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Artigo

Multivariate analysis of the effects of soil parameters and environmental factors on the flavonoid content of leaves of *Passiflora incarnata* L., Passifloraceae

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RESUMO: "Análise multivariada dos efeitos dos parâmetros do solo e fatores ambientais no teor de flavonoides das folhas de *Passiflora incarnata* L., Passifloraceae". O objetivo deste trabalho foi avaliar os efeitos do solo (pH, macro e micronutrientes), fatores ambientais (temperatura, umidade, época do ano e período da coleta) e condições meteorológicas (chuva, sol, nublado, nublado com chuva) no teor de flavonoides das folhas de *Passiflora incarnata* L. (Passifloraceae), através do tratamento quimiométrico dos dados por PCA (análise de componentes principais) e HCA (análise hierárquica de agrupamentos). Os flavonoides totais foram quantificados por cromatografia líquida de alta eficiência-detecção por ultravioleta (CLAE-UV/DAD). As análises por PCA e HCA mostraram que as amostras de *Passiflora* não apresentam uma classificação específica em relação às variáveis estudadas e que as variáveis do meio ambiente não são relevantes para descrever o modelo estudado, porém os elementos do solo Fe, B e Cu demonstraram correlação inversa à concentração dos flavonoides totais.

Unitermos: *Passiflora incarnata* L., cultivo de plantas medicinais, análise quimiométrica, flavonoides, CLAE-UV/DAD.

ABSTRACT: The aim of the present study was to evaluate the effect of soil characteristics (pH, macro- and micro-nutrients), environmental factors (temperature, humidity, period of the year and time of day of collection) and meteorological conditions (rain, sun, cloud and cloud/rain) on the flavonoid content of leaves of *Passiflora incarnata* L., Passifloraceae. The total flavonoid contents of leaf samples harvested from plants cultivated or collected under different conditions were quantified by high-performance liquid chromatography with ultraviolet detection (HPLC-UV/PAD). Chemometric treatment of the data by principal component (PCA) and hierarchic cluster analyses (HCA) showed that the samples did not present a specific classification in relation to the environmental and soil variables studied, and that the environmental variables were not significant in describing the data set. However, the levels of the elements Fe, B and Cu present in the soil showed an inverse correlation with the total flavonoid contents of the leaves of *P. incarnata.*

Keywords: *Passiflora incarnata* L., cultivated medicinal plants, chemometric analysis, flavonoids, HPLC-UV/PAD.

INTRODUCTION

The family Passifloraceae comprises more than four hundred species including *Passiflora incarnata* L., a plant that is widely employed as an herbal medicine and is described in some European pharmacopoeias. The principal components of the leaves of *P. incarnata* most likely associated with the pharmacological properties of the species are the harman alkaloids and the flavonoids (many of which are *C*-glycosylated) (Dhawan et al., 2004; Pereira & Vilegas, 2000). Total flavonoid content is a very important parameter with respect to the quality assessment of phytomedicines derived from *Passiflora* leaves. Moreover, these compounds have been chosen as analytical markers in the high-performance liquid chromatographic (HPLC) and high-performance thin layer chromatographic methods employed in distinguishing *P. incarnata* from *P. alata* Curtis and *P. edulis* Sims, the latter species being the most frequently cropped in Brazil (Pereira et al., 2004).

P. incarnata is native to the southern United States and, although the species can be cultivated in Brazil, its productivity is reportedly quite low (Bruckner & Picanço, 2001). In order to assess the feasibility of producing raw material for the phytopharmaceutical industry through

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commercial cultivation of this medicinal plant, the effect of soil parameters and environmental factors on the total flavonoid content of the leaves of *P. incarnata* grown on an experimental farm at Botucatu-SP, Brazil, were evaluated. The chromatographic data acquired were submitted to chemometric analysis, the employment of which provides a powerful tool for the quality assessment of medicinal plants (De Barros et al., 2006).

