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CHEMICAL AND BIOSENSORS

Comparison of Tyrosinase Biosensor and Colorimetric Method for Polyphenol Analysis in Different Kinds of Teas

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Abstract: We report the content of polyphenols, expressed as chlorogenic acid equivalents, in a variety of commercially available samples of yerba mate. The 48% of analyzed samples presented a 92 ± 8 mg of extracted chlorogenic acid equivalents per gram of dry base. Composites exhibited a lower extracted amount. Results from samples contained in tea bags are higher, indicating dependence on milling degree. The extracted chlorogenic acid, expressed as $mg \cdot g^{-1}$, was evaluated by three methods in a unique yerba mate sample: biosensor ($89.2\,mg \cdot g^{-1}$), Folin ($90.2\,mg \cdot g^{-1}$), and high-performance liquid chromatography (HPLC) analysis ($21.0\,mg \cdot g^{-1}$). Biosensing system validation was performed. Repetitiveness of genuine replicates was consistent with nature of the samples. Discrimination between yerba mate and other plants can be done using principal component analysis (PCA) and the corresponding physical and chemical descriptors. Flavor and taste alterations can be studied by means of analytical methods that involve low-cost

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instrumentation. It has been observed that some green tea samples present remarkable differences depending on the specie.

Keywords: Yerba mate, tea, biosensor, PCA analysis

INTRODUCTION

Polyphenols are natural products containing several hydroxyl groups directly associated with a cyclic benzene ring; they are widely distributed in plants. Important health benefits have been attributed to these compounds such as protection from heart disease and hypertension (Henry and Stephens-Larson 1984), cancer prevention (Shi et al. 1994), and antiviral activity (Sakamaka et al. 1992), due to antioxidant properties (Ho et al. 1992).

There are many phenolic compounds in plants. The most frequently found are flavonoids, which are molecules possessing two phenols joined by a pyran (flavanols, such as catechins plus proanthocyanidins, anthocyanins, and their oxidation products), and nonflavonoids (phenolic acids, stilbenes like trans-resveratrol) (Stoclet et al. 2004). The main polyphenol dietary sources are fruit and beverages (fruit juice, wine, tea, coffee, chocolate, and beer) and, to a lesser extent, vegetables, dry legumes, and cereals. The phenolic composition of wine has received particular attention (Souquet et al. 2000), because it is particularly high (0.01% in white and up to 0.2% in red wines). Also, fresh tea leaf is unusually rich in the flavonol group of polyphenols known as catechins, which may constitute up to 30% of dry leaf weight (Graham 1992) and phenolic content in olive oil has been reported as criteria of quality (Capannesi et al. 2000).

Ilex paraguayensis is a specie of tea from South America. This plant (leaves and twigs) is processed to obtain a final commercial product named *yerba mate*. The mate is a famous popular tea consumed in Argentina, Brazil, Uruguay, and Paraguay. Even though the therapeutic properties of yerba mate have been extensively explored (Ramirez-Mares, Chandra, and Gonzalez de Mejia 2004), there are few studies of the chemical composition of mate (Graham 1984); (Kawakami and Kovayashi 1991). They report the presence of flavonoids and phenolic compounds (*p*-coumaric and ferulic acid) in about 10% of dry weight.

In the present paper, we report the content of polyphenols in a variety of commercially available samples of yerba mate using a tyrosinase biosensor and other standard analytical methods. The former has been widely explored for the detection of phenolic compounds in different samples (Onnerfjord et al. 1995; Wang et al. 1997; Hedenmo et al. 1997; Campo Dall'Orto et al. 1999; Romani et al. 2000; Campuzano et al. 2003; Stanca and Popescu 2004). We have applied principal components analysis (PCA) to our dataset in order to discriminate among different tea and yerba mate samples.

In statistics, PCA is a technique that can be used to simplify a dataset; more formally, it is a transform that chooses a new coordinate system for the dataset such that the greatest variance by any projection of the dataset comes to lie on the first axis (then called the first principal component), the second greatest variance on the second axis, and so on. The PCA analysis has been used to distinguish between different tea types (black and green teas). Organic compounds, such as polyphenols (Liu et al. 1987); (Tomlins and Gay 1994) or catechins (Fernández et al. 2000), measured by high-performance liquid chromatography (HPLC), has been applied for classification of teas.

