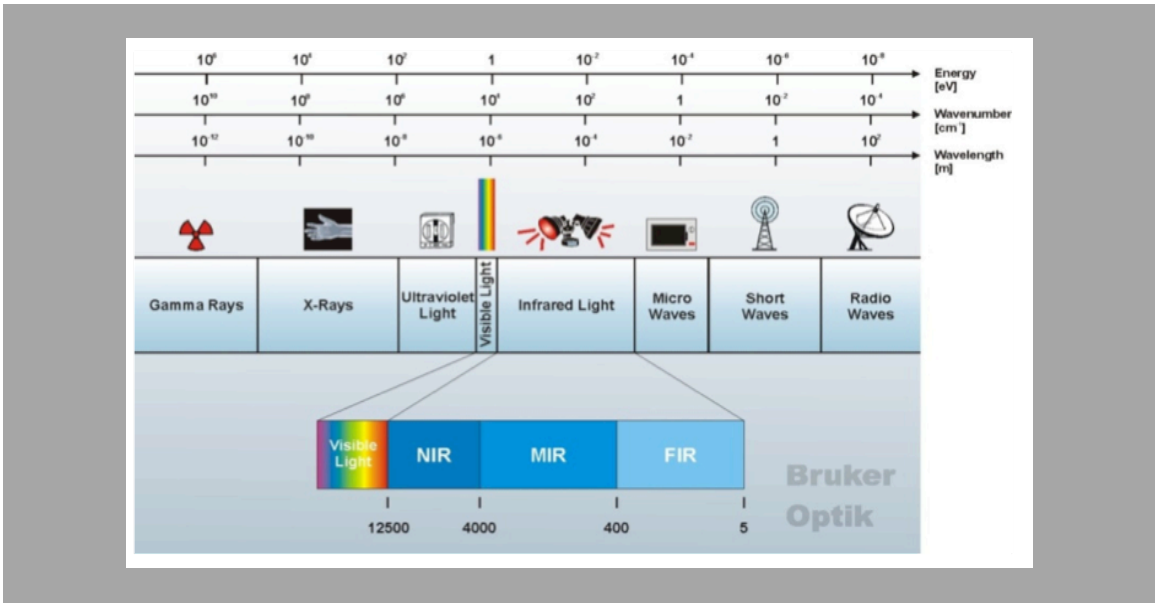


Figure 5. Reference connotation for the molecular vibrations (BRUKER OPTIK)

Absorbance intensity decreases with an increase in overtones and the combination bands are registered at lower energies than the overtones. NIR overtones are derived from the fundamental bands, whereas the combination bands are the sum of two or three fundamental bands (Romanach, 2011).

The correlation between structure and spectrum provides a base for a cause-and-effect relationship between the spectra (instrument response) and reference data (analyses), with the aim of providing a scientific basis for several multiple choices on the strength of infrared spectroscopy (Workman, 2005). The infrared spectral regions of the electromagnetic spectrum extend from 780 to 100,000nm ($12,821\text{ cm}^{-1}$ - 5 cm^{-1}) and are classified into near infrared (NIR), middle infrared (MIR) and far infrared (FIR) (Manley et al., 2008). The NIR regions occur between 780 and 2500nm ($12,821\text{ cm}^{-1}$ - $4,000\text{ cm}^{-1}$) (Figure 5).

Despite the development of the NIR technique, wet chemistry analysis is still considered the 'gold standard' for feed testing (Weiss and Pell, 2007). Many certified feed analysis laboratories have the capacity to conduct wet chemistry analyses or NIR analyses, or both. Certified feed analysis laboratories around the world use complex mathematical and statistical methods for predicting quality in all types of forages (Weiss and Pell, 2007).

NIR spectroscopy produces both qualitative and quantitative analyses. In order to develop a calibration model, a set of samples and a calibration algorithm for multivariable analysis are essential for NIR spectrometry. The calibration application usually means using a sophisticated chemometrics technique to extract information from data (spectra of measurements) derived from NIR spectral patterns in response to the chemical or physical properties of the samples used for calibration (Workman, 2005; Mark and Campbell, 2008). In order to perform multivariable calibration, the models need a valid analytical relationship between X (instrument response [spectrum]) and Y (reference analyse [wet chemistry]) (Workman, 2005). Using statistical probabilities, this allows researchers to confirm if X and Y are linked (Workman, 2005). The accuracy of the NIR method for assessing forage quality can be determined by the differences between NIR and reference methods that have a lower sampling error (Weiss and Pell, 2007). The ability to assess feed quality, especially in terms of useful energy content and the amount of feed an animal will take in voluntarily, is fundamental to any feed assessment. This also highlights the need for successful calibration models and for external validation to demonstrate that the model is robust (Walshaw et al., 1998).

In Europe, the NIR technique was first used by Biston et al. (1989), who focused on rapeseed moisture, protein and oil determination. This study showed that the technique could be adapted for a variety of plant breeding purposes, such as improving rapeseed quality, as well as in oil factories (Biston et al., 1989). Over the past decade, the NIR technique has become widely used as an analytical method in many areas (Mark and Campbell, 2008). Roberts et al. (2003) provided an extensive review of the uses of NIR technology in agriculture. The review describes using NIR successfully to predict the nutritive value of forage and hay. For studying grassland and meadow hay, Vazquez de Aldana (1996) developed a useful calibration model to assess ash content. Ash content reveals the minerals available and unavailable to animals, which is an important parameter in determining feed quality. NIR spectroscopy has also been used to predict moisture and protein content in wheat (Garnsworthy et al., 2000), to determine the chemical composition, *in vitro* OMD and *in vitro* gas production in feed and forage (Lavrencic et al., 2001), to estimate protein and net energy content in maize at various stages of development (Volkers et al., 2003), to assess the mineralized nitrogen (PMN) and nitrogen fractions in particulate organic matter (PSOM-N) (Moron and Cozzolino, 2004), to determine soil organic carbon concentration in meadows (Van Waes et al., 2005) and to investigate

aNDFom, ADF, lignin (sa), ash, CP, total phenol and tannin content, metabolisable energy, *in vitro* OMD and short chain fatty acids in sheep feed (Mahipala et al., 2009).

The development of a successful and accurate calibration model requires a database that covers the full diversity of field conditions (Decruyenaere et al., 2009). Currently, the emphasis is increasingly on *in vivo* OMD and the OM voluntary intake calibration models for forage (Decruyenaere et al., 2009) and on feed chemical composition and ingredients (Fernández Pierna et al., 2010).

The first agricultural research work in Romania to use the NIR technique, for assessing meadow and forage hay, was conducted by Vidican et al. (2000). Their study described the theory and principles of NIR spectroscopy within the Romanian context. NIR spectroscopy devices first appeared in the country's universities and research institutions in early 2008, although the concept dated back to the 19th century and had long been applied in industry and agriculture elsewhere, initially for flour analysis (Hart, et al., 1962). Several research efforts in Romania using the technique were soon being implemented, focusing on an assessment of total CP content in feed samples collected from a permanent meadow (Hărmănescu and Moisuc, 2009), maize quality and fertilization trials (Rotar et al., 2009), alfalfa quality and fertilization trials (Vidican et al., 2011) and the fatty acids profile of grassland forage (Hărmănescu, 2012).

Advantages and disadvantages of laboratory, animal-based and NIR spectrometry analyses of forage quality

Over the past decade, many researchers have noted the advantages and disadvantages of laboratory, animal-based and NIR spectrometry analyses (Table 1). When spectroscopic methods are used to determine the chemical composition of feed, it is still necessary to use laboratory and animal-based analytical methods as reference methods. The works carried out by Shenk, Westerhaus, Barton, Marten, Martin, Murray, Dardenne and several other researchers led to the wide use of the NIR technique in agriculture and the food industry, and scientists now accept it as a valid analytical technique (Workman, 2005). The accuracy of spectrometric methods needs to be assessed in comparison with the results derived from laboratory analyses of the performance of animal feed (Weiss and Pell, 2007). With the advent of new methodologies and a better understanding of the factors that limit animal performance, the chemical characterization of forage will continue to be an important field of study (Cherney, 2000).

Table 1. Advantages and disadvantages of laboratory, animal-based and NIR spectrometry analyses of forage quality

Advantages and Disadvantages	Laboratory analyses	Animal-based analyses	NIR spectrometry analyses
Advantages	<p>greater objectivity and accuracy of results (O'Neill, 2006); increasingly visible through simplification and standardization (Olteanu et al., 2005);</p> <p>less expensive and less time-consuming, does not require a fistulated animal as inoculum donor, allows experimental conditions to be maintained more precisely as <i>in vivo</i> trials (Getachew et al., 1998);</p> <p>employs prescribed procedures to ensure validity and readability (O'Neill, 2006);</p> <p>reproducibility, repeatability and comparable analyses can be made (O'Neill, 2006);</p> <p>not influenced by researchers personal bias (O'Neill, 2006);</p>	<p>animal welfare, small sample size needed, allows faecal analyses to be conducted to assess diet (Williams, 2000);</p> <p>enables kinetics of fermentation to be measured (Williams, 2000);</p> <p>offers benefits in terms of ethical considerations, no need for sophisticated devices (Johnson, 1966);</p> <p>errors of measurements removed due to residual washing, filtration and weighing (van der Baan, 2008);</p>	<p>fast, clean, easy, non-polluting, non-invasive, non-destructive, requires minimal sample preparation and no chemical supplies, (Vazquez de Aldana, 1996; Manley et al., 2008);</p> <p>enables multiple analyses to be performed in one operation, uses selection of wavelengths and mathematical treatments for equation calibration (Rotar and Carlier, 2011);</p> <p>multivariate and environmentally friendly analysis (Engelsen et al., 2001)</p> <p>can be applied to analysis both online and in the field (Osborne, 2000);</p>
Disadvantages	<p>laborious, time-consuming, generally expensive and destructive (Tilley and Terry, 1963; Jones and Hayward, 1975);</p> <p>limited results, univariate (Getachew et al., 1998), structural bias and false representation of the subject (O'Neill, 2006), does not provide information on the kinetics of forage digestion (Getachew et al., 1998);</p> <p>influence of processing methods on feed nutritive value cannot be evaluated (Krishnamoorthy et al., 1995);</p> <p>environmentally harmful (Engelsen et al., 2001);</p>	<p>expensive, time consuming, technically challenging (Corchan et al., 2007);</p> <p>large number of animals necessary to implement body condition score method (Bastin, 2013);</p> <p>gas production technique have limitation regarding health and behaviour (Williams, 2000);</p> <p>increases amount of handling required, adding to animal stress (van der Baan, 2008);</p>	<p>requires well-trained staff to develop new calibration model, sensitive to the signal, need to maintain calibration kit (Rotar and Carlier, 2011);</p> <p>high cost of device, particle size can influence the spectrum (Rotar and Carlier, 2011).</p>

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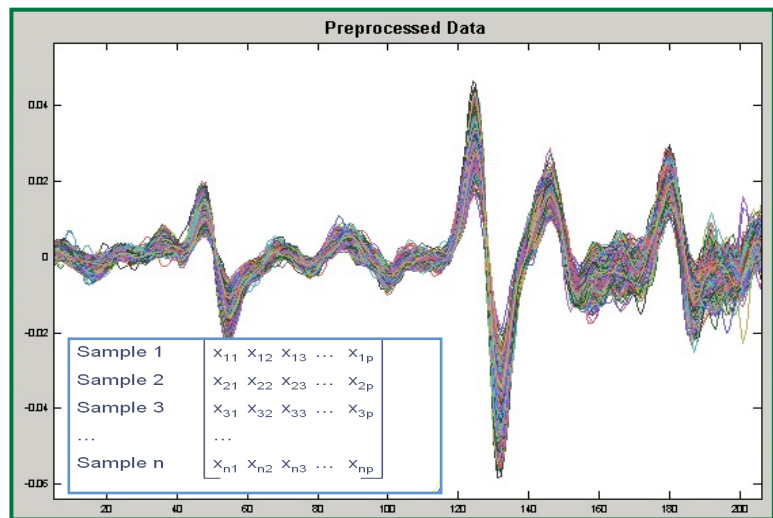
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Chapter 2



Review of the literature - 1

Chemometric tools for NIRS and NIR Hyperspectral Imaging

Review I — DALE *et al.* (2012)

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Running head: Chemometric tools for NIRS and NIR Hyperspectral Imaging

Abstract

Many new analytical tools based on spectroscopic technologies are currently being developed. Near infrared (NIR) spectroscopy is a well-known technology now being used in the agricultural sector to acquire chemical information from samples. NIR has many advantages: it is easy to use, provides fast and simultaneous analysis of several components, is non-polluting, non-invasive and non-destructive, and can be used both online and in the field. Recently, NIR spectroscopy has been combined with imaging technologies, creating the near infrared hyperspectral imaging (NIR-HSI) system. This technology provides spectral and spatial information from an object simultaneously. The main differences between

NIR-HSI and NIR spectroscopy is that many spectra can be recorded simultaneously from a large area of an object with NIR-HSI, whereas with NIR spectroscopy only one spectrum from a small area can be recorded for analysis. Both technologies are described here, with special emphasis on the main spectrum and image analysis methods. Several qualitative and quantitative applications of NIRS and NIR-HSI in agricultural products are outlined. Developments in NIRS and NIR-HSI will benefit agriculture greatly by providing high quality and safe agricultural products, better plant and grain selection techniques and improved compound feed production.

Keywords

NIRS, NIR-HSI, non-destructive methods, chemometric tools, agricultural applications

Introduction

In the agricultural sector, near infrared (NIR) spectroscopy is a well-known technology for acquiring chemical information from plant samples (Rodríguez-Otero, et al., 1997). Using this technology, C-H, N-H and O-H bonds are induced to vibrate to higher energy levels (Romanach, 2011). These vibrations appear in different overtones and combination bands and provide information on the relationship between the static and molecular vibration properties of a material (Miller, 2001). With its low sensitivity, NIR spectroscopy can detect different functional groups in the overtones and combination bands (Burns and Margoshes, 1992). The frequency of a combination band is the sum of the different vibration frequencies (Miller, 2001). This principle is used to identify and quantify components. The use of NIR spectroscopy enables the reflectance spectra of opaque milled or intact materials to be acquired. A typical NIR spectrum can be considered as the spectral signature or fingerprint of a material. NIR spectroscopy is a useful technique for analyzing important components in agricultural samples (e.g., chemical composition, microorganism detection and quantification) with minimum sample preparation.

NIR spectroscopy has been used in agriculture since the 1960s, when Karl Norris and his co-workers used it to study soybean moisture (Hart et al., 1962). In recent years, NIR technologies have been developed combining NIR systems and a microscope to create NIR microscopy (NIRM) (Baeten et al., 2012). NIR has also been combined with imaging technologies to create the NIR hyperspectral imaging (NIR-HSI) system (Fernández Pierna et al., 2004; Fernández Pierna et al., 2012). For many researchers, 'hyperspectral' is a new concept. 'Hyper' means 'many', 'spectroscopy' relates to 'seeing' and 'spectrometry' to 'measuring'; therefore, hyperspectral in this context can be defined as the simultaneous measurement of many wavelength bands (ElMasry and Sun, 2010). The NIR-HSI system provides spectral and spatial information about an object; this forms a three-dimensional 'hypercube' that contains the object's physical and chemical information. The images provide enough information to identify and distinguish spectrally unique materials. There are various types of NIR-HSI scans: point (staring) scans, push-broom (line) scans and plane (global) scans (Fernández Pierna et al., 2009). Until the late 1990s, only experts in remote sensing spectral images had access to hyperspectral systems or the software tools to explore such images. In the past decade, though, hyperspectral image analysis has become one of the fastest growing and most powerful technologies in remote sensing (Kavitha et al., 2012).

Objective

The objectives of this paper are to describe the advantages and disadvantages of NIR spectroscopy and NIR-HSI and to demonstrate how a spectrum or image can be analyzed. It concludes with a list of NIR spectroscopy and NIR-HSI applications in agriculture.

Advantages and disadvantages of spectroscopic techniques

Major advantages of spectroscopic techniques include: ease of use; repeatability and reproducibility; reasonable start-up costs; non-polluting, non-invasive and non-destructive; and the ability to be applied both online and in the field. With NIR-HSI, many spectra can be recorded simultaneously from one sample, as opposed to obtaining a unique, average spectrum, as is the case when using classical NIR spectroscopy (Figure 1). Hyperspectral images provide more spectral and spatial information than NIR technology does. One spectral image regroups numerous spectra, so that each pixel of the spectral image corresponds to one spectrum of the target sample. The spectrum or spectral signature can be used to characterize, classify or even identify any given material (Shaw and Manolakis, 2002).

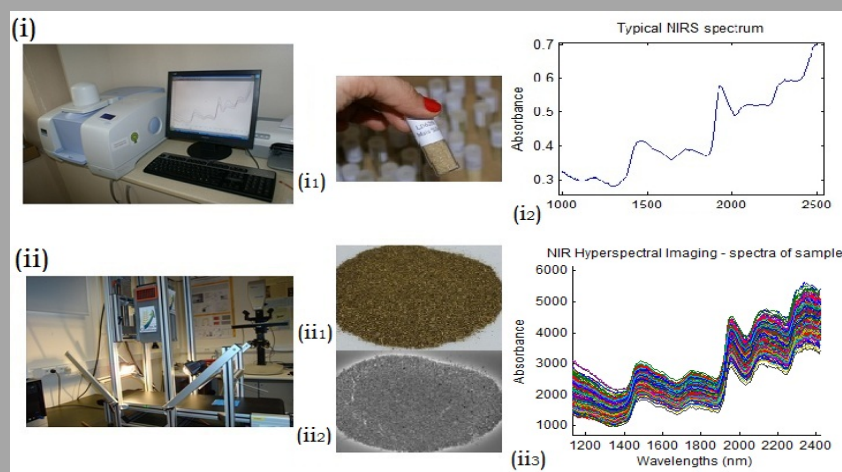


Figure 1. Acquisition of spectrum/spectra by (i) a conventional NIRS system and (ii) a laboratory-level NIR-HSI system (Source CRA-W, Gembloux, Belgium)

Legend: (i) NIR system (Forage Crops Lab, USAMV Cluj, Romania); (i₁) sample support for NIR system; (i₂) typical spectrum of an NIR system; (ii) laboratory-level NIR-HSI system (source CRA-W, Gembloux, Belgium); (ii₁) photograph of sample; (ii₂) hyperspectral image of sample; (ii₃) typical spectra obtained using a laboratory-level NIR-HSI system.

The disadvantages of NIR spectroscopy and NIR-HSI include: relatively high cost of instruments (but this could be offset by the reduction in analyses costs); fast hardware speed required; calibration models for standardization required; possible presence of pixels (spectrum) in the image that lack any chemical information (e.g., bad, dead, noise, or blinking or drifting pixels) (Chang, 2000; ElMasry and Sun, 2010).

Spectra analyses

Qualitative and quantitative analyses using NIR spectroscopy usually require the application of calibration algorithms based on physico-chemical measurements. In order to obtain efficient qualitative and quantitative information from data derived from NIR spectroscopy and NIR-HSI instrumentation, chemometric tools are required (Roggo et al., 2005). Chemometrics is the science of extracting and interpreting relevant chemical information from data using mathematical and statistical procedures (Massart et al., 1988) and it correlates quality parameters or physical properties with analytical instrument data. Since 1974, chemometrics has been the subject of an increasing number of published papers. The discipline is closely related to statistics, models, chemistry and data, and mastering it requires skills in all these areas (Wold, 1995). In order to study, understand and interpret experiments, data need to be connected and modeling approaches need to be simpler to interpret and better at providing predictions (Wold, 1995).

Building a calibration model starts with spectra pre-processing treatment. After collecting the spectra, it is necessary to carry out a pre-treatment procedure to remove high- or low-frequency interferences. Types of pre-processing treatments include: polynomial baseline correction, Savitzky-Golay derivative, Standard Normal Variate (SNV), mean centering and unit variance normalization (Gowen et al., 2007; ElMasry and Sun, 2010).

