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HPLC determination of catechins and caffeine in tea. Differentiation of green, black and instant teas

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A simple and fast high performance liquid chromatographic method for five catechins and caffeine using an ODS column and a water–acetonitrile–formic acid mobile phase system was developed. The catechins (epicatechin, catechin, epigallocatechin, epigallocatechin gallate, epicatechin gallate) and caffeine were separated by an acetonitrile gradient within 20 min. The detection limit of the method was approximately 10 ng for all the compounds (by injecting 10 μL). Several green, black and instant teas were analysed using this method. By using the studied compounds as chemical descriptors, linear discriminant analysis was performed and complete differentiation of the green, black and instant teas was achieved.

Introduction

Tea is one of the most popular beverages in the world and is obtained from the leaves of the plant *Camellia sinensis*. Green and black teas are the two most consumed types. Green tea is produced by drying and roasting the leaves and for black tea the leaves are additionally fermented.[1,2] Brewed tea, especially green tea, is the only food product known to contain significant levels of catechins.[3] The tea leaves used in the brewing process have been found to contain up to 30% of the dry weight of the leaf[4] and are an important factor in the taste of the tea.[5] High levels of catechins may render the tea bitter, whereas in black teas polymerized catechins such as theaflavins and thearubigins that result from the fermentation process are important factors in determining the overall quality of the tea.[2] Catechins have been suggested to possess various pharmacological properties, including being antihypertensive,[6] antioxidative,[7] antiarteriosclerotic,[8] anticarcinogenic[9] and hipocholesterolaemic,[10] and also to prevent dental caries.[11] Owing to these beneficial effects on the human health of the catechins present in the tea brews, especially in those made of green tea, there is increasing interest in studying the levels of these compounds in different types of tea samples. The methylxanthines, especially caffeine, are alkaloids present in the tea plant. Caffeine is known for its stimulatory effect[12] and is an important factor in the quality of the tea.

Several workers have reported the determination of catechins and caffeine in green tea by capillary electrophoresis,[13–15] although high performance liquid chromatography (HPLC) is currently the most useful approach for the routine analysis of and research on non-volatile tea constituents, including tea catechins and alkaloids.[1] Isocratic elution has been performed,[16] although gradient elution is the most commonly used approach,[3,17,18] control of the temperature being necessary to obtain an adequate resolution in a short time of analysis.

This paper reports a simple and rapid HPLC method in which the caffeine and the major catechins occurring in green, black and instant teas are separated at room temperature with a short time of analysis. The five catechins determined were (+)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG) (Fig. 1). By using the caffeine and catechin contents and applying pattern recognition (PR) methods, differentiation of those types of tea can be achieved.

Experimental

Reagents and standard solutions

Methanol and acetonitrile (Romil, Cambridge, UK) were of HPLC grade. Milli-Q (Millipore, Bedford,, MA, USA) treated

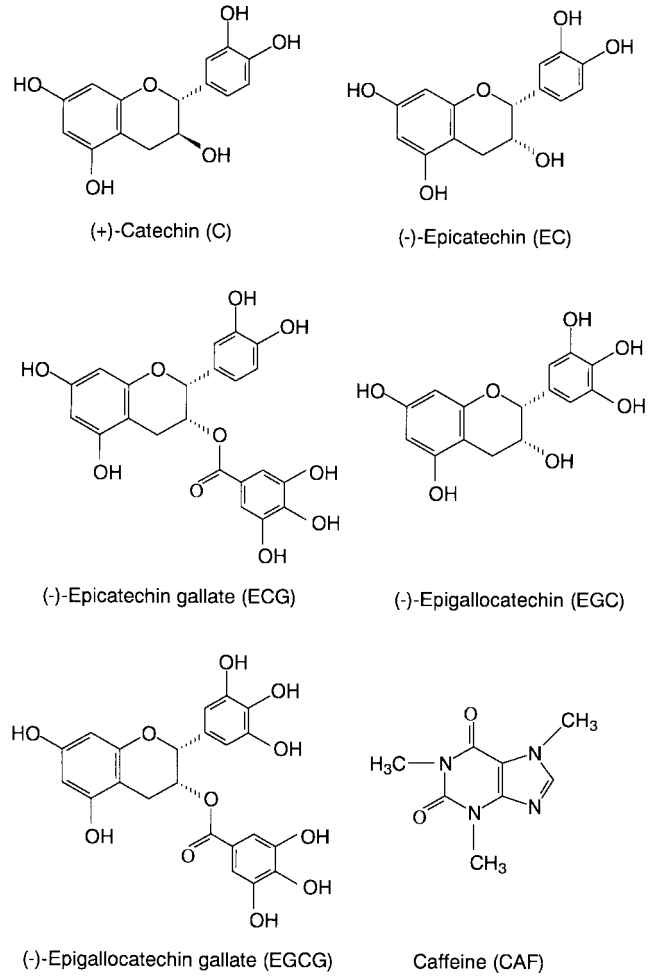

Fig. 1 Structures of catechins and caffeine.

