PC&CA – Tool for Polyphenols Content Monitoring at the End of Spring Depending on Organic and/or Mineral Fertilization of Perennial Grassland

Monica HARMANESCU*

Faculty of Agriculture. Banat's University of Agricultural Sciences and Veterinary Medicine "Regele Mihai I al Romaniei" from Timisoara, Calea Aradului, no.119, PC 300645, Romania.

*corresponding author, e-mail: monica.harmanescu@yahoo.com

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Abstract

The present research proposed to use Principal Components & Classification Analysis (PC&CA) as tool for the polyphenols content monitoring of perennial forages, at the end of May 2008, from a Romanian grassland fertilized since 2003. The grassland soil was Calcic Luvisol. StatSoft – STATISTICA Version 10 performed the matrix correlation of PC&CA. The ten trials unfertilized or with different fertilization were the PC&CA cases. The active variables of PC&CA were the floristic composition components: *Poaceae* (GMay08%), *Fabaceae* (FMay08%), others botanical families group (OMay08%), *Calamagrostis epigejos* (Ce%), *Festuca rupicola* (Fr%), *Lathyrus nissolia* (Ln%), *Lathyrus pratensis* (Lp%), *Filipendula vulgaris* (Fv%) and *Rosa canina* (Rc%). Perennial forages polyphenols content at the end of May 2008 and mineral nitrogen fertilization data were supplementary variables. The correlation coefficient for total polyphenols content (PMay08) and others botanical families group (OMay08%) was positive: 0.704. In addition, the correlation coefficients of PMay08 and Fv% or Rc% were positively and higher than 0.700. Mineral nitrogen application influenced positively the *Poaceae* component (correlation coefficient - 0.915). The PC&CA can represent a helpful tool for polyphenols content monitoring of grassland perennial forages at the end of spring, depending on fertilization.

Keywords: grassland, multivariate analysis, polyphenols.

Introduction

Nowadays the multivariate analysis techniques became like salt in dishes for statistic investigations of a high number of samples characteristics (variables). Principal components analysis, considered an "algorithm in biometrics" (Karamizadeh *et al.*, 2013), or an "exploratory tool" (Henderson, 2006), is one of the oldest and more utilized multivariate techniques over time. There are many examples that demonstrate the accuracy of information extracted from many experimental data by principal components multivariate analysis facilities: fraud detection in automobile insurance domain (Brockett *et al.*, 2002), digital images classification (Ehsanirad and Kumar, 2010; Ostaszewski *et al.*, 2015), missing data values identification based on probabilistic

formula of a theoretical mathematical model (Ilin and Raiko, 2010; Dray and Josse, 2015), pattern classification of drugs in pharmacology (Bober *et al.*, 2011), cancer diagnose (Bair *et al.*, 2006), pattern analysis of wine (Camara *et al.*, 2006; Giaccio and Vicentini, 2008; Fu *et al.*, 2012) or green tea (Fu *et al.*, 2012), animal behavior depending on environmental conditions (Budaev, 2010), quality evaluation of dairy products (Chapman *et al.*, 2000), fruits classification based on qualitative parameters (Zaragoza, 2015), plants diversity (Casas and Ninot, 2003; Henderson, 2006), foliage identification of plant species based on different characteristics (Ehsanirad and Kumar, 2010; Kadir *et al.*, 2012), genetic variability of plants germplasm (Evgenidis *et al.*, 2011;

Mahendran *et al.*, 2015), submergence tolerance of flooded plants in river floodplains (Mommer *et al.*, 2006), selection of the most important criteria of *Triticum aestivum* genotypes to improve genetically the yield of bread wheat (Beheshtizadeh *et al.*, 2013), etc.