The most common pre-processing treatments used for this thesis were SNV transformation, where the slope variation caused by scatter and variation in particle size was removed from the spectra (Candolfi et al., 1999), and derivative conversion, where irrelevant baseline signals from samples were removed by taking the derivative of the measured responses with regard to the variable number (index) or other relevant axis levels (e.g., wavelength, wavenumbers) (Wise et al., 2006). The first derivative was generally used to remove any offset from the sample and de-emphasize the low-frequency signals (Wise et al., 2006), whereas the second derivative accentuated the high-frequency signals, thus enhancing selectivity (Wise et al., 2006).

After spectra pre-processing treatment, the calibration algorithm can be applied for classification and quantification. In order to analyze data, many multivariate analytical tools are used, including principal component analysis (PCA), principal component regression (PCR), multi-linear regression (MLR), partial least squares regression (PLS), modified partial least squares regression (MPLS), partial least squares discriminant analysis (PLS-DA), linear discriminant analysis (LDA), support vector machines (SVM), artificial neural networks (ANN), soft independent modeling of class analogy (SIMCA) baseline shift (BLS) and spectral information and divergence (SID) (Chang, 2000; Fernández Pierna et al., 2006; Gowen et al., 2007; Gómez-Sanchis et al., 2012).

The most common mathematical algorithms used for this review were PCA, for data compression and information extraction (Wise et al., 2006); PLS, for establishing a linear link between two matrices, the spectral 'X' data and the reference 'Y' values (Roggo et al., 2005), in order to identify factors for capture variance and obtain correlation (Wise et al., 2006); and PLS-DA, for discriminating between classes (McGoverin et al., 2011). The spectral pre-processing treatments and calibration algorithms have been extensively reviewed in the literature (Dolmatova et al., 1997; Roggo et al., 2005; Fernández Pierna et al., 2006; Wise et al., 2006; ElMasry and Sun, 2010; Yao and Lewis, 2010; Fernández Pierna et al., 2011).

The PCA algorithm uses projections to extract, from large numbers of variables, a smaller number of new variables representing higher sample variability (Jørgensen, 2000). This addresses overfitting problems through spectral data reduction, and useful information is acquired (Osborne et al., 1993). The new variables, principal components or eigenvalues are a linear combination of original spectral data, containing information from the entire spectrum (Jørgensen, 2000). Using PCA, the new variables are fitted into the data space, with the direction determined by maximum variability. If the second new variable is uncorrelated with the first one, the orthogonal axis is chosen and the operation continues until 99% of variation is assimilated and the new variable can be justified by a sufficient amount of variation (Jørgensen, 2000). If the component have more variable, a higher loading of the variable on the principal component is registered. The loadings can be interpreted as correlations between the variables and the components, and the score can be the principal component value on the axis, where the object has been projected (Jørgensen, 2000).

The PLS algorithm was created as a way of assessing the structural relationships among blocks of variables (Wold, 1975) and has been applied in marketing and econometrics (Tenenhaus et al., 2005). In science and technology, one of the most common data-analytical problems has been to create a PLS model using a set of predictor 'X' variables with several dependent variable 'Y' responses (Wold et al., 2001). Wold et al. (2001) noted that in chemistry PLS could be used to relate the 'Y' properties of chemical samples to their chemical composition 'X', or the 'Y' quality and quantity of manufactured products to the conditions of the manufacturing process 'X', or the chemical properties, reactivity or biological activity of a set of 'Y' molecules to their chemical structure 'X'. The ability of PLS to analyze the response profiles could be extended in various directions, such as non-linear modeling, hierarchical modeling when the variables are numerous, and multi-way data (Wold et al., 2001).

The PLS-DA algorithm is useful for tasks classification and is based on the PLS model, having a category-dependent variable (Serrano-Cinca and Guiterrez-Nieto, 2011). In defining groups of individuals, PLS-DA caters only for one-response variables. In chemistry, this algorithm can be used in various ways, such as sample classification depending on origin in space or time, molecule classification depending on properties, or reactions classification depending on mechanism (Wold et al., 2004). PLS-DA is not affected by multicollinearity because of its orthogonal components (Serrano-Cinca and Guiterrez-Nieto, 2011.) and can be used as a standard chemometric tool (Barker and Rayens, 2003; Westerhuis et al., 2008).

NIR calibration model performances can be characterized by standard error of calibration (SEC) or standard error of cross validation (SECV) (Wise et al., 2006). When it is necessary to know if the model fits the calibration data, the root-mean-square error of calibration (RMSEC) is calculated by the equation:

$$\text{RMSEC} = \sqrt{\frac{\sum_{i=1}^n (\hat{y}_i - y_i)^2}{n}}$$

Where: \hat{y}_i = values of the predicted variable when all samples are included in the model formation; y_i = the value of the property of interest; and n = number of calibration samples.

Another common technique for selecting a significant number of factors involves the calculation of cross-validation by the model, as a training set of data, individual spectra or a class of spectra are removed from the model and tested against the calculated model (Cremers and Radziemski, 2013). Cross-validation involves selecting the type and number of splits and iterations to be made; the samples are left out in contiguous blocks and the starting position of the first block shifts randomly through the data, which helps reduce the likelihood of over-fitting (Wise et al., 2006). Over all the repetitions of the cross-validation procedure, a prediction error (root mean square error of cross-validation, RMSECV) can be obtained. In other words, RMSECV is a measure of a model's capacity for predicting samples not used in the calibration model (Wise et al., 2006). The exact RMSECV value depends not only on the number of factors used, but also on how the cross-validation test sets were formed. RMSECV can be calculated exactly as RMSEC, but y_i is the prediction for samples not included in the model formulation, or the sum of squares prediction errors related to a number of factors taken into account by the model. When cross-validation is selected, the RMSECV and sum of squares prediction errors are measured, indicating how a model built using all the calibration data will perform when applied to new data.

In order to assess calibration model performance, an independent set of samples is used to obtain the root-mean-square error of prediction (RMSEP) and the standard error of prediction (SEP) (Wise et al., 2006). These data are used to describe the NIR analytical error when analysing samples of unknown quantitative composition (Hartmann and Buning-Pfaue, 1998). When the calibration model is validated using a validation sample set, the RMSEP is obtained by the equation:

$$\text{RMSEP} = \sqrt{\frac{\sum(\hat{y}_i - y_i)^2}{n}}$$

where \hat{y}_i is a set of reference values and y_i is a set of predictions for n samples in a validation set.

The best choice for measuring calibration merit is the SEP, which estimates what a typical difference between prediction and reference values is likely to be when a calibration model is used (Davies and Fearn, 2006).

Agricultural applications of NIR spectroscopy and NIR-HSI

The first studies based on using the NIR spectroscopy were published between 1930 and 1940. By 1990, more than 1,000 articles on studies using this technique had been published (Burns and Margoshes, 1992). In 2012 alone, more than 1,000 articles on the agricultural applications of NIR spectroscopy were published (Google Scholar, consulted on 10.05.2012).

Table 1. Summary of measurement mode, product type, wavelength region and classification algorithm used in papers published on NIR spectroscopy and NIR-HSI applications in agriculture and agro-industry

NIR spectroscopy applications				NIR-HSI applications			
Products	Wave length (nm)	Model	Reference	Products	Wave length (nm)	Model	Reference
Forage quality: - ash content - digestibility - protein content	1,100- 2,500	MLR MLR PLS	Vazquez de Aldana et al., 1996; Vidican et al., 2000; Hărmanescu and Moisuc, 2009	Forage quality : - protein content - digestibility	1100- 2500	PLSDA	Dale et al., 2012
Compound feedstuffs	400- 2,500	MPLS	Pérez-Marín et al., 2004	Compound feed and impurities	1100- 2500	PCA SVM	Fernández Pierna et al., 2006 and 2012
Wheat quality: - protein content	1,100- 2,500	PCA SIMCA	Gatius et al., 2004	Barley, wheat and sorghum grains - moisture content	1000- 2500	PCA PLSDA	McGovern et al., 2011
Cereals: - modifications made by filamentous fungi and yeasts - damage caused by <i>Fusarium culmorum</i>	1,000- 2,500 570- 1,100	Review PCA	Santos et al., 2010 Pettersson and Aberg, 2003	Detection of <i>Fusarium</i> - in maize - in wheat	720- 940 400- 1000	ANN PCA	Firrao et al., 2010; Bauriegel et al., 2011
Detection of ergot bodies in wheat kernels	1,100- 2,400	Fisher coef. value	Vermeulen et al., 2009	Detection of ergot bodies in wheat kernels	900- 1700	Fisher coef. value	Vermeulen et al., 2009
Hardness in diverse corn germplasm	1,000- 2,500	BLS	Hoffman et al., 2010	Glassy and floury endosperm in maize	1000- 2498	PLSDA PCA	Williams et al., 2009
Alphamylase activity: - in malted barley - in wheat kernels	400- 2,500 1,235- 2,450	PLS MLR PCA PLS	Tarr et al., 2012 Xing et al., 2011	Alphamylase activity: - in wheat kernels	1,235- 2,450	PCA PLS	Xing et al., 2011
Potato constituents	1,100- 2,500	PCA MPLS	Hartmann and Buning-Pfaue, 1998	Detection of hollow heart in potatoes	900- 1,700	SVM	Dacal-Nieto et al., 2011
Detection of castor bean meal in flour-containing products	1,000- 2,500	MPLS	Rodríguez-Saona et al., 2000	Detection of meat-and-bone meal (MBM) in compound feed	960- 1,662	SVM	Fernández Pierna et al., 2004
Detection and identification of bacteria in an isolated system	750- 2,500	PCA PLSDA SIMCA	Alexandrakis et al., 2008	Beet cyst nematodes Microorganism in spinach (<i>E. coli</i>)	1,100- 2,400 400- 1,000	SVM PCA ANN	Vermeulen et al., 2011; Fernández Pierna et al., 2012 Siripatrawan et al., 2011

NIR was first used in agriculture to determine moisture in soybean (Norris et al., 1976). The HSI remote sensing system was initially used to detect and map vegetation and minerals. The first case study to use NIR-HSI focused on detecting meat-and-bone meal (MBM) in compound feed (Fernández Pierna et al., 2004). Both NIR spectroscopy and NIR-HSI are now used for a wide variety of agricultural and agro-industrial purposes, from large scale to the microscopic level. Table 1 lists some of the applications that have been developed in agriculture using NIR spectroscopy and NIR-HSI systems, in order to illustrate application diversity in animal nutrition, plant protection, and food and feed quality and safety.

Conclusions

This review has shown that NIR spectroscopy and the emerging NIR-HSI method are reliable, non-destructive and rapid techniques for predicting quantitative and qualitative chemical and physical properties. In order to extract the maximum amount of information, however, these techniques need to use chemometric tools, pre-processing treatments and calibration model assessments.

Low-cost NIR-HSI systems will be needed if the technique is to be used more widely in the future, including an NIR-HIS system that can easily identify optimal wavelengths/wavebands, depending on the application. More robust calibration and validation models are therefore necessary in order to develop a model that adequately represents the data. Good model reliability will encourage more widespread online and in-field use of this technology (via, for example, small portable instruments) in agriculture and could therefore contribute to improving agricultural productivity and reducing the cost of process monitoring and product inspection.

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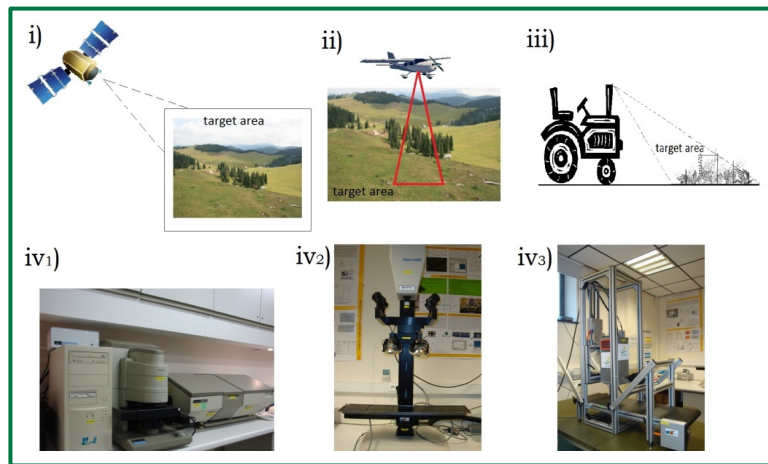
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Chapter 3



Review of the literature - 2

Hyperspectral imaging applications in agriculture and agro-food product quality and safety control: A review

Review II — DALE *et al.* (2013)
Applied Spectroscopy Reviews, 48 (2) (2013) 142-159.

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Running head: Hyperspectral imaging applications

Abstract

In this review, various applications of Near Infrared Hyperspectral Imaging (NIR-HSI) in agriculture and in the quality control of agro-food products are presented. NIR-HSI is an emerging technique that combines classical NIR spectroscopy and imaging techniques in order to simultaneously obtain spectral and spatial information from a field or a sample. The technique is non-destructive, non-polluting, fast and relatively inexpensive per

analysis. Currently, its applications in agriculture range from vegetation mapping, crop disease, stress and yield detection to component identification in plants and impurity detection. There is growing interest in HSI for the safety and quality assessment of agro-food products. The applications have been classified from the level of satellite images to the macroscopic, if not, molecular level.

Keyword

NIR spectroscopy, satellite system, airborne system, ground-base HSI, NIR-HSI, agriculture, agro-food industry.

Introduction

Agricultural materials are characterized by different chemical composition and internal physical structures, which mean that, when working with Near Infrared (NIR) spectroscopy, they reflect, scatter, absorb and/or emit electromagnetic energy in different ways at specific wavelengths. These differences are characterized by a typical NIR spectrum that can be considered as the spectral signature or spectral fingerprint of the material. NIR spectroscopy has been a well-known technology in the agricultural sector since the works conducted by Norris and co-workers in the 1960s (Hart et al., 1962). It is a non-destructive method of analysis based on the diffuse reflectance of samples, widely used for rapidly determining the concentration of nutrients and feeding value in dried and fresh crop materials (Murray, 1986; Biston et al., 1989; Dardenne et al., 1992; Vidican et al., 2000; Decruyenaere et al., 2009; Rotar et al., 2009), food and feed quality control (Chen et al., 2002; Salguero-Chaparro et al., 2012) and food safety (Fernández-Ibanez et al., 2009; Santos et al., 2010; Pei et al., 2011).

In recent years, new methods based on NIR spectroscopy technology have been developed, mainly based on a combination of techniques. NIR technology has been combined with a microscope to create NIR microscopy (NIRM) (Yang et al., 2011) and with imaging techniques to create Hyperspectral Imaging (HSI) methodologies. ElMasry and Sun (2010) defined HSI as a “combination of the strong and weak points of visible/near-infrared (VIS/NIR) spectroscopic techniques and vision techniques”. The images provide enough information to identify and distinguish spectra as individual material. A hyperspectral image offers the potential to extract more accurate and detailed information than that obtained when working with classical NIR technology. Burger and Geladi (2006) noted that NIR-HSI gives us a natural expansion of conventional spectroscopy as well as the spatial position information of the acquired spectra. They therefore concluded that NIR-HSI was a hybridization of spectroscopy and spatial imaging. With the decrease in wavelength resolution, the NIR-HSI spectrum is compensated by increasing the spectral quality obtained from thousands of spectra. NIR-HSI processing algorithms, known as Multivariate Imaging Analysis (MIA), are still being developed. Hyperspectral images have become one of the most common research methods in the exploration and monitoring technologies used in many areas of work (ElMasry and Sun, 2010). A NIR spectroscopy system provides one spectrum per measurement, whereas hyperspectral images provide thousands of spectra from one sample. In one measurement, each pixel corresponds to one spectrum. The image taken by NIR-HSI also gives a spectral signature of the sample that is unique and can be used to characterize and identify any given material (Shaw and Manolakis, 2002).

The initial uses of these hyperspectral images were for remote sensing applications (detection and mapping) because of the reflection characteristics of the spectra. HSI was used for the detection of military vehicles hidden in vegetation and for some NASA work (Shippert, 2003). It was also successfully used by geologists to identify and simultaneously analyse more than 150 materials, including minerals, vegetation, ice and snow (Clark and Swayze, 1995). Hyperspectral images give a good enough spectral range and spatial resolution for mapping and studying the Earth's surface, and for characterizing soil properties, including moisture, organic matter content and salinity (Ben-Dor et al., 2002). NIR-HSI is useful in the paper industry for sorting different types of materials (e.g., pulp, paper, cardboard, newspaper, and bleached and unbleached fibres) (Tatzer et al., 2005). HSI is very useful in the art domain, not only for artwork conservation (Fischer and Kakoulli, 2006), but also for identifying pigments in paintings and palimpsests (Rapantzikos and Balas, 2005). The technique has been used in the

medical sector to determine various diseases such as peripheral vascular diseases (Kellicut et al., 2004), and in ophthalmology and oncology (Harvey et al., 2002), immunohistochemistry (Levenson et al., 2002), latent fingerprinting and age assessment of bruises in forensic medicine (Bartic et al., 2002), and face recognition in biomedicine and human identification (Pan et al., 2003). Recent studies have demonstrated that HSI can be used in cancer diagnosis (Siddiqi et al., 2008). The HSI technique is a promising method for evaluating cervical cytologic preparations and, if used in conjunction with slide scanners, can assist in the automated detection of pre-cancerous and cancerous cells. NIR-HSI can be used for mapping compound distribution, testing active pharmaceutical ingredients and excipients for formulation uniformity, identifying contamination on tablet surfaces and detecting dissolution problems in solid pharmaceutical forms (Roggo et al., 2005; Gendrin et al., 2007).

Objective

The objective of this paper is to describe HSI and its principles, and to compare the advantages and disadvantages of NIR-HSI with the classical NIR spectroscopy technique. The applications described range from landscape or field scale, such as mapping a canopy or highlighting vegetation stress, to the more restricted microscopic, if not molecular, level, such as detecting contaminants or quantifying biochemical parameters.

Principles and instrumentation

The field of spectral imaging can be divided into three domains: multispectral imaging (MSI), hyperspectral imaging (HSI) and ultraspectral imaging (USI). MSI is a system where the image acquired has few separated wavelengths. In HSI, the image is acquired with an abundance of continuous wavelengths. USI is when one image is acquired with a low spatial resolution of several pixels (i.e., the system used has a very fine spectral resolution) (ElMasry and Sun, 2010).

Hyperspectral images or *hypercubes* are three-dimensional datasets containing light intensity measurements where two dimensions (X and Y) represent spatial positions and the third dimension (λ) represents absorbance at different wavelengths. The images can be interpreted, typically, as stacks of hundreds of two-dimensional spatial images at different wavelengths, or tens of thousands of spectra, aligned in rows and columns.

Three instrumentation approaches are used to acquire hyperspectral images. These approaches can be termed (a) *point (staring) scan*, (b) *push-broom (line) scan* or (c) *plane (global) scan*, depending on the orientation of the scanning dimension relative to the two-dimensional spatial sample axes. A *point scan* (or staring instrument) acquires a spectrum at a single spatial location using a Fourier Transform (FT) or grating-type spectrometer.