water with a resistivity higher than 18 MΩ cm was used throughout. Other chemicals were of analytical reagent grade (Merck, Darmstadt, Germany).

(−)-Epigallocatechin, (−)-epigallocatechin gallate and (−)-epicatechin gallate were purchased from Sigma (Steinheim, Germany) and (−)-epicatechin and (+)-catechin from Fluka (Buchs, Switzerland). These reagents were stored at −20 °C. Caffeine was obtained from Merck. Stock standard solutions (200 μg mL^{-1}) were prepared in acetonitrile and stored at 4 °C. Working standard solutions were prepared weekly from the stock solutions by dilution with acetonitrile.

Apparatus

The HPLC system consisted of an assembly of two Waters (Milford, MA. USA) Model 510 pumps controlled by a Waters AGC-680 automated gradient controller, a Rheodyne (Cotati, CA, USA) Model 7120 injection valve with a 10 μL sample loop, a Waters Model 486 tunable absorbance detector operated at 275 nm and a Carlo Erba (Milan, Italy) DP700 integrator. A 25 cm × 4.6 mm id LiChrosorb RP-18 5 μm column (Teknokroma, Barcelona, Spain) was used for the separation.

Chromatographic conditions

A two-solvent gradient elution was performed, with a flow rate of 1 mL min^{-1}. The solvents compositions used were (A) water–acetonitrile–formic acid (94.7 : 4.3 : 1 v/v) and (B) water–acetonitrile–formic acid (49.5 : 49.5 : 1 v/v). The mobile phase composition started at 90% solvent A and 10% solvent B, being increased linearly to 30% solvent B in 10 min, followed by a linear increase of solvent B to 80% in 5 min, the final conditions being held for an additional 5 min. All samples were microfiltered before injection.

Samples

A set of 37 samples of commercially available teas were selected for study. Green, black and instant tea samples were included and labelled as G, B and I, respectively. Table 1 includes a short description of the samples studied. Instant tea samples were included in the data set in order to assess whether or not the manufacturing process leads to a scatter of the samples beyond the varieties from which they are derived.

The samples were extracted by the method of Suematsu *et al.*[19] with slight modifications: 0.5 g of tea sample was extracted with 100 mL of acetonitrile–water (1 : 1 v/v) at room temperature for 40 min with constant stirring. The extract was filtered and diluted to volume in a 100 mL calibrated flask. Aliquots of this solution were adequately diluted and injected for HPLC analysis.

Data analysis

In the 37 tea samples (cases), the content of caffeine (CAF), catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) were determined. Each of these variables was considered as a chemical descriptor and their values are given in Table 1. A data matrix whose rows are the cases and whose columns are the variables was prepared and used in the chemometric calculations. PR methods utilised in this study include principal component analysis (PCA)[20] and linear discriminant analysis (LDA).[21]. PCA and LDA calculations were performed using the statistical package CSS:STATISTICA from Statsoft (Tulsa, OK, USA).

Results and discussion

Method development

HPLC separation using an ODS column was considered.[1,3,17,22] To obtain an adequate resolution of the peaks in a reasonable time of analysis, a gradient elution programme was developed. Several aqueous mobile phases were tested with acetonitrile, methanol and tetrahydrofuran as co-solvents. The best results were obtained with water–acetonitrile-formic acid at a flow rate of 1 mL min^{-1}, which allows the separation of the five catechins and caffeine within 20 min.

From the UV absorption spectra recorded for the studied catechins and caffeine, it was found that the highest signal appeared when 275 nm was used as the analytical wavelength. Fig. 2 shows a chromatogram of standards obtained under these conditions.

