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Differentiation of Green, White, Black, Oolong, and Pu-erh Teas According to Their Free Amino Acids Content

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In this paper, the differentiation of green, black, Oolong, white, and Pu-erh teas has been carried out according to their free amino acid contents. Alanine, arginine, asparagine, aspartic acid, glutamic acid, isoleucine, histidine, leucine, phenylalanine, serine, theanine, threonine, and tyrosine have been determined by liquid chromatography with derivatization with *o*-phthalaldehyde and fluorescence detection. The chromatographic separation was achieved with a Hypersil ODS column and gradient elution. The amino acid contents were used as chemometric descriptors for classification purposes of different tea varieties. Principal component analysis, *k*-nearest neighbors, linear discriminant analysis, and artificial neural networks were applied to differentiate tea varieties. Using back-propagation multilayer perceptron artificial neural networks, 100% success in the classification was obtained. The most differentiating amino acids were glutamic acid, asparagine, serine, alanine, leucine, and isoleucine.

KEYWORDS: Tea; amino acids; reversed phase high-performance liquid chromatography; derivatization; chemometrics; pattern recognition

INTRODUCTION

Tea is one of the most popular and widely consumed beverages in the world with an estimated per capita worldwide consumption of 40 L of beverage per year (*1*). It is made from the leaves of the plant *Camellia sinensis* (L.). Tea has important physiological properties and potential health benefits due to the presence of compounds such as polyphenols, amino acids, vitamins, carbohydrates, caffeine, and purine alkaloids (*2*). There are different tea varieties with different external qualities and inner qualities due to morphologic and chemical diversities (*3*). Green and black are the most popular categories. Drying and roasting the leaves produce green tea, and with black tea, leaves are additionally fermented. If this fermentation is partially carried out, Oolong tea is obtained. In the fermentation, the enzymatic oxidation of tea polyphenols takes place, leading to the formation of theaflavins and thearubigins, which are responsible for the characteristic aroma and color of black and Oolong teas (*4*). Black tea is consumed worldwide, and green and Oolong teas are consumed mainly in Asia and North Africa. Less consumed but also highly appreciated are white and Pu-erh teas. White tea is an unfermented tea made from new growth buds and young leaves of the plant. The buds may also be shielded from sunlight during growth to reduce the formation of chlorophyll, giving the young leaves a white appearance. Pu-erh teas are mostly unfermented from a variety of *C. sinensis* found in the mountains of southern Yunnan. They can undergo an aging process for several months or even years prior to produce raw Pu-erh tea. While unaged and unprocessed raw Pu-erh is technically an unfermented tea, aged raw Pu-erh has occasionally been mistakenly categorized as a subcategory of black tea due to the dark red color of its leaves and liquor. However, Pu-erh in both forms has undergone secondary oxidization and fermentation caused both by organisms growing in the tea and from free radical oxidation, thus making it an unique type of tea (*5*).

The most important chemical constituents that influence the taste and flavor of tea infusions are polyphenols, flavonols, caffeine, sugars, organic acids, amino acids, and volatile flavor compounds (*6*). It has been demonstrated that there is a relationship between the quality of tea and the amino acid contents (*7*). For instance, the brothy sweet umami taste note of the green tea is due to amino acids, especially theanine (Thea) (*8*). Thea (5-N-ethyl glutamine) exists only in the free form, is the most important amino acid in tea (*9*), and takes part in the biosynthesis of polyphenols (*10*). Thea also has many biological effects. It has been reported that it decreases the level of norepinephrine and serotonin in the brain and the blood pressure (*11*). Cooperative effects of antitumor agents and Thea on cancer have also been reported (*12*). Amino acids can be determined in tea by the ninhydrin assay method (*13*), although liquid

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chromatography (*10*, *14*) is most commonly used. Because of the lack of a suitable chromophore, chromatography with postcolumn or precolumn derivatization (*8*, *14*, *15*) is usually carried out. *o*-Phthalaldehyde (OPA) is commonly used as a derivatizing reagent for amino acids determination with fluorescence detection (*8*, *16*) although other reagents such as phenylisothiocyanate (*8*) for UV detection have also been proposed. Alternatively, integrated pulsed amperometric detection (*7*) and electrospray mass spectrometry (*17*) have also been used for analysis of amino acids in tea samples.

