

## A Multivariate Study on the Inhibition of the Xanthine Oxidase by Phenylpyrazole Compounds

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### Abstract

In this work, a set of phenylpyrazole compounds was studied in order to determine the molecular properties responsible for their previously tested ability to inhibit the xanthine oxidase (XO) enzyme. Several electronic, steric and lipophylic properties were calculated by quantum chemical methods from the geometries optimized by the DFT/B3LYP method. The chemometric methods PCA, HCA and SDA were employed to find the subset of variables that could correctly classify the compounds according to their inhibitory activity. The PCA, HCA and SDA results showed that four variables can be considered as relevant for the XO inhibitory activity:  $\mu$  (dipole moment),  $QR_2$  (total charge of the substituent  $R_2$ ),  $D_3$  (torsion angle formed by the atoms C6-C1-C2-R2) and MR (molar refractivity). From the PCA, HCA and SDA results in this work, a classification model was built with the aim to be used in the search for phenylpyrazole compounds that present XO inhibitory activity.

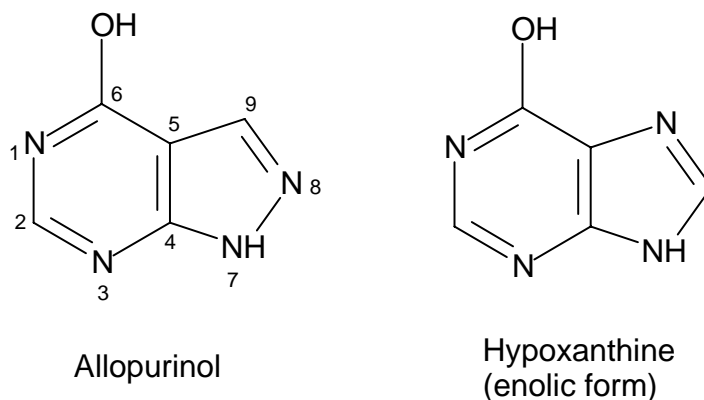
**Keywords.** Phenylpyrazole compounds, Xanthine oxidase, PCA, HCA, SDA.