Application of chemometric analysis may help to improve our understanding of the chemical composition of cultivated medicinal plants with respect to the determination of the most appropriate conditions for their commercial cultivation and subsequent utilization in the production of standardized phytomedicines. So, the goal of the present study was to differentiate leaf samples harvested from specimens of *Passiflora incarnata* cultivated or collected under different conditions in order to obtain information that could be applied to the commercial production of phytomedicines derived from this species. Although reports concerning the chemometric analysis of the volatile constituents of some aromatic species cultivated under different conditions are available (Economakis et al., 2002; Karioti et al., 2003), to the best of our knowledge the present study is the first relating to the application of chemometric methods to data derived from the HPLC analysis of flavonoids from a species of *Passiflora.*

MATERIAL AND METHODS

Chemicals and materials

Acetonitrile and methanol were of HPLC-grade (Omnisolv-Merck, Darmstadt, Germany), and ethanol and formic acid were of analytical grade (Merck, Darmstadt, Germany). HPLC-quality water was prepared using a Millipore Milli-Q system (Millipore, SP, Brazil). Rutin hydrate (95% pure; Sigma, St. Louis, MO, USA) was utilized as standard for the quantitative analysis of flavonoids. Sep-Pak C_{18} cartridges (400 mg) were supplied by Waters (Milford, MA, USA).

Cultivation plots and soil analyses

A total cultivation area of 295.68 m^2, comprising thirty three flower-beds (each of area 8.92 m^2) together with a native vegetation area, was employed in this study. Eleven different soil types, each replicated in the thirty three randomly located flower-beds, were investigated. A sample of soil from each flower-beds was collected, and the samples corresponding to the triplicated areas were bulked and homogenized to produce eleven representative soil samples. In order to determine the composition and the differences at composition of the eleven soil samples, analyses were performed as described by Raij et al. (2001) and carried out at the Laboratory of Soil Fertility, Universidade Estadual Paulista (UNESP), Botucatu-SP, Brazil, in August 2004 under the direction of Prof. Dr. Leonardo T. Bull and Prof. Dr. Dirceu M. Fernandes. The pH, content of organic matter (OM), cation exchange capacity (CEC), sum of bases (Svalue; Na, K, Ca and Mg), percentage base saturation (V%; given by Svalue/CEC x 100), content of micronutrients (B, Cu, Fe, Mn and Zn), content of the major exchangeable cations (K, Ca and Mg), level of P, level of Al, and total acidity (H + Al) were determined for each soil sample. The analyses of B, Cu, Fe, Mn and Zn were performed by atomic absorption spectrometry, the amounts of K, Ca and Mg were measured by flame photometric spectrometry, the level of H + Al was obtained by UV/VIS spectrometry, and ion exchange chromatography was utilized to determine the values of OM, P, Al, Svalue, CEC and V%.

Plant material

Commercial seeds of *Passiflora incarnata* L. Passifloraceae were provided by Johnny's Selected® Seeds (Albion, ME, USA) and sown in January 2003. Seeds were germinated in a green house at room temperature during September and October 2003. Seedlings (n=33) were selected and replanted in flower-beds in the absence of chemical fertilizers and pesticides. On the same day samples (approximately 9 g) of leaves of mature plants were harvested at three random flower-beds, with each one of collection being performed in duplicated during morning (*ca.* 7:00 am), afternoon (*ca.* 2:00 pm) and evening (*ca.* 5 pm) periods. Harvesting was performed during two non-consecutive periods (1st period-6th February until 14th May 2004 and 2nd period-15th October until 30th December) that were interrupted by the winter season when vegetative dormancy occurs. Table 1 specifies the flower-beds chosen in each week and the harvesting dates. Environmental factors (ambient temperature and humidity) and meteorological conditions (sun, rain, cloud or cloud/rain) during each collection of the material were also monitored.

