PC&CA - MIRROR OF GRASSLAND FEED FRAP ANTIOXIDANT ACTIVITY IN SUMMER, DEPENDING ON FERTILIZATION?

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Abstract

The topic of this research was to test a chemometric technique (Principal Components & Classification Analysis) to reflect some characteristics of a real case: the influence of fertilization management on FRAP antioxidant activity in summer of grassland perennial forages. An area of 25 m^2 for each experimental trial was organised, with five replications, in complete randomized blocks design. StatSoft – STATISTICA, version 10, performed the chemometric matrix. Ten cases were included, nine of them with different fertilization management and one unfertilized. It was applied fermented sheep manure and/or mineral fertilizers based on NPK since 2003. The active variables of PC&CA were: the fertilization data, soil pH and the gravimetric percent of the spontaneous plants *Calamagrostis epigejos* (Ce%), *Festuca rupicola* (Fr%), *Trifolium repens* (Tr%), *Inula britannica* (Ib%), *Filipendula vulgaris* (Fv%) and *Rosa canina* (Rc%). The supplementary variable was the perennial feed FRAP antioxidant capacity. The coefficient for soil pH and *Trifolium repens* (%) correlation was 0.8, while in *Calamagrostis epigejos* (%) and *Festuca rupicola* (%) cases the coefficients were negatively: -0.6, respectively -0.8. Positive coefficients were obtained in summer for the correlations between grassland perennial feed FRAP antioxidant activity and *Inula britannica* (%), respectively *Rosa canina* (%). Principal Components & Classification Analysis can reflect as a mirror the perennial forage FRAP antioxidant activity at low pH, as grassland functional feed for animal, depending on fertilization management.

Key words: FRAP antioxidant activity, functional feed, grassland, perennial forages, multivariate analysis.

Antioxidants are natural bioactive compounds from plants which can scavenge/ reduce the free radicals (O_2•-, O_2, HO•, NO•, ONOO-, HOCl, RO(O)•, etc.) responsible for many degenerative diseases (Prior *et al*, 2005; Molan *et al*, 2012; Nishaa *et al*, 2012; Araujo *et al*, 2013; Lu *et al*, 2014; Phatak *and* Hendre, 2014). Epidemiologic researches found an inverse dependence between the mortality caused by chronic diseases and vegetal products consuming (Pellegrini *et al*, 2003).

Medicinal properties of plants were associated generally with the antioxidant activity (Hărmănescu *et al*, 2008; Adedapo *et al*, 2009; Molan *et al*, 2012; Phatak *and* Hendre, 2014), depending on the edible part: herb (Phatak *and* Hendre, 2014), leaf (Adedapo *et al*, 2009; Dudonne *et al*, 2009), fruit peel (Araujo *et al*, 2013), seed, flower, pod, root, etc. (Dudonne *et al*, 2009). Burri *et al*. (2017) demonstrated also in their studies that antioxidant activity of plants can be improved significant by the cultivar selection.

There are many chemical methods nowadays for total antioxidant capacity determination (scavenging or reducing capacity), but one of the most used assays is FRAP - *Ferric reducing ability of plasma*. This method consists in total antioxidant activity quantification at low pH, based on ferric-tripyridiltriazine reagent (Benzie *and* Strain, 1996).

Pellegrini *et al.* (2003) quantified for example the highest antioxidant activity for spinach and peppers among vegetables, for different berries among fruits, for soybean oil and coffee. In plants cases the state of art indicates a direct significant correlation between the ferric reducing activity and phenolic compounds (Molan *et al*, 2012; Araujo *et al*, 2013). Arceusz *and* Wesolowski (2015) concluded that phenolic acids content can be a discriminator parameter for spices and herbs better than minerals content.

In the international context of food safety and security, also the Romanian low no.150/2004 has clear specifications regarding the feed safety used for animals' ratio to assure the quality of raw matter for food industry. That's way it is so important to monitor also the quality of forages from grassland, an inestimable feed resources for ruminants. Because of the high amount of experimental results obtained for the quality of grassland forages characterization in correlation with the environmental factors, the challenging task of this scientific research was to test chemometric PC&CA to reflect some realistic

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hypothesis about the impact of fermented sheep manure and/or NPK mineral fertilization management on grassland forages FRAP antioxidant activity in summer.