Discrimination of varieties of tea can be achieved according to several criteria. Luminescence (*6*), near-infrared reflectance (*3*), and infrared (*18*) spectroscopic measurements have been proposed. Electronic nose (*19*) and electronic tongue (*20*) have also been used. Nevertheless, the classification according to chemical composition has provided excellent results. The metal content (*21*−*24*), catechins and purine alkaloids (*25*), and volatile components (*26*) have been used as chemical parameters to differentiate between tea varieties. Till now, the amino acid composition has not been used for classification purposes. In this paper, the free amino acids alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), glutamic acid (Glu), isoleucine (Ile), histidine (His), leucine (Leu), phenylalanine (Phe), serine (Ser), Thea, threonine (Thr), and tyrosine (Tyr) have been determined in white, green, Oolong, black, and Puerh teas. Reversed phase high-performance liquid chromatography (RP-HPLC) with precolumn derivatization with OPA has been used. Those amino acids have been used as chemical descriptors to differentiate the tea varieties. Several pattern recognition methods such as principal component analysis (PCA), *k*-nearest neighbors (KNN), linear discriminant analysis (LDA), and artificial neural networks (ANN) have been applied.

MATERIALS AND METHODS

Chemicals and Reagents. Ala, Arg, Asn, Asp, Glu, His, Ile, Leu, Phe, Ser, Thr, and Tyr were purchased from Sigma-Aldrich (Steinheim, Germany). Thea standard was purchased from Tokyo Kasei (Tokyo, Japan). Amino acid stock solutions of 200 mg/L were prepared in 0.1 M HCl and stored at 4 °C. Diluted solutions were prepared weekly. OPA and mercaptoethanol were purchased from Sigma-Aldrich. Acetonitrile and methanol, HPLC grade, were purchased from Romil (Cambridge, United Kingdom). Hydrochloric acid, sodium hydroxide, boric acid, and disodium phosphate were obtained from Merck (Darmstadt, Germany). A 40 mM phosphate buffer (pH 7.5), prepared from disodium phosphate and hydrochloric acid, and a 0.4 M boric acid/borate buffer (pH 9.5) were prepared. The derivatization reagent was freshly prepared everyday by dissolving 50 mg of OPA in 1.5 mL of mercaptoethanol. Ultrapure water was used throughout and was obtained from a Milli-Q system from Millipore (Milford, MA).

Tea Samples. In this study, commercial tea, tea bags, and tea leaves were purchased from local retail shops. Tea infusions were prepared as follows: 0.2500 g of tea was extracted with 10 mL of distilled water at 80 °C for 25 min. After the tea was cooled at room temperature, the volume was taken to 10 mL with water. The sample solutions were filtered through a 0.45 μm nylon filter membrane.

HPLC Analysis. Amino acids were determined using an HPLC system, consisting of a quaternary pump with vacuum degasser, autosampler, thermostatted column compartment, and a fluorescence detector Agilent 1100 (Agilent Technologies, Santa Clara, CA). An Agilent ChemStation was used for instrument control and data acquisition. A reversed phase column Hypersil ODS (Agilent) 250 mm $\times$ 4 mm and 5 μm particle size was used. The column temperature was maintained at 40 °C. Gradient elution (**Table 1**) was used to obtain adequate separation. The composition of the optimized mobile phase A was methanol/acetonitrile/water (45/45/10). Mobile phase B was phosphate buffer of pH 7.5. The flow rate was 2 mL/min. Fluorescence detection was performed with excitation and emission at 340 and 450 nm, respectively. Precolumn derivatization was carried out (*27*, *28*). A 2.5 μL aliquot of tea infusion was mixed with 2.5 μL of borate buffer and 32 μL of OPA solution. After 5 min of incubation, 20 μL of the mixture was injected in the chromatograph.

