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Cluster Analysis and Artificial Neural Networks Multivariate Classification of Onion Varieties

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Eight cultivars of different colored onions (white, golden, and red) were evaluated for fresh bulbs cultivated and grown under the same environmental and agronomical conditions. Cluster analysis and principal component analysis, based on different flavonoids, total phenols, and pungency, data showed that the onions were not clustered according to variety (genetic similarity degree), whereas the color was the variable with the highest influence, ranging between 50 and 70%. Artificial neural networks were applied to study the possibility of discriminating among onion varieties. Characterization of the onion according to variety and procedence of the seeds was around 95−100%. Samples belonging to the Carrizal Alto procedence had an incorrect classification for 25% of the data.

KEYWORDS: Onion (*Allium cepa*); cluster analysis; ANN multivariate classification; flavonoids

INTRODUCTION

Several studies on the antioxidant activity of *Allium* vegetables have been conducted as a result of the current interest in health foods (*1*, *2*). The results suggest that onion (*Allium cepa* L.) and garlic (*Allium sativium* L.) are sources of numerous antioxidants that play an important role in physiological protection against reactive oxygen species generated in the body. Onion is a good source of quercetin, one of the most abundant flavonol-type flavonoids in fruits and vegetables (*3*).

Flavonoids are exclusive and widely distributed in the plant kingdom. They are mainly produced as a pigment and play an important role in the normal growth, development, and defense of plants. The ability of so-called dietary flavonoids to prevent lipid peroxidation in live cells has been described in the literature (*4*, *5*). At the biochemical level, flavonoids act as enzyme inhibitors, provide defense against ultraviolet radiation, are chelating agents for metals, and act as reducing agents (*6*). Flavonoids, particularly quercetin derivatives, have recently been the subject of special attention because of their role as dietary constituents. Epidemiological studies pointed to their possible role in preventing cardiovascular diseases and cancer (*7*−*9*). This health-promoting activity seems to be related to the antioxidant (free radical scavenging) activity of flavonoids (*10*). Quercetin is the major flavonoid present in onions (*A. cepa* L.). The flavonoid quercetin is a potent in vitro inhibitor of membrane lipid peroxidation and LDL oxidation. In fact, its antioxidant capacity (mM Trolox) has been demonstrated to be 5-fold higher than those observed for vitamin E and vitamin C (*11*). Almost 180 different glycosides of quercetin have been described in nature (*12*).

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Onions are widely used as vegetables in many countries, and they are easily cultivated in different environments. Over the past 10 years onions have been receiving more attention from consumers, who have shown an increasing interest in their bioactive or functional compounds that provide health benefits including disease prevention. Foods with a high content of flavonoids, in addition to their nutritive value, can have a protective effect on human health. Bordia et al. (*13*) and Dorant et al. (*14*) confirmed such medicinal properties and that onion consumption seems to prevent the rise in serum cholesterol after a fatty meal and to control the growth of *Helicobacter pylori*, which is one of the risk factors for pectic ulcer and stomach carcinoma.

Besides the nutritional and medicinal values, onions are consumed for their flavor and because their use is known to enhance the flavor of other foods (*15*, *16*). The pungency in onions is caused by volatile sulfur compounds, which can be related to the flavor intensity in onions. Some of these compounds affect the eyes, often producing the so-called lachrymatory effect. The flavor of onions and other *Allium* vegetables is dominated by organosulfur stemming from the enzymatic decomposition of *S*-alk(en)yl-L-cysteine *S*-oxide flavor precursors. The typical onion flavor comes from an enzyme called alliinase; this enzyme is released and instantly breaks down the flavor precursors. Besides which, chemical compounds related to the flavor, such as pyruvic acid as well as ammonia and chemically unstable sulfenic acids, are generated from precursors (*17*). The amount of enzymatically generated pyruvic acid on onion homogenization is therefore a good measure of the allinase action on the flavor precursors and has been shown to be correlated with perceived onion pungency (*18*−*20*). The balance between pungency and sugars influences the sweetness of onions (*15*, *21*). Phenolic compounds are secondary products of sugar catabolism. Environmental

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conditions can modify certain transformation speeds, sometimes to the point of upsetting the order of physiological changes in the ripening onion.