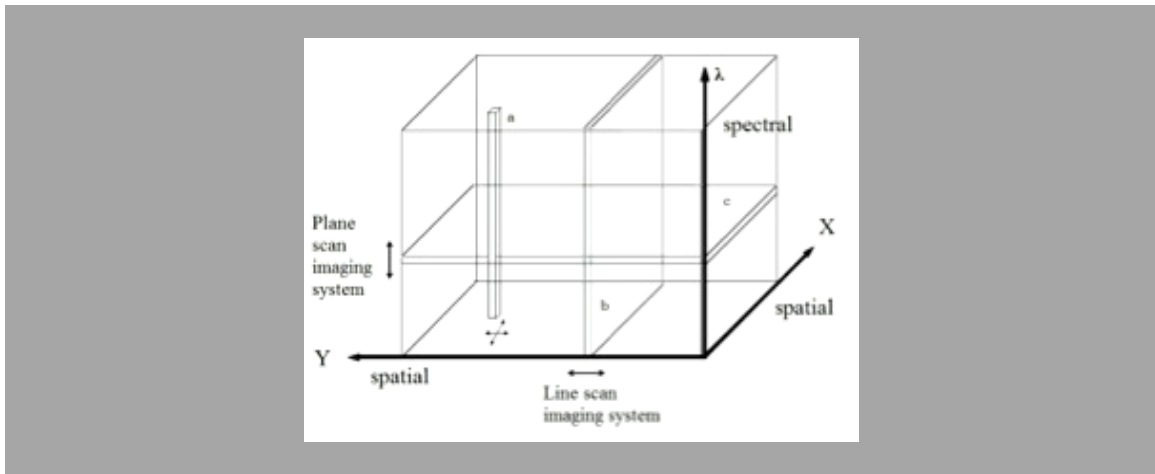


Figure 1. Hyperspectral image (hypercube) acquisitions technique (adapted from Vermeulen et al., 2010 (CRA-W))

Hyperspectral images are obtained by successively measuring spectra while the sample is repositioned in the X and Y spatial dimensions. This kind of instrument is often used in microscopy using a high precision X-Y motion stage. The *push-broom* system projects a line of light onto a two-dimensional Focal Plane Array (FPA), and is best suited for remote sensing by aircraft or online process measurement because the Y spatial axis may be arbitrarily long. The *plane scan* (or whiskbroom) imaging system positions the measurement camera parallel to the sample surface, obtaining X-Y spatial images with fixed sizes limited by the dimensions (pixels) of the camera detector. Hyperspectral images are obtained by modulating the radiation reaching the camera via the use of band pass or tuneable filters positioned in front of the camera (Fernández Pierna et al., 2009).

Advantages and disadvantages of NIR-HSI

For both classical NIR and HSI, the obvious **advantages** include simplicity of data acquisition, low cost per analysis, rapid inspection, and simultaneous analysis of several compounds, non-destructive method, and accuracy. The advantages of all NIR spectroscopy systems are reflected in NIR-HSI systems. In NIR spectroscopy systems, however, the samples usually have to be ground at less than 1 mm, but with NIR-HSI systems sample preparation is not necessary; the samples can be scanned without any grinding and can be used for other purposes (e.g., for germination assays or rescanning when the samples are in different vegetation stages in order to predict the optimal period for harvest) (ElMasry and Sun, 2010).

One of the strong points of NIR-HSI is the time saving, not only for sample preparation, but also for database registration (ElMasry and Sun, 2010; Vigneau et al., 2011). With conventional NIR techniques, one measure gives one spectrum (Figure 2). Thousands of spectra can be obtained with NIR-HSI, giving a complete picture of the distribution and concentration of chemical compounds at the pixel level, and the possibility of simultaneously obtaining the spectral and spatial description of the sample (Piqueras et al., 2012).

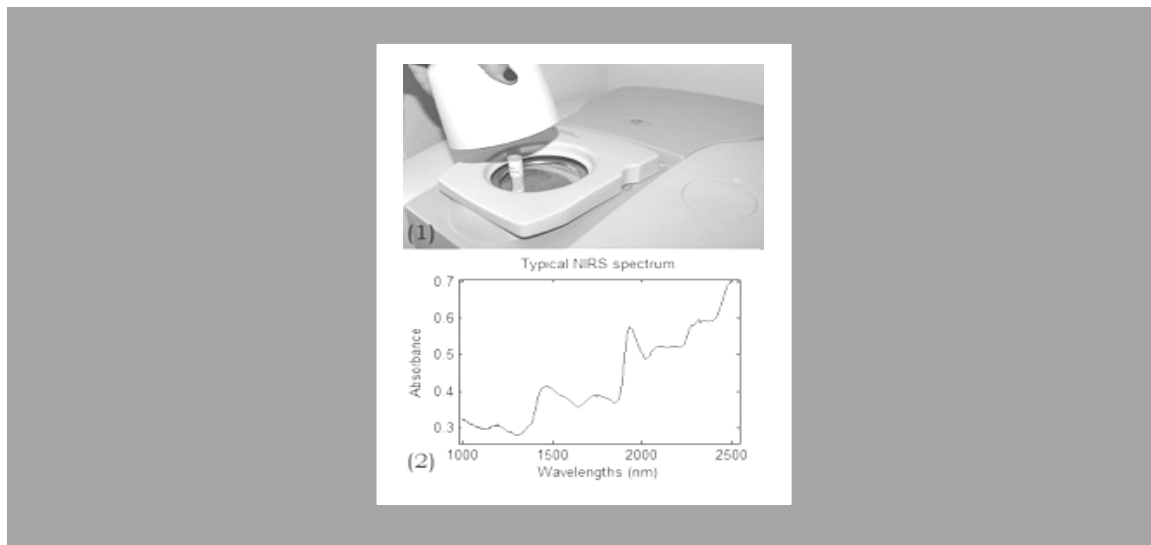


Figure 2. Acquisition of spectrum by conventional NIR spectroscopy system

Legend: (1) NIR spectroscopy system; (2) Typical spectrum of NIR spectroscopy system

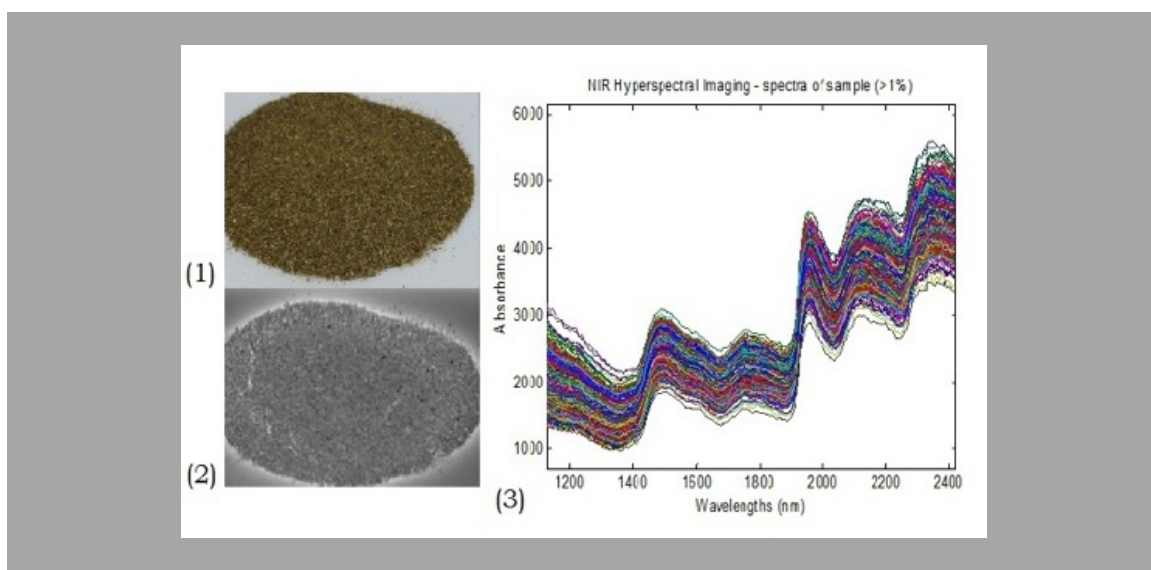


Figure 3. Acquisitions of spectra using a laboratory-scale NIR-HSI system (CRA-W)

Legend: (1) Photograph of sample; (2) Hyperspectral image of sample; (3) Typical spectra (<1%) of a laboratory-scale NIR-HSI system

Hyperspectral images can provide high-quality spectra of surfaces (Amigo et al., 2008) related to internal information (e.g., they can detect and quantify bacteria distribution inside the product) (Burger and Geladi, 2006; Siripatrawan et al., 2011).

Although this technique has the potential to detect diseases and defects in agricultural products and food, its application is limited because of the present cost of equipment, a clear

disadvantage of the method (Chen et al., 2002). In addition, for rapid image acquisition and analysis, NIR-HSI requires very high hardware speed, a major factor that limits its use (ElMasry and Sun, 2010). As in the case of NIR spectroscopy, NIR-HSI is an indirect method and calibration models are necessary. This is a disadvantage in both systems. To obtain efficient qualitative and quantitative analyses, NIR spectroscopy and NIR-HIS methods need to be combined with chemometric techniques (Roggo et al., 2005), a discipline that uses mathematical and statistical methods to extract and interpret chemical information from data (Fernández Pierna et al., 2009). In the literature there are many reviews and textbooks on chemometrics (Roggo et al., 2005; Fernández Pierna et al., 2006; Chang, 2000; Gowen et al., 2007; Gómez-Sancis et al., 2012). The disadvantages are that all this modelling and data processing is time consuming, that interpretation programs are very expensive and that specialists are needed for calibration and standardization. Another disadvantage of NIR-HIS is the registration of a series of successive overlapping bands; it is difficult assigning them to specific chemical groups, and working with what are seen as ‘bad pixels’ (also known as ‘spies’ – Figure 4) (ElMasry and Sun, 2010). In order to identify and detect different unambiguous spectra in the same image, it is necessary for a sample to have the same absorption characteristics (ElMasry and Sun, 2010). López-Alonso and Alda (2002) carried out a comprehensive study on ‘bad pixels’, defining them as pixels classified as anomalous (e.g., pixels that always produce the same signal and from which chemical information cannot be extracted). Blinking or drifting pixels with erratic behaviour can also be called ‘bad pixels’, being clearly different from those considered as ‘good pixels’. There are also ‘noisy pixels’ (i.e., pixels emitting a noise higher than a fixed level).

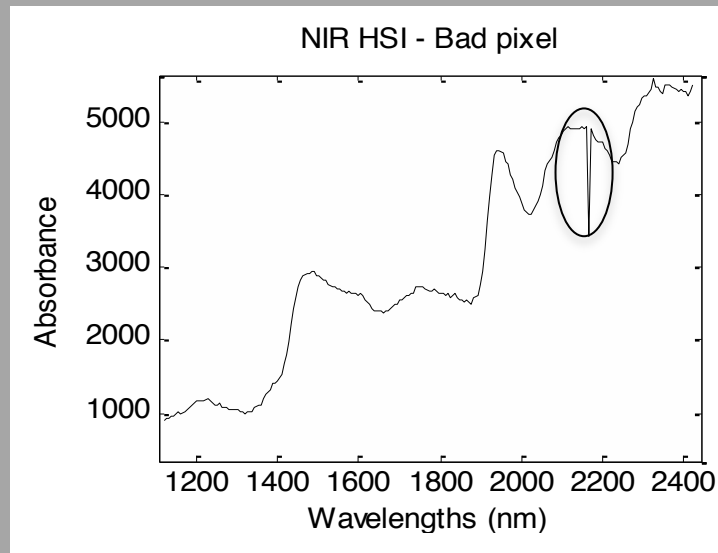


Figure 4. Spectrum of ‘bad pixel’ (spie) (CRA-W)

Applications of NIR-HIS systems

The applications are described here according to the system used, ranging from satellite images to small-scale studies: (i) Satellite HSI systems; (ii) Airborne VIS/NIR systems; (iii) Ground-based HSI systems; and (iv) Laboratory-scale HSI systems (Figure 5).

i) Satellite HSI systems

Many studies using satellite systems have been conducted since the 1960s in different domains. The pioneering studies were in the domains of mining and geology (van de Meer et al., 2012). In the following years the technique was adapted for agricultural uses, such as determining the physical properties of plant canopies (e.g., leaf size and leaf area index; wavelengths ranged between 400 and 2,400nm) (Landgrebe, 2002). Many studies focused on the relationship between optical properties and pigment concentration of leaves. For example, Johnson et al. (Johnson et al., 1999) conducted studies on leaf area index and chlorophyll determination and on discrimination between grass, weed and plastic objects. The focal plane-screening used had wavelengths ranging from 330nm to 1,100nm, with a 3nm spectral resolution. Broge and Leblanc (2001) investigated the application using satellite data for leaf area index and canopy chlorophyll density, under the same methodological conditions (wavelengths 550-1,000nm). Significant results were produced from monitoring plant growth and estimating the photosynthetic productivity potential.

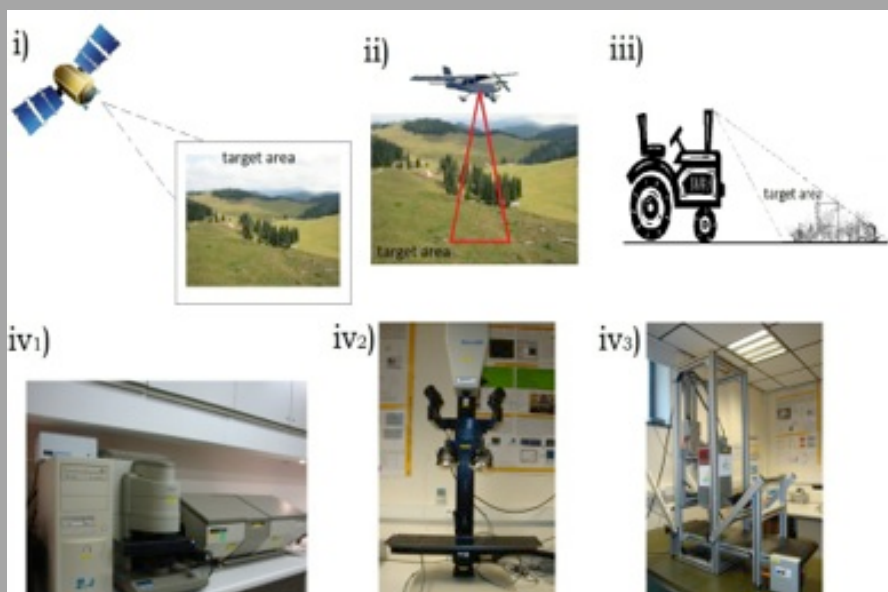


Figure 5. Hyperspectral Imaging Systems (photo original)

Legend: i) Satellite Hyperspectral Imaging Systems; ii) Airborne Visible/Near Infrared Imaging Systems; iii) Ground-based Hyperspectral Imaging Systems; iv₁) NIR Hyperspectral Imaging Systems – point (staring) scan (CRA-W); iv₂) NIR Hyperspectral Imaging Systems – plane (whiskbroom) scan (CRA-W); iv₃) NIR Hyperspectral Imaging Systems – push-broom (line) scan (CRA-W)

Other studies have focused on discrimination between plant stresses imposed by limiting water, insufficient nitrogen fertilizer, or both. El-Shikha et al. (2007) used the remote sensing monitoring system, Agricultural Irrigation Imaging System (AGIIs), and showed in 22 x 22m plots that the effects of nitrogen treatment were more pronounced on leaf area index, plant canopy width and fresh yield than the effects of water treatment on broccoli culture. Successful results were obtained at a reflectance band of 720 nm. El-Shikha et al. (2007) concluded, however, that it would be better for future studies to use airborne scanning or airborne imagery because it is more practical and less expensive than the satellite systems.

ii) Airborne VIS/NIR systems

Whereas satellite data focus on canopy studies, airborne hyperspectral data are addressed mainly to terrestrial vegetation (e.g., plant diseases, plant productions, biochemical parameters). In a study on vegetation community stress, (Merton, 1999) used NASA's Airborne Visible/Infrared Imaging Spectrometer (AVIRIS) to map multi-temporal trends; these were strongly correlated and were successfully used to predict the biochemical impact and geographical extent of vegetation. Zang et al. (2003) successfully used the same AVIRIS system in combination with Spectral Angle Mapping (SAM) to detect tomato stress induced by late blight disease. The wavelengths ranged from 400 to 2,500nm, and the spatial resolution was 4 m. The same technique was used by Parker Williams and Hunter Jr. (2002) to estimate leafy spurge (*Euphorbia esula* L.) cover in 66 circular vegetation plots with a radius of 23m (wavelengths 400-2,500 nm; spectral resolution was 10nm). It is possible to use AVIRIS, however, to estimate leafy spurge distribution and design abundance maps. The differentiation of individual plant species can be problematic because all green plants have similar spectral characteristics.

With a portable Hyperspectral Tunable Imaging System (PHYTIS package), composed of two liquid-crystal tunable filters (Varispec filters, Cambridge Research Instrumentation, Woburn, MA, USA), Fitzgerald (2004) successfully identified cotton (*Gossypium hirsutum* L.) fields and estimate cotton production. The absorption was centred on 400-720nm wavelengths passing from visible light to NIR radiation (650-1100nm wavelengths).

Several studies have demonstrated that airborne imaging systems can be successfully used for mapping invasive plants (Pengra et al., 2007; Andrew and Ustin, 2008). Pengra et al., (2007) mapped the common reed (*Phragmites australis* (Cav.) Trin. Ex Steud.) in saltmarsh vegetation (wavelengths 400 -2,500nm). They found spectral differences between *Phragmites* and 26 other saltmarsh vegetation associations at wavelengths below 1,100nm using the Spectral Angle Mapper (SAM), and obtained a classification accuracy of 81.40%. Andrew and Ustin (2008) used an airborne system (126 wavebands, 3m spatial resolution, and 450-2,500nm wavelengths) to map perennial pepperweed (*Lepidium latifolium* L.) on grasslands, using the cellulose absorption index (defined at 2,100nm). The model used Mixture Tuned Matched Filtering (MTMF), giving an accuracy higher than 85%. Fiorani et al. (2011) presented a number of selected wavelengths (around 970nm, 1600nm and 2100nm) for specific characteristics of plants, such as chemical composition or canopies.

iii) Ground-based HSI systems

With this system, the hyperspectral images are taken at field level, usually by fixing a camera on an agricultural vehicle. It allows, *inter alia*, field production to be estimated. Yang et al.

(2003) estimated grain sorghum yield variability using a CCD camera-based HSI (wavelengths 457-922 nm; 1.5m spatial resolution). The yield maps generated from the images taken with airborne and ground-based HSI corresponded closely with yield data measured after harvest. Similar results were reported by Schut et al. (2006) for grass yield and nutrient content in a field measured at 848-1680 nm. They reported that the system was very suitable for measuring large fields, especially for ground coverage, index of reflection intensity and wavelet entropy. Schut et al. (2012) reported a consistent correlation between dry matter content estimated with Partial Least Squares (PLS) and ground coverage, index of reflection intensity and mean reflection at 800 nm.

Using the same approach, Suzuki et al. (2012) predicted forage chemical composition (ImSpector V10, Specim, Oulu, Finland; wavelengths 360-1,010 nm; spectral resolution 10nm). The study was conducted in a field with the aim of mapping the grass chemical components by developing different calibration models; the coefficient of determination for crude protein and Total Digestible Nutrients was higher than 0.70. Okamoto et al. (2007) developed a model focusing on weed detection and plant classification of plants, under the same conditions described above. Initially, the plant species were classified, and then the plant leaves and background soil were separated. For plant discrimination, the Euclidean distance achieved with segmentation was used, achieving a classification accuracy of 75-80%, whereas discriminant analysis gave an accuracy of 90%. Mapping botanical composition and herbage mass in pasture using the same ground-based HSI systems has been developed by Suzuki et al. (2008). The herbage mass was first predicted by Linear Discriminant Analysis (LDA), and the plant species were then classified as perennial ryegrass, white clover, other plants and dead material, with a classification accuracy of 91.6%.

iv) Laboratory-scale HSI systems

Qualitative applications

One of the first studies using this technology in the agricultural sector focused on the detection of meat and bone meal in compound feedstuffs using HSI in the NIR Focal Plane MatrixNIR (Malvern Instruments Ltd., Malvern, Worcestershire, UK; wavelengths 900-1,700nm at increments of 6nm) (Fernández et al., 2004). The method was developed to enforce food legislation adopted after the European 'mad cow' crisis connected with Creutzfeldt-Jakob diseases in humans. Thousands of spectra in massive sample space had to be collected simultaneously. For detection, the chemometric Support Vector Machines (SVM) tools were used. This alternative method of NIR Focal Plane MatrixNIR was suggested as being more effective than methods used at that time, which were cumbersome and required a specialist. Similar studies were carried out by Riccioli et al. (2011) for discriminating between terrestrial and fish species in animal protein by-products used in livestock feed. The samples were analysed by NIR Chemical Imaging (NIR-CI), in the 1,000-1,700nm wavelengths range. Four algorithms – Mahalanobis distance, Kennard-Stone, spatial interpolation and binning – were applied in order to select an appropriate subset of pixels for further Partial Least Squares Discriminant Analysis (PLSDA). For the four algorithms used, the classification accuracy obtained was higher than 99.61%.