EXPERIMENTAL

Preparation of Tyrosinase Biosensor

Tyrosinase from Mushroom with an activity of 3900 units (mg solid)$^{-1}$ was purchased from SIGMA. The enzyme was dissolved in deionized water in order to obtain a 10 μg. μL^{-1} solution that was fractioned in 2.5 μL aliquots and stored at −25°C until its use. A 1/10 dilution from 25% glutaraldehyde solution was made in 50 mM phosphate buffer, pH = 7.00.

A 3 mm glassy carbon working electrode for thin-layer cell was used to prepare the tyrosinase biosensor.

A glassy carbon working electrode was polished with 0.3 and 0.05 μm alumina particles on polishing clothes, rinsed with distilled water, and dried with paper before use. Immobilization of tyrosinase was accomplished by addition of 1.5 μL aliquot of 10 μg. μL^{-1} tyrosinase solution and 1.5 μL of 2.5% glutaraldehyde solution on the electrode surface. The mixture was allowed to dry for 1.5 h at room temperature (20 ± 2°C) for polymerization. The enzyme electrodes were rinsed with deionized water and stored in pH = 7.00, 50 mM phosphate buffer at room temperature for at least 2 h before use. These electrodes cannot be stored for more than 12 h due to low adherence, and they were prepared every day over a 4 months period.

Flow Systems

A HPLC system consists of an isocratic pump P100 Spectra from Thermo Separation Products, peek tubing of 0.010′ × 1/16′, a MicroPack guard column (4 cm × 4 mm), a model 7125 Rheodyne syringe loading sample injector with a 20 μL sample loop, and a 200 μL spacer connecting the injection valve to the flow cell were used in FIA experiments.

For HPLC separations, a 5 μm particle size Lichrosorb RP-18 HPLC column (200 mm × 4.6 mm; HP) was introduced between the injection valve and the detector.

Cells

The 7 μL, thin-layer flow cell contains a glassy carbon working electrode (model MF-1000, BAS), a Ag/AgCl reference electrode (model MF-2078, BAS), a 16 μm thin-layer gasket (model MF-1055, BAS), and a stainless steel auxiliary electrode block on which the working electrode is mounted. It was used for FIA (with the enzyme immobilized on working electrode surface) and HPLC measurements.

The electrochemical cell for stationary solutions and electrodes consisted of a 3 mm glassy carbon working electrode (MF-2012, BAS), a Ag/AgCl reference electrode (MF-2079, BAS), and a platinum wire as an auxiliary electrode.

Detection

Cyclic voltammetry and amperometric measurements were performed with a microprocessor-controlled electrochemical analyzer. Cyclic voltammetry was used to study the electrochemical behavior of phenolic compounds for further PCA analysis by cycling the electrode potential over the oxidation waves, between 0 and 1.5 V at a scan rate of $50\,mV.s^{-1}$.

All amperometric measurements were carried out by applying the specific operational potential and allowing the transient current to decay to steady-state values before the analysis.

The applied potential for tyrosinase electrode in FIA mode was determined by hydrodynamic voltammetry and was fixed in −0.1 V vs. Ag/AgCl. The flow rate was $0.3\,mL.\,min^{-1}$.

For HPLC experiments, the flow rate was $1\,mL.\,min^{-1}$, and an amperometric detector was used. The optimal electrode potential for phenolic compound detection was determined in the mobile phase (see the next section, Reagents and Solutions) by cycling the potential over the oxidation waves, between 0 and 1.5 V at a scan rate of $50\,mV.s^{-1}$. The bare electrode potential was set in +1.0 V vs. Ag/AgCl according to these preliminary results.

PCA was performed with Matlab 5.3 software.

A HP8452 diode array spectrophotometer and a quartz crystal cell were used to obtain ultraviolet (UV) spectra of sample extracts.

Reagents and Solutions

Buffer solutions were prepared with deionized water from a Millipore Milli-Q purification system, and all chemicals were of analytical-reagent grade.