Table 1. Scheme of Elution Gradient

time (min)	solvent A (%)	solvent B (%)
0	10	90
10	18	82
15	24	76
25	60	40

Table 2. Sensitivity and Recovery for the Determination of Amino Acids

amino acid	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	recovery (%)
Ala	11.7	39.0	96 ± 5
Arg	10.7	35.8	97 ± 4
Asn	17.0	56.7	101 ± 5
Asp	44.6	148.7	96 ± 7
Glu	32.8	109.3	100 ± 2
His	14.3	47.8	94 ± 8
Ile	23.5	78.3	95 ± 5
Leu	15.8	52.7	99 ± 2
Phe	15.5	51.8	92 ± 8
Ser	11.3	37.8	96 ± 7
Thea	12.3	40.8	95 ± 6
Thr	21.0	69.9	94 ± 6
Tyr	20.7	69.0	99 ± 5

HPLC Method Validation. Selectivity, accuracy, precision, detection and quantification limits, and linearity ranges were considered. The selectivity criterion for an assay method is that the analyte peaks will have a chromatographic baseline with a suitable resolution from all of the other sample components. In our case, the peaks showed resolutions $\geq$1.5 for all of the determined compounds. The accuracy was assessed by recovery experiments. Known amounts of standards of amino acids were added to tea extracts. Recoveries were calculated comparing the obtained amounts with those added, and their values ranged between 92.1 and 101.5%. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as three and 10 times, respectively, the ratio between the standard deviation of the regression and the slope of the calibration line. The obtained LOD and LOQ values ranged between 10.7−44.6 and 35.8−148.7 ng mL^{-1}, respectively. A calibration line for each amino acid was constructed, and the on-line linearity (*29*) was >95% in all cases. **Table 2** summarizes the linear range, LOD, LOQ, and recovery values. To evaluate the precision of the method, seven replicate analyses of a standard solution on different days were performed. The precision expressed as relative standard deviation always remained <2% for all of the compounds studied.

Data Analysis. For the chemometric study, each tea sample was characterized by 13 chemical descriptors, which are the contents of the 13 amino acids analyzed. Pattern recognition methods were applied to the data matrix, composed of 13 columns (the analyzed amino acids) and 94 rows (tea samples). PCA was used as a visualization technique, and supervised learning methods, such as KNN, LDA, and ANN, were applied to obtain classification rules. Statistica 7.0 software package (StatSoft Inc., 2004) was used for the statistical data analysis.

Chemometric Methods. *PCA.* This technique can be applied to visualize the data trends and to provide a first evaluation of the discriminant efficiency of the variables. PCA is based on the derivation of linear combinations of the variables to produce new variables called principal components (PCs) that are uncorrelated (*30*). PCs are obtained sequentially, and each successive PC accounts for as much of the remaining variability as possible, and each new variable must be totally independent of all other variables. PCA attempts to condense the information of measured data explained by the first PCs. Hence, data plots using PC1 and PC2 as variables (scores plot) enable us to visualize the data trends of the data matrix with a lesser dimensionality (*31*).