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## 1 INTRODUCTION

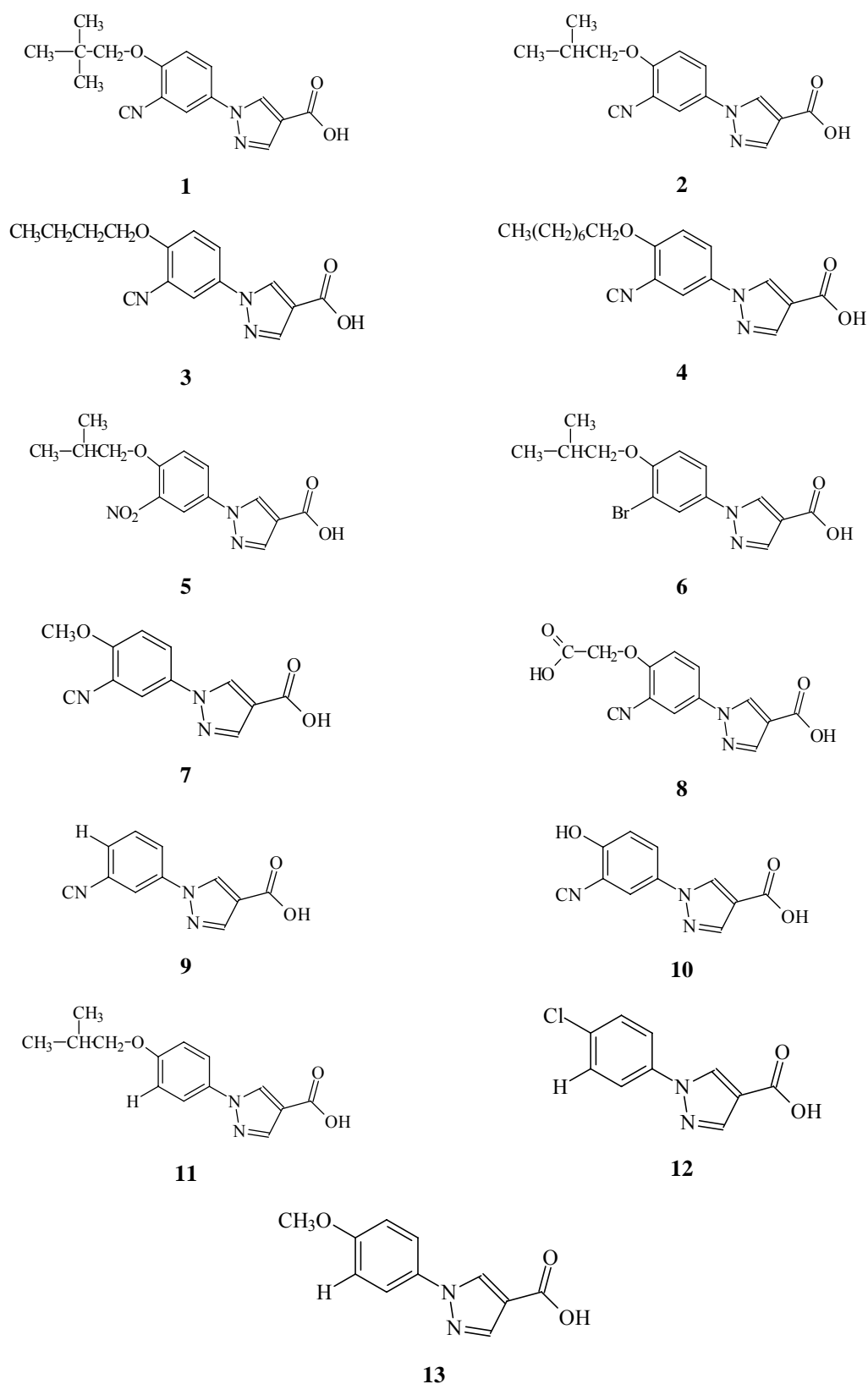
The enzyme xanthine oxidase (XO) is responsible for the production of uric acid from hypoxanthine and xanthine, which are originated from the degradation of exogenous or endogenous purine basis [1]. The accumulation of uric acid in the body is responsible for the formation of several diseases, for example gout [2,3]. Another process occurs in hypoxia conditions where the enzyme XO is also involved in the liberation of reactive oxygen species (ROS) such as the free radicals hydroxyl (HO•), peroxide nitrite (OONO<sup>-</sup>), superoxide (O<sub>2</sub><sup>-</sup>) and especially hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). These reactive species are associated with some diseases as Parkinson's, Alzheimer's and other pathological processes [4-9].

Both processes (urate and free radicals productions) are very dangerous to human organism and should be avoided. The allopurinol molecule has structural similarity with hypoxanthine and xanthine (see Figure 1). So, it is a powerful competitive inhibitor of the XO enzyme and is widely used to control the uric acid levels in the plasma and in the treatment of gout [10]. However, several people have developed serious allergic reactions, called Allopurinol Intolerance Syndrome (AIS), which is characterized by fever, renal and hepatic compromising, erythematous lesions and other symptoms associated with the secondary metabolite oxypurinol (which has a half-life markedly longer than the allopurinol [11,12]).



**Figure 1.** Structural analogy between allopurinol and hypoxanthine.

An alternative for the treatment of this syndrome (AID) is the search for compounds without the purine rings which prevent the formation of oxypurinol. Ishibuchi *et al.* [10] synthesized a series of phenylpyrazole compounds that presented different capacities of inhibition of the XO enzyme. The chemical structure of each compound synthesized by Ishibuchi *et al.* [10] is showed in Figure 2 and the IC<sub>50</sub> values for the phenylpyrazole compounds studied are displayed in Table 1.



**Figure 2.** Chemical structure of the 13 phenylpyrazole compounds synthesized by Ishibuchi *et al.* [10].

**Table 1.** The two groups of the phenylpyrazole compounds studied: Group A (compounds with high activity), and Group B (compounds with low activity)