Kim et al. (2004) used Fluorescence HSI for the detection of skin tumors (ulcerous lesions surrounded by a rim of thickened skin and dermis) on chicken carcasses, replacing the time-consuming, expensive and uncomfortable organoleptic inspection

method. They used an HSI system from the USDA Instrumentation and Sensing Lab (ISL-HSI), which includes a CCD camera, a spectrograph, a sample transport mechanism and lighting sources (wavelengths 425-711nm). The detection rate was 76%, indicating that the method needs to be improved; some spots were irrelevant for tumors and some carcasses were not filtered out in the spatial classifier, giving a false positive rate that was too high. Various studies were conducted to detect the contamination of poultry carcasses with visceral content (Lawrence et al., 2003; Park et al., 2005). In order to demonstrate that it is possible to detect faecal and ingested contaminants using NIR-HSI, Lawrence et al. (2003) and Park et al. (2005) used an imaging camera consisting of a focusing lens, a prism-grating spectrograph and a high-resolution CCD camera. Park et al. (2005) used the Region of Interest (ROI) algorithm at wavelengths of 290-1,000nm and obtained an accuracy of 96.6% using Principal Component Analysis (PCA). The imaging system operated from wavelengths of about 400 to 900nm (Lawrence et al., 2003). Similar studies were carried out by Wang and ElMasry (2010) for apple bruising detection based on physical and chemical changes compared with unbruised fruits (wavelengths 400-1,000nm). They developed a model using different algorithms: Minimum Noise Fraction Transform (MNF); ROI; PCA; PLS; and Artificial Neural Networks (ANN). The 750, 820 and 960nm wavelengths were chosen for bruising detection. In order to determine the potential of the selected wavelengths for bruising detection, PCA was conducted with successful results, such as 93.25% of the variance between normal and bruised spectral data (Principal Component 1 [PC1]: 70.01% and Principal Component 2 [PC2]: 23.94%). Nagata et al. (2004) and Nagata and Tallada (2008) worked successfully on strawberry bruising detection. The LDA algorithm was used at a range of 825nm and 980nm and the percentage rate of discrimination was more than 90.70% for the calibration model, whereas for validation it was more than 86.50%. Other applications of the ISL-HSI system were used for vegetables. The system was used successfully for detecting cucumber chilling injury, with recognition rates of 93.30% for injured cucumbers and 88.30% for uninjured cucumbers (Cheng et al., 2004). With this system, the hyperspectral images were acquired at wavelengths of 448-951nm, with a 4.5nm interval. For detection, PCA and Fisher's Linear Discrimination (FLD) were used.

NIR-HSI is also used to measure food quality, particularly fruit quality. For consumer acceptance and fruit shelf life, firmness is very important and it is necessary for the industry to use a non-destructive sensing system to evaluate it. High scattering from a surface depends on the cell structure of the food and is related to the texture. Scattering profiles can therefore be used to predict fruit firmness. A visible/near infrared hyperspectral imaging (VIS/NIR-HSI) system based on a Varispec Liquid Crystal Tunable Filter (LCTF, Cambridge Research and Instrumentation, Inc., Woburn, MA, USA) was used for firmness detection, and LDA, normalized difference (ND) and ANN algorithms were used to analyse the spectra (Nagata and Tallada, 2008). For strawberry firmness detection, wavelengths of 665-685nm, 755-870nm and 955-1000nm were shown to be optimal (Standard Error of Prediction [SEP] around 0.258 in the case of 70% to fully ripe strawberries and 0.350 in the case of 50% to fully ripe strawberries) (Nagata and Tallada, 2008). A similar technique was used by Lu and Peng (2006) to determine peach firmness. The most important bands for predicting peach firmness were found to be around 677nm, 710-850nm and 950 nm. The Lorentzian Distribution (LD) parameter combinations for firmness calibration of two types of peaches (*Red Haven* and *Coral Star*) were chosen. The

coefficients of determination (R^2) obtained with MLR were between 0.51 and 0.58 and between 0.67 and 0.77, respectively. In both cases, further analyses are needed to improve the method.

Beef color and tenderness are two major parameters of beef quality. Beef quality evaluation by a trained panel is expensive, time-consuming and difficult to organise. The development of a non-destructive, fast, accurate, online technology for predicting beef color and tenderness is therefore highly desirable. As noted above for fruit firmness, hyperspectral scattering profiles would be useful for assessing beef quality because light scattering from a surface is closely related to product texture. Recently, a VIS/NIR-HSI (400-1,100nm) was used to predict the beef color parameters and tenderness (*Warner-Bratzler Shear Force*, *WBSF*) of Luxi cattle between 25 and 36 months old, at carcass weights of 280-450 kg (Wu et al., 2012). These authors fitted scattering profiles derived from hyperspectral images to the LD function in order to extract parameters that were used to predict the tenderness and color of 7-day-old cooked beef steaks. The LD function parameter contributions of optimal wavelengths were used to establish MLR models. The R^2 for calibration and cross-validation was higher than 0.91 and the overall accuracy of classification in tender and tough groups was 93.8%. Previously, using a pushbroom HSI with a diffuse flood lighting system, Naganathan et al. (Naganathan et al., 2008) classified beef tenderness into categories (tender, intermediate and tough) with a classification accuracy of 96.4%, while Kim et al. (2008), using an optical scattering feature of lights, obtained an accuracy of 98.4%, although they did not give details about the type of beef, the age or the genotypes of the animals and the carcass weights. These results highlight the potential of HSI optical scattering for the online detection of beef meat quality.

Other studies on contaminant determination have been conducted by Gómez-Sanchis et al. (2012) on citrus fruits to detect *Penicillium* fungi. They used a Hyperspectral Vision System based on Liquid Crystal Tunable Filters (LCTF) (Schneider's Xenoplan optics), with ranges between 400nm and 720nm for VIS spectral wavelengths and between 650nm and 1100nm for NIR spectral wavelengths. The aim of the study was to prevent or at least reduce the *Penicillium* associated economic losses in citrus culture. The accuracy of the classifying methods such as ANN and decision trees was about 98%. The same technique, but with LDA and Classification and Regression Tree models (CART), was used for mandarin fruit and the classification accuracy of rotten fruit was more than 91% (Gómez-Sanchis et al., 2008).

Another application of HSI in the field of fruit diseases is the detection of citrus canker. Caused by bacteria, *Xanthomonas axonopodis* Hasse, this is a severe and devastating disease that can affect the peel (conspicuous and erumpent lesions) of some citrus varieties in infected regions of the world. Currently, there is no really effective treatment or prevention to eradicate the disease. The Spectral Information and Divergence (SID) algorithm was recommended by Chang (2000) to classify the fruits. It is based on a stochastic approach called 'spectral information measure', which has been shown to outperform classical spectral matching techniques. In this application, SID is interesting for differentiating canker disease from normal and other common diseased peel lesions (Qin et al., 2009). The HSI wavelengths ranged from 450 to 930nm and the overall classification accuracy for grapefruit was 96.2%, using an optimal SID threshold value of 0.008 based on the assumption that the false

negative and false positive errors were equally weighted. The method could therefore be used to discriminate citrus canker from other peel diseases, but future development is needed to improve detection accuracy and make it suitable for online and routine application.

NIR-HSI was used by Fernández Pierna et al. (2006) to screen compound feeds. A model was constructed by sorting the particles, using a classification tree where every node encountered a discriminating step. These measures are complemented by discriminating equations created from hyperspectral databases for each class of materials obtained in the MatrixNIR (wavelengths range 900-1,700nm). Discriminating equations were constructed using chemometric SVM tools and classification accuracy was more than 99% for calibration data and more than 88% for validation data. Later, Fernández Pierna et al. (2012) carried out a similar study on impurity discrimination (straw, broken grains, grains from other crops, weed seeds, insects, plastic, stones, pieces of wood and paintings, animal faeces) in cereals (wheat, spelt and barley). SVM was used as a chemometric tool and classified the impurities with an accuracy of more than 95%.

Burger and Geladi (2006) used MatrixNIR at wavelengths of 960-1,662nm and different algorithms (PLS, ROI and PCA) successfully to discriminate different types of cheeses on the basis of the protein, fat and carbohydrate content. The same technique was used to discriminate salt and sugar granules by the peaks centred at 1,130nm, 1,438nm and 1,470nm, which correspond to salicylic acid, sugar and citric acid, respectively. Abdel-Nour and Ngadi (2011) used HSI to detect omega-3 fatty acids in designer eggs. ROI was used to select the specific spectral region (994 and 1109nm), and for discriminant analysis the kernel means algorithm was used, with 100 % accuracy. For egg classification, the PLS algorithm was used. The R^2 coefficients were 0.89, 0.54 and 0.75 and the residual predictive deviations (RPDs) were 2.85, 1.30 and 2.00, respectively for linolenic acid, eicosapentaenoic acid and docosahexaenoic acid.

Quantitative applications

Peirs et al. (2002), Peirs et al. (2003) and Menesatti et al. (2008) applied the hyperspectral technique to determine the starch index of apples as a maturity parameter for predicting the optimal harvest period. They used NIR-HSI equipped with a scanner and spectrophotometer at wavelengths of 1,000-1,700 nm. The PLS-DA algorithm was used with a correlation coefficient higher than 0.94. Weinstock et al. (2006) used the same technique to predict oil and oleic acid concentration in individual corn kernels. The germ from the endosperm was first distinguished by the PCA algorithm, and then the PLS algorithm was used successfully to predict the total oil and oleic acid concentration, with a Root Mean Standard Error of Prediction (RMSEP) of 0.7% and 14%, respectively. Alpha-amylase activity was also predicted in Canadian Western Red Spring wheat samples; the analyses were performed by FT-NIR and Short-Wavelength Infrared HSI (SWIR-HSI); the wavelength range was 1235-2450nm and the PCA and PLS algorithms were used (Xing et al., 2009; Xing et al., 2011). A spectral information divergence was registered at a wavelength of 1900nm, where water, starch and protein were believed to be the combination vibrations of overtones of alpha-amylase activity (Fernández-Ibanez et al., 2009; Xing et al., 2011; Burns and Ciruczak, 2008). It was pointed out that models built with both systems were similar; the R^2 for FT-

NIR was lower than for SWIR-HSI (0.82 and 0.88, respectively) and the root mean square error (RMSE) was 0.90 for FT-NIR and 0.52 for SWIR-HSI.

A MatrixNIR camera (Malvern Instruments Ltd., Malvern, Worcestershire, UK) and a sisuChema SWIR-HSI system (Specim, Spectral Imaging Ltd., Oulu, Finland) were used to differentiate glassy from floury maize endosperm (wavelengths 960-1,662nm MatrixNIR camera and 1000-2,498nm SWIR-HSI) (Williams et al., 2009). The results showed the ability of the model (PCA and PLSDA) to predict glassiness and flouriness correctly with an RMSEP of 0.294. Bauriegel et al. (2011) used HSI (wavelengths 400-1000 nm; spectral resolution 2.5nm) for the early detection of fusarium infection in wheat. During the development stages, the healthy and diseased tissues could successfully be distinguished with 87% accuracy and were in a $\pm 10\%$ range of tolerance. Similarly, Firrao et al. (2010) used the HSI technique for maize fumonisin detection. As mycotoxins are known to be difficult to detect directly using optical methods, the aim of the study was to scan the contaminated samples at wavelengths from 720 to 940 nm. The authors considered that the HSI could provide a reliable contamination estimation (Standard Error of Prediction [SEP] was 0.1895) within a few minutes and be used to assist in lot selection at various stages of the maize processing chain. Del Fiore et al. (2010) built a discrimination model for healthy and diseased maize kernels produced by toxic fungi with HSI (Spectrometer ImSpector Specim V10). The VIS/NIR range was 400-1,000 nm. Using the PCA algorithm, toxic fungi in maize kernels at specific wavelengths (410nm, 535nm and 945nm) was detected. Also in cereals, Vermeulen et al. (2009) used MatrixNIR (wavelengths range 900-1,700) to discriminate the ergot bodies from wheat kernels. The Fisher coefficient calculated on the pre-processed data (wavelength range 1,220-1,440nm) allowed contaminated samples to be detected ($P < 0.05$).

Plant diseases remain the major problem in crop production, leading to yield and quality loss (Zijlstra et al., 2011). An NIR line scan or pushbroom imaging spectrometer (Burgermetrics), which uses a cooled, temperature-stabilized MCT (Mercury-Cadmium-Telluride) detector (Xenics), combined with a conveyor belt (wavelengths range 1,100-2,400nm), was used to quantify crop parameters, such as beet cyst nematodes (*Heterodera schachtii* A. Schmidt) (Fernández Pierna et al., 2012, Vermeulen et al., 2011). The specific pixels corresponding to the conveyor belt showed higher absorbance around wavelengths of 1690 and 1970 nm; the chemometrics tool used in both studies was SVM. The R^2 calculated were rather low: 0.65 and 0.71, respectively. HSI has been used successfully in food and water bacterial contamination detection (Gowen, et al., 2007). Some fresh vegetables are common vehicles for foodborne pathogens (e.g., *Salmonella* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*). NIR spectroscopy has been used for the analysis of micro-organisms in vegetables, but does not give information on bacteria distribution on the product. Siripatrawan et al. (2011) have developed an HSI method for the detection of *E. coli* K12 in packaged fresh spinach with minimal human interference. They used a hyperspectral camera with a wavelength range of 400-1000nm and a spectral resolution of 5nm to acquire hyperspectral images. Chemometrics, including PCA and ANN, were then used to analyze the pre-processed data. The predicted number of *E. coli* vs. true values was closely fitted ($R^2=0.97$) and the prediction mean square error was very low (MSE=0.038).

Conclusions and future developments in HSI

In this review, a wide range of HSI applications in agriculture and agro-food quality and safety, from a macroscopic approach to a more limited field area, has been described. Among the various HSI systems, satellite and airborne HSI show a weakness in the conformity between the information acquired through interpretation of the images and the data acquired in the field, leading to possible errors. With ground-based HSI, it is simpler to determine if the data are similar or not, but errors are still possible because not only the samples are registered. The background can be intercepted also and it is necessary to improve the technique to obtain lower prediction errors. Finally, with NIR-HSI, errors are not registered in big scale because data are collected directly on samples, on sample surfaces or from the inside of samples for specific component detection or discrimination. However a lot of errors are registered such as sampling, pixel overlapping, penetration depth, lack of fit...

Compared with classical analytical methods (e.g., HPLC, MS), as a non-destructive, fast, non-polluting method, HSI is an emerging technology for diversified applications in agriculture and in food quality and safety. Its ability to determine the internal constituents of food products is of prime importance in the food industry. Also, compared with the other imaging systems, NIR-HSI is able to give spatial and spectral information as well as multi-constituent information, and it is sensitive to minor constituents. But if the analyte of interest is concentrated in spots and if it is homogeneously distributed the limit of detection will be determined as with the conventional, normal NIR. There is now a growing interest in using HSI in agriculture and the agro-food industry for controlling and predicting agro-food quality through specific components analyses, but also for the online detection of diseases and chemical, microbial or biological contaminants. This paper has shown that HSI can be used successfully in grassland studies on a large scale, but the system is not yet developed enough for species discrimination on dried and milled samples. HSI could be used for the discrimination of botanical families and plant species and for the detection of toxic and invasive plants from mixed meadows. With regard to future developments other than applications in agriculture and food quality and safety, it is necessary to develop low-cost HSI systems for dedicated applications (e.g. by identifying optimal wavelengths/wavebands depending on the application for which it is intended). An improvement in pre-processing speed and robustness, particularly in the reliability of models, could encourage more widespread online utilization of this technology in agriculture and the agro-food industry which could therefore reduce the cost of process monitoring and product inspection.

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Chapter 4



Research strategy

Research strategy

Thesis Aim

The aim of this thesis was to develop innovative methods for characterizing: (i) the forage quality of the grasslands in the Gârda area of the Apuseni Mountains in Romania, using near infrared (NIR) spectroscopy; and (ii) the botanical composition of these grasslands, using NIR-hyperspectral imaging (NIR-HIS).

Research concept

One of the most important aspects of NIR spectroscopy is the validation of the calibration model. Strong emphasis is put on the use of non-destructive methods for determining the quality of feed, and developed countries have done a considerable amount work on developing such methods, as well as using the technique to build spectral databases, develop NIR spectroscopy calibration models and validate these models. We planned to conduct all these tasks within the context of Romania.

Objective

The objective of the thesis was to develop non-destructive methods for evaluating the quality of feed originating from the Gârda area of the Apuseni Mountains. The first task was to study the potential of NIR spectroscopy for building a spectral database for forage quality based on a large collection of semi-natural grassland samples, using a 'local' calibration model built by the Walloon Agricultural Research Centre (CRA-W) in Belgium to determine various parameters (e.g., protein, dry matter, ash, fibre, fat, aNDFom, ADF, lignin, digestibility, crude energy) from samples collected worldwide, outside Romania. The second task, which involved larger spectral data registration as an image, was to develop calibration models for an NIR-HSI system. Distinguishing samples of pure grassland species can be time consuming, and it was therefore decided to build a spectral database of pure samples and then discriminate these samples into binary and ternary artificial sample mixtures. The main objective of these tasks was to identify the botanical families to which the samples belonged (*Poaceae*, *Fabaceae* and other botanical families [OBF]). The originality of this thesis is that it focused on developing calibration models not only for quality assessment, but also for sample discrimination.

Using NIR spectroscopy to determine the quality of grassland forage samples

There is very little information in the literature on the forage quality of the Apuseni Mountains grasslands. This study sought to improve the forage quality database and to develop the use of NIR spectroscopy for evaluating forage quality. 'Local' NIR models developed by CRA-W were used to analyse semi-natural grassland forage from the Apuseni Mountains. Three experiments were conducted, using different organic and mineral fertilizer combinations, and the samples collected were analysed using classical methods. They were scanned in the NIR spectrometry wavelength band and the CRA-W models were established using mountain grassland samples from throughout Europe. Due to the presence of *Arnica montana* L. in the Apuseni Mountains, which has similar characteristics to tropical plants, tropical data were also used in the calibration model. The effect of different fertilization regimes on the nutritive quality of these meadows was also analysed.

These experiments were described in a paper published in the journal *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*.

Using NIR-HSI to determine the quality of grassland forage samples

Hyperspectral images enable researchers to extract more accurate and detailed information than is possible with any type of NIR spectroscopy. This study sought to discriminate pure grassland species (*Festuca rubra* L., *Trifolium repens* L., *Agrostis capillaris* L., *Hieracium aurantiacum* L. and *Arnica montana* L.) from binary and ternary species using an HSI system (ImSpector N25E). The samples were collected from natural grasslands in the Apuseni Natural Park and, using images obtained previously of the floristic composition of the experimental plots, analysed to determine if the pure species were in the spectral database. Later, a model built by MatLab based on PLS-DA was used to discriminate pure species from mixtures of binary and ternary species. This work was carried out to determine the floristic composition of a meadow, which is possible only if the spectral database of grassland species contains spectra for each identified species.

The first part of the study was published in *Scientific Papers Journals. Agronomy Series*, University of Agricultural Science and Veterinary Medicine Iasi, Romania and also was presented at the 24th General Meeting of the European Grassland Federation (EGF 2012) held in Lublin, Poland, 3-7 June 2012.

A third article was published on using NIR-HSI in the laboratory to discriminate the botanical families (Poaceae and Fabaceae). The plants were collected from the semi-natural grasslands and, after drying and milling, were evaluated using NIR-HSI. The spectra obtained from the NIR images were used to construct PLS-DA models. This method was used to determine whether or not toxic species were present in the natural meadow and to see, in future images of the floristic composition of the experimental plots, whether or not the pure species were in the spectral database.