The catechins and caffeine were identified in the tea samples by comparing their retention times with those of standard solutions. Under the selected operating conditions, the retention times (in minutes) for the studied compounds were as follow: 10.3 (EGC), 11.6 (C), 13.1 (CAF), 14.0 (EC), 14.6 (EGCG) and

Table 1 Contents (% w/w, dry base) of catechins and caffeine in green, black and instant tea samples[a]

Sample	Origin[b]	EGC	C	CAF	EC	EGCG	ECG
1G	China	2.253	0.557	2.663	0.552	4.683	0.571
2G	China	2.263	0.831	3.285	0.576	4.705	0.545
3G	China	2.773	0.844	3.092	0.589	4.724	0.649
4G	China	2.501	0.369	2.630	0.605	2.649	0.436
5G	China	3.499	0.340	2.837	1.102	4.155	0.800
6G	Unknown	2.052	0.458	2.333	0.436	1.762	0.277
7G	Unknown	2.025	0.310	2.385	0.444	1.575	0.259
8G	China	2.889	0.228	2.413	0.790	3.978	0.674
9G	China	1.327	0.297	2.067	0.426	0.581	0.107
10G	Japan	4.826	0.340	3.414	1.211	3.981	0.579
11G	Darjeeling	3.226	0.602	3.355	0.693	5.675	0.936
12G[c]	China	2.763	0.229	2.369	0.718	2.317	0.377
13G	China	2.775	0.476	3.139	0.839	1.831	0.242
14G	Japan	2.509	0.264	1.844	0.735	1.094	0.176
15G	Japan	2.227	Nd	1.780	0.666	1.088	0.157
16G	China	1.145	Nd	1.258	0.407	0.213	0.098
17G	China	1.933	Nd	0.774	0.492	Nd	0.091
18G	Unknown	1.457	Nd	1.723	0.675	0.483	0.188
1B[c]	Unknown	0.568	0.486	2.862	0.469	0.696	0.527
2B	China	0.656	0.343	2.903	0.459	0.525	0.283
3B	Blend[e]	1.910	0.507	3.297	0.424	0.658	0.440
4B	Unknown	1.363	0.326	2.672	1.004	0.316	0.389
5B	Ceylon	1.616	0.383	2.634	0.288	0.997	0.612
6B	Assam	1.601	0.462	3.288	Nd	0.456	0.450
7B	Ceylon	1.446	0.348	2.914	0.369	1.546	0.767
8B	Blend[f]	1.690	Nd	3.060	Nd	0.634	0.443
9B	Kenya	2.203	0.422	3.422	0.201	1.144	0.707
10B	Blend[g]	1.849	0.323	3.052	Nd	0.644	0.574
11B[c]	Blend[h]	0.963	0.193	2.771	Nd	0.238	0.214
12B	Unknwon	0.588	0.193	2.604	Nd	0.304	0.264
13B[d]	Unknown	1.165	0.167	2.572	0.396	0.814	0.532
14B	Ceylon	0.502	0.502	3.286	Nd	0.367	0.377
15B	Unknown	1.639	0.189	2.886	Nd	0.527	0.344
16B	Unknown	1.340	0.284	2.664	Nd	0.399	0.291
17B	Unknown	1.608	0.325	2.778	Nd	0.406	0.423
1I		1.397	0.843	3.694	0.455	1.791	0.985
2I		3.426	0.731	3.834	0.570	1.509	1.005

[a] EGC, epigallocatechin; C, catechin; CAF, caffeine; EC, epicatechin; EGCG, epigallocatechin gallate; ECG, epicatechin gallate; G, green tea; B, black tea; I, instant tea; Nd, not detected. [b] According to the label claim. [c] Bergamot flavoured. [d] Lemon flavoured. [e] Ceylon and India. [f] Kenya and India. [g] Ceylon and Kenya. [h] Ceylon, China and Kenya.

17.4 (ECG). In Fig. 3 a chromatogram of a tea sample is presented.

Performance and characteristics of the HPLC method

In order to check the performance of the method, the selectivity, linearity, detection and quantification limits, accuracy and precision were evaluated.

The selectivity criterion for an assay method is that the analyte peaks will have a chromatographic baseline with a suitable resolution from all the other sample components. In our case, the peaks showed resolutions ≥ 1.5 for all the determined analytes.