This multivariate technique use a linear model in orthogonal projection for extracting essential observations based on amount of the data variance (Casas and Ninot, 2003; Henderson, 2006; Giaccio and Vicentini, 2008; Ilin and Raiko, 2010; Karamizadeh *et al.*, 2013; Dray and Josse, 2015; Mahendran *et al.*, 2015; Ostaszewski *et al.*, 2015). Briefly, it can be process using principal component analysis many experimental data transposed in matrix form: correlation matrix or covariance matrix. As noted Martin *et al.* (1996), this technique reduce the multidimensional results to smaller dimensions (some principal components), for easier human understanding, without losing the most important information (Henderson, 2006; Karamizadeh *et al.*, 2013). It can be exploited the contribution of each variable to the principal component linear model interpreting the factor loadings (Martin *et al.*, 1996). According to Budaev (2010), when principal component analysis is performed, it is recommended to note all the information regarding the type of data matrix, the numbers of principal components (factors) which describe the studied process, the eigenvalues, the factor loadings, the factor scores, the correlations of the original matrix, etc.

The topic of the present study was to use the Principal Components & Classification Analysis multivariate technique, implemented in StatSoft - STATISTICA Version 10, as a tool for total polyphenols content monitoring of grassland perennial forages, at the end of May 2008 (Romanian end of spring), depending on organic and/or mineral fertilization. Polyphenols are natural compounds synthesized in plants as secondary metabolites, with antioxidant and free radical scavenging properties (D'Archivio *et al.*, 2007; Shivashankara and Acharya, 2010; Ozcan *et al.*, 2014; Surai, 2014; Reis *et al.*, 2016). According to Bennick (2002), polyphenols appear as vegetal defense mechanism to survive against stress factors as predator's damages or microbial population attacks, including for the seeds protection during green stage to maturity. There are a lot of polyphenols compounds as phenolics acids,

flavonoids, hydrolysable tannins, condensed tannins, etc. (Bennick, 2002; D'Archivio *et al.*, 2007; Ovaskainen *et al.*, 2008; Amararathna *et al.*, 2016) mainly in fruits, leaves and seeds (Petti and Scully, 2009).

Polyphenols became important in many research fields especially in the last twenty years (Scalbert *et al.*, 2005; D'Archivio *et al.*, 2007). It is well-known their benefic role for human health to prevent different type of cancer, cardiovascular and degenerative diseases (Scalbert *et al.*, 2005; D'Archivio *et al.*, 2007; Petti and Scully, 2009; Shivashankara and Acharya, 2010; Yang *et al.*, 2012; Balekar *et al.*, 2014; Amararathna *et al.*, 2016; Reis *et al.*, 2016). Furthermore, there were elaborated many studies with regard to the effects of polyphenols from vegetal sources in animal nutrition (Bennick, 2002; Brenes *et al.*, 2010; Lipinski *et al.*, 2017). Sharma *et al.* (2011) affirmed, for instance, that in early lactation period the dairy cow's organism is exposing to a high risk of mastitis and others diseases because of a high level of oxidative stress correlated with a low antioxidant protection comparatively with advanced pregnant cows. Salami *et al.* (2015) spoke as well in a review about the importance of antioxidants compounds on the well-being of animals, including on the quality of the products with animal origin (food shelf life, organoleptic and nutritional values). They proposed the improvement of UE legislation regarding the antioxidants content for animals feed and highlighted the necessity to classify them in a defined category: additives for feed or drugs for veterinary usage.

In Mediterranean countries, ruminants as cattle, sheep or goat beneficiate of polyphenols especially by grazing from grasslands forages (Lamy *et al.*, 2011). In Romania, the pastures and hay meadows forages represent also an important nutritional source for animal feeding. That's why the polyphenols content monitoring of Romanian grasslands forages is required.

Materials and methods

StatSoft - STATISTICA Version 10 performed the Principal Components & Classification Analysis (PC&CA). The results of gravimetric floristic components from ten studied trials of a hill permanent grassland, with five replications for each case, were used as active variables of PC&CA model: GMay08% - *Poaceae*, FMay08% -

Fabaceae, OMay08% - others botanical families group, Ce% - *Calamagrostis epigejos*, Fr% - *Festuca rupicola*, Ln% - *Lathyrus nissolia*, Lp% - *Lathyrus pratensis*, Fv% - *Filipendula vulgaris* and Rc% - *Rosa canina*. As supplementary variables of PC&CA were introduced the total polyphenols content of grassland perennial forages (PMay08) and mineral nitrogen fertilization data (N%). The eigenvalues of the first two principal components (Factor 1 and Factor 2) of PC&CA were 3.31 and 2.76, which described around 67.5% of total variance of experimental results (PC1 - 36.8%; PC2 - 30.7%). The eigenvalue of the third principal component was 1.19 and described with PC1 and PC2 more than 80.7% of total variance.