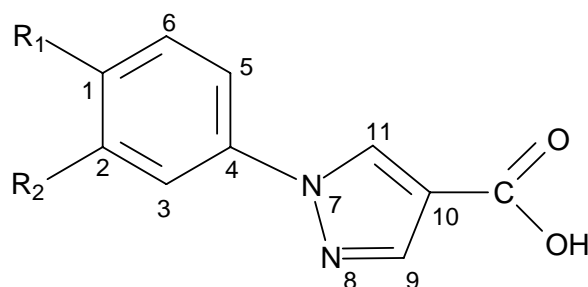
<b>Grupo A<sup>a</sup></b>				
<i>Compound</i>	<i>IC<sub>50</sub> value (nmols/L)</i>	<i>Log IC<sub>50</sub></i>	<i>R<sub>1</sub></i>	<i>R<sub>2</sub></i>
1	5.8	0.76	OCH <sub>2</sub> C(CH <sub>3</sub> ) <sub>3</sub>	CN
2	7.1	0.85	OCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	CN
3	15	1.18	OC <sub>4</sub> H <sub>9</sub>	CN
4	21	1.32	OC <sub>8</sub> H <sub>17</sub>	CN
5	33	1.52	OCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	NO <sub>2</sub>
6	43	1.63	OCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	Br
<b>Grupo B</b>				
7	460	2.66	OCH <sub>3</sub>	CN
9	1500	3.17	OCH <sub>2</sub> CO <sub>2</sub> H	CN
9	3300	3.52	H	CN
10	3600	3.56	OH	CN
11	4700	3.67	OCH <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	H
12	NA	NA	Cl	H
13	NA	NA	OCH <sub>3</sub>	H

<sup>a</sup> From Ishibuchi *et al.* [10]; NA = No Activity (less than 50% of activity)

Several molecular properties of chemical substances can be correlated with biological activity data and this correlation is called as structure-activity relationship (SAR) [13-16]. The SAR methods have been used successfully in pharmaceutical applications [17] and in this work we performed a structure-activity relationship study using quantum chemical and chemometric methods with the aim to understand which molecular properties of the phenylpyrazole compounds listed in Table 1 are responsible for the inhibitory activity of the XO enzyme presented by these compounds.

## 2 MATERIALS AND METHODS

The general structure of a phenylpyrazole compound and the numbering used in this work are displayed in Figure 3. The molecular structure of each phenylpyrazole compound studied is showed in Figure 2. The compounds were divided into two groups: *actives* (Group A - compounds 1 to 6 in Figure 2), and *inactives* (Group B - compounds 7 to 14 in Figure 2). The inhibitory activity of each phenylpyrazole compound was determined by Ishibuchi *et al.* [10] using a spectrophotometric measurement to accompany the uric acid production. The inhibitory activity was expressed in terms of  $IC_{50}$  (compound concentration that produces 50% of the maximum inhibitory effect) obtained from a mixture containing the XO enzyme, the xanthine (substrate) and each compound to be tested [10]. The classification between active (Group A) and inactive (Group B) phenylpyrazole compounds considered the  $\text{Log } IC_{50}$  values (see Table 1), i.e. compounds with  $\text{Log } IC_{50}$  lower than 2.0 were considered as having a high XO inhibitory activity (i.e. they were considered in this work as active compounds) and compounds with  $\text{Log } IC_{50}$  higher than 2.0 were considered as having a low XO inhibitory activity (i.e. they were considered in this work as inactive compounds).



**Figure 3.** General structure of the phenylpyrazole compounds studied and the numbering used in this work.

The initial molecular geometry of each compound was obtained by using the molecular mechanics method (MM+) [18,19]. Afterwards, the Density Functional Theory (DFT) with the B3LYP functional (DFT/B3LYP) [20-22] was used for a final optimization. The calculation of the molecular properties used to describe the main features of the compounds were performed by using the DFT/B3LYP method and the 6-31G\* basis set.

We considered in this work that the biological activity of a drug depends mainly on three different kinds of molecular properties: electronic, steric (and/or topological) and lipophilic [23]. So, we decided to calculate a large set of physicochemical descriptors (parameters or variables) by using the available computational packages. The following descriptors were calculated:  $Q_n$  (atomic charges derived from the electrostatic potential),  $E_{\text{HOMO}}$  (the highest occupied molecular orbital

energy),  $E_{LUMO}$  (the lowest unoccupied molecular orbital energy),  $\chi$  (Mulliken's electronegativity),  $\mu$  (dipole moment),  $\alpha$  (molecular polarizability),  $\Delta H_f$  (heat of formation), MR (molar refractivity), A (molecular surface area), Vol (volume),  $E_T$  (total energy),  $E_{el}$  (electronic energy), E.A. (electronic affinity), log P (partition coefficient),  $B_n$  (bond order),  $D_n$  (dihedral angles),  $A_n$  (interatomic angles),  $L_n$  (bond length) and a series of topological descriptors.