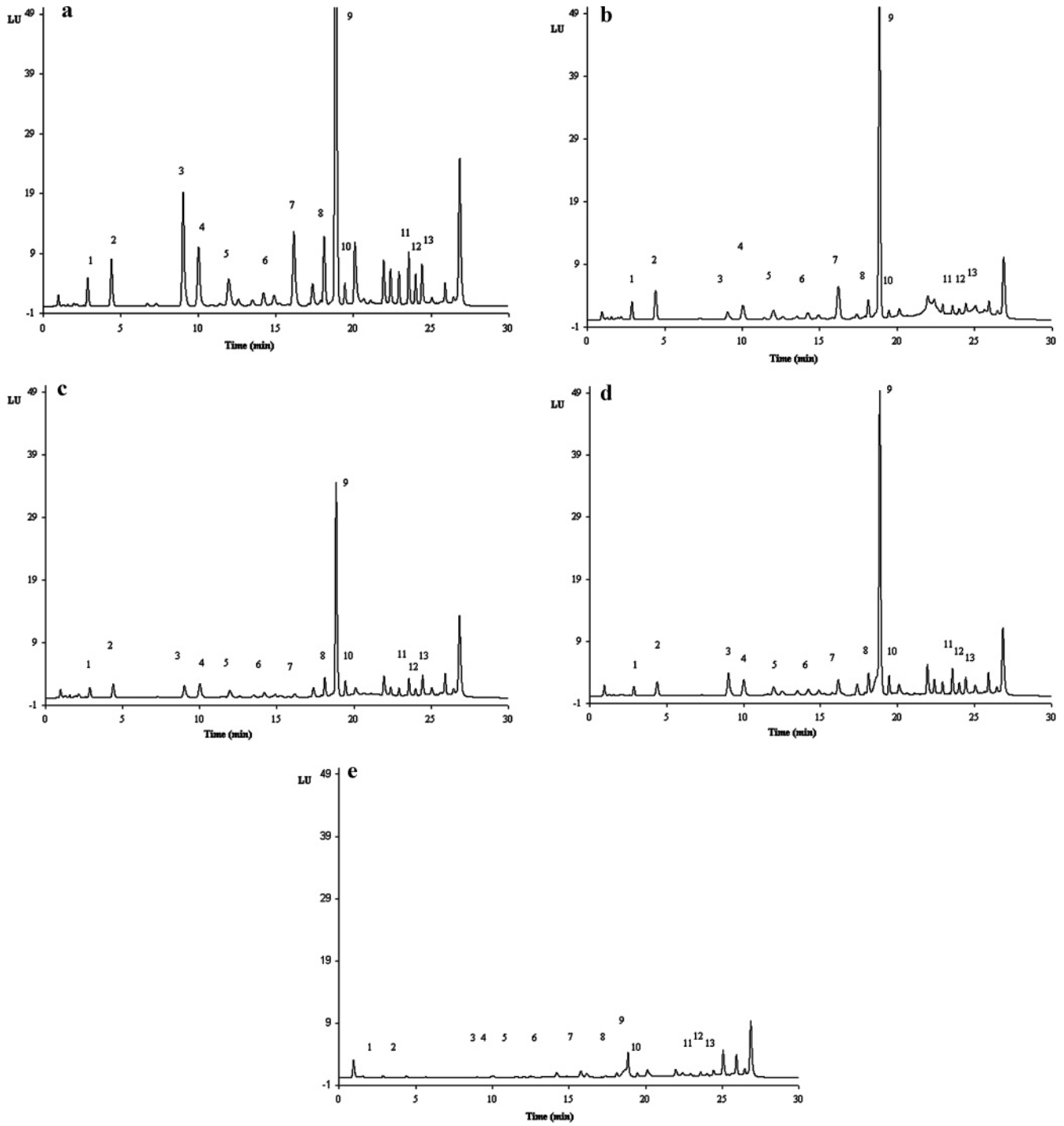

Figure 1. Chromatograms of amino acids in different tea varieties. (**a**) White, (**b**) green, (**c**) Oolong, (**d**) black, and (**e**) Pu-erh. Column, C-18, 250 mm × 4 mm, 5 μm; 40 °C, and flow rate, 2 mL/min. Gradient elution (see **Table 1**). Order of elution and retention time: 1, Asp, 2.89; 2, Glu, 4.44; 3, Asn, 9.11; 4, Ser, 10.11; 5, His, 12.17; 6, Thr, 14.99; 7, Arg, 16.24; 8, Ala; 18.17; 9, Thea, 18.84; 10; Tyr, 19.49; 11, Phe, 23.60; 12, Ile, 24.04; and 13, Leu, 24.45.

KNN. This is a nonparametric method that can be used to classify an unknown object when the class membership of a training set of objects is known. The distance of this object is computed to all of the training, and it is assigned to the class of the majority of its KNN. This method does not take into account the class distribution, and it can be used when nonplanar boundaries between classes can be established (*32*).