A pH = 7.00, 50 mM phosphate buffer was used as carrier and supporting electrolyte in FIA measurements. Phenolic compound stock solutions were prepared daily by dissolving 10 mg of the substance in 50 mM phosphate buffer solution, pH = 7.00. Dilutions were made in the same media.

Voltammetric experiments for PCA analysis were made in 50 mM phosphate buffer solution, pH = 7.00.

Extracts were diluted with deionized water for UV spectra.

The mobile phase for chromatographic separations was a mixture of 5 mM H_3PO_4 and acetonitrile 85 : 15. Phosphoric acid was an analytical reagent provided by Mallinckrodt, and it was diluted with deionized water. Acetonitrile was HPLC grade and purchased from Baker. The solutions were degassed before use. Standard solutions were prepared using mobile phase as solvent.

Samples

Twenty-five commercially available herbal products of *Ilex paraguayensis St. Hil.* (yerba mate) traditionally used to prepare infusions and other plant species were selected for study. These products are presented in Table 1.

Dry leaves (1.000 ± 0.001 g) were extracted in 20 mL of pH = 7.00, 50 mM phosphate buffer, and sonicated for 10 min at room temperature to avoid phenolic compound degradation during the extraction process. The

Table 1. *Ilex paraguayensis* commercial products and other plant species selected for study.

Yerba Mate (*Ilex paraguayensis*) Samples
Presentations
Common: Y01, Y02, Y05, Y07, Y10, Y15, Y16, Y18
In bags: Y03, Y06, Y09, Y14, Y20
With twigs: Y13 (stabilized), Y17, Y19
Without twigs: Y08, Y22
Light: Y04, Y21
Composite: Y11 (in bags), Y12, Y24
Others: Y23 (from farm, exposed to sunlight 1yr, without milling), Y25 (selection)

Tea (*Camellia sinensis*) Samples
Presentations
Black, in bags: S26, S27, S28, S30, S32, S36, S39, S41
Black, leaves: S29
Green, in bags: S33, S35, S40
Green, leaves: S31, S38

Other Plant Samples
Mentha piperita (in bags): S34; *Matricaria Chamomilla* (in bags): S43;
Tilia platyphyllos (in bags): S44; *Lippia turbinata* (in bags): S45;
Composite (in bags): S46; *Aloysia triphylla* (in bags): S47, S49;
Boldea boldus (in bags): S48; *Jasminum polyanthum* (leaves): S37;
Rosa rubiginosa (milled): S42.

suspension was filtered in order to separate leaves and tissues from liquid. The volume was brought to 25 mL with buffer solution, and it was centrifuged for 5 min. The supernatant was filtered with a 0.2 μm pore size membrane filter (K06001; Renner GMBH). The filtrate was properly diluted with buffer solution for analysis.

The yerba mate infusion is drank in a particular way in South America—it is sipped from a cup using a metal or wood straw and filter, doing repetitive extractions with small volumes of hot (80–84°C) water, during short periods of time. A laboratory procedure that closely reproduces the extractive process was 10 min sonication at room temperature instead of high-temperature extraction at 80°C.

Standards

Analytical grade rutin, epigallocatechin gallate (EGCG), chlorogenic acid, caffeic acid were (Aldrich), and phenol and catechol (Mallinckrodt) were used as standards.

RESULTS

Polyphenol Assessment

Different trademark products listed in Table 1 were analyzed with tyrosinase biosensor in a flow amperometric detection system.

The influence of pH on amperometric response was tested using standards of chlorogenic acid and EGCG and several yerba mate samples. Chlorogenic acid and EGCG are the principal electroactive compounds in yerba mate and tea leaves, respectively. The response was reproducible up to pH = 7.00, and if decreased during measurement at pH = 9.00 due to spontaneous degradation of polyphenols.

It was observed that tyrosinase enzyme in the biosensor must be induced by substrate before use, as indicated in previous reports (Onnerfjord et al. 1995; Stanca and Popescu 2004).

Table 2 summarizes analytical parameters in the linear range of calibration plots for different enzyme substrates. It must be pointed out that rutin was not detected, and EGCG was detected with a very low sensitivity. For caffeic acid and EGCG, a significant variation in sensitivity can be found for different sensors, defining two groups of results.