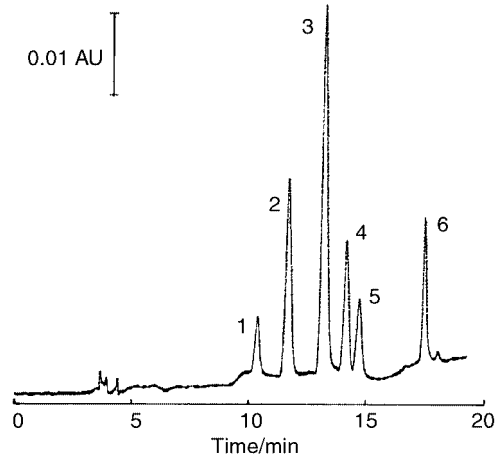

Fig. 2 Chromatogram of a standard solution. (1) Epigallocatechin (60 μg mL^{-1}); (2) catechin (30 μg mL^{-1}); (3) caffeine (5 μg mL^{-1}); (4) epicatechin (20 μg mL^{-1}); (5) epigallocatechin gallate (10 μg mL^{-1}); and (6) epicatechin gallate (5 μg mL^{-1}).

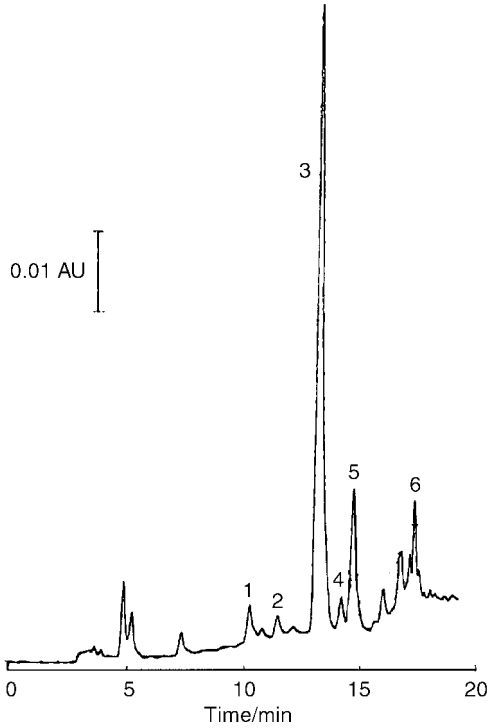

Fig. 3 Chromatogram of a green tea sample. (1) Epigallocatechin; (2) catechin; (3) caffeine; (4) epicatechin; (5) epigallocatechin gallate; and (6) epicatechin gallate.

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.[23] The obtained LOD and LOQ values, respectively, are 1.3 and 4.6 μg mL^{-1} for EGC, 0.4 and 1.3 μg mL^{-1} for C, 1.1 and 3.6 μg mL^{-1} for EC, 1.4 and 4.8 μg mL^{-1} for EGCG, 0.2 and 0.9 μg mL^{-1} for ECG and 1.9 and 6.3 μg mL^{-1} for CAF. Hence the proposed method allows catechins and caffeine to be suitably determined in tea samples.

The accuracy of the method was evaluated from recovery assays, preparing spiked tea samples in triplicate at several levels of concentration higher than the LOQ. The average recoveries were calculated according to Cuadros *et al.*[24] and ranged between 90.0 and 103.6%. By applying Student's *t*-test to the recoveries obtained, the null hypothesis was accepted at the 5% significance level, and consequently it is possible to state that the proposed method is accurate.

In order 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 $< 1\%$ for all the compounds studied.

Determination of catechins and caffeine in green, black and instant tea

The catechin and caffeine content in green, black and instant tea samples was determined by applying the proposed method. Previously, by using acetonitrile-water at room temperature, an extraction step was performed. In this way, possible degradation of the catechins with temperature was prevented. The results obtained are given in Table 1. The major catechin contents correspond to EGCG and EGC whose concentrations reach up to 5% in some green tea samples. EGC is the catechin that appears at a major percentage in black teas, although these contents are lower than the corresponding values in green tea. The caffeine level ranges between 1.0 and 3.5%. Instant tea samples have a higher content of caffeine of up to 3.8%; in these samples the major catechins are EGCG and EGC.