The calculated descriptors were selected so that they could represent the electronic ( $Q_n$ ,  $E_{HOMO}$ ,  $E_{LUMO}$ ,  $\chi$ ,  $\mu$ ,  $\alpha$ ,  $\Delta H_f$ , MR, E.A.,  $E_T$  and  $E_{el}$ ), steric (A and Vol), lipophilic (log P) and topological (several indexes) features of the compounds studied. These features are supposed to be important in the XO inhibitory activity presented by the phenylpyrazole compounds studied. The correlation between the molecular properties calculated and the biological activity studied was done by using the pattern recognition methods (PCA, HCA, and SDA) built in the computational package Pirouette [24].

The descriptors  $Q_n$ ,  $E_{HOMO}$ ,  $E_{LUMO}$ ,  $\mu$ ,  $\alpha$ ,  $E_T$ ,  $E_{el}$  and  $\Delta H_f$  were calculated by using the DFT/B3LYP and the 6-31G\* basis set of the molecular package Gaussian 98 [22]. The descriptors MR, A, Vol and log P were calculated with the program ChemPlus [25] and the descriptors  $\chi$  and E.A. were obtained according to Mulliken's theory [26] and they are defined as

$$\chi = \frac{1}{2}(IP + EA) = \frac{1}{2}(-E_{HOMO} - E_{LUMO}) \quad (1)$$

$$E.A. = -E_{LUMO} \quad (2)$$

The topological descriptors were calculated with the program Dragon 2.1 [27] which evaluates a large variety of descriptors that represent different sources of chemical information. These descriptors contain information on the whole 3D molecular structure in terms of size, shape, symmetry and atomic distribution [28].

### 3 RESULTS AND DISCUSSION

#### 3.1 PCA results

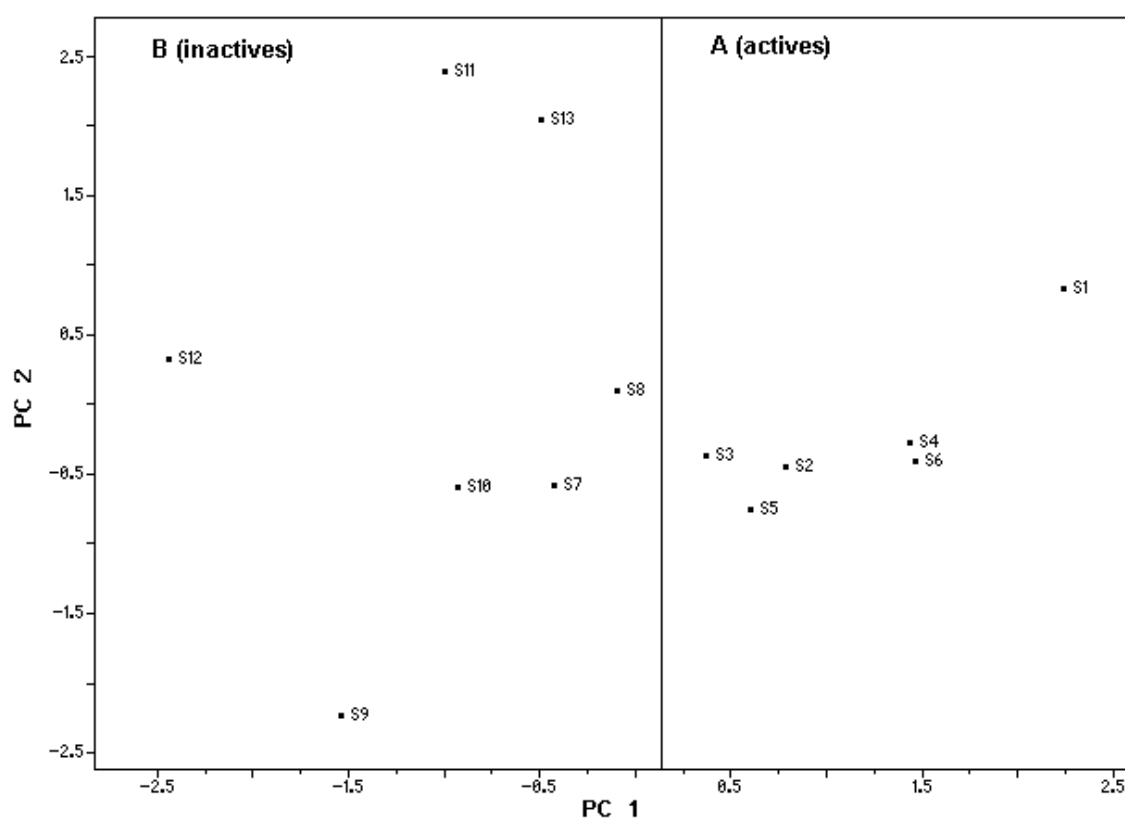
The main purpose of the Principal Component Analysis (PCA) is to determine a few linear combinations of the original variables which can be used to summarize the data set without losing information [29]. This is achieved by a linear transformation of the original data set of variables into a smaller number of uncorrelated principal components (PCs). Geometrically, this transformation represents the rotation of the original coordinate system and the direction of the maximum residual variance is given by the first principal component axis. The second principal component, orthogonal to the first one, has the second maximum variance and so on. In this way, projections conserving maximum amounts of statistical information can be plotted in order to show us a more detailed study of the data structure [30-32].