The sensitivity decreased in the sequence: phenol, caffeic and chlorogenic acid > α-methyl DOPA > EGCG.

Detection limits were calculated according to $3 \times S_b \times m^{-1}$ criterion, where m is the sensitivity, and S_b was estimated as the standard deviation of

Table 2. Analytical characteristics of linear portions for calibration graphs obtained with tyrosinase biosensor

Phenolic compound	Linear range (μM)	Slope and standard deviation (nA μM^{-1})	r^2	Detection limit (μM)
Phenol	30–200	0.2769 ($n = 3$), $S = 0.0725$	>0.9808	1.25
Caffeic acid	20–200	0.4563 ($n = 3$), $S = 0.0281$	>0.990	2.68
	50–370	0.2653 ($n = 3$), $S = 0.0529$		1.82
Chlorogenic acid	70–400	0.1673 ($n = 7$), $S = 0.0593$	>0.992	2.52
EGCG	300–600	0.01296 ($n = 7$), $S = 0.001$	>0.984	143.08
		0.02158 ($n = 6$), $S = 0.002$		82.16
α-methyl DOPA[a]	100–500	0.0284	>0.998	6.42

[a]At 0 mV vs. Ag/AgCl; the rest at −100 mV vs. Ag/AgCl, and flow rate of 0.3 mL min^{-1}.

signals from each tested substrate solution at the lowest concentration level of the corresponding calibration plot. The regression confidence level is 95% for confidence range limits of slope value.

Figure 1 shows the responses obtained for a series of 10 consecutive 20 μL injections of Y01 sample extract dilution. The R.S.D. was 1.3% for a mean signal value of 70.35 nA (peak height) and for a σ_{n-1} value of 0.90.

R.S.D was not higher than 3.3% (Y09) for injection triplicates.

The genuine replicates exhibited a larger difference. For nine Y01 genuine replicates, R.S.D. in chlorogenic acid equivalent extraction was 6.6%. This result is attributed to a high dispersion in particles size distribution, even if the material is homogenized before sampling.

Changes in the electrode response were compensated by periodic calibration with an EGCG standard. It was injected every 15 min between sample extracts in order to evaluate response stability and to correct variations.

In order to evaluate matrix effect, the chemical calibration of the biosensor was made by two different methods. The phenolic concentration, resulting from direct interpolation in the calibration plot using six concentration levels was compared with that obtained by standard addition. Four concentration levels were employed in the last case, where chlorogenic acid was added to yerba mate Y01 extracts.

The results expressed as chlorogenic acid equivalents did not exhibit significant difference between interpolated values ($90.0 \pm 2.9\ mg \cdot g^{-1}$) and the standard addition calibration method ($89.2 \pm 6.4\ mg \cdot g^{-1}$), according to significance tests with $P < 0.05$.

According to this evidence, the biosensor can be used to analyze this type of plant, and matrix compounds do not interfere in polyphenol detection.