Chemometric approach to classification of teas

Multivariate data analysis (MDA) can be useful for processing chemical data dealing with n samples (here the tea samples) featured by p chemical descriptors (here the contents of EGC, C, EC, EGCG, ECG and CAF) leading to a data matrix of $n \times p$ dimensions. Some techniques of MDA can be used to investigate the data set with respect to diverse objectives such as the data visualisation/reduction and grouping/classification. MDA methods for data visualisation and dimensionality reduction and for sample classification include PCA and LDA, respectively. PCA is based on the derivation of linear combinations of the measured chemical descriptors to produce new variables called principal components (PCs) that are uncorrelated. PCs are obtained sequentially: the first PC (PC1) accounts for the largest portion of explainable variability in the measured data, the second PC (PC2) accounts for the next largest portion of explainable data variability, and so forth. In other words, PCA attempts to condense the information (variability) of measured data explained by the first PCs. Hence data plots using PC1 and PC2 as variables (scores plots) enable us to visualise the data trends of the data matrix with a lesser dimensionality ($n \times 2$).[25]

LDA differs from data reduction methods such as PCA in that it is concerned with determining the so-called discriminant functions as linear combinations of the chemical descriptors

which best separate the classes according to minimisation of the ratio of within-class and between-class sum of squares. The number of discriminant functions (t) is the minimum value between the number of classes less one ($k - 1$) and the number of descriptors, p ($t = \min\{k - 1, p\}$).[26] An *a priori* knowledge of the number of classes and the class membership of each sample in the data matrix is assumed. Before calculating the discriminant functions, the variables that should be included in the analysis must be selected. For this, stepwise discriminant analysis (SDA)[27] is performed. The forward stepwise approach was followed in this case. In a first run there are no variables in the model, and in each step the variable with more discriminant power is successively added. The discriminant power of a variable is given by Wilk's λ statistic test.[25] Classes are then separated by hyperplanes into subspaces within the space of the discriminant functions and samples are classified according to falling in one of the class subspaces (classification rule). In our case we have three classes for classification purposes ($k = 3$), black, green and instant teas, and $n = 37$ samples to be classified.

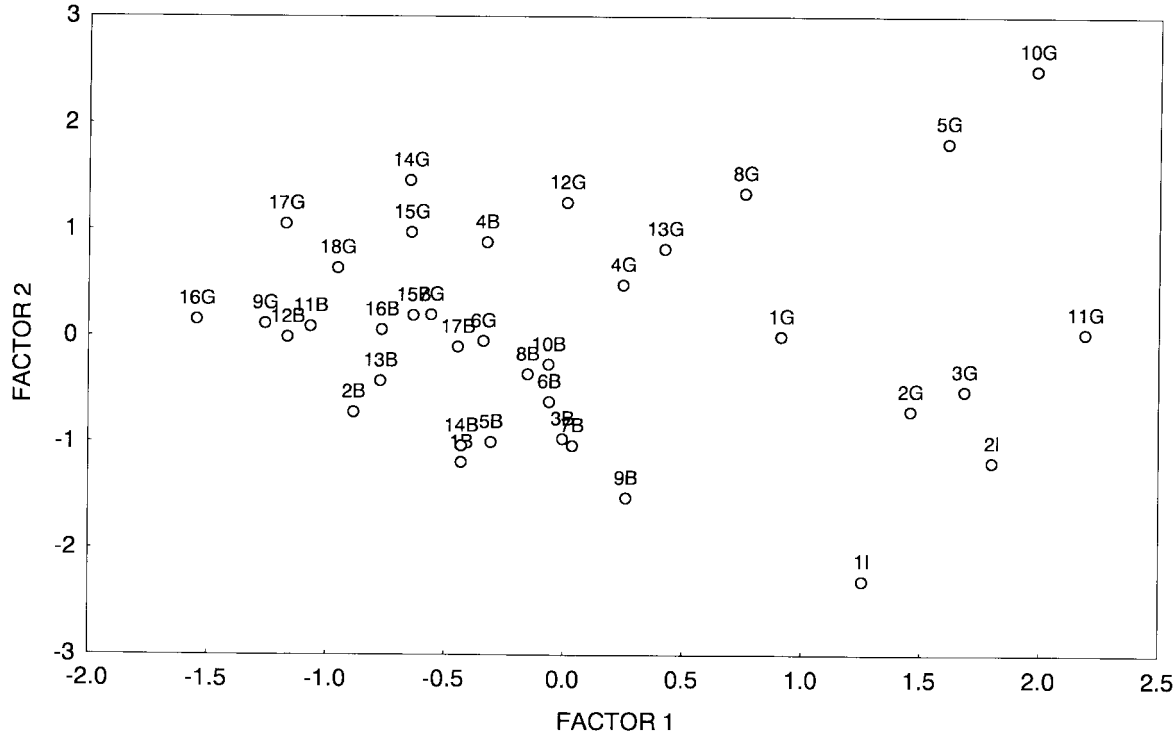

Fig. 4 Scores plot for the first PCs.