The aim of this work was to differentiate onion cultivars by applying multivariate analysis techniques such as principal component analysis, discriminant analysis, and artificial neural networks on their quercetin, kaempferol, and glycoside quercetin contents, total phenols, and pungency and to study the influence of variables such as cultivar and the procedence of the onion seeds.

MATERIALS AND METHODS

Onion Sampling and Sample Preparation. The five traditional cultivars Guayonje (n = 24), San Juan de la Rambla (n = 12), Carrizal Alto (n = 6), Carrizal Bajo (n = 12), and Masca (n = 18) from Tenerife Island (Canary Islands) were studied, along with a commercial cultivar, Texas Early Grano 502 (n = 6), where n corresponds to the number of samples. The onion cultivars had two different colored skins: (i) yellow, belonging to the Texas and San Juan de la Rambla cultivars, and (ii) red, for the rest of the cultivars. The seeds of the analyzed onion cultivars came from different regions of the island. The seeds for the Texas cultivar were acquired from Z-seeds, the Masca cultivar seeds were from El Turrón, the Guayonje cultivar seeds were from Puerto de la Madera (n = 12) and Juan Fernández (n = 12), the San Juan de la Rambla cultivar seeds were from Las Aguas (n = 6) and El Rosario (n = 6), and the Carrizal Alto and Carrizal Bajo cultivar seeds were from Carrizal Alto and Carrizal Bajo, respectively.

The onion seeds were cultivated on the experimental farm in Tacoronte (Tenerife, Spain). All of the onion cultivars were cultivated using the same agronomic, soil, and climatic conditions. As the planting took place under the same agricultural conditions, the changes on the analyzed variables are likely to be related to the influence of the varietal factor. The experimental onion plantation was designed by adopting a randomized block design with 3 repetitions and with 15 random accessions. Two onion samples containing three to five bulbs were collected from each experimental block, and six replicates by sample in each variety were analyzed. The onions were collected in the same maturation stage, when between 50 and 80% of the plants had had their leaves flattened (*22*−*24*). Harvesting took place in the months of June and July, and the onions were supplied by the Agricultural by Biodiversity Conservation Center of Tenerife (CCBAT).

Analytical Methods. Experimental details for the determination of flavonoids, total phenols, and pungency have been previously reported (*24*), and only certain specific details are given here for the eight variables used in this study.

Total Phenols. The extraction of total phenolic compounds was made by mixing the sample with methanol at 80% and sonicating for 10 min (*23*). The phenol content in the onion samples was spectrophotometrically analyzed at 750 nm using a Folin−Ciocalteu (Sigma Chemical Co., St. Louis, MO) colorimetric method described by Kujala et al. (*25*). A UV−vis (diode array) Hewlett-Packard 8453 spectrophotometer equipped with a Hewlett-Packard Vectra XA computer was used. Gallic acid (Sigma-Aldrich) was used as a standard for the quantification and expression of the results.

HPLC Determination of Flavonoids. The HPLC method used was based on the method proposed by Lombard et al. (*26*) with slight modifications. About 1 g of the frozen homogenized onion puree was weighed directly in polypropylene tubes and mixed with 2 mL of methanol 80% with water acidulated with trifluoroacetic acid to pH 2.5. Afterward, the tubes were put into an ultrasound bath for 30 min at 40 °C and then centrifuged at 4000 rpm for 10 min. The supernatant was carefully recovered to prevent contamination with the homogenized onion puree pellet. This liquid phase was stored at −80 °C in the freezer. A milliliter of this dissolution was passed through a 0.45 μm filter GHP (Waters, Milford, MA) prior to HPLC analysis. Duplicate injections were performed, and average peak areas were used for quantification.