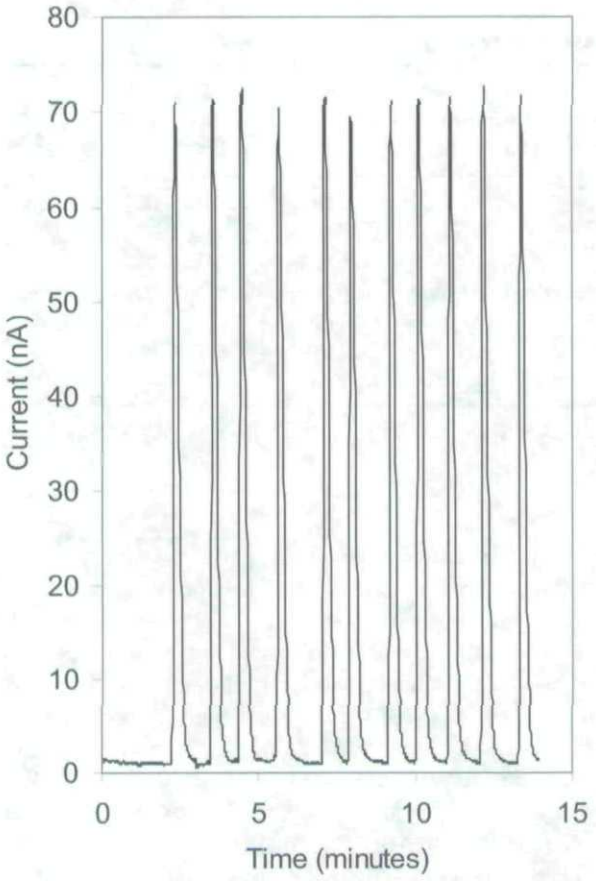

Figure 1. Amperometric response obtained with tyrosinase biosensor for a series of 10 repetitive 20 μL injections of Y01 sample extract dilution in pH = 7.00, 50 mM phosphate buffer. Flow rate: 0.3 ml. min^{-1}. Applied potential: − 100 mV vs. Ag/AgCl.

Polyphenol content in yerba mate Y01 extract was also obtained by chromatographic analysis. Chlorogenic acid concentration obtained by direct interpolation in the calibration plot was 20.0 $mg \cdot g^{-1}$, and by the standard addition method was 21.0 $mg \cdot g^{-1}$ (Fig. 2). These values markedly differed from results obtained with tyrosinase biosensor (89.2 $mg \cdot g^{-1}$), because this device responds to a variety of soluble polyphenolic compounds. Nevertheless, results are expressed as chlorogenic acid equivalents.

Polyphenolic compounds extracted from leaves were analyzed with biosensor for 25 commercially available samples (Fig. 3). We have analyzed two main groups of samples, yerba mate (*Ilex paraguayensis*) leaves and composites, which are a mixture of *Ilex paraguayensis* with other kinds of teas (Table 1).

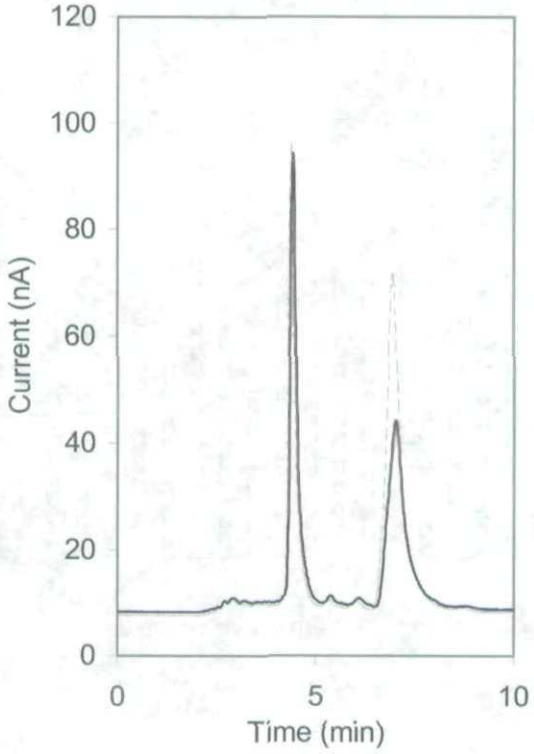

Figure 2. Chromatograms of yerba mate extract and chlorogenic acid standard. Stationary phase: RP-18; mobile phase: 5 mM phosphoric acid–acetonitrile 85 : 15. Working electrode potential: +1.0 V. Flow rate: 1 mL. min^{-1}. Diluted Y01 extract (solid line); 9.5 μM chlorogenic acid standard added to diluted Y01 extract (dashed line).

The results indicated that the amount of polyphenols extracted is highly dependent on milling degree, relative percentage of leaves in sample composition, and yerba mate percentage when the sample is composite.

A value of 92 ± 8 mg of chlorogenic acid extraction (CHE) per gram of the sample was obtained for most of samples (48% of analyzed extracts). A mean value of 9.8% was found for RSD in CHE for the 25 samples.