All the recorded data were obtained at the end of May 2008 in Caras-Severin district, Gradinari (N: 45°12' latitude; E: 21°60' longitude). According to the *Meteorological Station Archive* from Oravita, for December 2007-May 2008 period the average value of rainfall was more than 60 mm and of the temperature around 7°C (higher in April-May 2008: around 15°C). There were more than 15 frost days in December 2007-February 2008. In 2003, it was for the first time when the permanent grassland was fertilized with fermented sheep manure and/or mineral. The complete randomized block design was chosen for the experimental field, with 25 m² for each trial. Five replications for each fertilization case/trial were organised. For the present study the unfertilized case was noted Z. The organic fertilization was marked as O cases, dependent by the sheep manure doses: 20 t/ha (O1), 40 t/ha (O2) and 60 t/ha (O3).

The organic-mineral fertilization was noted as OM cases, all heaving a constant dose of 20 t/ha sheep manure and different doses of mineral fertilizers in addition: OM1 - 50 P₂O₅ (kg/ha); OM2 - 50 P₂O₅ (kg/ha) + 50 K₂O (kg/ha); OM3 - 50 P₂O₅ (kg/ha) + 50 K₂O (kg/ha) + 50N (kg/ha). The mineral fertilization was noted as M cases (M1, M2 and M3), fertilized with constant doses of mineral phosphorus (50 kg/ha) and potassium (50 kg/ha) and different doses of mineral nitrogen in addition: 100, 150, and respectively (100+100) kg/ha. It was applied yearly the ammonium nitrate, superphosphate, potassium salt and NPK complex mineral fertilizers, while the organic fertilizer at each two years, on the Calcic Luvisol. The soil hydrolysis pH values (SR ISO 10390/1999) in May 2008 were

between 5.7 - 6.3, while the humus and the soil total nitrogen contents varied in 5.6 - 8.5% and 0.3 - 0.5% range.

It was determined the total polyphenols content of grassland perennial forages by VIS-spectrophotometry using Folin Ciocalteu reagent, prepared with lithium salts to eliminate the samples solutions turbidity, as was described by Folin and Ciocalteu (1927). The work protocol respected the samples and reagents quantities adapted by Gergen (2004). The samples ethanol extracts (70%) were prepared for 1 g of sample. The ethanol extracts of perennial forages were diluted 1/20 before the reagents were added. The samples absorbance monitoring was made by UV/VIS Specord-205. The results for total polyphenols content were calculated as µM gallic acid/g. The linearity of calibration curve was obtained ($R^2 > 0.99$).

Results and discussions

In the local soil and climatic conditions of Gradinari (Caras-Severin) at the end of May 2008 (the Romanian end of spring), the grassland perennial forages growth without fertilizers application, in natural conditions of Z case, accumulated the highest total polyphenols content (181 µM gallic acid/g) comparatively with the others nine studied cases with different organic and/or mineral fertilization. Smaller than in Z case, but higher than others fertilization cases, it was the average value of total polyphenols contents of perennial forages of organic fertilization cases with 20 t/ha sheep manure, exclusively or with mineral fertilizers (O1, OM1, OM2 and OM3): 98.5 µM gallic acid/g. For the perennial forage of O1 (exclusively fertilized with 20 t/ha sheep manure) it was obtained the highest total polyphenols content (106 µM gallic acid/g) reported to all others O and OM cases. The different doses of mineral nitrogen fertilization, mixed with organic or others mineral fertilizers (OM3, M1, M2 and M3), decreased the average value of total polyphenols contents of perennial forages from Gradinari grassland: 78.5 µM gallic acid/g. The average value of total polyphenols contents of perennial forages from exclusively mineral fertilization (M1, M2 and M3) was 74.7 µM gallic acid/g. The smallest total polyphenols content (60 µM gallic acid/g) was found in exclusive mineral fertilization case (M2), with the

Table 1. The correlation coefficients of PC&CA (active & supplementary variables)