HPLC grade methanol (A) and 0.05% trifluoroacetic acid (B) were used as the mobile phases in an isocratic regimen in the proportions of 45% of A and 55% of B. Absorbance was monitored at 362 nm. The injection volumes of the samples were 15 μL, the flow rate was 0.9 mL/min, and the temperature of the column was 30 °C. Quercetin dihydrate (Sigma-Aldrich) and kaempferol (Sigma-Aldrich) were used as standards for identifying the free quercetin and kaempferol. We used the calibration corresponding to isoquercetin (quercetin-3-*O*-glucopyranoside) (Extrasynthèse, Genay, France) because it is chemically similar to the major quercetin glucosides (*26*) for the quantification of quercetin monoglucosides.

Pungency. One bulb from each onion sample was sliced in half longitudinally, and one portion was homogenized using a model T-25 Basic Turmix (Ika-Werke, Staufen, Germany) at a ratio of 1 mL of added water/g of onion. The homogenate was left to stand for 10 min at room temperature and then filtered. Colorimetric determination of pyruvic acid was performed using a modified Schwimmer and Weston method (*27*). An aliquot of 25 μL of the onion filtrate was added to 1 mL of water in a test tube. One milliliter of 0.25 g/L 2,4-dinitrophenylhydrazine in 1 M HCl was added to this, and the sample was placed in a 37 °C water bath. The samples were removed from the water bath after 10 min, and 1 mL of 1.5 M NaOH was added. The absorbance at 515 nm was then determined. A blank and standards were prepared by adding 25 μL of sodium pyruvate solution, ranging in concentration from 0 to 6 mM.

STATISTICAL DATA ANALYSIS

Eight variables, quercetin (Q), quercetin diglucoside (QDG), quercetin monoglucoside 1 (QMG1), quercetin monoglucoside 2 (QMG2), isoquercetin (IQ), kaempferol (K), pungency (P), total phenols (TP), and average weight (W), were studied to differentiate six commercial cultivars of different colored onions according to their flavonoid content. Each datum was standardized according to $z_{ij} = (x_{ij}\text{-average}_j)/(\text{standard deviation})_j$, to give each variable equal weight in the statistical analysis for the analysis. The effect of procedence and cultivar on onion plants in the field on the flavonoids, total phenol content and analyzed pungency was assessed using variance analysis (ANOVA).

To explore the chemical data for pattern recognition, principal component analysis (PCA) was applied with the aim of narrowing the focus of our attention to the main variables and natural trends in the samples. When the PCA was performed, the information about the samples contained in the original variables was projected onto a smaller number of new variables called principal components (PCs) (*28*).

Cluster analysis (CA) is one of the most useful statistical tools used in chemometrics for discovering groups and localizing (identifying) interesting distributions and patterns in the underlying information contained in the data (*29*). There are several clustering algorithms; Ward's method, for our purposes, was selected as the linkage method and with Euclidean distance being the measure of similarity. Linear discriminant analysis (LDA) is based on the extraction of discriminant functions of the independent variables by means of a qualitative dependent variable and several quantitative independent variables.

All of the statistical analyses have been performed using Statistica V.6 (StatSoft Inc., Tulsa, OK) and Trajan 3.0 software package Trajan Neural Network Simulator, release 3.0 D (copyright Trajan Software Ltd., 1996−1998).

Artificial Neural Networks (ANN). Seventy-eight onion samples of 6 cultivars, characterized by 8 variables, have been used in the training process. The onion samples were classified using ANN techniques according to cultivar. The multilayer perceptron (MLP) applying perceptron learning algorithms was used. The lowest root mean squared (rms) error was reached by a combination of backpropagation and quick propagation algorithms. The data set is randomly split into training, test, and validation sets. A typical three-layer neural network consisting of an input, hidden, and output layer was planned. The neural network was processed to generate a function that models the underlying relationship present in the onion data, between the input and the output layers.