Collected material was immediately dried at 40 ^{o}C to constant weight, powdered using a domestic blender and ground. Only particles between 0.5-1.0 mm were utilized for the extractions, and these were stored in glass flasks protected from light and humidity until required for analysis. Specimens of mature plants were identified by Dra. Inês Cordeiro (Instituto Botânico de São Paulo, SP, Brazil) and a voucher was deposited in the herbarium of the Instituto Botânico de São Paulo with the identification code RG SP374018.

Table 1. Total flavonoid content of leaves of *Passiflora incarnata* L. harvested during different weeks of the year and periods of the day.

Weeks of collection	Days of harvesting	Flower-beds of each harvesting*
1	Feb, 6	9, 1, 7
2	Feb, 13	3, 6, 10
3	Feb, 20	11, 2, 7
4	Feb, 27	2, 7, 10
5	Mar, 6	3, 5, 8
6	Mar, 12	4, 6, 8
7	Mar, 19	9, 6, 5
8	Apr, 2	4, 8, 10
9	May, 6	9, 10, 4
10	May, 14	10, 9, 4
11	Oct, 15	1, 4, 9
12	Oct, 22	10, 5, 9
13	Oct, 29	8, 3, 6
14	Nov, 6	9, 4, 3
15	Nov, 12	10, 9, 4
16	Nov, 19	10, 9, 4
17	Dec, 4	10, 9, 4
18	Dec, 10	10, 9, 4
19	Dec, 20	10, 9, 4
20	Dec, 30	10, 9, 4

*numbering of the flower-beds triplicate

Sample preparation and HPLC-UV/PAD analyses

Samples of powdered leaves of *P. incarnata* were submitted to extraction and clean-up procedures and analysed by quantitative HPLC following an optimised method that has been previously described (Pereira et al., 2004). HPLC analyses were performed utilizing a Alliance model 2795 liquid chromatograph (Waters Corporation, Milford, MA, USA) equipped with a Waters model 996 photodiode array detector (PAD) and a Supelcosil C_{18} column 250 x 4.6 mm i.d.; 5 µm (Supelco, Bellefonte, PA, USA). The mobile phase consisted of 2% formic acid in water (solvent A) and acetonitrile (solvent B), initially at 15% B, with linear gradients from 15-30% B between 10-40 min, and from 30-15% B between 40-50 min: the flow rate was 0.8 mL min^{-1} and chromatography was carried out at room temperature (~25 °C). Samples were manually injected using 10 µL loop (Rheodyne, Rohnet Park, CA, USA), and UV/PAD spectra were recorded between 200 and 400 nm. All samples were analysed in duplicate and the collected data were processed using Waters Empower Pro® version 5.0. For quantitative analysis, detection was at 337 nm and methanolic solutions of rutin (from 25 to 250 $mg.L^{-1}$) were utilized in the construction of the calibration curve.

Chemometric analysis

Exploratory analysis of the data using principal component analysis (PCA) and hierarchical cluster analysis (HCA) was performed with the aid of Minitab software version 14 (Minitab Inc., State College, PA, USA). PCA was used to establish the simplest mathematical model capable of describing the data set satisfactorily, and represents the most appropriate statistical approach when the goal is to establish the relative importance of individual variables in determining the data structure. HCA is a pattern recognition technique utilized to reveal the structure residing in a data set and to disclose the natural groupings that exist between samples characterized by the values of a set of measured variables. The concepts of dissimilarity and similarity are basic to cluster analysis of a sample. Typically, a hierarchical agglomerative cluster analysis of the studied variables is performed and the square of the Euclidean distance taken as a measure of similarity between samples (Chipman & Tibshirani, 2006). In order to decide a distance or dissimilarity between two groups, popular methods of measuring distance between groups can be applied as: (1) single linkage, which uses the minimum distance between points in different groups; (2) complete linkage, which uses the maximum distance; (3) mean linkage, which uses the average of all distances

between points in the two groups and (4) centroid linkage, which uses the distances between group centroids (*e.g.* group means).