Variables	G% May08	F% May08	O% May08	Ce%	Fr%	Ln%	Lp%	Fv%	Rc%	*N%
FMay08%	-0.964									
OMay08%	-0.229	-0.039								
Ce%	0.825	-0.719	-0.448							
Fr%	0.339	-0.458	0.385	-0.228						
Ln%	-0.586	0.509	0.366	-0.362	-0.334					
Lp%	-0.364	0.454	-0.301	-0.164	-0.315	-0.274				
Fv%	0.110	-0.314	0.710	-0.067	0.242	-0.035	-0.232			
Rc%	-0.012	-0.148	0.591	-0.170	0.226	-0.020	-0.356	0.524		
*N%	0.915	-0.848	-0.333	0.897	0.063	-0.456	-0.298	0.154	-0.187	
*PMay08	-0.222	0.032	0.704	-0.378	0.216	-0.047	-0.017	0.710	0.873	-0.347

Note: *PMay08 as μM gallic acid/g

Figure 1. The PC&CA projection of the 10 studied cases on PC1xPC2 plane

highest mineral nitrogen dose applied in one step (150 kg/ha). Sereme *et al.* (2016) highlighted also that organic fertilisation with fermented manure (livestock) increase the phenolics compounds content of plants comparatively with mineral N P K fertilizers.

The floristic composition of perennial forages was monitored gravimetric in May 2008 and some components were used as active variables for PC&CA multivariate technique. The *Poaceae* species (GMay08) were presented in perennial forages, depending on fertilization, as following (average values): 41% (Z), 21% (O cases), 24% (OM cases) and 74% (M cases). *Calamagrostis epigejos* (Ce) was not present in unfertilized (Z) and in 20t/ha exclusive sheep manure fertilization (O1) cases, but covered (average values) the

grassland soil of: O2 and O3 (5.5%); OM1, OM2 and OM3 (5%); M1, M2 and M3 (45%). *Festuca rupicola* (Fr) was quantified (average values) in Z case perennial forage around 37%, 10% in O cases, 16% in OM cases and 24% in M cases. The *Fabaceae* family (Fmay08) was stimulated by organic fertilization, heaving the following average values: 54% in O and OM cases (smaller in OM3 - 42%), and 4% in M cases. Without fertilization, the *Fabaceae* family quantified 21% (Z). *Lathyrus nissolia* (Ln) and *Lathyrus pratensis* (Lp) were generally in maximum 4%, respectively 36%, in perennial grassland covering, higher in organic fertilized cases and smaller in unfertilized and mineral fertilization cases. The perennial plants species of others botanical families group (OMay08) covered around 38% of

Figure 2. The PC&CA projection of active and supplementary variables on the PC1xPC2 plane

Table 2. The factor loadings of PC&CA (correlations between PC1, PC2 and variables)

Variables	PC 1 (Factor 1)	PC 2 (Factor 2)
G% May08	0.974	-0.201
F% May08	-0.986	-0.052
O% May08	-0.055	0.938
Ce%	0.725	-0.491
Fr%	0.450	0.462
Ln%	-0.605	0.310
Lp%	-0.435	-0.462
Fv%	0.280	0.731
Rc%	0.170	0.735
*N%	0.857	-0.323
*P May08 (μM gallic acid/g)	-0.024	0.776

Note: *P May08 as μM gallic acid/g

permanent grassland soil in unfertilized case (Z), heaving smaller percents in the others cases: 23% (O cases), 25% (OM cases) and 23% (M cases). *Filipendula vulgaris* (Fv) was quantified in maximum 10%, the highest content being in Z and M3 cases. The highest gravimetric percents of *Rosa canina* (Rc) were in Z case (11%).

The correlation coefficients of active and supplementary variables of PC&CA are in Tab. 1, while the both projections of the 10 studied cases and of the active & supplementary variables on the PC1xPC2 plane are in Fig. 1 and Fig. 2:

All the recorded data noted for the gravimetric floristic composition of the ten perennial

forages trials, used as active variables of PC&CA multivariate technique, were actually the green mirror of the ten permanent grassland microecosystems conditions at the end of spring (May 2008), unfertilized or influenced anthropically by fertilization (organic and/or mineral). Analyzing objectively Fig. 1 and Fig. 2, it can be noticed that total polyphenols content (P May08) was positively influenced by the existing normal ecological conditions of permanent grassland, without fertilizers application (Z case). A possible explanation for these observations can be the nutritional stress which affect the perennial plants in unfertilized case conditions of perma-

nent grassland, especially regarding the presence of bioaccessible forms of nutrients in soil.