Principal components analysis was used often in the last decades to extract useful realistic information about the complex natural phenomena (Wesołowski *and* Konieczynski, 2003; Okporie, 2008; Arceusz *and* Wesolowski, 2015). The applications of this flexible multivariate analysis technique are recommended in the future including for permanent grassland forages quality characterization as functional feed for animals.

MATERIAL AND METHOD

The Principal Components & Classification Analysis (PC&CA) chemometric matrix included active and supplementary variables of a real case: spontaneous forages from a hill grassland ecosystem located in Romanian Caras-Severin district. The supplementary variable was the grassland forages FRAP antioxidant capacity. The active variables were: the exclusive organic fertilization data with fermented sheep manure (OI, OII and OIII cases), the exclusive mineral fertilization data based on NPK (MI, MII and MIII cases), the organic – mineral fertilization data (OMI, OMII and OMIII cases), the soil pH, and the gravimetric percents of *Calamagrostis epigejos* (Ce%), *Festuca rupicola* (Fr%), *Trifolium repens* (Tr.%), *Inula britannica* (Ib%), *Filipendula vulgaris* (Fv%) and *Rosa canina* (Rc%) of grassland spontaneous forages. StatSoft – Statistica, version 10, was chosen for PC&CA chemometric analysis.

The chemical determination of grassland perennial forages total antioxidant capacity, as reducing activity at low pH, was made respecting the FRAP assay based on ferric-tripyridiltriazine reagent (Benzie&Strain, 1996). Ethanol extracts (70%) were obtained from analyzed forage samples, diluted 1/20 before the FRAP reagent addition. For the method calibration Mohr salt was chosen, as described Gergen (2004). The absorbance was monitored at 593 nm at UV/VIS Specord-205 spectrophotometer of AnalyticJena. SR ISO 10390 (1999) was respected for soil pH measurements. The floristic matrix of grassland spontaneous covering (%) was determined gravimetric.

The experimental field selected as studied real case to test the PC&CA model facilities was situated in a Romanian hill ecosystem from Caras-Severin. Around 200 m was the hill altitude. The grassland coordinates were: 45°12' latitude (N) and 21°60' longitude (E). Calcic Luvisol was the permanent grassland soil. Temperate continental with Mediterranean influences climate characterize the hill region. The complete randomized blocks design was used for the grassland experimental field. 25m^2 was the area of each experimental trial, with five replications for each fertilization system. OI, OII and OIII were fertilized with fermented sheep manure in 20-60 t/ha range, at each 2 years. The exclusive mineral fertilization (MI, MII and MIII), based on NPK, was made yearly, based on 50kg/ha dose differences: it was applied for all three cases constant doses of $P_{50}K_{50}$, and different doses of mineral nitrogen varied between 100 (one step) and 200 kg/ha (two steps). The OMI, OMII and OMIII trials were fertilized mixed, with organic and mineral NPK doses; the fermented sheep manure was applied in constant dose (20t/ha) for all three cases; in OMI was added P_{50}; in OMII was added $P_{50}K_{50}$; in OMIII was added $N_{50}P_{50}K_{50}$. The fertilization of permanent grassland began in 2003. One trial remained unfertilized (U), to compare the results obtained in different fertilization systems to those from normal environmental conditions without human's interventions. The plants and soil were sampled in summer (June 2009).

RESULTS AND DISCUSSIONS

The PC&CA model was fitted based on chemometric matrix, to extract relevant information regarding the perennial forages FRAP antioxidant capacity at low pH, depending on fertilization systems. It was included in multivariate data matrix one supplementary and ten active variables, which characterised the ten cases of PC&CA. Nine principal components described 100% the quality of PC&CA model. The Gutman's lower bound is considered very important by Verma *et al.* (2016). For the PC&CA chemometric model of the perennial forages FRAP antioxidant capacity, only the first three principal components had the eigenvalue higher than unit. The total variance of the first two principal components (PC1 and PC2) summarized more than 76%, PC1 heaving 55%. First three principal components (PC1, PC2 and PC3) described more than 87% of the PC&CA total variance.