During the training step numerous networks with different architectures were examined. The number of input neurons as

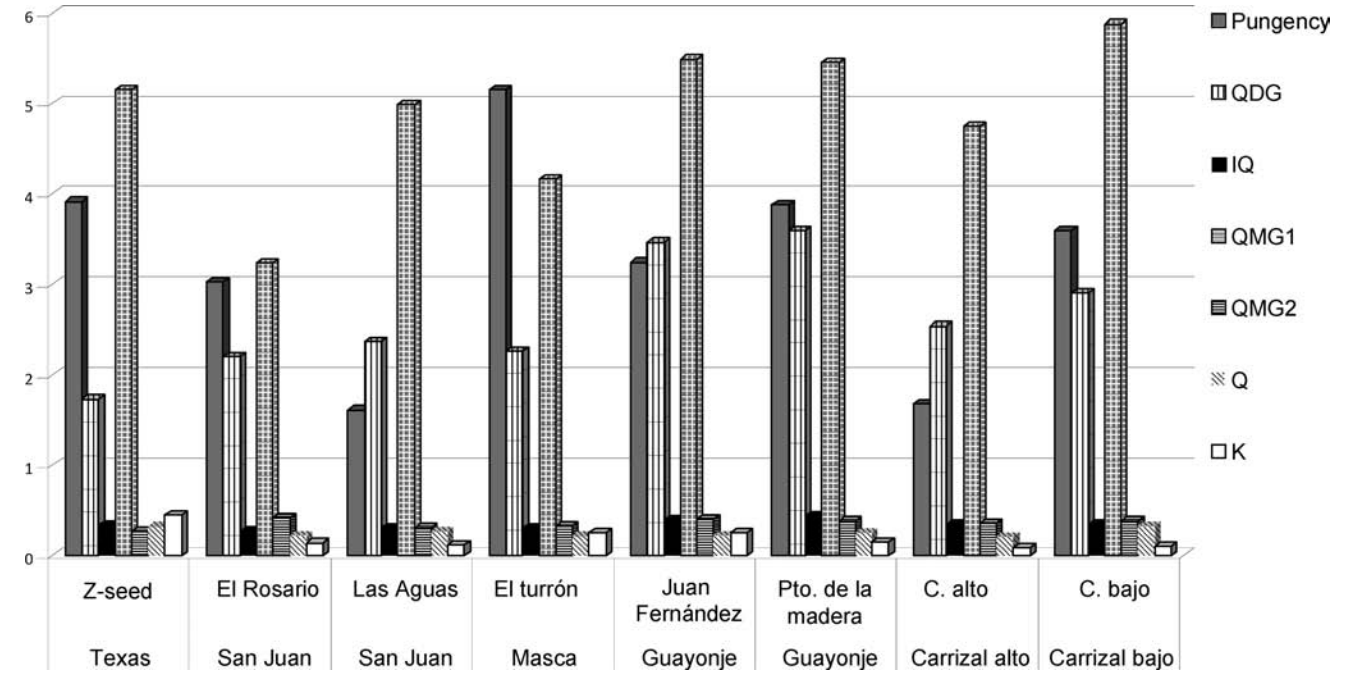

Figure 1. Summary of flavonoids, total phenol content, and pungency according to factors such as variety and procedence of onions.

well as output neurons was set by the number of variables and cultivars, respectively. The network selection was based on the appropriate selection of (a) the number of hidden layers in multilayer perceptrons and (b) the number of hidden neurons in the network. The number of hidden neurons, N_h, was found by examining several types of the 3-MLP with regard to the corresponding final rms error. The optimal number of hidden units was found at the break on the rms versus N_h dependence for all of the studied problems. The decision about the network design was based on the corresponding training sets but not individually for each validation set in the leave-one-out procedure (*29*, *30*).