The highest levels of extracted polyphenols were found in particulate material, with large surface-to-volume ratios. On the other hand, the lowest levels were obtained for samples with higher amounts of twigs, lower degrees of milling, or mixtures of different species. Furthermore, the difference in polyphenol content was evident when the same trademark products but different milling procedures were compared, as can be seen in Table 1 and Fig. 3. Y02 and Y03 samples are from the same trademark, but Y03 is

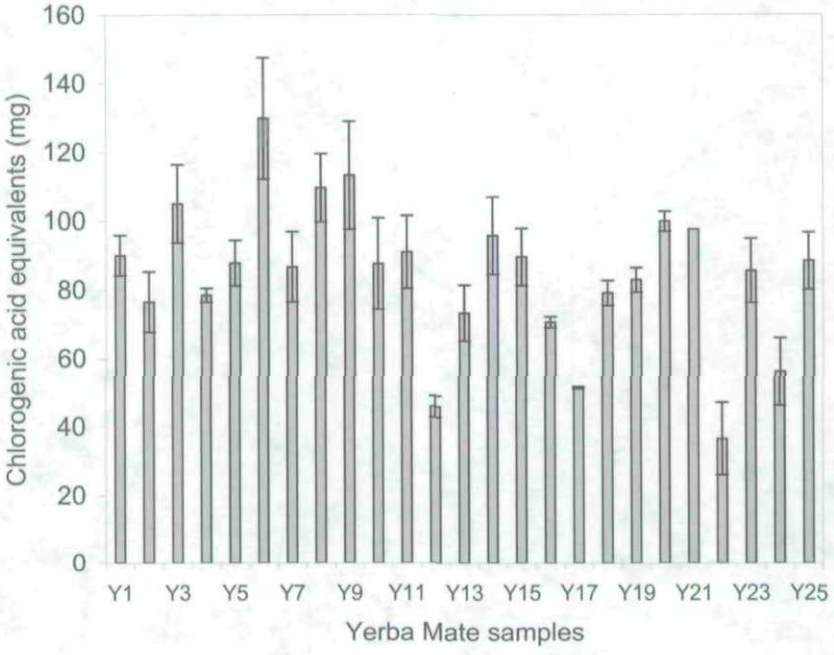

Figure 3. Extracted amount of polyphenolic compounds (expressed as mg equivalent of chlorogenic acid) in 1 g of dry yerba mate leaves (Y01 to Y25).

packed in bags and has a larger milling degree. The same behavior is observed for Y07 and Y06 samples.

Polyphenol content was also determined by Folin–Ciocalteau method in Y01 sample, obtaining a value of $90.2 \pm 8.9\,\mathrm{mg \cdot gr^{-1}}$ (dry base). No significant difference was observed between biosensor (by standard addition calibration, $89.2 \pm 6.4\,\mathrm{mg \cdot g^{-1}}$) and Folin–Ciocalteau colorimetric methods, according to significance tests with $P < 0.05$. Nevertheless, polyphenol analysis by colorimetric method was extended to other yerba mate trademarks, finding lack of correlation for some samples. Y11, Y14, and Y20 exhibited a significantly higher polyphenol level with the colorimetric method. On the contrary, for Y06 (also packed in bags), the higher value corresponded to the biosensor. Other tea species have been tested with biosensor and colorimetric methods. In this case, EGCG was chosen as standard, because it is the principal phenolic compound present in tea (*Camellia sinensis*). Results are shown in Fig. 4, where lack of correlation between methods can be observed for most of samples.

The colorimetric method leads to higher amounts of analyte in black tea compared to the biosensor results. This could be attributed to the low selectivity of Folin reagent. High correlation between methods was only observed in *Aloysia triphylla* and yerba mate.