In this work, we autoscaled all calculated variables so that they can be compared in the same scale. Afterwards, PCA was used to reduce the number of variables and select the most relevant ones, i.e. those responsible for the XO inhibitory activity. Several tests were performed and we obtained a good separation between active and inactive phenylpyrazole compounds using four variables:  $\mu$ ,  $QR_2$ ,  $D_3$  and MR (see Table 2).

**Table 2.** Calculated values for the most important variables that classify the compounds studied ( $\mu$ ,  $QR_2$ ,  $D_3$  and MR)

Compound	$\mu$ (Debye)	$QR_2$	$D_3$ ( $^\circ$ )	MR ( $\text{Å}^3$ )
1	5.409	-0.024	178.315	43.18
2	3.851	-0.055	178.863	38.78
3	4.080	-0.069	179.253	38.91
4	4.152	-0.063	179.201	57.32
5	4.055	-0.115	179.206	39.02
6	3.790	-0.040	178.396	40.33
7	3.809	-0.071	179.232	25.04
8	4.452	-0.043	179.213	28.28
9	1.865	-0.110	179.390	18.62
10	3.501	-0.047	179.267	20.00
11	5.428	0.151	179.793	33.02
12	2.954	0.110	179.714	17.35
13	5.292	0.129	178.940	19.28

From PCA results, we can see that the first three principal components (PC<sub>1</sub>, PC<sub>2</sub> and PC<sub>3</sub>) describe 95.05% of the overall variance as follows: PC<sub>1</sub> = 43.66%, PC<sub>2</sub> = 36.54% and PC<sub>3</sub> = 14.86%. Since almost all of the variance is explained by the first two PCs, their score plot is a reliable representation of the spatial distribution of the points for the data set studied. The most informative score plot is presented in Figure 4 (PC<sub>1</sub> versus PC<sub>2</sub>) and we can see that PC<sub>1</sub> alone is responsible for the separation between active and inactive compounds. Looking at Figure 4, we can see that the thirteen compounds studied were separated into two groups: A (active compounds – compounds 1 to 6 in Table 1) and B (inactive compounds - compounds 7 to 13 in Table 1) where PC<sub>1</sub>>0 for the active compounds and PC<sub>1</sub><0 for the inactive ones.