The results of grassland spontaneous forages FRAP antioxidant capacity were included as supplementary variable in the PC&CA data matrix. The perennial forage harvested from unfertilized trial (U) quantified the highest FRAP antioxidant capacity: 240 µM Fe^{2+}/g. For all the others studied fertilization cases, the forages FRAP antioxidant capacity was smaller. In exclusive organic fertilization system, the grassland forages had an average value of FRAP antioxidant capacity around 220µM Fe^{2+}/g. The fermented sheep manure, applied alone or in addition with P_{50} and/or K_{50}, assured an average value of feed FRAP antioxidant capacity around 228 µM Fe^{2+}/g. The mineral nitrogen application, in organic or conventional (non-organic) systems, decreased the average value of feed FRAP antioxidant capacity to 175 µM Fe^{2+}/g.

The soil pH, included as active variable in PC&CA data matrix, varied in the ten studied cases between 5.4 and 6.0, the soil being the most acidified in the exclusive mineral fertilization case with the highest mineral nitrogen application in two steps: N_{200}. The average value of soil pH for exclusive mineral fertilization system was 5.5, while in unfertilized case the soil pH was 5.7. The exclusive fermented sheep manure application increased the soil pH average value (5.8) comparatively with unfertilized case.

Six from the total active variables of the PC&CA matrix characterized the grassland spontaneous flora. *Calamagrostis epigejos* (%) had the highest average percent in exclusive mineral fertilization system (12%) and the smallest in organic system (7%). The perennial *Festuca rupicola* (%) had the same distribution profile as *Calamagrostis epigejos* reported to fertilization systems, but its presence in grassland spontaneous flora was around four times higher than *Calamagrostis epigejos* (%). *Trifolium repens* average percent was the highest in organic system (30%), while the mineral NPK in addition with 20t/ha fermented sheep manure decreased its distribution to 22 %, more significant for mineral N_{50} application case (OMIII). In unfertilized or exclusive mineral fertilized cases the *Trifolium repens* quantified under 1%. The others three spontaneous plant species, *Inula britannica*, *Filipendula vulgaris* and *Rosa canina*, were gravimetric identified under 5%, 10%, and respectively 20%.

Analysing statistically the multivariate response of data matrix according to the factor loadings, organic system had a high positive impact on the first principal component (PC1): fermented sheep manure fertilization data (0.79), *Trifolium repens* (%) presence in perennial grassland covering (0.86) and soil pH (0.89). Both the *Rosa canina* (%) distribution in grassland perennial covering and feed FRAP antioxidant capacity had also positive coefficients in PC1 structure: 0.51, respectively 0.31. High negative impact on PC1 had the mineral fertilization system: mineral nitrogen fertilization data (-0.90), mineral P_{50} fertilization data (-0.75), mineral K_{50} fertilization data (-0.85), *Calamagrostis epigejos* gravimetric percent (-0.84) and *Festuca rupicola* distribution (-0.87). In the second principal component (PC2) structure the highest negative impact had *Filipendula vulgaris* (%) and *Rosa canina* (%) distribution: -0.97 and -0.65.

The variables projection (active and supplementary) on PC1xPC2 plane of PC&CA chemometric model is presented in *figure 1*.