An aspect of this experiment was described at the 16th International Conference on Near Infrared Spectroscopy (ICNIRS) organized by the National Research Institute of Science and Technology for Environment and Agriculture, and held in La Grande-Motte, France, 2-7 June 2013. The whole experiment was described in a paper published in *Talanta*.

As explained in previous chapter the forage quality is very important. Forage quality or nutrition value is a function of chemical composition, which can be determined by laboratory methods. The NIR technique in comparison with classical methods is non-destructive, non-polluting, fast and relatively inexpensive per analysis. Investigations on nutritional quality of Carpathians Apuseni Mountains (Romania) grasslands are rarely performed with NIR technique. Therefore in the previous reviews the NIR technique and the chemometrics tools are described. To address the NIR technique issue, in Romania, the first study, article of the present PhD thesis proposed the investigation regarding forage quality by NIR Spectrometry. 'Local' NIR spectrometry models from CRA-W Gembloux were used to analyze the forages quality from the grassland. And then the second objective was to assess the effect of different fertilization regimes on the nutritive quality of these meadows.

Chapter 5



Article 1

*Fertilization effects on the chemical composition
and in vitro organic matter digestibility of Romanian
semi-natural mountain meadows as predicted
by NIR spectrometry*

Fertilization effects on the chemical composition and in vitro organic matter digestibility of Romanian semi-natural mountain meadows as predicted by NIR spectrometry

Article I — DALE *et al.* (2013)

Article adapted from *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 41 (1) (2013) 42-48.

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Running head: Grassland forages quality

Abstract

Management of livestock grazing in highly-productive mountain meadows is an important aspect for the economic viability and the environmental impact of a grassland-based farm. The main aim of this study was to build near infrared models to determine the chemical composition and in vitro organic matter digestibility of Romanian meadow forages. The treatments were organic and mineral fertilizer combinations, and forage samples were obtained from three fertilization experiments conducted in the Apuseni Mountains; these samples were analysed using classical and NIR methods. The samples were scanned in the NIR wavelength band. The CRA-W Gembloux 'local' calibration models were validated with Romanian meadow forages and then used in order to predict the forage quality of samples. A second objective of the study was to determine the effects of fertilization on forage quality. The results showed a decrease in crude protein content from the NPK treatment (150:75:75), which can be explained by a reduction of *Fabaceae* plants with this treatment from 17.25% of the populations in the control (semi-natural meadow not fertilized) to 6.25% in the fertilized plots. The decrease in protein content and in vitro organic matter digestibility was related to a reduced *Fabaceae* presence. Our recommendation is to use mineral fertilization with NPK doses less than 100:50:50 to improve meadow productivity; meanwhile organic fertilization can also be used to complement and maintain biodiversity and forage quality.

Keywords

Forage quality, semi-natural meadows, chemical composition, in vitro organic matter digestibility, NIR, Apuseni Mountains

Introduction

In the Romanian Apuseni Mountains, specifically in the Gârda area, more than 34% of grasslands are used as a source of hay to for livestock (Gârda, 2010). Meadow management is an important aspect for the economic viability and the environmental impact of grassland-based farms in this region. Good meadow management reduces the need to buy expensive concentrate feeds and limits the environmental impact by using appropriate levels of fertilizers and choosing optimal grazing periods.

A semi-natural meadow is defined as one that may be mowed for fodder or provide grazing for livestock (Rotar et al., 2010). The determination of forage quality from semi-natural meadows is important for both nutritional and economic reasons (Rotar et al., 2010). Generally, forage quality is influenced by many factors, including soil, climatic conditions, floristic composition of the meadow, stage of maturity of plants when harvested, and forage preservation methods. The nutritive value of forage depends on its chemical composition including: crude protein [CP], crude ash [ash], ether extract [EE], crude fibre [CF], neutral detergent fibre [aNDFom], acid detergent fibre [ADF], lignin [lignin (sa)] and in vitro organic matter digestibility [OMD].

Currently, new analytical tools for agriculture based on spectroscopic technologies are being developed to extend the work of Norris and coworkers in the 1960's (Hart et al., 1962). Near Infrared (NIR) Spectroscopy is a well-known technology in the agricultural sector allowing the acquisition of chemical information from the samples with a large number of advantages, such as: an easy to use tool, fast and simultaneous analysis of several components, non-polluting, non-invasive and non destructive technology, and the possibility of online or field implementation (Dale et al., 2013).

Although the development of the NIR technique has advanced the science of forage evaluation, wet chemistry analysis is still considered the “gold standard” for forage testing. Many certified feed analysis laboratories are capable of performing wet chemistry, NIR analyses, or both (Weiss and Pell, 2007). Certified feed analysis laboratories around the world use complex mathematical and statistical methods for predicting forage quality in all types of forages. The accuracy of the NIR method to determinate forage quality is determined by the differences between NIR predictions and results of reference methods that have to be less than the sampling error (Weiss and Pell, 2007).

In some developed countries several investigations on forages were initiated, which aimed at developing non-destructive methods; assessing the possibilities to use the NIR spectrometry technique in order to build a spectral database; building models of NIR spectrometry calibrations and validation for various parameters (protein, dry matter, ash, fiber, fat, NDF, ADF, lignin, digestibility, crude energy, etc).

In some countries of the European Union, NIR spectrometry has been used since 1980, although in many other countries it is still in an early stage of development. NIR spectrometry is widely used for rapidly determining the concentration of nutrients and feeding value in dried and fresh crop materials (Decruyenaere et al., 2009; Rotar et al., 2009), or in grassland products (De Boever et al., 1999; Bovolenta et al., 2008; Mahipala et al., 2009). In Romania, NIR spectrometry was used first in 2000 (Vidican et al., 2000).

Various researchers in Central and Eastern Europe conducted long-term mineral fertilizer experiments (Hejcman et al., 2007); all these experiments showed that NPK fertilizers changed meadow species richness, reduced soil fertility and increased meadow forage value. Păcurar (2005) pointed out that prior to 2001, only organic fertilizers were applied to Romanian Apuseni Mountains. Mineral fertilizers were unavailable and forage quality was not analysed (Păcurar, 2005). To address the local fertility problems of the Romanian Apuseni Mountains, different levels of fertilization were applied to semi-natural meadows in the Gârda area (Păcurar et al., 2005). It was shown that fertilization based on different organic and mineral fertilizer levels contributed to higher production of forages from semi-natural meadows and to a modification of plant species frequency (Păcurar et al., 2010).

As there is a paucity of information regarding forage quality of Apuseni Mountains grasslands in the published literature, the purpose of this study was to improve the forage quality database and to develop the use of NIR spectroscopy to evaluate forage quality. Our concerns were not related to monitoring forage quantity, but rather to determine the quality of forages from stationary experiments performed on the semi-natural grasslands in the Apuseni Mountains.

Objective

The main means of this study was to use Walloon Agricultural Research Centre (CRA-W) Gembloux (Belgium) 'local' NIR models for analysing the semi-natural meadow forages from the Apuseni Mountains. The CRA-W Gembloux (Belgium) 'local' NIR models were established using different mountain grassland samples from throughout Europe. Due to the presence of *Arnica montana* L. in the Apuseni Mountains, known also as leopard's bane, which apparently share similar characteristics with tropical plants, tropical data were also used in the calibration model. A second objective was to assess the effect of different fertilization regimes on the nutritive quality of these meadows.

Materials and methods

The study was conducted in the Romanian Apuseni Mountains, at an altitude of 1130m in the Glacier Plateau-Poiana Călineasa area near Glacier village (GPS coordinates of Ghețari Research Centre: Latitude: 46.500 N - Longitude: 22.816 E). This area is in the Bihor Mountains, in the central and highest part of the Apuseni Mountains.

Three different fertilization trials were carried out simultaneously. All the experiments were conducted on a red clay soil and were established in spring 2001 using either manure (i.e., organic), mineral fertilizer, or a combination of both. The experimental design for the first experiment used five combination treatments with four replicate plots per treatment: T1 - semi-natural meadow (control); T2 - 20 t/ha cow and horse manure; T3 - 10 t/ha cow and horse manure + 50N 25P₂O₅ 25K₂O/ha; T4 - 100N 50P₂O₅ 50K₂O/ha; and T5 - 10 t/ha cow and horse

manure + 100N 50P₂O₅ 50K₂O/ha. The experimental design for the second experiment was four organic treatments with four replicate plots per treatment: O1 - control; O2 - 10 t/ha cow and horse manure; O3 - 20 t/ha cow and horse manure; O4 - 30 t/ha cow and horse manure. The experimental design for the third experiment was four mineral treatments with four replications plots/treatment: M1 - control; M2 - 50N 25P₂O₅ 25K₂O/ha; M3 - 100N 50P₂O₅ 50K₂O/ha; and M4 - 150N 75P₂O₅ 75K₂O/ha.

Each plot measured 10 m² and the annual fertilization input has been applied each spring from 2001. The organic fertilizer came from a local farm in the Gârda area. Cattle and horse manures were mixed (50%/50%), producing a fertilizer containing 0.40% N, 0.39% P and 0.45% K (on a fresh matter basis) (Păcurar et al., 2012). The mineral fertilizer used was a NPK complex applied at 20:10:10 kg/ha, respectively.

The results presented in this paper refer to samples collected in 2010. The floristic composition was characterized following the Braun-Blanquet method (Braun-Blanquet, 1964) and the results were published by Păcurar et al. (2012).

The grass, which was in flowering stage, was cut in the field in July 2010. Using a drill, 500 g samples were collected on each replicate plot (5 x 100g samples collected randomly from the total grass collected) in duplicate. The 104 samples (52 plots x 2 samples) were air-dried for 1 week and then in a drying stove at 60°C for 2 days. They were then milled first using a 5 mm sieve (Grindomix GM 200, Retsch, Haan, Germany) and then a 1 mm sieve (Cyclotec™ 1093, Tecator, Sweden) as required for the NIR technique.

The spectra of samples were acquired in duplicate on a NIR system 6500 (Foss NIRSystems, Silver Spring, MD, USA). The NIR spectra were collected at 2nm intervals between 400 and 2500nm, with two replicates, using the WinISI® 1.50 software (Infrasoft International, Port Matilda (now State College), PA, USA). Each spectrum was a mean of 32 scans/sample.

Based on the CP and OMD predicted values by the CRA-W Gembloux (Belgium) 'local' NIR calibration model, 20 contrasted samples were selected to carry out the reference 'wet chemistry' analysis: for CP, the Kjeldhal method - AOAC (1990); for ash, method 942.05 - AOAC (1990); for EE, method 920.39 - AOAC (1990); for CF, method 73/46/CEE - Fibre Cap (FOSS, DK); for aNDFom, ADF and Lignin (sa), the Van Soest - Fibre Cap method (FOSS, DK); and for OMD, the De Boever method (De Boever et al., 1986).

The 20 samples were used to develop an external validation of CRA-W Gembloux NIR 'local' calibration model. The WinISI® 1.50 software was used in the treatment of the spectral data obtained and for establishing the mathematical models. The spectra (trimmed to 1300-2400nm) were treated as following: the standard normal variate and detrend scatter correction (SNVD) procedure was applied to the spectral data; the spectra were then transformed through a mathematical first order derivation (1, 4, 4, 1 [1st derivative, 4nm gap, 4 points smoothing, and 1 point second smoothing]); a cross validation based on 'leave one out' was used; and 12 Partial Least Squares (PLS) factors were chosen. The algorithm used for the calibration model was the modified PLS algorithm. The validation statistics were performed according to ISO/FDIS 12099:2010(E).

The validated model was then used to predict the chemical composition of the 84 remaining samples. The predicted nutritional quality parameters were analyzed by the GLM procedure (version 9.2; SAS Institute Inc., Cary, NC), using fertilization treatment in a randomized block design with blocks containing all treatment combinations with 4 replicates. Factors in the model were block and treatments. Chemical analyses were determined from each replicate plot,

with two sub-samples for each plot, and statistical analyses were performed on mean values for each plot. When GLM indicated a significant effect of treatment ($P < 0.05$), the differences were compared by the Waller–Duncan multiple range test.

Results and discussions

The CRA-W Gembloux NIR ‘local’ calibration model (Sinnaeve et al., 1994) characteristics are presented in Table 1. ‘Local’ means that each sample is predicted with its own model calculated on a subset of samples selected on the basis of the closest calibration samples (highest correlations between the spectrum to be predicted and the spectra of the library).

Table 1. NIR calibration performances and statistical results of the external validation

Content	Mean	N	SD	SEC	R ²	RCV ²	SECV	Bias	SEP	SEP(C)	SEP(C)/ SECV ratio	TUE
CP	10.44	1036	5.65	0.84	0.98	0.98	0.87	-0.608	0.561	0.396	0.455	1.139
Ash	8.87	1096	3.98	1.39	0.88	0.87	1.45	0.196	0.843	0.594	0.410	1.885
EE	3.84	65	1.24	0.60	0.76	0.60	0.78	-1.003	0.552	0.389	0.499	0.852
CF	29.86	849	8.68	1.87	0.95	0.95	1.97	-0.411	1.176	0.829	0.421	2.538
ADF	60.32	746	15.21	3.16	0.96	0.95	3.36	0.008	1.949	1.374	0.409	4.291
Lignin (sa)	35.82	708	8.30	2.48	0.91	0.90	2.60	0.419	2.341	1.651	0.635	3.368
aNDFom	6.74	513	4.37	1.42	0.89	0.86	1.58	-0.038	2.328	1.642	1.039	1.931
OMD	47.81	139	14.21	3.01	0.96	0.94	3.48	1.659	4.168	2.939	0.845	4.156

Legend: CP, crude protein; EE, ether extract; CF, crude fiber; aNDFom, neutral detergent fiber; ADF, acid detergent fiber; Lignin (sa), acid detergent lignin; OMD, in vitro organic matter digestibility; SD, standard deviation; SEC, standard error of calibration; R², coefficient of determinations; RCV², cross-validation coefficient of determination; SECV, standard error of cross validation; Bias, errors derived from predicted-actual component values; SEP, standard error of prediction; SEP(C), standard error of prediction corrected for bias; SECV, standard error of cross validation.

For the validation of the CRA-W Gembloux NIR ‘local’ calibration model, the analysed values for CP, ash, EE, CF, aNDFom, ADF, Lignin (sa) and OMD from 20 samples were used. The calibration models (Table 1) gave the best results for CP ($RCV^2=0.98$), followed by aNDFom, CF, OMD, ADF, ash, Lignin (sa) and finally EE. The best calibration models were obtained for CP, with high R² and RCV² coefficients and low SECV. Good results were also obtained for OMD, CF and aNDFom, with R² and RCV² > 0.94. The results obtained for the calibration

models were similar to those published by other authors for the same type of biological material. The R^2 obtained in our experiment was similar to De Boever et al., (1999) and Andueza et al., (2011) for CP, how obtained R^2 values: 0.92 and 0.98, respectively; for CF and ADF the R^2 was similar to De Boever et al., (1999) and Koukolova et al., (2010), how obtained R^2 values of 0.98 and 0.74, 0.84 and 0.87 respectively. Similarly for aNDFom and for OMD value the R^2 values we obtained were the same as reported by De Boever et al., (1999) and Andueza et al., (2011) how obtained 0.84 and 0.88, respectively.

The poorest results were observed for the EE calibration model due to the low fat content in forages, the high variability in chemical analysis (Amari and Abe, 1997; Berardo et al., 1997; Park et al., 1998), and low the low sample size. Also another reason can be the method used for classical analysis, because EE determines not only the fat content (i.e., triglycerides), but also soluble plant pigments. For example, another method that could be used for plant tissue is the chloroform-methanol extraction method based upon the principle of Bligh and Dyer (Fishwick and Wright, 1977). Moreover, the low number of samples used in the calibration model (i.e., 65 vs. at least 1000) and also the narrow range in EE variability could be the reason for the poor results. In most cases, EE is not determined on forage samples. In conclusion, it can be said that more classical analyses for better correlations needed to be conducted, particularly for EE, ash, Lignin (sa) and the ADF calibration models.

Bias values are also important in the evaluation of equation performance (Stuth et al., 2003). A high bias indicates systematic errors between calibration and prediction datasets (Roggo et al., 2003). Concerning the validation test, based on 20 samples (Table 1), for CP, ash, CF, aNDFom, ADF, Lignin (sa) and OMD, the calibration models gave good results, with a bias less than 1.7.

The standard error of prediction (SEP) is used for evaluating calibration model accuracy by indicating the variability in deviation of the reference data from NIR spectral data (Manley et al., 2008). The standard error of prediction corrected for the bias (SEP[C]) is an average difference between predicted and reference values. In this study, SEP[C] values were lower than 1.651 for CP, ash, EE, CF, aNDFom, ADF and Lignin (sa) and 2.939 for OMD, indicating that the prediction potential was rather low in the case of OMD. The SEP(C)/SECV ratios were lower than one for all parameters measured (Table 1) indicating that the prediction models developed were robust. The unexplained error confidence limit (TUE) is a limit that a validation SEP must exceed in order to be significantly different from the SEC at the confidence limit specified ($P < 0.05$). In our case the TUE was higher than SEP for all the parameters except for OMD and aNDFom.

The predicted NIR results for the chemical analyses (CP, ash, EE, CF, aNDFom, ADF, Lignin (sa)) and OMD values were improved when using the external validation set shown in Table 2.

In the first combination (T) experiment, CP content was relatively low content (Table 2). The EE and Lignin (sa) content of forages from the first fertilization trial were similar ($P > 0.05$) for all the treatments, with mean values of 3.76% and 8.68%, respectively. The second experiment trial, fertilized by organic (cow and horse) manure, showed significant differences in all the treatments compared with the control without fertilization for CP, higher content was 15.42% ($P < 0.001$), for ash, higher content was 10.06% ($P < 0.01$), for CF, lower content was 23.79% ($P < 0.01$), for ADF, higher content was 31.68% ($P < 0.01$), for Lignin (sa), higher content was 8.62% ($P < 0.05$) and for OMD, higher content was 73.22% ($P < 0.01$), whereas no differences were recorded for EE. By contrast, the aNDFom content declined (35.54%) with organic fertilization treatment ($P < 0.05$).

In the third experiment, the main effect of mineral fertilization decreased CP ($P<0.05$), ash ($P<0.01$), and OMD ($P<0.001$), whereas CF ($P<0.01$), aNDFom ($P<0.001$), Lignin (sa) ($P<0.01$) and ADF ($P<0.01$) all increased.

Table 2. Effects of fertilization on chemical composition and *in vitro* organic matter digestibility of meadow predicted by NIR spectrometry (N=2)

Treatment	CP	Ash	EE	CF	aNDFom	ADF	Lignin (sa)	OMD
T1	13.39b	10.55a	4.00	22.18c	40.44c	29.59a	8.41a	65.68a
T2	14.88a	10.76a	3.75	22.39bc	41.00c	31.65a	8.30a	66.79a
T3	15.23a	9.91ab	3.61	23.71abc	44.23b	29.83a	8.40a	64.61a
T4	12.33c	8.79b	3.79	26.21a	54.70a	32.44a	9.11a	56.67c
T5	13.01bc	8.62b	3.66	25.51ab	55.17a	32.48a	9.18a	59.28b
MSE_T	0.72	0.94	0.49	1.92	1.35	1.92	1.21	1.31
O1	13.37c	9.06b	3.58	24.89a	40.40a	30.67ab	7.85b	65.51c
O2	15.66b	9.80ab	3.56	23.79b	38.68b	29.44b	7.32c	68.96b
O3	16.71a	10.06a	3.35	24.00ab	36.33c	31.00a	8.62a	71.50ab
O4	15.42b	9.88a	3.29	24.21ab	35.54c	31.68a	7.84b	73.22a
MSE_O	0.58	0.44	0.33	0.88	1.02	0.83	0.30	1.99
M1	14.29a	10.68a	3.65	21.36b	35.66d	29.28b	8.19b	69.44a
M2	13.84ab	9.73ab	3.65	23.18b	41.20c	30.29b	8.26b	67.17b
M3	12.39bc	8.82ab	3.22	26.55a	50.68b	34.31a	9.41a	57.04c
M4	11.56c	8.17b	3.32	27.71a	55.39a	34.23a	8.64b	56.27c
MSE_M	1.02	1.27	0.38	1.53	0.42	1.54	0.44	1.16

Note: Different letters between treatments indicate significant differences (Waller Duncan test, $P<0.05$).