RESULTS AND DISCUSSION

The determination of individual flavonoid contents in onion bulbs is an important chemical analysis that is performed to evaluate their quality. **Figure 1** shows a summary of the qualitative and quantitative information obtained about the composition free flavonols (quercetin, kaempferol), glycosides of quercetin (QDG, QMG1, QMG2, and IQ), and pungency. Quercetin and its derivatives were the dominant flavonoids in the onions, whereas the flavonoid kaempferol was a minor flavonoid. QMG1 was the most abundant quercetin glycoside in all onion cultivars studied. This flavonoid could be hyperoxide or quercitrin according to the chemical species identified by other authors (*25*, *31*). The onion cultivar that showed the maximum pungency was Masca; in contrast, the Carrizal Bajo cultivar showed the lowest pungency. There were numerous significant differences in many of the analyzed flavonoids, total phenols, and pungency between the onion cultivars (*32*).

As shown by Rodriguez et al. (*24*) the univariate data treatment was not able to differentiate/classify the onions on the basis of their flavonoid contents according to cultivar or seed procedence; thus, a multivariate approach is considered in this work. CA, PCA, LDA, and ANNs were applied to unravel the hidden patterns present in the data.

Cluster Analysis. CA, one of the unsupervised learning methods, which is designed to identify natural groupings of objects in the data set, was applied to discover possible similarities or affinities among the onion samples. Owing to its unsupervised character, CA is a pattern recognition technique that can be used to reveal the structure residing in a data set (*33*). Because of the high number of samples considered ($n = 78$), the obtained dendrogram became very complex when each sample was identified, and therefore, this is not shown. CA did not cluster the samples according to either the cultivar or seed procedence. Great variation was found for samples belonging to the Guayonje and Masca cultivars. The classification rules given by the supervised chemometrics techniques were validated by means of a cross-validation procedure, which was performed by dividing the complete data set into a training set and an evaluation set.

CA failed to differentiate the onions according to different skin color of the (figure is not shown here due to its size). Because no natural groups of samples were observed, CA was performed to study random similarities in the behavior of the variables being considered. The dendrogram showed two main groups of variables A and B. The first group (A) includes two main subgroups: (i) Q and (ii) IQ, QDG, QDG1, and QMG2, whereas the second group (B) contains pungency, total phenols, and kaempferol variables.

Principal Component Analysis. PCA was performed using the autoscaled pungency variables, Q, IQ, QDG, QDG1, QMG2, total phenols, and kaempferol. Hence, the first seven PCs explained up to 95.6% of the total variance (eigenvalue ≥ 1). The loading of the variables used for the characterization of the onions in the space of the first three PCs is shown in **Figure 2**. It is clear in the loading plot that the variance of the system is mainly associated with QDG, QMG1, QMG2, and IQ, whereas pungency, kaempferol, total phenols, and mean weight load to a large extent to PC2. Moreover, pungency is the variable with the largest contribution to PC2, with Q being the flavonoid that has the highest load on PC3, and the contribution of the total phenols can be considered to be very low. Therefore, Q and quercetin glycosides are associated with different PCs, which could be explained by differences in the solubility of these compounds. Therefore, glycosylation increases the polarity of quercetin. Free quercetin is slightly less water-soluble than the quercetin glycosides.

The onion samples were projected on the score subspace with the aim of following underlying trends in the samples. The score

plot of the first two PCs (**Figure 3**), which accounted for 68.02% of the total variance, revealed that most of the samples tended to group into one large cluster, where some small groups of the samples are seen. Further differentiation was not possible in these clusters. One can observe, in **Figure 3**, that the onion samples belonging to San Juan de la Rambla, Carrizal Alto, and Carrizal Bajo cultivars had negative values of tPC2. These cultivars showed lower values for pungency, kaempferol, and total phenols than the other three onion cultivars considered (Masca, Guayonje, and Texas cultivars). The onion samples were not grouped according to color or mean weight.