LDA. LDA differs from data reduction methods such as PCA in that it is concerned with determining the so-called discriminant functions (DFs) as linear combinations of the chemical descriptors, which best separate the classes according to minimization of the ratio of within-class and between-class sum of squares. The number of DFs (t) is the minimum value between the number of classes less one ($k - 1$) and the number of descriptors, p [$t = \min(k - 1, p)$] (*33*). An a priori knowledge of the number of classes and the class membership of each sample in the data matrix is assumed. Once the DFs have been calculated, the classes are separated by hyperplanes into subspaces within the space of the DFs, and the samples are classified according to falling into one of the class subspaces (classification rule).

ANNs. ANNs are efficient tools for classifying and discriminating food products (*34*). Multilayer perceptron ANNs (MLP-ANN) are feedforward multilayer networks consisting of neurons arranged in layers (an input layer, one or more hidden layers, and an output layer) being the connections (weights) unidirectional from input to output (*35*,

Table 3. Amino Acids Content[a] (mg/g, w/w Dry Base) in Tea Varieties

amino acid	white	green	black	Oolong	Pu-erh
Ala	1.17–2.09	0.19–0.68	0.14–0.81	0.29–0.60	0.02–0.12
Arg	1.09–2.74	0.12–1.31	0.07–0.62	0.07–0.46	0.00–0.07
Asn	3.82–11.09	0.30–1.37	0.25–1.64	0.40–1.02	0.00–0.05
Asp	0.82–1.96	1.12–2.33	0.30–1.79	0.32–0.84	0.03–0.32
Glu	1.27–2.86	1.43–2.61	0.47–2.28	0.63–1.34	0.02–0.26
His	0.99–3.11	0.29–1.17	0.07–1.41	0.15–0.96	0.00–0.05
Ile	0.85–1.66	0.17–0.46	0.16–0.65	0.19–0.53	0.03–0.10
Leu	0.66–1.34	0.12–0.27	0.11–0.52	0.25–0.55	0.02–0.12
Phe	0.96–2.18	0.20–0.50	0.16–1.34	0.16–0.70	0.02–0.11
Ser	1.48–3.25	0.36–0.77	0.22–1.12	0.33–0.63	0.02–0.08
Thea	5.3–33.37	1.62–3.37	0.49–4.12	0.85–2.82	0.07–1.15
Thr	0.41–0.75	0.15–1.25	0.07–0.41	0.10–0.31	0.00–0.10
Tyr	0.51–1.11	0.16–0.50	0.10 1.45	0.28–0.82	0.01–0.07

[a] Obtained from triplicate analysis.

36). Because ANNs learn from the data set, it is necessary to divide the cases in different subsets, a training set that allows learning the relationship between inputs and outputs and a test set that shows the prediction ability of the network. When the network learns not only the gross structure of a data set but also the fine one, it leads to bad performance of the constructed model. To avoid these overfitting problems, a third subset of cases, verification set, may be included. MLP-ANNs are usually trained by back-propagation (BP), minimizing the prediction error made by the network (*37*, *38*).

RESULTS AND DISCUSSION

Free amino acids were determined in white, green, Olong, black, and Pu-erh teas by applying the proposed RP-HPLC method. The presence of Ala, Arg, Asn, Asp, Glu, His, Ile, Leu, Phe, Ser, Thr, Tyr, and Thea was confirmed by standard addition. **Figure 1a−e** shows chromatograms corresponding to samples of different varieties of tea. Other peaks appearing in the chromatograms can correspond to other amino acids present in tea leaves such as lysine, methionine, valine, and glutamine (*7*, *10*). The results obtained are given in **Table 3**. Thea was the most abundant amino acid and accounts for more than 50% of the amino acid fraction of the tea samples. White and green teas had the highest content of amino acids. Pu-erh teas had the lowest content. A clear relation between the amino acids content and the elaboration process of teas can be observed. Higher amounts of free amino acids are present in white and green teas that are not fermented ones. From this fact, it can be inferred that amino acids could be used as good chemical descriptors to differentiate tea varieties.