Another important aspect that can be remarked is that the highest total polyphenols content was related to the highest gravimetric percent of Fv% and Rc% (species from others botanical families group), which was quantified in stress conditions of unfertilized Z case. These observations can be sustained superposing Fig.1 on Fig. 2 and reflecting on the correlation coefficients of *PMay08 (as supplementary variable) with O%May08, Fv% and Rc% (as active variables), presented in Tab.1: 0.704, 0.710, and respectively 0.873. Hamouz *et al.* (2010) concluded also that the presence of stress factors (different climatic and soil conditions) during vegetation stage of plants increase the polyphenols concentration.

It must be mentioned also that supplementary variable *PMay08 and active variables O%May08, Fv% and Rc% were the main components with the highest positive influence in principal component PC2 (Factor 2) as shown Tab. 2: 0.776, 0.938, 0.731, respectively 0.735.

On the other side, the principal component PC1 (Factor 1) was characterised as positive components mainly by G%May08 and Ce%, both active variables, and N% fertilization data as supplementary variable (Tab. 2): 0.974, 0.725, respectively 0.857. Nitrogen mineral fertilizer application tilting the balance in favour of *Poaceae* species in grassland perennial floristic covering, the correlation coefficients *N% - G%May08 and *N% - Ce% being 0.915, respectively 0.897, as it was shown in Tab. 1, Fig. 1 and Fig. 2. Balcerek *et al.* (2009) found small quantity of phenolics in the aerial part of some *Poaceae* species. Regarding the correlation between the supplementary variable *N% and the active variable F%May08, the correlation coefficient was high and negatively: -0.848 (Tab. 1, Fig. 1 and Fig.2). The negative balance of *Poaceae*/ *Fabaceae* ratio of grassland perennial floristic list, depending on fertilizers application, was confirmed also by the high negative correlation coefficient of active variables G%May08 - F%May08: - 0.964 (Tab. 1).

The present study reflects that PC&CA (correlation matrix, factor loadings, both projections of cases and active & supplementary variables fitted on PC1xPC2) can be a helpful tool to extract relevant information for total polyphenols content monitoring of perennial

forages from grassland depending on mineral and/or organic fertilization, even when a high number of experimental results (variables) characterize the cases.

In the future, because the forages of Romanian pastures and meadows represent natural resources for herbivores feeding, perhaps the polyphenols content of forages will be monitor for routine as one of the most important qualitative parameter, especially in perennial grassland herbaceous covering. It will be possible also to obtain perennial forages as functional feed with antioxidant activity, cheaper than others sources, by implementing a smart management system with minimum investment individualized for each permanent grassland territorial administrative unit, depending on natural seed stock and ecosystem conditions. It will be possible as well to improve the quality of perennial functional feed from grazed pastures or hay meadows by seeding in addition herbaceous plant species with high polyphenols content, consumed by animals, to assure the animal welfare.

Conclusions

The highest total polyphenols content of perennial forages at the end of May 2008 (Romanian end of spring), in ecological conditions of Gradinari grassland (Caras-Severin), was quantified in unfertilized case (181 μ M gallic acid/g), related directly to the highest gravimetric percent of species from others botanical families group.

The fertilization with 20 t/ha fermented sheep manure (O1 and OM cases) decreased

the average value of total polyphenols content of perennial forages reported to unfertilized environmental conditions (Z case), but it remains higher than in mineral nitrogen fertilization trials (OM3 and M cases).

In the exclusive mineral fertilization conditions (M cases) it was produced perennial forages with the average value of total polyphenols content smaller than in unfertilized, organic fertilization and organic-mineral fertilization cases. The exclusive mineral fertilization with the highest nitrogen dose in one-step (150 kg/ha) increased the *Poaceae* gravimetric percents and decreased the total polyphenols content to the smallest concentration for the studied period.

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