Theoretically, three hundred sixty samples of leaf material from plants grown in each of the thirty three different flower-beds should have been available for analysis: however, owing to accidental losses only two hundred ninety one samples could be considered. A total of twenty one variables were defined with respect to: (1) environmental factors, *i.e.* ambient temperature and humidity during the collection of the material, harvest period (Table 1), time of collection (morning, afternoon or evening) and meteorological conditions (sun, rain, cloud or cloud/rain); (2) soil parameters, *i.e.* pH, OM, CEC, Svalue, V%, B, Cu, Fe, Mn, Zn, K, Ca, Mg, P, (H + Al); and (3) total content of flavonoids (expressed in rutin equivalents). In order to remove possible distortions arising from the different magnitudes of the numerical values of the variables, each was standardized by subtracting the mean value and dividing by the standard deviation. For each sample set, a 291 x 21 data matrix was constructed in which the rows represented the samples of *P. incarnata* and the columns represented the variables.

RESULTS AND DISCUSSION

In the present study, HPLC-UV/PAD analyses were performed at room temperature such that the chromatographic profiles of the extracts of *Passiflora incarnata* L. Passifloraceae (Figure 1) were similar to those reported previously but with slight differences in the retention times of individual components (Pereira et al., 2004). Using the described system, the calibration curve ($y=1.53 \times 10^4 x - 6.79 \times 10^4$; $r^2=0.9866$) constructed with standard rutin was found to be linear in the range 25-250 $mg.L^{-1}$ and presented a coefficient of variation < 4%. All of the peaks that could be identified, on the basis of their characteristic absorption bands (Mabry et al., 1970), as being associated with flavonoids were quantified (for UV/PAD spectra see Figure 1). The range of the total flavonoid contents of two hundred ninety one leaf samples of *P. incarnata* collected during different weeks, soil and periods of the day were 0.02-4.89%. The results of soil samples analysis are displayed in Table 2.