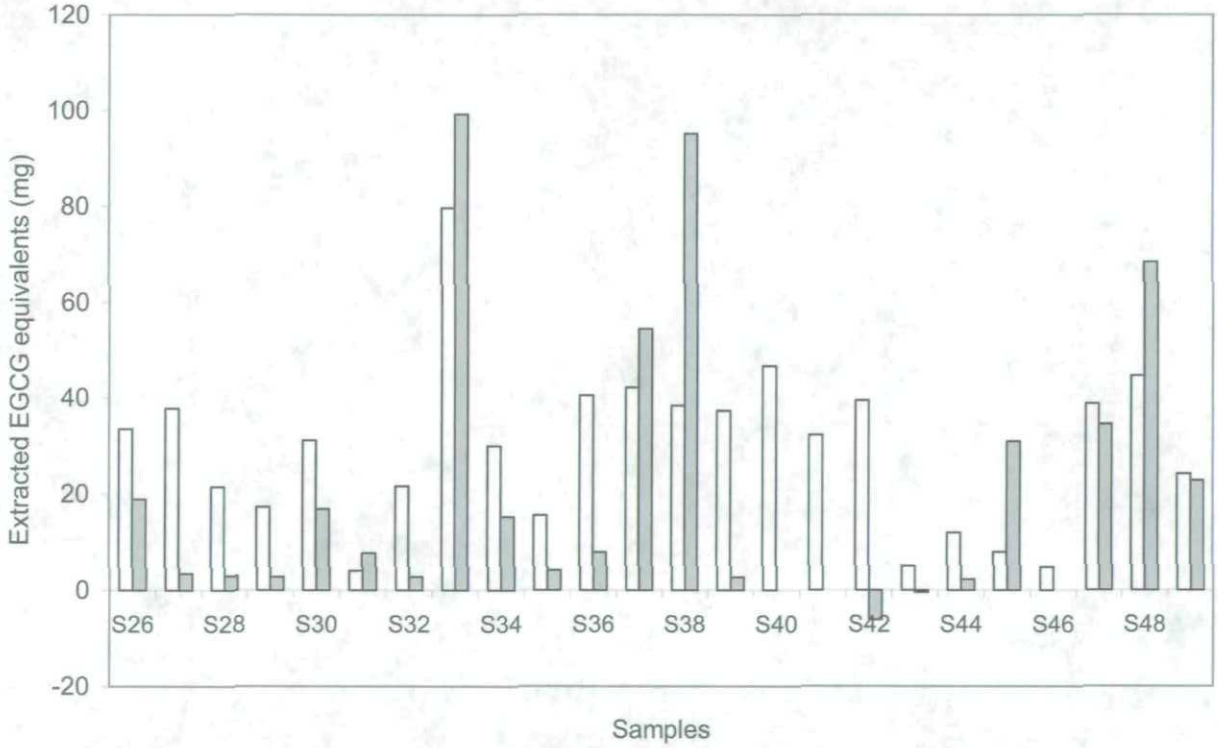

Figure 4. Extracted polyphenols analysis for S26 to S49 plant species trademarks. Results employing biosensor (■) and colorimetric (□) methods are presented for each sample.

PCA Approach

PCA is a multivariate data analysis technique that can be employed to visualize sharing features among groups of samples. PCA reduces the original chemical and physical sample descriptors to a few dimensions or variables, condensing the information and simplifying classification procedure. In this sense, discrimination of plant extracts can be achieved by simple, universal, and fast analytical methodologies followed by statistical analysis.

In this case, we compare yerba mate product with other plants of general consumption. Forty-nine samples were included (Table 1). Physical and chemical descriptors were peak potential and current potential from voltammetric experiments, absorbance and maximum wavelength in three regions of UV spectra, extracted polyphenols obtained with biosensor, and extracted polyphenols obtained with colorimetric method.

PC1 vs. PC2

The first two uncorrelated variables, PC1 and PC2, were compared in the biplot (Fig. 5) and involve 85.7% of the total variance.