Legend: CP, crude protein; EE, ether extract; CF, crude fiber; aNDFom, neutral detergent fiber; ADF, acid detergent fiber; Lignin (sa), acid detergent lignin; OMD, *in vitro* organic matter digestibility; T1, semi-natural meadow (control); T2 - 20 t/ha cow and horse manure; T3 - 10 t/ha cow and horse manure + 50N 25P₂O₅ 25K₂O; T4 - 100N 50P₂O₅ 50K₂O; T5 - 10 t/ha cow and horse manure + 100N 50P₂O₅ 50K₂O; O1, control; O2, 10 t/ha cow and horse manure; O3, 20 t/ha cow and horse manure; O4, 30 t/ha cow and horse manure; M1, control; M2, 50N 25P₂O₅ 25K₂O; M3, 100N 50P₂O₅ 50K₂O; M4, 150N 75P₂O₅ 75K₂O; MSE, standard error of the mean.

There were treatment effects on the botanical composition of the plots subjected to the T5 treatment (10 t/ha cow and horse manure + 100:50:50), as the *Fabaceae* species covered 9.25% of the plots, whereas in the T4 treatment (100:50:50) plants from this family covered only 4.75% (Păcurar et al., 2011). As species in the legume family are noted for their high nitrogen content, this reduction helps to explain the relatively low CP content (12.33%) that was measured and predicted in our samples. The results obtained by Păcurar et al. (2012) on samples collected in the same trials indicated that organic fertilization induced an increase in the presence of *Fabaceae* species, whereas mineral fertilization resulted in a decrease in these species. Similar studies of meadow fertilization approaches carried out by Lee and Lee (2000) showed that mineral (nitrogen) fertilization simulated grass growth and reduced legume growth. Using mineral (nitrogen) application, Aydin and Uzun (2005) noted that botanical composition was negatively affected, resulting in forage with lower CP content, but a higher dry matter yield.

Organic fertilization apparently regenerated the vegetation, providing an inexpensive and non-destructive approach to accelerating succession in biodiversity compared with mineral fertilization (Rowe et al., 2006). In addition, cow and horse manure fertilization leads, in the short term, to an increase in the cover of competitive dominant plant species favored for growth (Rowe et al., 2006). After the organic fertilization application, major changes were observed in botanical composition; with applications of the O3 and O4 treatments, the *Trisetum flavescens* L. - *Agrostis capillaris* L. meadow type changed to the *Festuca rubra* L. - *Agrostis capillaris* L. meadow type (Păcurar et al., 2011). After mineral fertilizer application on *Festuca rubra* L. - *Agrostis capillaris* L. there was a change to the *Agrostis capillaris* L. - *Trisetum flavescens* L. meadow subtype in the M3 and M4 treatments (100:50:50 and 150:75:75 respectively) (Păcurar et al., 2012).

Like in the other experiments, the EE was not affected by the treatments. Mineral fertilization is known to increase the nutrient content in leaves and improves fodder quality and productivity in the short term (Hudewenz et al., 2012), which was confirmed by our results. Hejman et al., 2007 reported that typical meadow species (*Alopecurus pratensis* L., *Trifolium repens* L. in Slovakia, all *Carex* and *Juncus* in sub-montane grassland in the Czech Republic, *Dactylorhiza majalis* P.F. Hunt & Summerh in *Juncus acutiformis* meadow in the Netherland) disappear from plots receiving high NPK fertilizer applications. In this study, the species number was reduced from 36 species in the O1 treatment (semi-natural meadow-control) to 24.25 species in the O4 treatment (150:75:75) (Păcurar et al., 2012).

The chemical composition of meadow forages predicted by NIR for other regions of the world were similar to those reported in our experiments: De Boever et al., (1999; western Europe) obtained similar CP (5.60%-14.10%), ash (7.60%-22.80%), EE (1.40%-3.90%), CF (21.50%-34.90%), aNDFom (51.80%-73.60%), ADF (24.50%-42.10%), and Lignin(sa) (1.30%-5.60%) values. In the Italian mountain region, Bovolenta et al., (2008) obtained similar CP (6.70%-13.20%), ash (9.70%-23.20%), ADF (19.80%-34.70%), Lignin(sa) (2.60%-13.10%) and OMD (30.50%-77.80%) values. Finally, in Australia, Mahipala et al., (2009) obtained similar aNDFom (55.50%-63.50%), ADF (34.10%-48.20%), Lignin(sa) (3.90%-19.10%) and OMD (30.10%-62.80%) values.

Păcurar (2005) established and conducted three experimental studies in 2001-2003 in the same meadow as ours, with the same treatments. His study investigated the ecology and sustainable management of these meadows. CP, CF and ash were analyzed over 3 years (2001-2003) and the sward was studied extensively. The CP, ash and CF content were between 7.80% and 9.95%, 9.11% and 15.03% and 26.68% and 30.17%, respectively.

Higher CP and ash content and lower CF content were noted in the T5 treatment in the first experiment during the first year of the trial, as well as in the T2 treatment in the following 2 years. In our study, after 9 years of treatments, no differences in ash content across all treatments were recorded. Regarding CP content, positive differences were recorded in the T3 treatment ($P < 0.01$); for CF content, positive differences were recorded in the T4 treatment ($P < 0.01$). In conclusion, the differences obtained in 2010 were due to changes in the herbaceous layer. The proportions of *Poaceae* and *Fabaceae* species changed (Păcurar et al., 2012) substantially in response to the fertilization regimes. Organic fertilization induced an increase in *Fabaceae*, whereas mineral fertilization led to an increase in *Poaceae* (Aydin and Uzun, 2005).

In the second experiment, however, the results obtained in 2001 by Păcurar (2005) showed that in the O4 treatment, CP and ash content were highest and CF content was lowest compared to the other treatments. Results obtained in 2002 and 2003 were different from 2001. In 2002, CP content decreased in all treatments compared with the O1 treatment (semi-natural meadow - control) ($P < 0.05$), but in 2003 it increased in the O2 treatment (10 t/ha cow and horse manure) ($P < 0.01$). Compared with our results for CP content, significant differences were found in the O3 and O4 treatments ($P < 0.05$, $P < 0.001$ respectively) compared to the control.

After short-term mineral fertilization, the results obtained by Păcurar (2005) for the third experiment in 2001-2003 showed a slight increase in CP content in the M3 treatment ($P < 0.05$) in 2001 and in the M4 treatment in 2002-2003 ($P > 0.05$). In 2010, after 9 years of mineral fertilization, CP content was decreased ($P < 0.01$) and the botanical composition also changed compared to the control. CF content decreased in 2001 ($P < 0.05$), but in 2002-2003, it increased in the M4 treatment and reached its highest value in the M3 treatment ($P < 0.01$); in 2010, the M4 treatment (150:75:75) showed the highest CF content ($P < 0.001$).

Concerning fiber determination, the original CF analysis has been largely replaced with the aNDFom analysis. As in our trial, De Boever et al. (1999) observed that aNDFom content was almost double of the CF content with mineral treatments. This was explained by higher aNDFom and lignin levels in *Poaceae* species compared to *Fabaceae* species (Merchen and Bourquin (1994), Collins and Fritz (2003) and Gosselink et al. (2004)). Bovolenta et al. (2008) pointed out that CF and aNDFom contents are indirectly proportional to CP content and influence the OMD value. It should also be noted that the mineral treatments induced large differences in aNDFom content, with much higher increases than for ADF content. This suggests that, with mineral treatments, aNDFom, ADF and CF content increased rather than CP content and the OMD values decreased. For organic treatments, however, lower aNDFom content was recorded in all fertilization treatments compared to the control plots, whereas CP content and OMD were higher.

Conclusions

The originality of the present paper is due to the use and successful validation of the CRA-W Gembloux NIR 'local' calibration model for a specific area from Eastern Europe that was not involved in the development of the model. The 'local' calibration model successfully confirms and predicts results found through traditional wet chemistry (i.e. proximate analysis), and additionally for organic matter digestibility of Apuseni semi-natural meadow forages. Regarding the calibration statistics it is recommendable to perform more chemical analyses for

OMD and aNDFom to improve the accuracy. For ash, EE, aNDFom, Lignin (sa) and ADF, the calibration model should be improved by increasing the number of samples for reduced variability and heightened sensitivity. In summary, mineral fertilization decreased species richness but increased the meadow productivity. Mineral fertilization reduced the CP content of the forages as a result of a decrease in the number of *Fabaceae* species in the herbaceous component. CF, aNDFom and ADF content showed a higher variability with mineral or organic-mineral fertilizers than with organic fertilizers only. The organic fertilization used alone induced an increase in OMD. Our recommendation is to use mineral fertilization with NPK doses less than 100:50:50 to improve meadow productivity; meanwhile organic fertilization can also be used as a way to complement and maintain biodiversity and forage quality.

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The previous chapter was focused on forage quality and NIR spectrometry of Occidental Carpathians, Apuseni Mountains (Romania). The study results showed that NIR Spectrometry could be successfully applied to predict the classical composition (crude protein, crude ash, ether extract content, crude fiber, NDF, ADF, lignin) and in vitro organic matter digestibility of forages from Romanian semi-natural mountain meadows. Because the percenage of Fabaceae species is reduced in the grassy sward, to determine exactly the dominant species of the meadows samples a methodology of NIR Hyperspectral Imaging approach will be developed in the next chapter. The aim of article is to develop a discriminating method to differentiate the species Festuca rubra L., Trifolium repens L., Agrostis capillaris L., Hieracium aurantiacum L., Arnica montana L. as pure samples species, and then the second aim is to discriminate the same species in the binary and ternary mixtures.

Chapter 6



Article 2

*Pure species of grass discrimination with the help
of NIR Hyperspectral Imaging*

Pure species of grass discrimination with the help of NIR Hyperspectral Imaging

Article I — DALE *et al.* (2011)

Article adapted from *Scientific Papers Journals. Agronomy Series*, 54 (2) (2011) 23-27.

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Running head: Grassland forages quality and chemometrics

Abstract

The objective of this study was to assess the ability of an SWIR ImSpector N25E hyperspectral imaging (HSI) system to discriminate pure species (*Festuca rubra* L., *Trifolium repens* L., *Agrostis capillaris* L., *Hieracium aurantiacum* L. and *Arnica montana* L.) in grass mixtures. All the samples were collected from natural grasslands in the Gârda area of the Apuseni Natural Park in Romania. They were dried naturally and then prepared for analysis using NIR spectroscopy. The model based on partial least squares discriminant analysis (PLSDA) was used to investigate whether or not class discrimination between pure species was

possible. Using the MatLab program, the study sought to establish if the pure species could be recognized in other images in the spectral data base. The potential of using the NIR-HSI system (Camera NIR) to discriminate or identify pure species was confirmed. A percentage of more than 96% correct prediction for species discrimination was obtained. This study should lead to work on another important issue, which is to identify toxic species in grasslands used as forage for animals. The floristic composition of a meadow can be determined only if the database contains the dates for each identified species in the grass mixtures.

Keywords

Discrimination, camera NIR, pure species, meadow.

Introduction

About 35% of the grasslands in the Gârda area in Romania's Apuseni Mountains is used for hay (Gârda, 2010). Grassland management is an important determinant of the economic viability and environmental impact of grassland-based agriculture. Good grassland management reduces the need to buy expensive feed and limits environmental impact by applying fertilizer and selecting the grazing and mowing periods. Forage contains a mixture of chemical, physical and structural characteristics that determine grassland quality and the accessibility of nutrients to the target animals (Cochran et al., 2007). The term 'forage quality' relates not only to nutritive value, but also to forage intake, particularly because forage plant characteristics change with maturity. Regular and timely analyses of forage are required to determine whether or not the forage meets the daily nutritional requirements of the animals (Cochran et al., 2007).

Near infrared (NIR) spectroscopy is based on the diffuse reflectance of ground samples and is widely used for laboratory measurements of nutrient concentration and feed value in dried and fresh crop material (Berardo, 1997; Park et al., 1998; Paul et al., 2002; Ruano-Ramos et al., 1999). Fernández Pierna et al. (2006) showed that the data obtained with NIR imaging instruments corresponded with those obtained using conventional NIR spectroscopy, and that the imaging instruments had all the advantages of conventional NIR spectroscopy. These instruments provide both spectral and spatial information. In recent years, emphasis has been put on developing non-destructive methods for determining feed quality. Developed countries have undertaken a considerable amount of work aimed at developing such methods based on using NIRS technology to build spectral databases and calibrate and validate new methods.

In the past 20 years, NIR spectroscopy has been increasingly used for forage analyses. Most NIR instruments used for this purpose cover the 1,000-2,500nm part of the spectrum. The imaging instruments detect adequate NIR data, provide a quick answer and offer good repeatability. Perspective NIR cameras and spectroscopes can detect fractions of plant species and properties of plant materials (Schut et al., 2006). Imaging spectroscopy provides a way of measuring light reflectance with an array of detectors, combining spectral and spatial resolution. Imaging spectroscopy takes the NIR spectroscopy concept one step further, as it measures *in situ* leaf reflectance with high spectral and high spatial resolution in the NIR area of the spectrum. This could be useful not only for variety discrimination, but also for individual sample discrimination.

Objective

The study sought to assess the ability of the NIR-HSI system to discriminate *Festuca rubra* L., *Trifolium repens* L., *Agrostis capillaris* L., *Hieracium aurantiacum* L. and *Arnica montana* L. as pure species and measure the proportions of these species in the grass mixtures.

Material and methods

Samples were collected in the Gârda area of the Apuseni Mountains, which is part of the Apuseni Natural Park. The grass in the fields was cut in July 2010 and left to dry for 6 hours. The grass samples were mixed, and samples of the pure species (*Festuca rubra* L., *Trifolium repens* L., *Agrostis capillaris* L., *Hieracium aurantiacum* L. and *Arnica montana* L.) were extracted, dried naturally in laboratory and, after a month, milled using a Retsch Grndmx Gm200 mill and then a Cyclotec™ 1093 sample sill. The samples were placed on a piece of teflon and scanned using an SWIR ImSpector N25E HSI system (Specim Ltd) (Figure 1). These NIR line scans (or push-broom imaging spectrometer scans) involved using a cooled, temperature-stabilized Mercury-Cadmium-Telluride (MCT) detector (Xenics), combined with a conveyor belt (Burgermetrics).

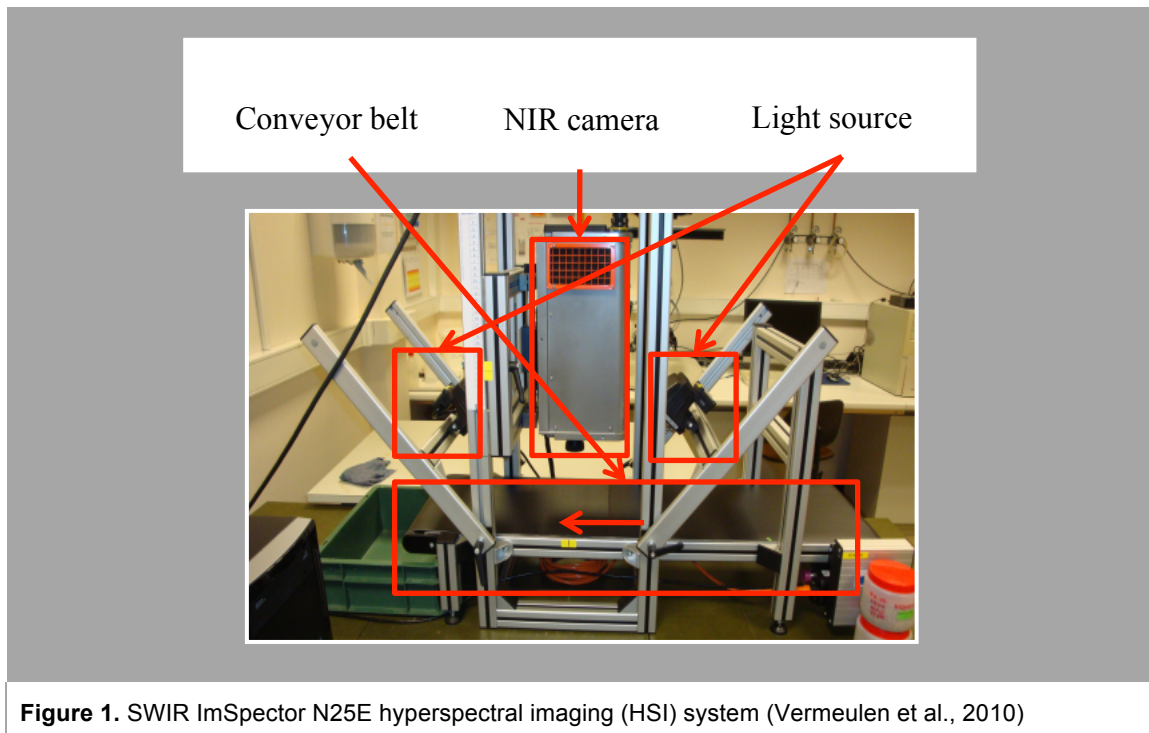


Figure 1. SWIR ImSpector N25E hyperspectral imaging (HSI) system (Vermeulen et al., 2010)

The parameters for the line scan instrument were: 1,100-2,400nm wavelength range with 6.3nm steps; 209 wavelength channels; an average of 32 scans/images; and an acquisition time of 0.05 sec/frame = 20 frames/sec = 25sec/image (Vermeulen et al., 2010).

Results and discussion

Each sample was scanned in order to register the images (Figure 2) and from these images 1,000 spectra were selected to be used in building a calibration model to highlight the samples. The calibration model contained the species listed in Table 1.

The MatLab program was used to build the model, creating a class for each species. Since the PLSDA model needs both the 'X' and 'Y' variables, the spectra, it was necessary to build the 'Y' variable with a name for each class.

Table 1. Class composition in the calibration model

Classes	Species	Botanical family
1	<i>Arnica montana</i> L. (AM)	<i>Asteraceae</i>
2	<i>Trifolium repens</i> L. (TR)	<i>Fabaceae</i>
3	<i>Hieracium aurentiacum</i> L. (HR)	<i>Asteraceae</i>
4	<i>Festuca rubra</i> L. (FR)	<i>Poaceae</i>



Figure 2. Image of a sample before being scanned with Camera NIR

After the dataset was generated, then the 'X' and 'Y' axes were built. The probability was generated using PLSDA model, and the ConfMatrix algorithm was used to visualise the equation. Table 2 presents the model for the equation and Table 3 presents the cross-validation model.

Table 2. Calibration model

Classes	AM	TR	HA	FR
AM	100	0	0	0
TR	0	100	0	0
HA	0	0	100	0
FR	0	0	0	100

Table 3. The cross-validation model

Classes	AM	TR	HA	FR
AM	98.40	0.30	1.20	0.10
TR	0.00	99.90	0.10	0.00
HA	0.80	0.40	98.80	0.00
FR	0	0	0	100

For the external validation, five more classes with a specific number of spectra for each species were registered. Table 4 presents the composition of the classes and Table 5 shows the external validation of the model. Because *Agrostis capillaris* L. was not in the calibration model, the prediction was 0.