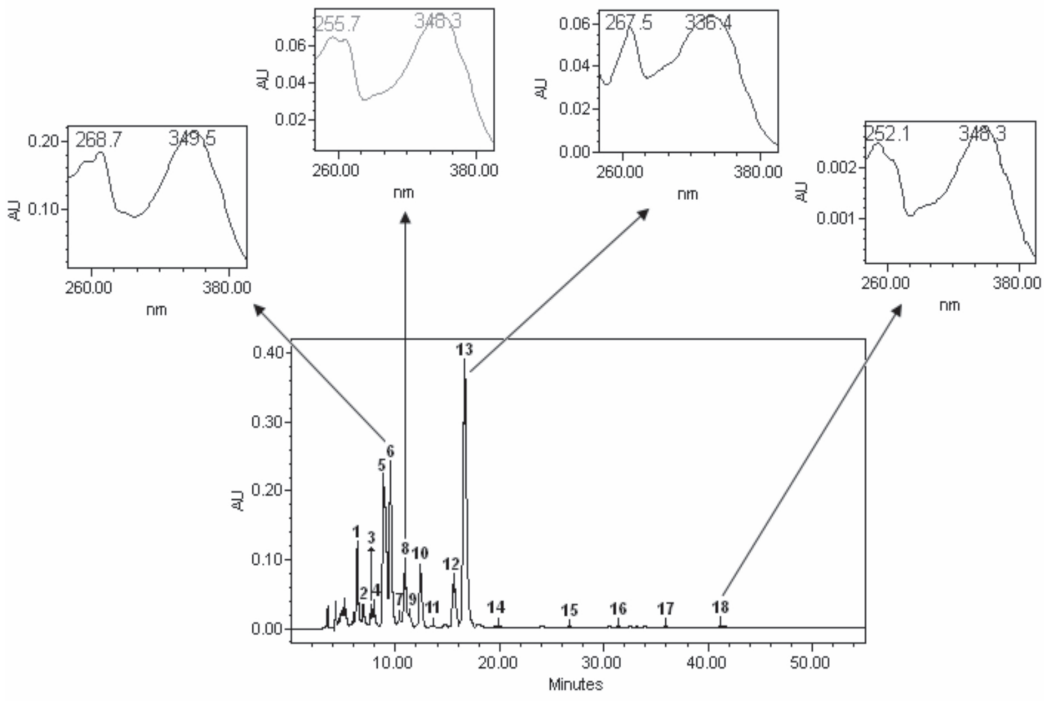

Figure 1. Representative HPLC-UV/PAD (λ = 337 nm) chromatogram of leaves of *Passiflora incarnata* L and UV/PAD spectra of flavonoids peaks identified as (**6**) orientin, (**8**) homoorientin, (**12**) vitexin and (**18**) luteolin.

The ranges of the measured parameters were: pH, 4.2-5.3; P, 3-16 $mg\ L^{-1}$; K, 0.9-2.2 $mmol.L^{-1}$; Ca, 4-25 $mmol\ L^{-1}$; Mg, 3-15 $mmol.L^{-1}$; V%, 19-65 $mmol\ L^{-1}$; B, 0.13-0.21 $mg.L^{-1}$; Cu, 0.8-1.2 $mg.L^{-1}$; Fe, 79-133 $mg.L^{-1}$; Mn, 0.7-2.8 $mg.L^{-1}$; and Zn, 0.3-2.9 $mg.L^{-1}$. Significant differences in the quality of the soils used in the plantation areas were revealed from the diversity of soil composition. Aluminium was not detected in any of the soil samples analysed and so this variable could not be considered in the analysis of the data.

In order to investigate possible correlations between all the variables studied, and to evaluate hypothetical models for the classification of samples, both PCA and HCA techniques were employed. Initially an evaluation of the relationships among the twenty one variables relating to the two hundred ninety one samples was conducted by PCA on the basis of a correlation data matrix in which the variables were standardized

Table 2. Characteristics of the eleven different type of soil employed in the present study.

Sample*	pH	OM[a]	Pa	Al[b]	H + Al[b]	K[b]	Ca[b]	Mg[b]	Svalue[b]	CEC[b]	V%	B[a]	Cu[a]	Fe[a]	Mn[a]	Zn[a]
1	4.3	16	4	0	33	0.9	5	3	9	42	21	0.15	1.2	91	0.7	0.7
2	5.0	27	15	0	28	1.4	25	12	39	67	59	0.21	1.0	133	2.5	2.2
3	5.3	22	11	0	24	2.2	21	11	34	57	59	0.15	0.9	102	1.6	2.1
4	4.2	19	4	0	38	1.0	5	3	9	47	19	0.17	1.0	123	0.7	0.4
5	4.2	17	3	0	36	1.2	4	4	9	45	19	0.18	0.9	118	0.7	0.3
6	5.2	23	12	0	24	1.3	21	13	35	59	60	0.16	1.0	89	1.7	2.5
7	5.2	27	16	0	25	1.1	25	13	40	65	61	0.16	0.9	121	2.8	2.9
8	5.2	19	11	0	25	2.1	22	12	36	61	59	0.18	0.9	87	1.4	1.8
9	4.8	13	5	0	26	1.1	11	9	21	48	45	0.14	0.9	86	0.9	0.5
10	5.0	18	5	0	26	1.3	13	10	25	51	49	0.13	0.8	79	0.8	0.6
11	5.3	24	8	0	21	1.3	23	15	39	61	65	0.15	0.8	112	2.3	2.0