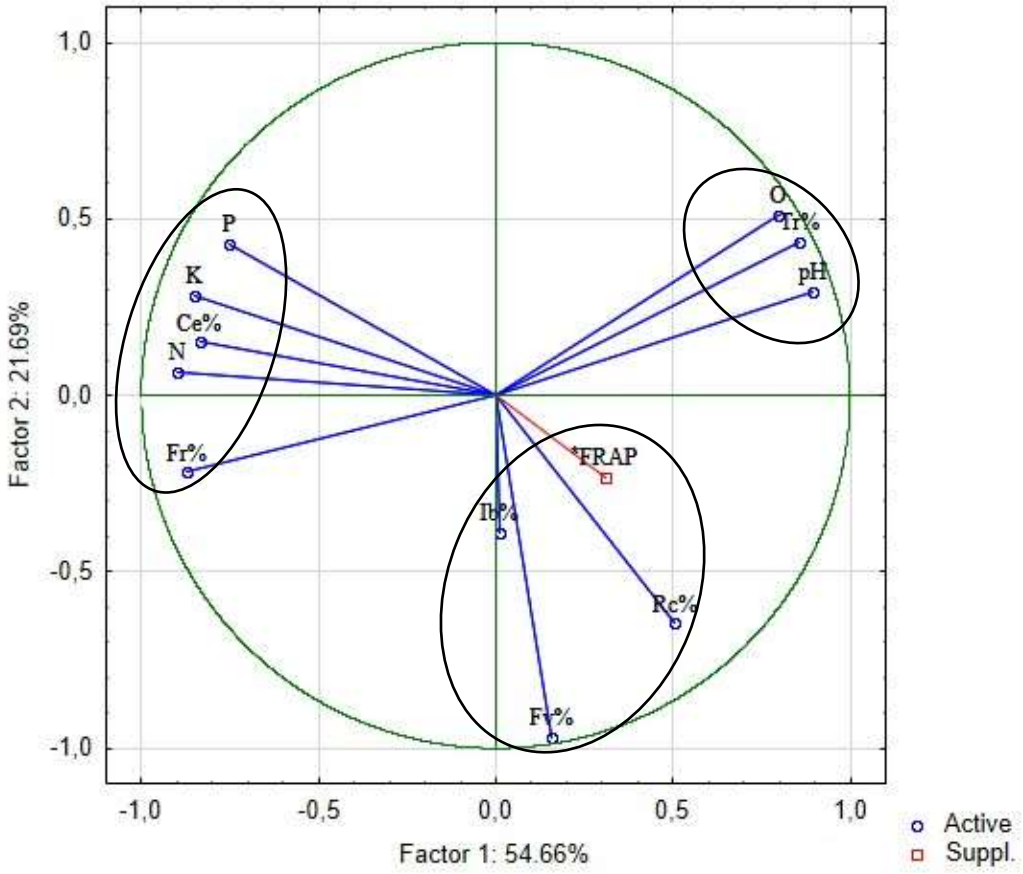

Figure 1 **The supplementary and active variables projection of PC&CA on PC1xPC2 plane**

Taking into consideration both the active and supplementary variables of PC&CA data matrix and their projections on PC1xPC2 plane, it was possible to extract some relevant information

about the influence of fertilization management on the perennial forages FRAP antioxidant capacity. It was three different situations reported to feed FRAP antioxidant capacity (*figure 1*). The perennial *Festuca rupicola* (%) and *Calamagrostis epigejos* (%) were positively influenced by mineral fertilization system. The coefficients for mineral NPK fertilizers and *Festuca rupicola* gravimetric percent correlations were 0.64, 0.54 and 0.70. Fermented sheep manure applied at each two years influenced negatively the presence of *Festuca rupicola* in grassland covering (-0.80). Analogously, the mineral NPK fertilization influenced positively the *Calamagrostis epigejos* gravimetric percent of perennial feed (0.89, 0.59 and 0.65). The grass species *Calamagrostis epigejos* (%) and *Festuca rupicola* (%) did not improve the forages FRAP antioxidant capacity (-0.18; -0.47).

Reported to the mineral fertilization system, on the opposite part of the plot of supplementary and active variables projection of PC&CA on PC1xPC2 plane, it was placed the organic fertilization system. A high impact of fermented sheep manure on *Trifolium repens* (%) distribution in grassland trials was highlighted by the PC&CA model, the correlation coefficient being 0.86. Mineral nitrogen application decreased significant the *Trifolium repens* (%) presence in the grassland perennial feed (-0.72). The coefficient for the forages FRAP antioxidant capacity and *Trifolium repens* (%) correlation was positively (0.32).