Table 4. Class composition for external validation

Classes	Species	Botanical Family	Number of spectra
1	<i>Agrostis capillaris</i> L.(AC)	<i>Poaceae</i>	1188
2	<i>Arnica montana</i> L. (AM)	<i>Asteraceae</i>	1225
3	<i>Trifolium repens</i> L. (TR)	<i>Fabaceae</i>	1016
4	<i>Hieracium aurentiacum</i> L. (HA)	<i>Asteraceae</i>	975
5	<i>Festuca rubra</i> L. (FR)	<i>Poaceae</i>	1225

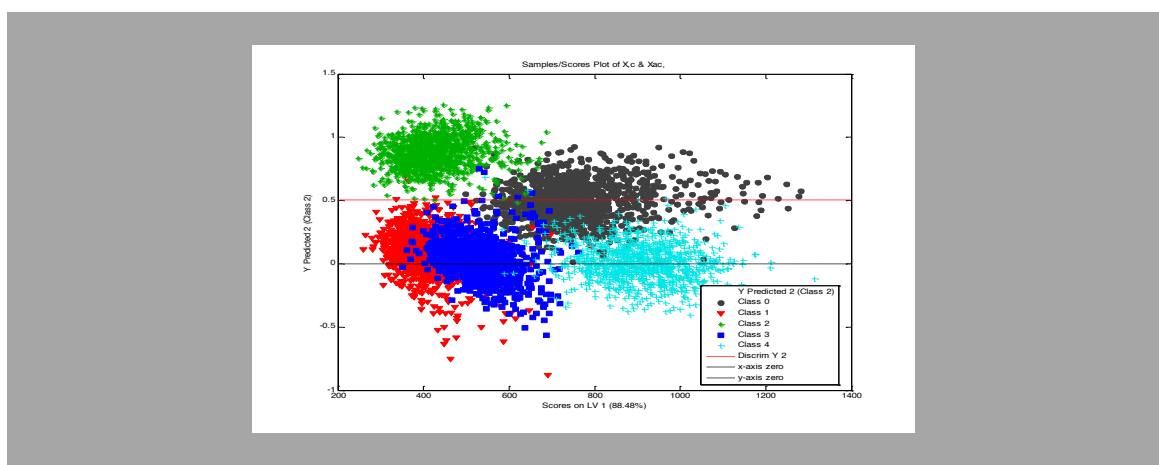
Table 5. External validation model

Classes	AM	FR	HA	TR
AC	0.00	0.00	0.00	0.00
AM	97.62	1.59	0.79	0.00
FR	2.07	96.73	1.19	0.00
HA	1.37	0.00	98.63	0.00
TR	0.00	0.00	0.60	99.40

For the external validation, the PLS Toolbox program was used. The 'X' Block axis ('X' axis = classes XAC, XAM, XTR, XFR, XHA) was the first to be validated and the model was then prepared for calibration.

Table 5 shows that the FR, TR, HA and AM species were included into initial model and, because of this, errors were registered. Class TR (*Trifolium repens* L.) had a 0.60% chance of being predicted as HA; class HA (*Hieracium arantiacum* L.) had a 1.37% chance of being predicted as AM; class AM (*Arnica montana* L.) had a 1.59% chance of being predicted as FR and a 0.79% chance of being predicted as HA; and class FR (*Festuca rubra* L.) had a 1.19% chance of being predicted as HA and a 2.07% chance of being predicted as AM.

As Figure 3 shows, class 0 (i.e., class AC, for *Agrostis capillaris* L.) is clearly visible among the other classes listed in Table 4. The AC species was used with other species only in the external validation model and is positive in this interval. It is shown in black. The figure also shows that it could not be confused with any of the other four species. This image was possible because AC was not in the calibration system and thus the confusion matrix could not be determined.

**Figure 3.** *Agrostis capillaris* L. species discrimination

More than 96% of the predictions obtained using the PLSDA model were correctly predicted, as shown by the external validation model. Schut et al. (2006) achieved similar results in the field and Daughtry et al. (2005) and Nagler et al. (2003) used imaging spectroscopy to predict yield, nutrients concentration and nutritive value. These imaging spectroscopy methods are based on a mathematical description of the differences between instruments or between recording dates, which are determined by recording a set of reference standards before measurement.

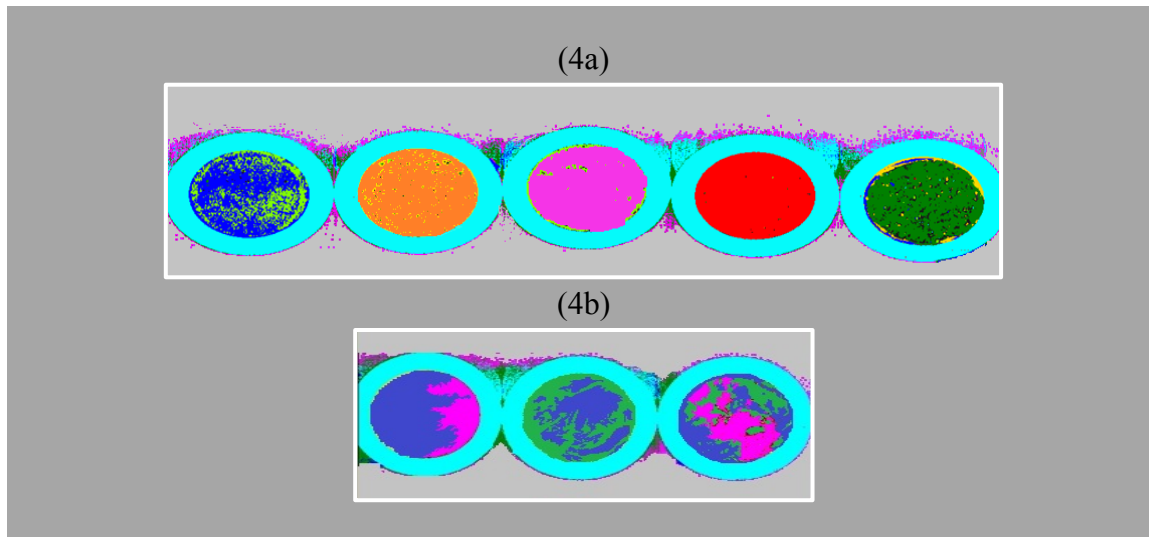


Figure 4. (a) Calibration and predicted images for the pure species samples (b) for different binary and ternary sample mixtures.

Legende: Orange = *Hieracium aurantiacum* L., blue = *Trifolium repens* L., magenta = *Arnica montana* L., red = *Festuca rubra* L., dark green = *Agrostis capillaris* L., cyan = small ring cup and grey = background.

The MatLab PLSDA model was then used to discriminate pure species from the mixtures of two or three species. It created images of pure samples, giving each species a different colour: *Hieracium aurantiacum* L. = orange, *Trifolium repens* L. = blue, *Arnica montana* L. = magenta, *Festuca rubra* L. = red and *Agrostis capillaris* L. = dark green; the small ring cup was represented by cyan and the background by grey (Figure 4a-b). This analysis was conducted to see, on previously obtained images of the floristic composition of the experimental plots, whether or not the pure species were recognized in the spectral database for the semi-natural grasslands of Romania's Gârda area. Table 6 presents the confusion matrix for this model. On average, a correct classification percentage of 91.65% was obtained. The percentage for *Arnica montana* L. was 98.76%, which shows the great ability of this procedure to detect pure species.

Table 6. Confusion matrix for the PLSDA model for discriminating pure samples

Classes	AM	FR	HR	TR	BKG	SUPPORT	AC	TOTAL
AM	98.74	0.08	0.76	0.00	0.00	0.17	0.00	99.75
FR	0.00	76.09	0.09	0.00	0.00	0.00	21.92	98.10
HR	0.00	0.08	94.94	0.48	0.00	0.00	0.24	95.74
TR	0.00	0.00	1.31	95.56	0.00	0.00	1.22	98.09
BKG	0.00	0.00	0.04	0.00	99.72	0.25	0.00	100.0
SUPPORT	0.00	0.00	0.00	3.94	0.00	91.00	4.23	99.17
AC	0.00	0.00	0.00	0.00	0.00	0.00	92.92	92.92
TOTAL	98.74	76.28	97.03	103.13	99.72	91.44	118.71	98.09

Legend: HR- *Hieracium aurantiacum* L., FR- *Festuca rubra* L., TR- *Trifolium repens* L., AC- *Agrostis capillaris* L. AM- *Arnica montana* L., BKG= background and SUPPORT= small ring cup.

PLSDA was used to determine whether or not it was possible to discriminate between the mixtures and to build a model that could be used to predict future images. The potential of using NIR-HIS to distinguish between pure species was also confirmed using a line-scan system with binary and ternary mixtures (Figure 4a-b). Figure 4a shows the image of pure samples scanned with the line-scan system. The pure samples are distinguished correctly, which is evident in the confusion matrix. Figure 4b shows that it was possible to distinguish pure samples of AM from other samples, as well as between binary and ternary mixtures of pure samples.

Conclusions

The standard prediction error (SEP) in discriminating pure species using an NIR imaging instrument (Camera NIR) was very good (99.4% for FR, 96.73% for TR, 98.63% for HA and 97.62% for AM). The objective of the next study will be to see whether or not the pure species are recognized in other images in the spectral database, and thus to assess the potential of using NIR-HSI to identify pure species. The floristic composition of grasslands can be determined only if there are data for each identified species in the species mixture in the database. Our study has shown that the calibration model can be used to discriminate species, and also can discriminate species within mixtures of two or three species. The method proposed in this paper to discriminate one species in species mixtures has real potential for determining botanical composition. The advantages of the NIR-HSI method compared with current commercial NIR spectrometry are the speed of analysis and the ability to conduct line-scans of thousands of spectra/25 s. This study help determine whether or not toxic species are present in natural grasslands used as forage for animals.

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The goal of previous chapter was to discriminate the pure grassland species as pure species and in binary and ternary mixtures. These analyses were carried out in order to see, on images obtained previously from the floristic composition of experimental parcels, if the pure species are recognized according to the spectral database or not. The next chapter aim will be to discriminate pure grassland species according to the spectral database as botanical families with the potential of NIR Hyperspectral Imaging system. The objective was to discriminate, at laboratory scale, using a line scan NIR-HSI system, different botanical families typically present in seminatural meadows. The second objective is to discriminate different grassland species according to botanical family: Poacea family (POA), Fabaceae family (FABA) and other botanical families (OBF) by NIR-HSI system. An important application for this technology is the potential discrimination of toxic plants.

Chapter 7



Article 3

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

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

Article III — DALE *et al.* (2013)
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Running head: Grassland forages quality and chemometrics

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.

Keywords

Hyperspectral imaging NIR, discriminant analyses, distinct grassland species, toxic species

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 (Nice, 2008). 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 (Nice, 2008).

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 (Mierliță, 2008), if forage quantity is limited, and when animals are very thirsty or hungry (Kline et al., 2000). 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 (Pleșa et al., 2011).

Up to now the analyses regarding determination of invasive and toxic plants presence 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 (Mierliță, 2008). 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 (Anadón et al., 2012).

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 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) (Bruneton, 2009). Alkaloids are any chemical compound, which contains group of heterocyclic nitrogenous substances of vegetable origin (Aniszewski, 2007). The toxicity of alkaloids may be variable, from low toxicity to fatal (Stegelmeier, 2011). The phenolic compounds are not uniformly distributed in plants at the tissue, cellular or subcellular levels (Naczka and Shahidi, 2004). 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 (Ignat et al., 2011). Urbas (2004) states that terpene compounds represent the largest and functionally most diversified natural group; they are also known as plant essential

oils. The toxicity of terpene compounds consist of allergic reactions at the skin or mucus levels (Bruneton, 2009). The sterols derived from plant sources are named phytosterols (Parish et al., 2008). They can be identified by the integral constituents in the lipid layers; the sterols are involved in the regulation of membranes (Kim et al., 2002). The most often found phytosterols are stigmasterol, sitosterol, brassinolide, clerosterol, cycloartenol and diosgenin (Parish et al., 2008).

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 (Fernández Pierna et al., 2012; Vermeulen et al., 2012). NIR-HSI brings a new dimension in spectroscopy: the spatial resolution. Hyperspectral images allow identification and quantification of chemical constituents within samples (Dale et al., 2013). 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. (2008) used a ground-based HSI system to map the grass chemical components directly in the field. Similar studies were made by Okamoto et al. (2007) 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 (Suzuki et al., 2012). 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. (2012) 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%.

Objective

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).

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 Ghețari 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 (Burrows and Tyrl, 2001). 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 (Dale et al., 2012).

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 Ghețari Research Centre: Latitude: 46.500 - Longitude: 22.816, Elevation: 1212 m.

Poaceae	Fabaceae	Other Botanical Families
<i>Agrostis capillaries</i> L.	<i>Lathyrus pratensis</i> L.	<i>Adonis vernalis</i> L.
<i>Anthoxanthum odoratum</i> L.	<i>Lotus corniculatus</i> L.	<i>Arnica montana</i> L.
<i>Briza media</i> L.	<i>Trifolium montanum</i> L.	<i>Clematis integrifolia</i> L.
<i>Cynosurus cristatus</i> L.	<i>Trifolium pratense</i> L.	<i>Clematis recta</i> L.
<i>Festuca pratensis</i> L.	<i>Trifolium repens</i> L.	<i>Conium maculatum</i> L.
<i>Festuca rubra</i> L.		<i>Datura stramonium</i> L.
<i>Trisetum flavescens</i> L.		<i>Equisetum palustre</i> L.
<i>Nardus stricta</i> L.		<i>Euphorbia cyparissias</i> L.
<i>Deschampsia flexuosa</i> L.		<i>Hieracium aurantiacum</i> L.
		<i>Hypericum perforatum</i> L.
		<i>Pteridium aquilinum</i> L.
		<i>Stellaria media</i> L.
		<i>Veratrum album</i> L.

Legend: These species were harvested from a semi-native meadow in Romania, Gârda area, GPS coordinates of Ghețari Research Centre: Latitude: 46.500 - Longitude: 22.816, Elevation: 1212 m.

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 1100-2400nm 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.

Table 2. List of toxic grassland species, their identified toxic compounds and some reactions that are caused by each toxic compound group (Burrows and Tyrl, 2001)

Toxic compounds	Grassland species
Alkaloids	<i>Conium maculatum</i> 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.
	<i>Datura stramonium</i> L. contains a neurotoxin, causing reduced GI mobility and appetite. Contains potent anticholinergic tropane alkaloids L-hyoscyamine and scopolamine. Similar toxicity to <i>Atropa belladonna</i> L. May also contain calystegin B2 in low concentrations.
	<i>Veratrum album</i> 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 derivatives	<i>Hypericum perforatum</i> 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).
	<i>Equisetum palustre</i> 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.
Terpenoids	<i>Clematis integrifolia</i> L. and <i>Clematis recta</i> 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 <i>Ranunculus</i> .
	<i>Pteridium aquilinum</i> 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.
	<i>Adonis vernalis</i> 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.
	<i>Euphorbia cyparissias</i> L. has 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.
	<i>Stellaria media</i> L. an annual weed, is not known to be toxic but is an invasive plant for meadows. Related genera (e.g., <i>Saponaria</i>) contain steroidal saponins (often githagenin) that are glycosides of pentacyclic oleananes.

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" (Wise et al., 2006). The PLS-DA calibration model created was validated using the "leave one out" cross validation method 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 (Wise et al., 2006). 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 (Silva et al., 2012).

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 (McGoverin, et al., 2011).

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 (%)	Poa	Faba	OBF
Predicted Poa	100	0.09	0
Predicted Faba	0	99.91	0
Predicted OBF	0	0	100
External validation results (%)	Poa	Faba	OBF
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.

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 (Wang et al., 2012). The results indicated that after the outliers were eliminated (2 % of all spectra) by Mahalanobis distance calculation, 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.

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.

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

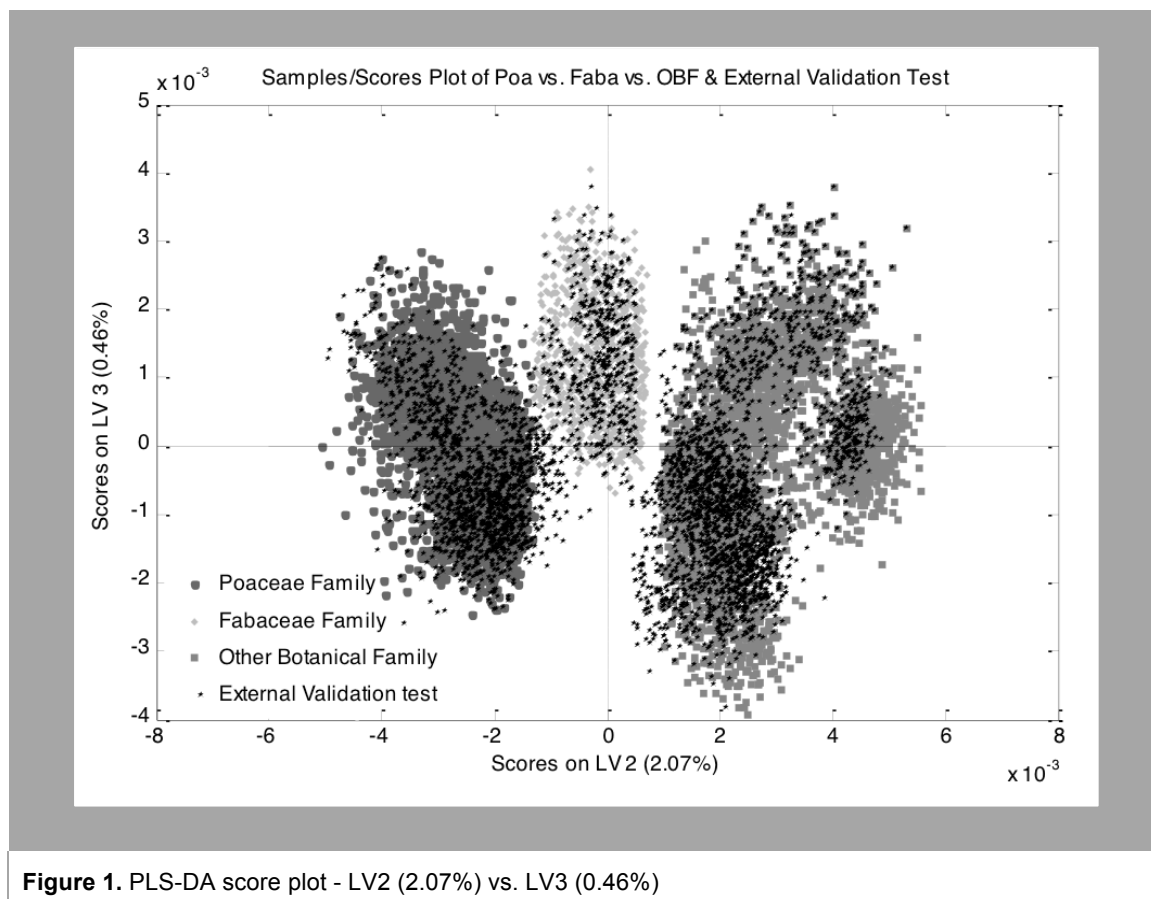


Figure 1. PLS-DA score plot - LV2 (2.07%) vs. LV3 (0.46%)

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 wasn't 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 (Osborne and Fearn, 1986).

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.

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 (1356nm), combination band of C-H stretching and deformation vibration in CH_2 (1395nm) and following by combination band of O-H stretching and deformation vibration (1482nm) have been associated to constituents as: polyphenones, alkaloids, protein and volatile and non-volatile acid (Chan et al., 2007) (Fig. 2). The same combination bands have been correlated to constituents observed by Chan et al. (2007) (2275nm, 2320nm and 2090nm respectively), because both present bands of combination and of hydroxyl groups. The band around 1193nm (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. (2008) have correlated a band in the vicinity of 1197nm (8351 cm^{-1}) to alpha-pinene alkaloid (Fig. 2).