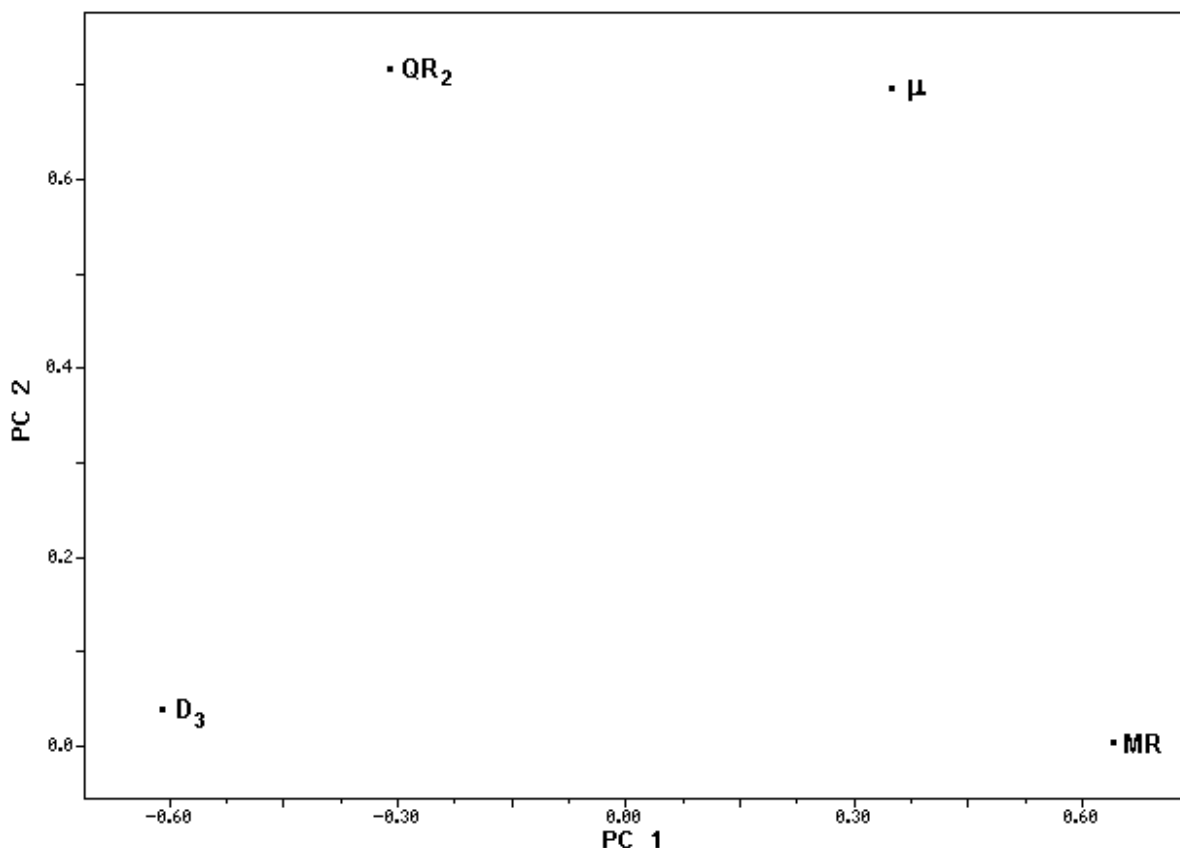


**Figure 4.** Score plot for the thirteen compounds studied.

The loading vectors for the first two principal components (PC<sub>1</sub> and PC<sub>2</sub>) are displayed in Figure 5. According to Figure 5, PC<sub>1</sub> can be expressed through the following equation:

$$PC_1 = 0.350 [\mu] - 0.309 [QR_2] - 0.609 [D_3] + 0.640 [MR] \quad (3)$$





**Figure 5.** Loading plot for the four variables responsible for the classification of the thirteen compounds studied:  $\mu$  (dipole moment),  $QR_2$  (total charge of the substituent at the atom C2),  $D_3$  (torsion angle formed by the atoms C6-C1-C2-R2) and MR (molar refractivity).

From Eq. (3) we can see that active molecules ( $PC_1 > 0$ ) can be obtained when we have higher  $\mu$  and MR values (notice that  $\mu$  and MR have positive coefficient in  $PC_1$  equation) combined with negative  $QR_2$  values and lower  $D_3$  values than those obtained for the inactive compounds. In this way, some important features on the active compounds can be observed:

- (i) dipole moment ( $\mu$ ): active compounds need to have high values for  $\mu$  and this indicates that the active molecules need to present electronegative substituents in order to influence the electron density distribution in the overall structure, avoiding the interaction between the phenylpyrazole compounds and the biological receptor;
- (ii) total charge of the substituent  $R_2$  ( $QR_2$ ): for a compound to be active, it needs to have negative  $QR_2$  values, i.e. the active compounds need to present electrophilic substituents at atom C2;
- (iii) torsion angle formed by the atoms C6-C1-C2-R2 ( $D_3$ ): the orientation of the substituent  $R_2$  is an important feature so that the phenylpyrazole compounds present XO inhibitory activity, i.e. active compounds need to have lower  $D_3$  values than those obtained for the inactive compounds;