For the agronomic point of view, it is important to specify that in organic farms, in soil and climatic growth conditions of studied Romanian hill permanent grassland, between *Trifolium repens* (%)-*Calamagrostis epigejos* (%) and *Trifolium repens* (%)-*Festuca rupicola* (%) correlations it was a high negative interdependence (-0.70, respectively -0.94). The grassland soil pH values increased in organic system (0.85) and decreased in conventional (non-organic) system (-0.84), heaving a strong positive impact on *Trifolium repens* (%) content of grassland forages (0.84).

The third situation referred to the studied plant species from others botanical families. The correlation coefficients between perennial forages FRAP antioxidant capacity in June (summer) and the distribution in grassland covering of *Inula britannica* (%), *Rosa canina* (%) and *Filipendula vulgaris* (%) were positively: 0.49, 0.40 and 0.21.

It can be useful to make a comparison between the PC&CA variables and cases projections on PC1xPC2 plane. It was projected on PC1xPC2 plane all the ten grassland trials, used as cases of PC&CA data matrix (*figure 2*):

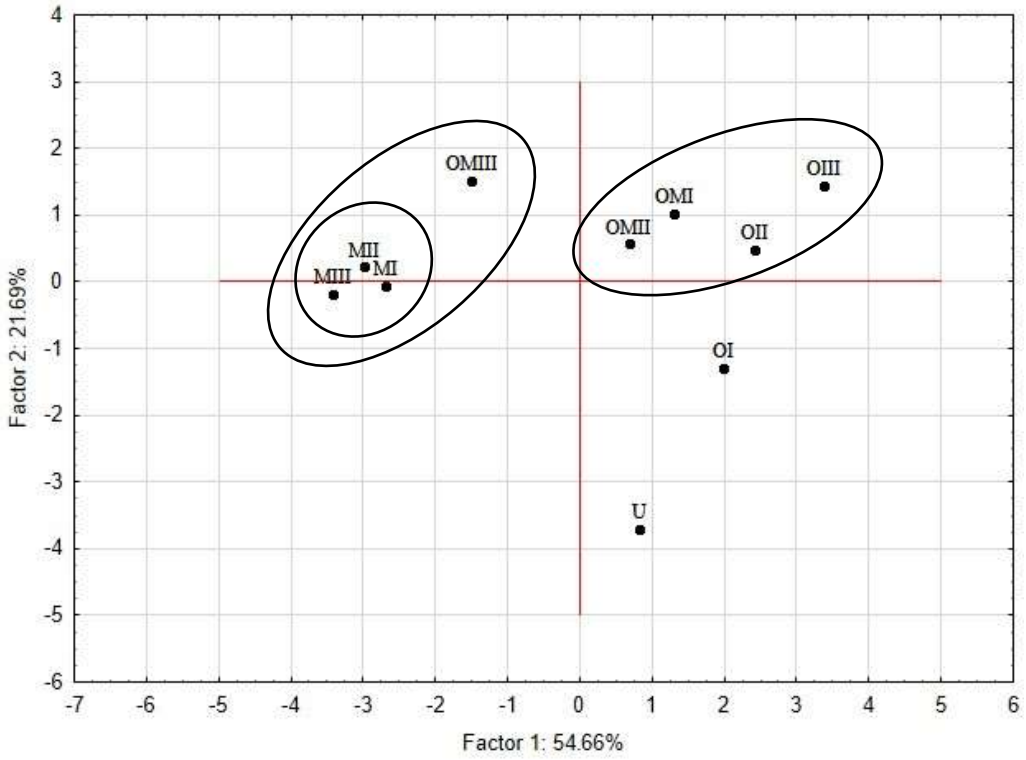

Figure 2 **The cases projection of PC&CA on PC1xPC2 plane**

PC&CA multivariate technique can be very helpful for the differentiation between the grassland microecosystems of organic and/or conventional (non-organic/ mineral fertilization) systems. Principal Components & Classification Analysis reflected as a mirror the changes of

spontaneous floristic matrix of perennial grassland and the forages quality depending on fertilization management of microecosystems.