To study the importance of each of the above variables for the chemical and nutritional interpretation of the results, variable selection strategies already applied in the literature were used (*34*−*36*). The variable selection strategies used here combined with PCA and factor analysis (FA) made it possible to discard those with the lowest contribution to the principal components (lowest loadings). By applying FA and, after application of the varimax rotation, the loading of variables in the first rotated factor are QDG, IQ, QMG1, QMG2; total phenols has a high weight in the second factor, whereas Q, kaempferol, and pungency have the greatest influence on the third, fourth, and fifth factors, respectively (**Table 1**). Mogren et al. (*36*) reported that there are only minor effects on onion properties, such as yield and size, from effects of the higher levels of nitrogen fertilizer. This study shows that where the onion is grown is a major environmental factor in determining quercetin concentration; the authors said that the highest variation in quercetin was correlated with global radiation in August. The glycosides QDG, IQ, QMG1, and QMG2 are grouped in the first factor, which is in agreement with the results observed in PCA. In accordance with that previously commented, such grouping may be due to the fact that the quercetin glycosides are more water-soluble than quercetin itself, which is necessary for the storage of the glycosides in the plant cell vacuoles (*37*, *38*).

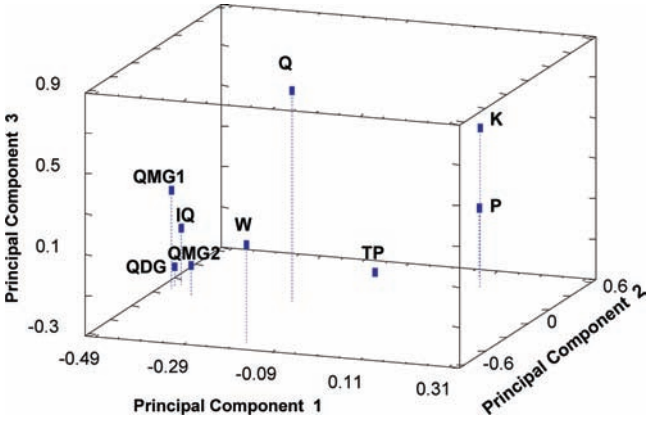

Figure 2. Results of principal component analysis. The loading plot is projected on the space of PC1 versus PC2.

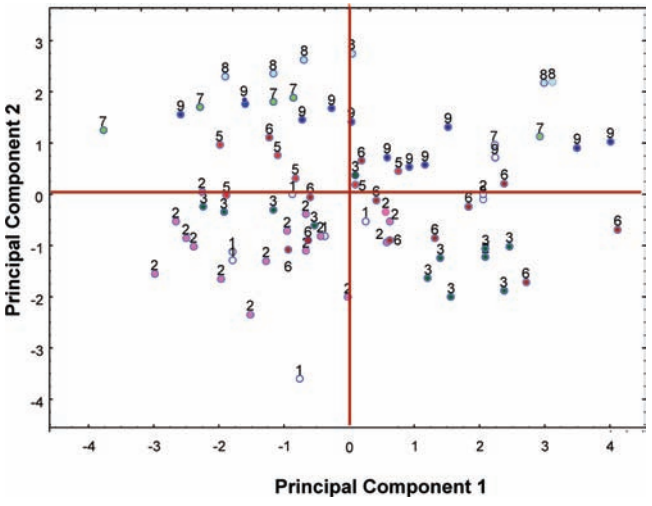

Figure 3. Scores plot of the onion samples projected on the space of PC1 versus PC2.