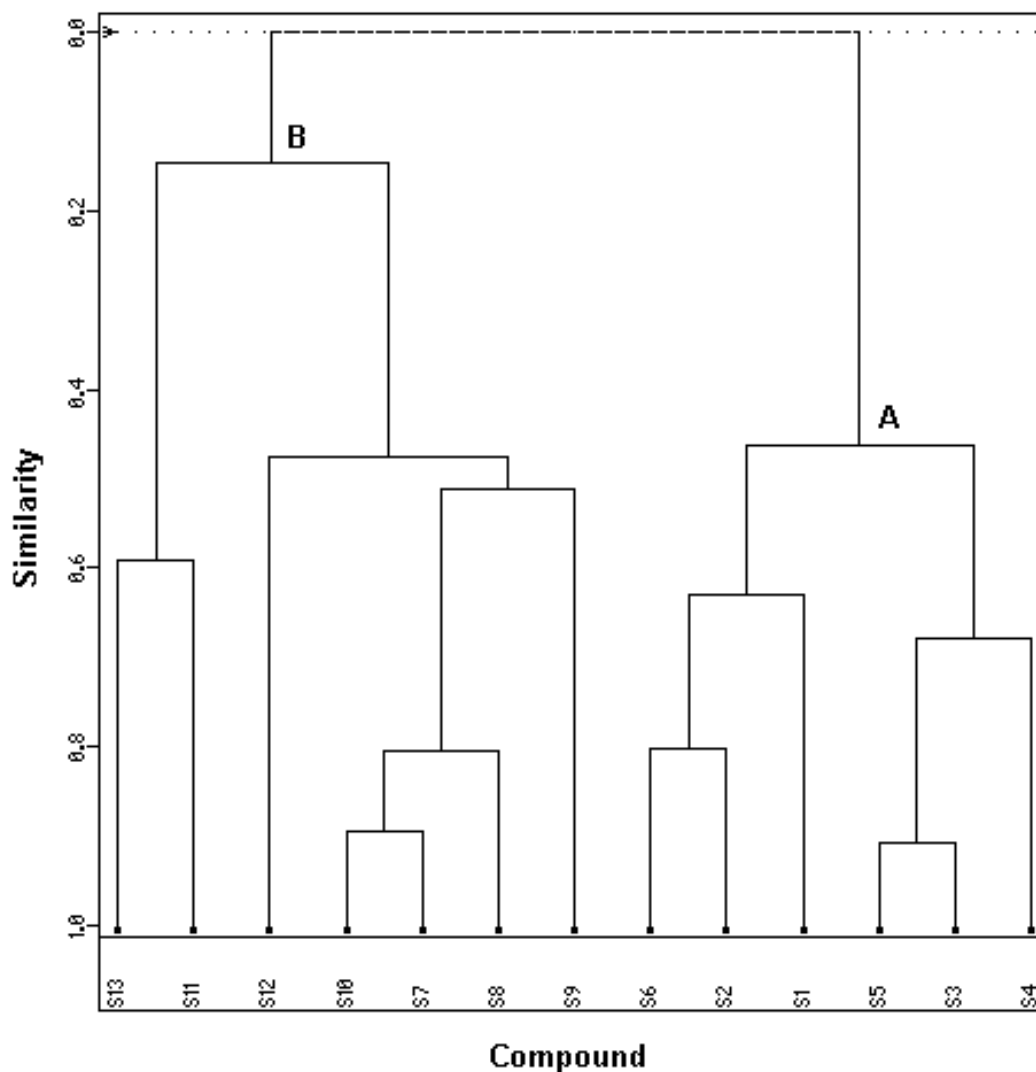
- (iv) molar refractivity (MR): for the active compounds, MR needs to have high values and this indicates that some substituents in the phenylpyrazole compounds studied can interact with the biological receptor by two ways: (a) through polar groups localized at the molecules studied (due to polarizability effects); (b) through steric effects, according to the size of the substituents. These two ways of interaction can occur as the presence of certain substituents could cause modifications on the receptor structure, avoiding the interaction between the phenylpyrazole compounds and the biological receptor.

Analyzing the four variables responsible for the XO inhibitory activity caused by the phenylpyrazole compounds studied, we can classify them in two classes: electronic ( $\mu$ ,  $QR_2$  and MR) and steric ( $D_3$  and MR). From this fact we can say that the main forces that rule the interaction between the phenylpyrazole compounds under study and the biological receptor are electronic and steric effects, mainly due to the presence of certain substituents that influence the electron density and the geometric structure of the compounds studied.

### 3.2 HCA results

Hierarchical cluster analysis (HCA) was used in this work as it groups the compounds based on their similarity degree. In this technique, each compound is initially assumed to be a lone cluster and one similarity matrix is built, generally calculating the Euclidean distance among all of the objects. Then, the compounds are clustered together and treated as a single cluster and successive iterations lead to the total clustering of all compounds according to their similarity level generating a dendrogram [32].