Differentiation of Teas. For chemometric calculations, the tea samples were divided into five groups, white (11 samples), green (21 samples), black (28 samples), Oolong (13 samples), and Pu-erh (21 samples). Pattern recognition procedures were applied to the data set trying to differentiate the five tea varieties according to their amino acid contents. Previously, data were autoscaled.

PCA. PCA was applied as a visualization technique. By applying this method, the two first PCs were extracted, explaining up to 88.13% of the total variance. **Figure 2** shows the corresponding scores plot obtained from eigenanalysis of the covariance matrix of autoscaled data. As can be seen, white teas appear in a scattered group at the positive scores of PC1 separated from samples of other varieties. Green teas appear grouped at the left upper side of the plot at positive scores of PC2. Black and Oolong samples form a mixed group, and Pu-erh teas appear as a compact group at negative values of PC1 and PC2. The variables with higher contribution to PC1 were Ala, Arg, Asn, Ile, Leu, Phe, Ser, and Thea and, for PC2, Asp and Glu. Although there is no clear separation between the samples, some trends can be observed. Nonfermented teas, white and green, are almost completely separated from the others. In the case of white teas, a complete separation is observed, even from the green tea samples. No separation was attained between fermented (black) and semifermented (Oolong) teas. Pu-erh teas are almost separated from the others, but they are located closer to fermented teas. PCA calculations using a reduced number of variables were performed, but no better differentiation was achieved.

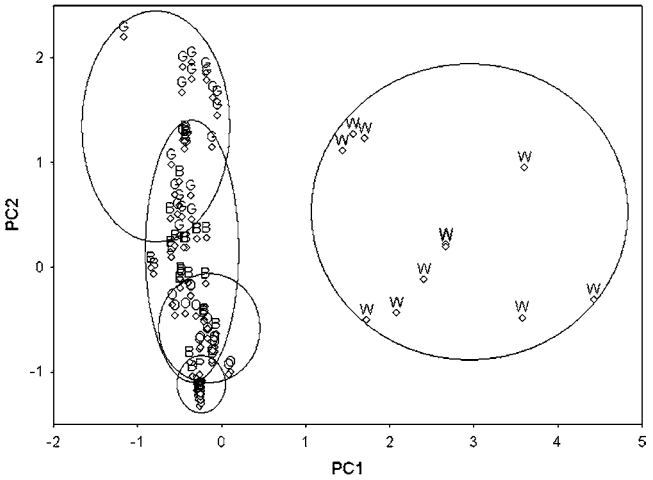

Figure 2. Scores plot of the tea samples for the first PCs. B, black; G, green; O, Oolong; P, Pu-erh; and W, white.

KNN. The data set was randomly divided in two subsets: training ($N = 72$) set to construct the KNN model and test set ($N = 22$) to evaluate the classification performance. KNN calculations were carried out considering the most discriminant variables extracted from PCA. A recognition ability of 100% for white, green, Pur-erh, and Oolong teas and 66.66% for black ones was obtained. Misclassified black teas were erroneously assigned as green and Oolong.

LDA. Backward stepwise LDA was followed in this case. In a first run, all of the variables are considered to construct the model, and in each step, the variable with less discriminant power is successively discharged. A random division of the samples into training ($N = 72$) and test ($N = 22$) set was performed. Accordingly, four DFs were calculated. We began LDA calculations using the variables with higher loadings in PC1 and PC2. Then, the four DFs were obtained using the variables Asn, Ala, Leu, Ile, Glu, and Ser. The recognition ability obtained using these classification rules were 100% for white, green, and Pu-erh teas, whereas for black and Oolong, they were 90.48 and 83.33%, respectively. **Figure 3** shows the plot of the samples in the plane defined by DF1 and DF2. As can be seen, complete separation of white and Pu-erh teas was accomplished. Groupings of green, black, and Oolong teas can be observed, with some black and Oolong samples misclassified.