Linear Discriminant Analysis. LDA, a supervised pattern recognition method, was applied to differentiate the onion samples. The method supplies a number of linear discriminant functions to provide a method for predicting the group into which a new case will most likely fall. A stepwise LDA using the forward selection approach was performed and, to assess its discriminating capacity, Wilk's λ was applied as selection criterion, as well as an F statistic to determine the significance of the changes in λ when a new variable is tested. LDA, when using cultivar as a classification factor, gave high recognition percentages for the classification (95% confidence limits for each cultivar) of the onion samples with the Texas and Carrizal Alto cultivars reaching a recognition of 100% and the Carrizal Bajo cultivar reaching a recognition of 91.7% (see **Figure 4**). However, prediction ability was not so high, especially for the onion samples belonging to the Masca and Guayonje cultivars, which had classifications of 77.8 and 54.2%, respectively. The trend of grouping of samples belonging to the Guayonje and Masca cultivars together indicates some similarities between the cultivars and some of the onions. These results are in agreement with the results found in other previous studies (*32*).

When LDA was applied to differentiate the onions according to seed procedence as the classification factor, 79.5% of the samples were correctly classified. All eight seed procedences were considered. The procedences corresponding to Puerto de la Madera, Juan Fernández, and El Turrón are grouped in the plane defined with the two first discriminant functions. When the characteristics of the samples are studied, there were some factors that defined the distribution of the onion samples. The yellow onions, the Texas and San Juan de la Rambla cultivars, which have the smallest bulb size (mean weight < 180 g), tend to differentiate.

Table 1. Factor Analysis after Varimax Rotation

variable	rotated factor 1	rotated factor 2	rotated factor 3	rotated factor 4	rotated factor 5
pungency	−0.0554	0.1285	0.0182	0.1189	0.9762
QDG	0.8983	0.1971	0.0076	−0.1190	−0.0330
IQ	0.8356	0.2558	0.1782	0.0179	−0.1179
QMG1	0.8310	0.0176	0.3767	0.0102	−0.0623
QMG2	0.8428	−0.1371	−0.0425	−0.1461	0.0540
Q	0.1358	−0.0059	0.9761	0.0496	0.0221
kaempferol	−0.0873	0.0077	0.0472	0.9854	0.1160
phenols	0.1234	0.9593	−0.0076	0.0088	0.1398

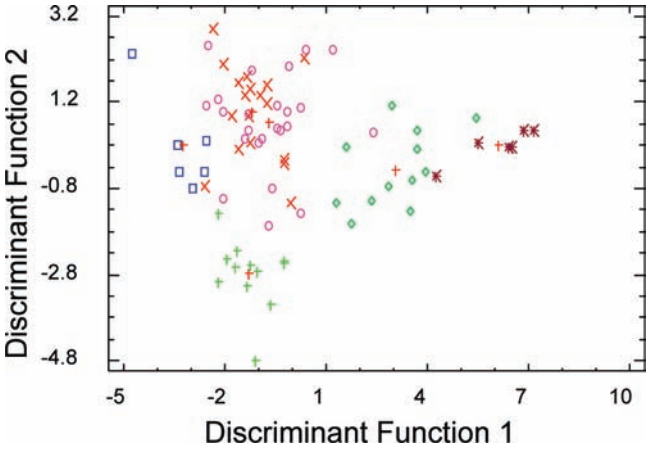

Figure 4. Scattered plot of the samples projected in the plane defined by the two discriminant functions according to cultivar as classification variable. Varieties: (□) Z-seed; (×) El Turrón; (○) Puerto de la Madera; (+) El Rosario; (∗) Juan Fernández; (◇) Carrizal Alto; (+) centroids.

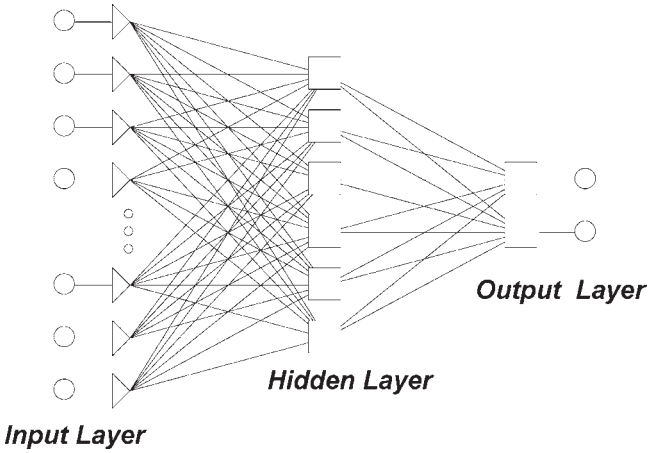

Figure 5. Optimized neural networks architecture.