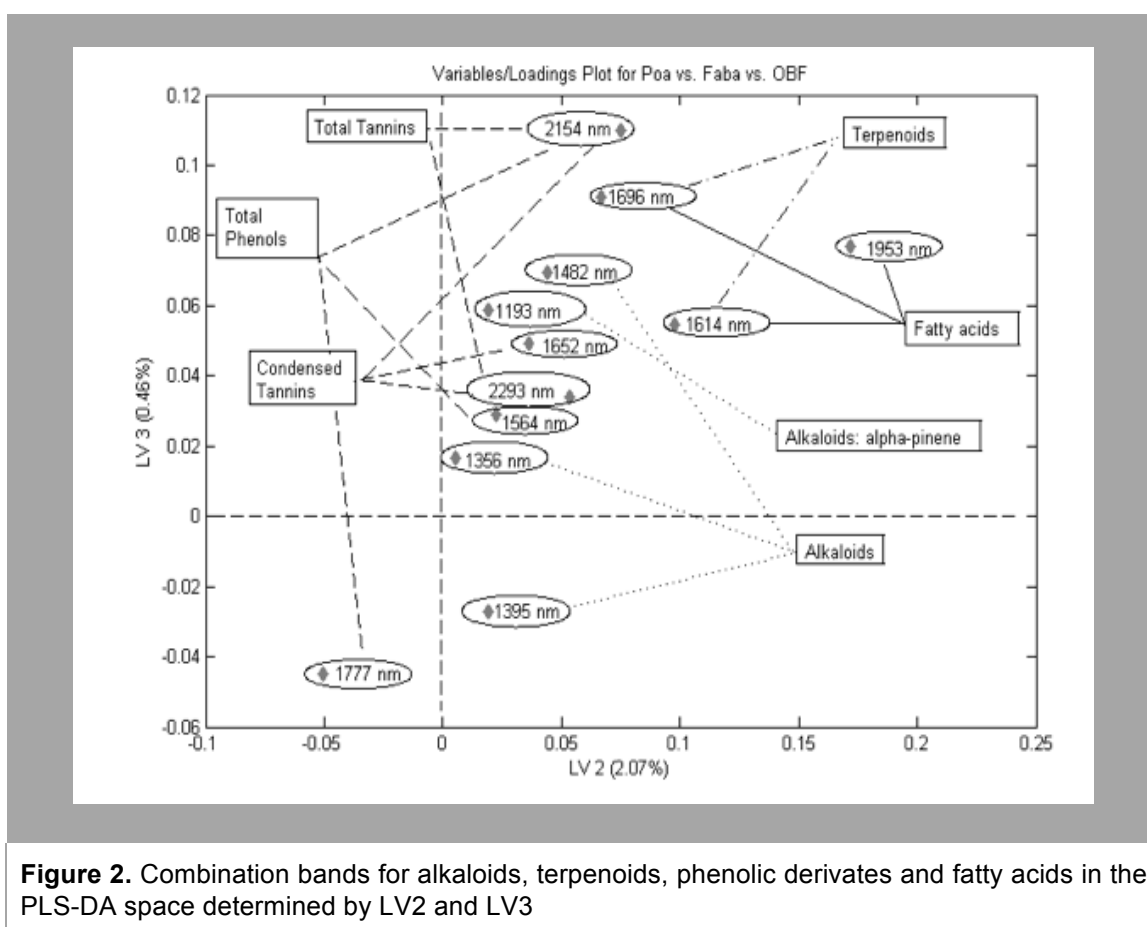


Figure 2. Combination bands for alkaloids, terpenoids, phenolic derivatives and fatty acids in the PLS-DA space determined by LV2 and LV3

Regarding the spectral regions close to 1564nm LV 2 (+), 1777nm LV 2 (-) and 2154nm 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 (Goodchild et al., 1998) (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[-]) (Chan et al., 2007). Roberst et al. (1993) noted that the main interest in

identification of tannins in *Lotus corniculatus* L. was in the vicinity of 2140nm wavelength range, which in our case could be the 2154 nm.

Another similar study to Roberts et al. (1993) was performed by Sinnaeve et al. (1993) and the specific wavelengths for condensed tannins were reported at 1460 and 2144nm for bands of O-H bonds and 1132, 1650, 2144, 2306 and 2350 for bands of C-H bonds. The 2154nm, 1652nm and 2293nm wavelengths have been also highlighted in the discrimination PLS-DA model and are similar wavelengths to 2150nm, 1604nm and 2312nm, respectively which were attributed by Goodchild et al. (1998) to condensed tannins (Fig. 2).

Phenolic compounds are omnipresent in most plant species and play an important role in resistance of plants disease (Schulz and Baranska, 2007). The spectral bands between 1250-2500nm 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 (Li and Qu, 2010). The wavelengths close to 1100-1300nm 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 (Bokobza, 2007; Ferrer Gallego et al., 2011).

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 (Cotreau et al., 2000; Crozier et al., 2000; Malking and Niyogi, 2000; Tsopmo and Kamnaing, 2011). Isoprene spectra are combinations of C-H stretching and deformation vibrations from -CH₃ groups (1696nm), C-H stretching and deformation vibrations from =CH₂ groups (1614nm) [35] (Fig. 2). Also the combinations and overtones of more fundamental bands such as C-H stretching and deformation vibrations -CH₃ groups (1696nm), C-H stretching and deformation vibrations =CH₂ groups (1614nm) and C-O stretching and deformation vibrations CO₂R groups (1953nm) can be considered as fatty acids (Cotreau et al., 2000) (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.

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 be 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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Chapter 8



General discussion and future prospects

General discussion and future prospects

General discussion

The development of infrared spectroscopy devices, techniques and methods of analysis began in the 1940s, focusing initially on organic farming (Shifrin, 2005). Later, NIR spectroscopy devices were developed for forage analyses (Hart et al., 1962). In recent decades, new approaches have been developed based on NIR hyperspectral imaging (HSI) systems (Fernández Pierna et al., 2004) and microscopic imaging systems (Yang et al., 2011). The NIR and NIR-HSI systems enable fast and accurate assessments to be made of forage quality, bringing many economic advantages, as reported by several authors in the past decade.

Various studies have been conducted over the past 13 years in Romania's Apuseni Mountains on the botanical composition of the semi-natural grasslands and their levels of fertilization. These studies provided very little useful information on forage quality, however, mainly because the grasslands are in the Natural Park of Apuseni Mountains and are part of the Natura 2000 project, where the objectives were to determine the area's biodiversity and botanical composition evolution. The initial objective of Chapter V in this thesis was to create external validation models of CRA-W's 'local' calibration model. This 'local' model was developed using mountain grassland samples from across Europe. In order to obtain better results, tropical plants were also used because of their similarity with *Arnica montana* L., a common species in the Apuseni grasslands. The use of the CRA-W calibration model in a specific area in Eastern Europe that had not been involved in the development of the model produced acceptable results. The calibration statistics, however, showed that it was necessary to conduct further chemical analyses of organic matter digestibility (OMD) and neutral detergent fiber (aNDFom) in order to improve the accuracy and to increase the number of samples. For ash, crude fat (EE), aNDFom, Lignin (sa) and acid detergent fiber (ADF), the calibration model could be improved by increasing the number of samples in order to reduce variability and increase sensitivity.

In summary, mineral fertilization reduced species richness in the grasslands, but increased productivity. The optimal fertilization proportion used to maintain plant diversity in the grasslands had been studied by Vîntu et al. (2008), Păcurar et al. (2010), Morea et al. (2011) and Rotar et al. (2012). A rational fertilization application could substantially increase production and biodiversity and improve fodder quality (Vîntu et al., 2008). Hejcman and Schellberg (2009) provided an extensive review on the long-term fertilization of the grasslands. It showed that the effects of short- and long-term fertilizer application on plant species composition differed substantially. It also described the differences between grasslands at lower altitudes and those in the sub-alpine vegetation zone, where the effects of fertilization varied from zone to zone. The review suggested that the key to successful grassland management lay in understanding the impact of fertilization applications on inter-specific plant competition. Our objective was to create a viable NIR model for predicting and determining

the chemical composition and OMD of these grasslands, whatever the botanical composition or fertilization treatments. Organic and mineral fertilization has been applied in the Apuseni Mountains over a long period, creating changes in grassland biodiversity (Păcurar et al., 2010). In extreme cases, after long-term fertilizer application the plant community will need several decades, or more, to adapt. In some cases the changes in plant species composition are irreversible (Hejcman and Schellberg, 2009). Hejcman and Schellberg (2009) pointed out that, on oligotrophic soils, long-term N application could lead to a predominance of unproductive species and/or low forage quality. Mineral fertilization reduces crude protein (CP) content because of the limited presence of Fabaceae species in the grasslands (Fabaceae coverage in the control treatment was 17.25% vs. 6.25% in the 150:75:75/ha NPK treatment). Morea et al. (2011) showed that biodiversity decreased considerably in the Apuseni Mountains after long-term fertilization, especially with regard to species treated with greater quantities of fertilization (*Carex pallescens* L., *Luzula multiflora* (Ehrh.) Lej., *Arnica montana* L., *Carlina acaulis* L., *Gymnadenia conopsea* L., *Hieracium aurantiacum* L. and *Prunella vulgaris* L.). Under extreme soil and weather conditions (average precipitation 997.4 mm/year), organic matter mineralization slowed down as a result of long-term NPK application (Hejcman and Schellberg, 2009).

In the Apuseni grasslands, low proportions of botanical families were registered over a long period (8 years) of NPK fertilization, and species richness contributed to changes in protein content. There was greater variability in crude fiber (CF), aNDFom and ADF content in the mineral or organic-mineral fertilizer treatments than in the organic ones, and the OMD values were higher in the organic treatments than in the mineral or organic-mineral ones. When applying organic, mineral or mixed fertilizers, the highest CP recorded was about 15.03% (in the 100:50:50/ha NPK treatment).

Organic-mineral fertilization has not been used by farmers in the Apuseni Mountains. Our recommendation is to use mineral fertilization with NPK doses of less than 100:50:50 in order to improve productivity. Organic fertilization could also be used to complement and maintain biodiversity and forage quality. Both our experience and the literature suggest that a realistic approach would be to apply 10 t/ha cow and horse manure + 50:25:25/ha NPK treatment in order to maintain the oligotrophic grassland in this mountainous environment and to increase fodder quality and productivity. Our analysis could help scientists in a range of disciplines to provide answers to practical and economic issues relating to fodder crops. Hejcman and Schellberg (2009) also noted that the long-term manipulation of plant communities, continuous observation and the documentation of long-term fertilization experiments are indispensable. Existing models and predictions require continuous validation.

Chemometric tools and pre-processing treatments were applied to the spectra in order to maximize the advantages of NIR spectroscopy and recent NIR-HSI systems. NIR and NIR-HSI are reliable, non-destructive and rapid systems for predicting chemical composition and the OMD value, both quantitatively and qualitatively. Knowledge of chemometric tools, pre-processing treatments and calibration model assessments is important for extracting the maximum amount of informations from the analyzed samples (Wise et al., 2006). Greater model reliability will encourage online and in-field use (especially with increased instrument miniaturization and portability) of these technologies in agriculture, which should improve productivity and reduce the costs of process monitoring and product inspection. More robust calibration and validation models are therefore needed in order to create models that cater to the data provided (Wold, 1995; Wise et al., 2006).

HSI applications in the agricultural range from macroscopic approaches to more focussed ones. If better predictions are to be obtained, it is necessary to improve this technique (Yang et al., 2003). In the NIR-HSI systems, data are collected directly from the samples, mainly from the surface or the inside, where the wavelength of penetration is about 100-200 μm maximum (Dahm and Dahm, 2007). For specific component detection, this system comprises some weak points (they relate, for example, to sampling, pixel overlapping, penetration depth and lack of fit). In addition, unlike other imaging systems, NIR-HSI can provide spatial and spectral informations, as well as multi-constituent information, and it can identify minor constituents.

The discrimination between pure plant samples, binary and ternary mixtures, as well as among botanical families, was demonstrated. Future studies need to be conducted using NIR-HSI to see if pure species can be detected in other images in different spectral databases, alongside the detection of toxic or invasive species. The differences between Poaceae and Fabaceae relate to differences in protein, starch and fiber content. High correlation and low error ($R^2=0.88$ and $\text{SECV}=0.135$) suggest that the botanical composition of meadows could be determined. This technique allows spatial information as well as information on sample heterogeneity to be obtained. The technique's originality lies in sample preparation (dry powder) and plant discrimination. More work is needed to refine the discrimination among these families and to demonstrate unequivocally which chemical compounds account for the differences. It is important to emphasize that the floristic composition of grasslands can be determined only if the spectra of each identified species are included in the databases used to calibrate the system. This preliminary study indicated that this technique could be very useful in determining the floristic composition of hays harvested from grazing lands. In future work on the species discrimination based on toxic components, chemical analyses should be conducted on all targeted compounds, to show clearly the differences in the NIR images. This approach could be of great interest for the management by the farmers for the security of grasslands.

An important objective of this thesis was to use NIR-HSI to discriminate species in the semi-natural grasslands of the Gârda area in Romania's Apuseni Mountains, the first time such work had been undertaken in the country. Two studies demonstrated the successful use of NIR-HSI to identify the main species (*Festuca rubra* L., *Trifolium repens* L., *Agrostis capillaris* L., *Hieracium aurantiacum* L. and *Arnica montana* L.) and discriminate among them in two- or three-species mixtures. The standard error of prediction (SEP) in the discrimination of the pure species using the NIR-HSI system was very good: 99.40% for *Festuca rubra* L., 96.73% for *Trifolium repens* L., 98.63% for *Hieracium aurantiacum* L. and 97.62% for *Arnica montana* L. The *Agrostis capillaris* L. species was not used for the calibration model, but it was used for the validation model in order to establish whether or not the method was working. Once good results had been obtained with the validation model, the next study objective was to see if the distinct grassland species were discernible in other images of the two- or three-species mixtures. Based on the spectral database for the Apuseni Mountains and using NIR-HSI to identify distinct species, on average a correct classification of 91.65% was obtained for the species mixtures. For *Arnica montana* L., the percentage was 98.76%, indicating the great ability of this procedure to detect distinct species.

Following the success of the procedure, a new study focusing on the discrimination of botanical families (Poaceae, Fabaceae and other botanical families [OBF]) was conducted. Poaceae and Fabaceae species dominate in the Apuseni grasslands. The OBF species include mainly toxic plants. The partial least squares discriminant analysis (PLS-DA) method based on NIR-HSI was used successfully to discriminate the grassland species according to botanical families.

This thesis highlights the growing interest in using HSI in the agricultural. HSI has been used successfully for controlling and predicting food quality based on specific parameters of food component analyses of firmness, tenderness, color parameters, protein, fat and carbohydrate contents and optimal harvest period. Various HSI applications have been described in the bibliographic review. Increasingly, scientists are facing the new challenge of online detection of diseases and chemical, microbial or biological contaminants (Gowen et al., 2007). The HSI system has been used successfully as both a ground-based and satellite-mounted technique to describe crop canopy variations. Its disadvantages, however, include its cost and its limitations in measuring. Because it can be used successfully in grassland studies on a large scale, the NIR-HSI system appeared worth investigating for species discrimination in dried and milled samples. To date, this issue has not received much attention (Dale et al., 2013). The HSI system has been used to develop discrimination techniques and calibration models.

Future prospects

With regard to future applications other than those in the agriculture and in food quality and safety, low-cost NIR or portable NIR systems, as well as NIR-HSI systems, need to be developed for specific tasks. One of the advantages of the NIR and NIR-HSI techniques is their economic value compared with classical wet chemistry methods, particularly with regard to savings in time and in reagent and waste costs. With the NIR technique, it has been shown that a good calibration model can predict dry matter forage samples with a low SEP (less than 2.5).

A new challenge for researchers in Romania, as well as for research institutions around the world, is to use these 'local' calibration models in order to perform NIR analyses to determine forage quality and chemical composition and nutritional value (energy and protein of forages) for ruminants feeding. Work could also be done on ameliorating or developing new calibration models by applying different fertilization levels to grasslands worldwide or by adding different environmental situation in order to obtain a robust model. Work could also be done exploring the use of NIR techniques in creating calibration and external validation models for different forage types (e.g., fresh forage, hay, sward or forage silage). The potential complexity of samples would provide a challenge, and it would be interesting to verify if the calibration and validation models gave equivalent statistical results for fresh forages as for dry matter samples.

The NIR technique could also be used to determine the botanical composition of forage in terms of the Leguminosae and Grass percentages. Similar work on calibration strategies, based on diverse artificial hand-sorted samples, was conducted in the 1980s for botanical family determination, based on many artificial mixtures. Other studies using NIR calibrations to determine botanical composition were conducted using artificial samples of species in known proportions. A set of samples was scanned under different conditions for example on different days with different temperatures and humidities. Relatively good calibrations and validation statistics were achieved with independent samples (Locher et al., 2005). In some cases, the use of a calibration strategy based on real samples is not possible (e.g., because the species for which the calibration is built is difficult to separate). In this case, a calibration strategy based on the spectra of botanically pure samples can be used, so long as some important points are borne in mind. There is not much gain in physically mixing only a few botanically pure samples in order to obtain a series of artificial samples ranging widely in composition. Although good calibrations for botanical composition based on botanically pure samples using

NIR have been described, a major challenge of this thesis was to perform similar applications using NIR-HSI systems.

The results reported using PLS-DA models based on the method described in this thesis offer great potential for determining botanical composition. They provided the answer to the question: Can the floristic composition of grasslands be determined using NIR-HSI? The answer is: Yes, as long as the database contains data for each identified species in the sample mixtures. With regard to botanical family determination, which constituted an initial step in the development of a procedure for detecting and quantifying toxic species in forage harvested from natural or semi-natural grasslands, the results indicated the need for further studies in this area. The NIR-HSI technique has been shown to be an interesting tool for discriminating among plant species harvested from grazing lands. In the future the quantitative aspect of the grassland species and also the complexity of samples mixtures (more than four samples) could be an interesting subject for the NIR-HSI applications.

The use of the technique should facilitate the identification of optimal wavelengths/wavebands in different applications and thus provide the low-cost NIR-HSI systems necessary for the more widespread use of this technology in the future. This NIR-HSI system could be used successfully to measure crop N status during the development of the plant, to detect over-fertilized crops due to chlorophyll saturation and to determine the biomass per unit of soil surface or the plant tissue electrical properties that could be used for medical purposes (Muñoz-Huerta et al., 2013). Although the initial application of NIR-HSI for pure grassland species has produced satisfactory results, new analyses need to be conducted to predict pure grassland species, forage quality and safety attributes. The NIR-HSI instruments offer real-time prediction with an online system that needs specific wavelengths in order to determine quantitative and qualitative analyses for chemical, nutritional, physical or sanitary parameters for human aspects (characteristics such as bruises, tenderness, freshness, color, pH) or agricultural aspects (chemical components, the OMD value, biomass, yield, nematode, fungi, foreign materials and botanical composition). Given that the prediction of grassland floristic composition is very important regarding animal dry-matter intake, the next stage would be to standardize reference methods for pure sample detection. To date, analyses to determine plant species have been based on botanical composition evaluation, including visual observation, which is a subjective and time-consuming method involving identifying plants directly in the field.

Based on the spatial and spectral information obtained using the NIR-HSI system, the spectral database is quickly collected and hundreds of spectra are registered, a great advantage compared with NIR systems. There are some disadvantages with the NIR-HSI technique, however, that could limit its application (e.g., purchase cost, computational challenges posed by the production of a substantial amount of informational data, varying computer capacity affecting image acquisition time, and time-consuming data processing and classification).

The industrial application of this technique for food inspection is limited mainly by the hardware speed needed for rapid image acquisition and the analysis of a huge amount of data. The technique could be used as a key component of computer-integrated systems and provide opportunities for several practical applications related to, for example, chemical issues of feedstuffs and food (protein, moisture and fat concentrations) or safety aspect (microbial and fungal contamination or parasite detection) or physical analyses of powder mixtures (e.g. cereals, flours,). An improvement in pre-processing speed and robustness, particularly in terms of model reliability, could encourage more widespread online use of this technology in

the agricultural and agro-food industries, reducing the costs of the process monitoring and product inspection.

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- Dale, L.M., Thewis, A., Boudry, C., Rotar, I., Dardenne, P., Baeten, V., Fernández Pierna, J.A. (2013). Hyperspectral imaging applications in agriculture and agro-food product quality and safety control: A review, *Appl Spectrosc Rev.* 48 (2) 142-159.
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Conferences

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