*numbering of the flower-beds triplicate.
[a]Values expressed in mg L^{-1}
[b]Values expressed in mmol L^{-1}

and attributed equal weight. The score plot so obtained revealed a discrimination of the samples into four different classes, but without a specific classification of samples in relation to an independent variable or group of variables. The first principal component (PC1) was the most significant for describing the model, accounting for 96.4% of the overall variance. The loading plot, however, showed that the environmental variables were not significant in describing variation in the data set, i.e. the contributions of these variables were very close to zero in PC1 and PC2. The loading plot also revealed that the soil ions (K, Mg, Ca, P, Zn, Mn, B, Fe, Cu), OM and Al + H were the most significant variables, and similarity among some variables could be observed. Thus, the environmental variables, Svalue, CEC, pH and V% were selectively removed from the data set. In this step, the Minitab procedure for the detection of outlier samples was applied showing forty nine outlier samples, and these forty nine samples were removed leaving two hundred forty two samples. In order to evaluate correlations between the remaining variables and the total flavonoid content, PCA and HCA analyses were conducted using the 242 x 16 data matrix data.

From the scree plot it may be seen that the first three principal components (PCs 1-3) are the most significant in describing the model and, together, account for 80.3% of the overall variance (Table 3). Indeed, most of the information concerning the system (*i.e.* 72.4% of the overall variance) is conveyed by PC1 (49.5% of the variance) and PC2 (22.9% of the variance). However, PC4 is the principal component that describes the total flavonoid variable (*cf.* Table 4), hence PCs 1-4 (accounting for 87.8% of the total variance) were considered further in this study.

Table 3. Eigenanalysis of loadings of the four principal components of the dataset.

	PC1	PC2	PC3	PC4
Eigenvalue	5.9356	2.7480	0.9537	0.8969
Proportion	0.495	0.229	0.079	0.075
Cumulative	0.495	0.724	0.803	0.878

In the PC1 *versus* PC2 loading plot (Figure 2), it is clear that the variables K, Mg, Ca and Al + H are not correlated with the flavonoid variable, whilst Fe, B and Cu display an inverse correlation with total flavonoid content contributing, respectively, 53.1, 43.0 and 27.1% of the total effect (Table 4).

Table 4. Principal component analysis of the soil variables and total flavonoid content.

Variable	PC1	PC2	PC3	PC4
Total flavonoids (FL)	0.037	0,228	0.362	0.892
OM	-0.274	-0.364	-0.173	0,188
P	-0.384	-0.174	0.036	0.075
Al + H	0.328	-0.327	0.015	0.130
K	-0.244	0.139	0.421	-0.088
Ca	-0.408	0.025	0.042	0.009
Mg	-0.378	0.204	-0.023	-0.061
B	0.016	-0.271	0.796	-0.297
Cu	0.103	-0.430	0.087	0.017
Fe	0.084	-0.531	0.074	0.191
Mn	-0.366	-0.209	-0.110	0.089
Zn	-0.384	-0.182	-0.008	0.042