Artificial Neural Networks. CA and LDA failed to show any clear classification for the onion samples when considering either the varietal or the seed procedence factors. Thus, the use of ANN as a mathematical tool by which the data processing can be characterized by analogy with biological neurons was considered. The ANN architecture with three-layer neural networks where the input layer included the variables Q, QDG, QMG1, QMG2, IQ, K, total phenols, and average weight and the ouput layer was the cultivar variety (V) was optimized. The optimal structure was found to be 9:6:2. The architecture of the 9:6:2 neural networks was selected using as the criterium the rms error as a function of the number of nodes in the hidden layer (**Figure 5**). With such a structure different algorithms such as backpropagation, conjugate gradient, and quick propagation were applied. The samples of five cultivars and of eight seed procedences were classified by means of the network and algorithms mentioned. The optimum network architecture was found to be 9:6:2 (input layer/hidden layer/output layer), according to the rms error. Calculations for the varietal and seed procedence factors, using learning rate 0.5, momentum 0.9, and 20.000 epochs, followed by the leave-one-out validation were performed under the described conditions of the training process. The program randomly assigned the samples to the training, test, and validation sets, and the criteria of minimal rms was kept during the prediction. The results shown in **Figure 6** give a 95−98% classification success for both classification criteria for the onion

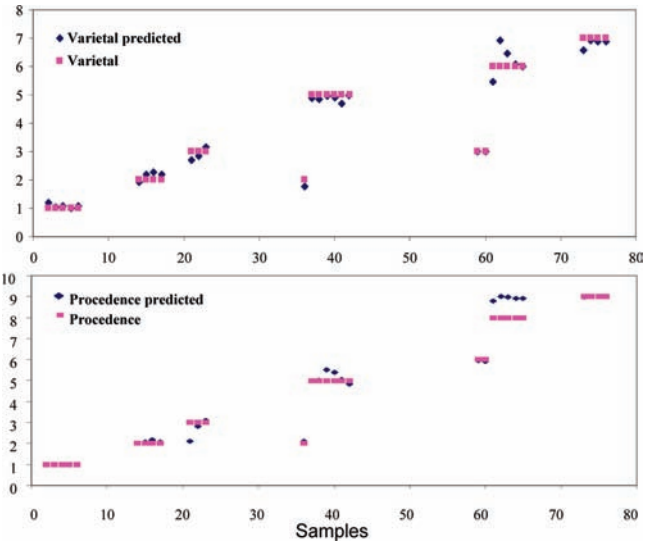

Figure 6. Classification using ANN of onion samples according to cultivar and procedence as classification variable.

samples with the exception of those belonging to the procedence named Carrizal Alto (8) and varietal (6).

To arrive at the classification of the onions from different cultivars and seed origin, the onions were characterized on the basis of their flavonoid and total phenol contents and pungency. PCA, two clustering methods (unsupervised and supervised analysis), and ANNs were used for chemometrical data processing to arrive at the classification of the onions. In light of the obtained results CA and LDA failed to classify the samples, based on the use of the variables Q, QDG, QMG1, QMG2, IQ, K, total phenols, and average weight for classifying the onions belonging to different varieties. A significant improvement in the classification of the onions to their varietal label was achieved using ANNs when the varietal and procedence variables were considered as classification factors. The developed analysis procedure may contribute to an improved characterization and control of onion production in the Canary region.

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