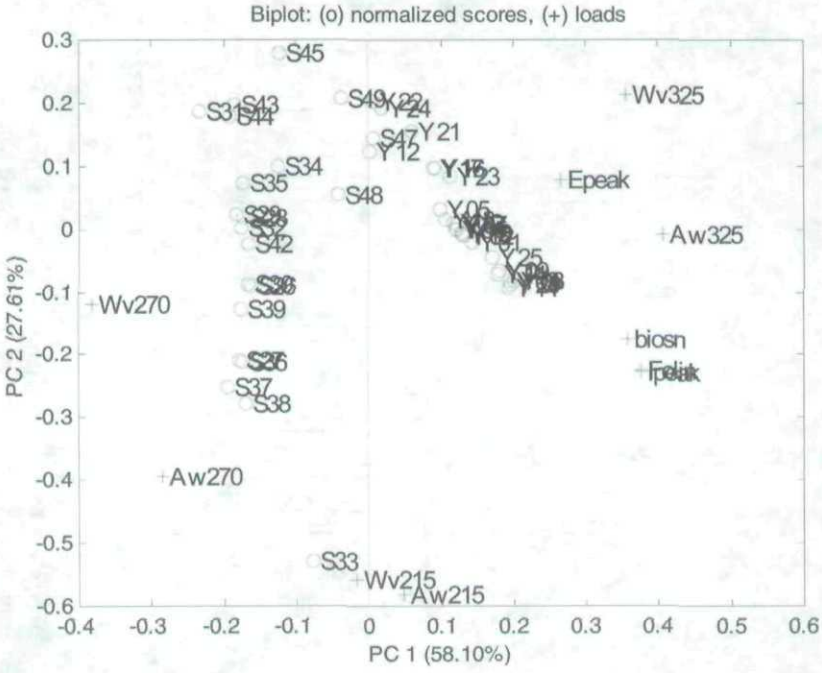

Figure 5. Biplot obtained from five descriptors and 49 samples.

Yerba mate samples formed a compact group between quadrants IV and I. At positive scores of PC1 and negative scores of PC2, yerba samples of large milling degree were localized. Around PC2 = 0, yerba samples of common presentation were found. Light with twigs and composites presentations are in the first quadrant, near PC2 = 0. According to load position, a yerba mate group was formed from an extracted amount of polyphenols (highly dependent on biosensor method), peak current, and absorbance at the 324–340 nm region.

A number of different species (*Aloysia triphylla, Lippia turbinata, Boldea boldus, Mentha piperita, Matricaria chamomilla*, and *Tilia platyphyllos*) were spread on quadrant II, not included in either the yerba or tea groups.

Tea samples were found between quadrants II and III, clearly apart from yerba mate. They were characterized by absorbance in the 272–286 nm region.

The S33 sample was a Java green tea that had a remarkably high absorbance value at 210 nm (and longer wavelength). S35 (Argentinean green tea) and S38 (Chinese green tea) did not present a clear difference with respect to black teas.

PC1 vs. PC3

This plot allowed separation of two green tea leaves samples (S31 and S38) from the rest (results not shown). Green tea in bags S35 is found together with black teas, and it exhibited low polyphenol concentration and low UV absorbance. Concerning green tea organoleptic features, S35 had a lighter green color, and it was odorless.

PC2 vs. PC3

This plot allowed separation of the two S31 and S38 green tea leaves samples and the S37 *Jasminum polyanthum* sample (high PC3 positive scores) from the rest (results not shown). Extracted polyphenols obtained with biosensor determined the difference between them. S33 (green tea from Java) and S35 (Argentinean green tea) have a PC3 score close to zero.

CONCLUSIONS

Polyphenols extracted from yerba mate and other plants were evaluated employing a tyrosinase biosensor. This device is known to recognize naturally occurring phenolic compounds and can easily be prepared with minimal environmental pollution and cost.

The selected methodology resulted in an useful tool to compare different sets of a particular product or to check chemical changes in tissues during elaboration steps that might bring flavor alteration.

Biosensing system validation was performed. Repetitivity for genuine replicates was consistent with the nature of the samples. A lack of modifier stability in time, probably due to low adherence, was detected. Sensitivity variations were corrected by means of chemical recalibration or internal standard use. It also resulted dependent on substrate molecular features.

Regarding samples, the biosensor method presented an optimal sensitivity for chlorogenic acid from yerba mate extracts. Milling degree determined polyphenol levels in yerba mate extracts. A 48% of analyzed samples presented a 92 ± 8 mg of extracted chlorogenic acid equivalents per gram of dry base. Composites exhibited a lower extracted amount.

The values obtained for extracted chlorogenic acid in Y01 yerba mate sample were $89.2\,mg \cdot g^{-1}$ with biosensor, $90.2\,mg \cdot g^{-1}$ with Folin method, and $21.0\,mg \cdot g^{-1}$ with HPLC analysis, indicating that the first two mentioned methods detected other soluble polyphenols besides chlorogenic acid.

Using PCA and the mentioned physical and chemical descriptors, discrimination between yerba mate and other plants was possible. Flavor and taste variations or composition alterations could be studied by means of analytical methods that involve low-cost instrumentation. Besides, it was observed that some green tea samples presented remarkable differences depending on the specie group.

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