Figure 6 shows our results obtained with the HCA analysis. The horizontal lines represent the compounds and the vertical lines the similarity values between pairs of compounds, a compound and a group of compounds and among groups of compounds. The similarity value between the two classes of compounds was 0.0 and this means these two classes are distinct. From Figure 6, we can see that the HCA results are very similar to those obtained with the PCA analysis, i.e. the compounds studied were grouped into two categories: *actives* (compounds 1 to 6 in Table 1) and *inactives* (compounds 7 to 13 in Table 1).



**Figure 6.** Dendrogram obtained for the thirteen phenylpyrazole compounds studied: Group A (actives) and Group B (inactives).

### 3.3 SDA results

The Stepwise Discriminant Analysis (SDA) is a multivariate technique used to build *discriminant functions* that represent linear combinations of the variables under study. These functions are supposed to divide groups of samples as distinctly as possible. SDA is an useful method to choose variables with high discriminant power (an indication of the influence of the variable over the separation of groups). After previous selection of the variables, the SDA method generated discriminant functions by using one variable at a time until the best discriminant function is obtained (the one that divide the samples in the most distinct groups). After the statistical

validation of the model, the discriminant function can be used to make predictions about the class of unknown samples [33,34].

For the set of compounds studied in this work, two discriminant functions were obtained: Group A (active compounds) and Group B (inactive compounds):

$$\text{Group A} = -2.34 - 1.17\mu + 0.03QR_2 - 1.85D_3 + 4.42MR \quad (4)$$

$$\text{Group B} = -2.14 + 1.15\mu + 0.04QR_2 + 1.64D_3 - 4.34MR \quad (5)$$

The allocation rule derived from our SDA result, when the XO inhibitory activity of a phenylpyrazole compound is investigated, is: (a) initially one calculates, for the compound studied, the value of the most important variables obtained with the SDA methodology, i.e.  $\mu$ ,  $QR_2$ ,  $D_3$  and  $MR$ ; (b) substitute these values in the two discriminant functions obtained in this work (Eqs. (4) and (5)); (c) check which discriminant function presents the higher value. The phenylpyrazole compound is active if the higher value is related to the discriminant function of Group A and vice versa. The results for the SDA classification are presented in Table 3. From Table 3 we can see that all compounds studied were correctly classified in their respective groups.

We also used in this work a cross-validation test using the “leave-one-out” methodology with the aim to determine if our SDA model (Eqs. (4) and (5)) is reliable. In this procedure, one compound is omitted of the data set and the discriminant functions (Eqs. (4) and (5)) are built based on the remaining compounds. Afterwards, the omitted compound is classified according to the generated discriminant functions (Eqs. (4) and (5)). In the next step, the omitted compound is included and a new compound is removed and the procedure goes on until the last compound is removed. The results obtained with the cross-validation methodology are summarized in Table 4. From Table 4 we can see that our models obtained with PCA, HCA and SDA are reliable, as the cross-validation error is equal to 0%.

## 4 CONCLUSIONS

In this work we used multivariate methods to understand the inhibitory activity of the enzyme xanthine oxidase (XO) presented by phenylpyrazole compounds. The PCA (Principal Component Analysis), HCA (Hierarchical Cluster Analysis) and SDA (Stepwise Discriminating Analysis) results showed that these statistical methodologies are powerful tools to be used in structure-activity relationship studies, as they give information on the main properties responsible for the biological

activity presented by the compounds studied. The four properties selected in this work were:  $\mu$  (dipole moment),  $QR_2$  (total charge of the substituent  $R_2$ ),  $D_3$  (torsion angle formed by the atoms C6-C1-C2-R2) and MR (molar refractivity). These molecular properties represent electronic ( $\mu$ ,  $QR_2$  and MR) and steric ( $D_3$  and MR) effects and should be considered in the design of new phenylpyrazole compounds presenting XO inhibitory activity. This conclusion is supported by the fact that the three chemometric methods (PCA, HCA and SDA) used in this work provided reliable models, i.e. they were built with the same variables and presented consistent results. The SDA model, which presented a classification error of 0% (with cross-validation), can be especially used in the classification of new phenylpyrazole compounds, furnishing directions for the synthesis of new phenylpyrazole compounds that present XO inhibitory activity.

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