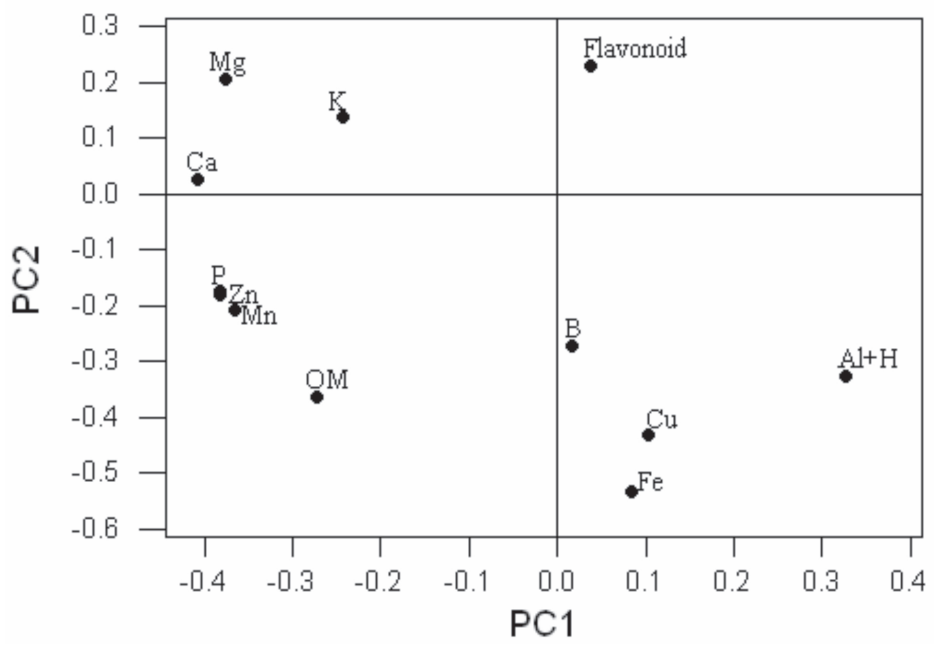

Figure 2. Loading plot of the variables soil ions (K, Mg, Ca, P, Zn, Mn, B, Fe, Cu, Al + H), organic matter (OM), and total flavonoid content.

HCA analysis of the data was conducted with the aim of grouping similar samples, and three distinct clusters may be observed in the resulting dendrogram (Figure 3). The first cluster is formed by the total flavonoid variable, the second by the variables Fe, Cu, B and Al + H, and the third by all other variables with a similarity value of 60.0% between them. Analysis of the dendrogram in conjunction with the PCA results confirmed that the total flavonoid content variable is independent and not influenced by environmental variations. The elements Fe, Cu and B are the variables most similar to flavonoid content but with the opposite effect, i.e., in the presence of these elements the total flavonoid content of leaves of *P. incarnata* tends to be lower and *vice versa*. Thus, the leaves of *P. incarnata* presenting the largest total flavonoid content were those cultivated in soil containing the lowest levels of Fe, Cu e B (sample 10, Table 2).

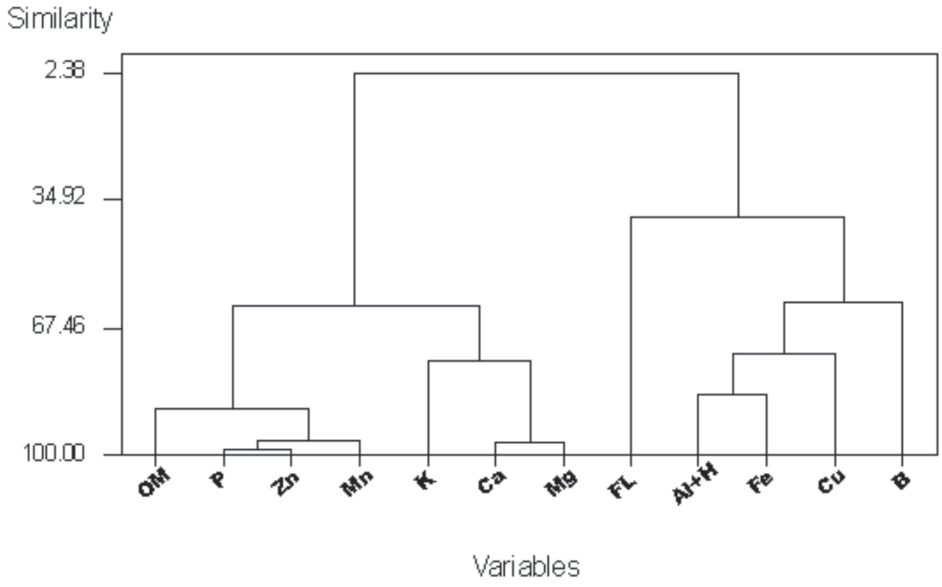

Figure 3. Dendrogram obtained by HCA analysis for the variables soil ions (K, Mg, Ca, P, Zn, Mn, B, Fe, Cu, Al + H), organic matter (OM), and total flavonoid content (FL).

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