ANNs. Considering that the linear model did not provide a complete solution to the classification problem, a nonlinear approach such as ANN was used. Back-propagation multilayer linear perceptron (BP-MLP) was used to construct a classification model to differentiate tea varieties. The data set was divided into three subsets, training ($N = 50$), verification ($N = 22$), and test ($N = 22$). The most discriminating variables obtained from LDA, Asn, Ala, Leu, Ile, Glu, and Ser, were used as input variables. The architecture of the network was 6:13:5. The network was trained by back-propagation during 1000 epochs with a learning rate and momentum of 0.25 and 0.5, respectively.

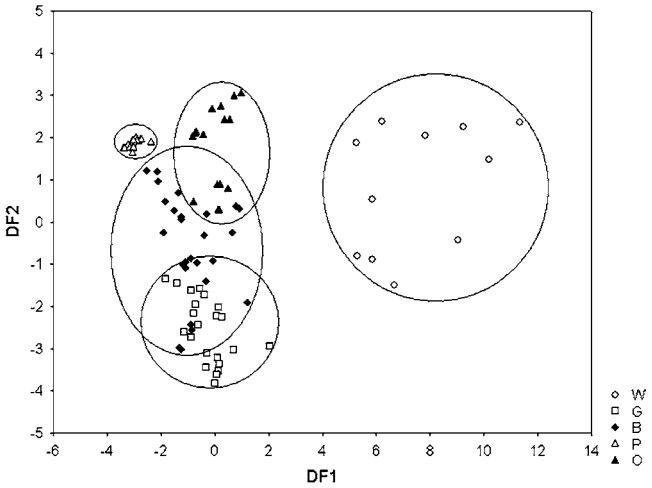

Figure 3. Scatter plot of the tea samples in the plane of the two first DFs. B, black; G, green; O, Oolong; P, Pu-erh; and W, white.

Table 4. Confusion Matrix BP-MLP Corresponding to Test Set[a]

		predicted									
		W		G		B		P		O	
		−	+	−	+	−	+	−	+	−	+
actual	−	19	0	17	0	16	0	18	0	18	0
	+	0	3	0	5	0	6	0	4	0	4

[a] Test set includes 22 samples from the white (W), green (G), black (B), Oolong (O), and Pu-erh (P) varieties.

After the model was constructed, a test set of samples was used to validate the classification procedure. **Table 4** shows the confusion matrix corresponding to the test set. As can be seen, all of the cases were correctly classified. Using the test set, two parameters, sensitivity (SENS) and specificity (SPEC), were calculated (*39*). Sensitivity of a class is referred to the number of objects belonging to this class that are correctly classified. Specificity of a class corresponds to the number of objects not belonging to this class that are correctly considered as belonging to different classes. Both parameters were 100%, indicating that MLP models the class distribution better than LDA.

Consequently, amino acids can be considered as good chemical descriptors to differentiate white, green, Oolong, black, and Pu-erh teas; Asn, Ala, Leu, Ile, and Glu are the most discriminating parameters. Although PCA, KNN, and LDA show some trends, a complete separation of the five classes considered could not be achieved. Using BP-MLP, 100% of success in the classification was obtained. The possible cause of this behavior can be the intrinsically nonlinear nature of the class distribution.

If we compare amino acids with other chemical parameters used in the discrimination, some conclusions can be inferred. Trace metal data (*21*, *22*, *24*) have been demonstrated to be very useful to discriminate types and geographical origin of teas, although in all cases a time-consuming acid digestion is necessary. Catechins together with caffeine (*25*) and volatile components (*26*) have also been used to differentiate tea categories. The discrimination of teas based in the content of free amino acids obtained by HPLC is advantageous because a very simple treatment of the sample is necessary. On the basis of the free amino acids content, further work can be addressed to study the geographical origin of teas.

ABBREVIATIONS USED

ANN, artificial neural network; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; BP-MLP, back-propagation multilayer linear perceptron; Glu, glutamic acid; His, histidine; Ile, isoleucine; LDA, linear discriminant analysis; Leu, leucine; LOD, limit of detection; LOQ, limit of quantification; OPA, *o*-phthalaldehyde; PCA, principal component analysis; Phe, phenylalanine; RP-HPLC, reversed phase high-performance liquid chromatography; Ser, serine; Thea, theanine; Thr, threonine; Tyr, tyrosine.

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