It was classified mainly five groups of microecosystems. First group was formed by the exclusive mineral fertilization system cases (MI, MII and MIII). This small group of exclusive mineral fertilization trials was included in a larger one, the second group, together with the microecosystem OMIII (20 t/ha fermented sheep manure + $N_{50}P_{50}K_{50}$). The N_{50} application changed the OMIII microecosystem conditions of plants growth, generally being more closely to those of exclusive mineral fertilization system than those of organic management cases (exclusive or in addition with P_{50} and K_{50}). The third group classified generally the organic fertilization cases, exclusive with the highest doses (OII, OIII) and also the microecosystems of 20t/ha fermented sheep manure with P_{50} and K_{50} (OMI and OMII). The last two groups included OI (organic microecosystem fertilized with 20 t/ha fermented sheep manure) and U (microecosystem without human's interventions).

Superposing the PC&CA plots (*figure 1* and *figure 2*), it is possible to observe, in a comprehensive way, the complex profile of the forages FRAP antioxidant capacity from permanent grassland. The microecosystems conditions of first and second groups did not influence significant the feed FRAP antioxidant capacity in June (summer), stimulating the presence of grass species *Festuca rupicola* (%) and *Calamagrostis epigejos* (%). The microecosystems of the third group had optimum growth conditions for *Trifolium repens* (%), also without a significant influence on forages FRAP antioxidant capacity in the studied period of the year. The last two microecosystems groups (the fourth and the fifth) had a nutrients dynamic which stimulated generally the highest presence of spontaneous species from others botanical families, as *Inula britannica* (%), *Rosa canina* (%) and *Filipendula vulgaris* (%), predicted the increasing of forages FRAP antioxidant capacity from permanent grassland. *Rosa canina* is not consumed by animals, but *Inula britannica* and *Filipendula vulgaris* can be taken in consideration in the future to obtain grassland functional feed with an improved FRAP antioxidant capacity.

The monitored multiresponse data sets (the variables) for the grassland microecosystems (cases) recommend the Principal Components & Classification Analysis as a useful statistic tool with many facilities to make differentiations based on feed FRAP antioxidant capacity, depending on grassland fertilization management. The PC&CA plots can be fitted easy with performing software package, playing a key role to generate hypotheses using high amounts of experimental results from the complex grassland ecosystem.

In the future it will be realistic to establish good practices to obtain functional feed for animals from the grassland spontaneous plants, especially for ruminants, cheaper than others sources. In this context, it will be necessary to identify new parameters to asses the feed quality and also the specific qualitative and quantitative methods for monitoring procedures, with low cost in terms of money and time.

While in humans and animals organism the oxidative stress generates free radicals, with high negative impact on health because are responsible for chronic diseases, plants fight against the stress factors biosynthesizing antioxidants which can reduce/neutralize the free radicals. That's way the FRAP antioxidant capacity can became a very important parameter to characterise the reducing activity of grassland forages, as functional feed, on free radicals, at low pH.

CONCLUSIONS

Principal Components & Classification Analysis chemometric technique reflected that *Trifolium repens* (%) had optimum growth conditions in organic farming system (fermented sheep manure), while *Calamagrostis epigejos* (%) and *Festuca rupicola* (%) were dominants in non-organic farms (exclusive NPK mineral fertilization, or organic with N_{50} system).

The highest forages FRAP antioxidant capacity was quantified for grassland unfertilized trial (240 μM Fe^{2+}/g). Different fertilization management reduced the average values of feed FRAP antioxidant capacity: around 220 μM Fe^{2+}/g in fermented sheep manure cases, 228 μM Fe^{2+}/g in organic-mineral without mineral nitrogen application, and 175 μM Fe^{2+}/g conventional (non-organic/exclusive mineral) systems. The forages FRAP antioxidant activity was correlated positive with *Inula britannica* (%) and *Rosa canina* (%) in the hill permanent grassland climate and soil conditions of summer.

Principal Components & Classification Analysis can reflect with success, as a mirror, the perennial forage FRAP antioxidant activity, at low pH, as grassland functional feed for animal, depending on fertilization management.

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