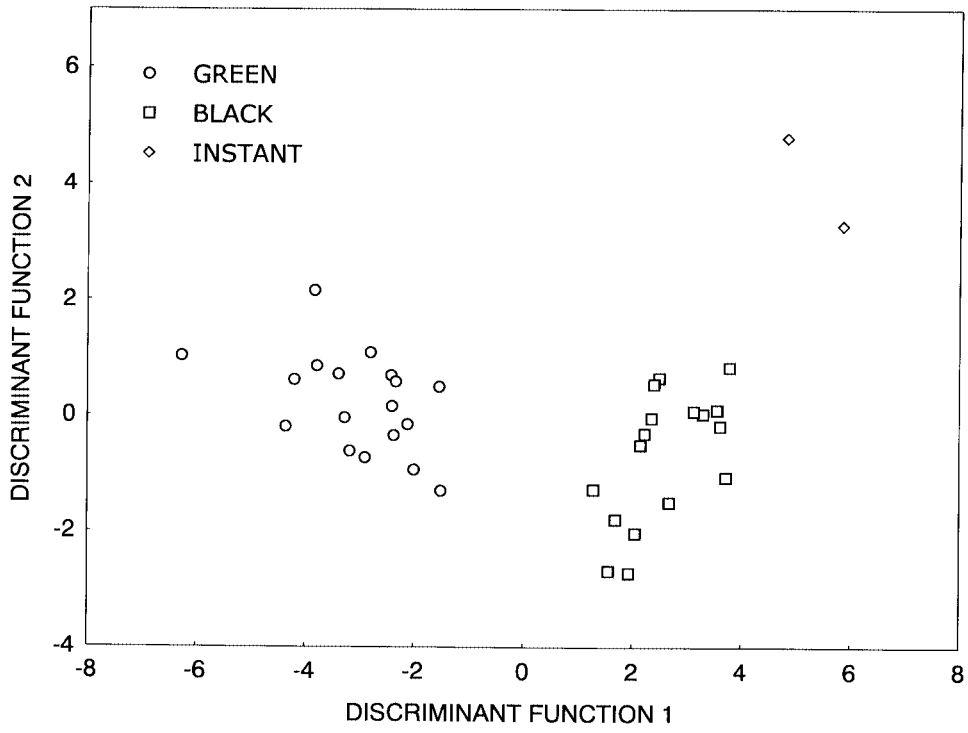

Fig. 5 Plot of the two discriminant functions.

PCA-based display methods

PCA-based display methods allow a more detailed study of the data trends. The first two PCs were calculated explaining up to 76.3% of the total variance. PC1 explains 46.6% and PC2 explains 29.7% of the total information. Fig. 4 shows the resulting scores plot. As can be seen, a larger dispersion of the green teas appears whereas black teas form a more compact group. Otherwise, most of the black tea samples are almost grouped at the negative scores of PC1 and PC2. Samples 9B and 4B are located at the positive side of PC1 and PC2, respectively.

An important feature that can be noted is that there is a quasi-linear separation of the green and black tea samples. However, no total separation between the two classes was observed. In the case of instant teas, both samples are closely located at positive values of PC1 and negative values of PC2. Apparently, they are not included in either the green or black tea groups.

Linear discriminant analysis

In order to obtain suitable classification rules and because of the quasi-linear separation observed in the scores plot, SDA analysis was applied to the data set. In our case, the selected descriptors were EGCG, ECG, C, EGC and CAF ($p = 5$), obtaining classification rules with a recognition ability of 100%. The number of discriminant functions is $t = 2 = \min\{2,5\}$. Fig. 5 shows the plot of the discriminant functions obtained. As can be seen, complete separation of the green and black tea samples was accomplished. Instant teas appear as an independent group separated from the other two classes.

Conclusions

The proposed HPLC method is suitable for the determination of CAF, EGC, C, EC, EGCG and ECG in green, black and instant tea samples. Using LDA and after a feature selection, discrimination between these classes of tea is possible. The chemical descriptors used in the LDA analysis were CAF, EGC, C, EGCG and ECG.

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