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# Comparative Study of Leaf Morphology, Phenolics and Methylxanthines in *Camellia sinensis* Teas from the Italian Market

**Anna Piovan*, Raffaella Filippini, Francesca Dalla Vecchia, Rosy Caniato**

**ABSTRACT**

In this study, we compared leaf morphology and the phenolic and methylxanthine contents of different teas (*Camellia sinensis* (L.) Kuntze). Different tea types in loose leaf form available in the Italian market were investigated. The presence of numerous fine silvery-white hairs on the young leaves and buds, and the small-clavate glands on the leaf margins, light-colored and gelatinous in young leaves while dark and leathery in the old ones easily detached, showed to be diagnostic characters useful to distinguish the young leaf from the old one. White tea types were the richest of caffeine (mean value 4.42 g/100 g dry weight); in the other teas the mean caffeine levels were in the range from 2.56 to 3.38 g/100 g dry weight, and the differences were not statistically significant. Phenolics were comparable in white and green types (around 15 g/100 g dry weight) and significantly higher than oolong and black types (around 2.9 g/100 g dry weight). The principal component analysis allowed description of results with 84.02 % of variance establishing a clear grouping for each tea type. The results show that leaf morphology coupled with chemical data could be useful for tea type characterization.

**Keywords:** *Camellia sinensis* (L.) Kuntze, Leaf morphology, Methylxanthines, Phenolics, Principal component analysis.

## 1. Introduction

*Camellia sinensis* (L.) Kuntze (Theaceae) is probably one of the most investigated plant for its medicinal and food applications, and its main use is to prepare tea, a very popular beverage made by brewing leaves and buds. Global trends in tea consumption are evolving; FAO predicts world black tea and green tea production growth respectively at 1.87 and 7.2 % annually over the next 10 years [1]. The reason of the increasing diffusion of this beverage is due to its health benefits which are being extensively investigated and have received a great deal of attention in recent years. *C. sinensis* leaves are mainly used in obtaining tea, but food supplements containing green tea extracts are also available. In Usa FDA has approved Veregen, the first "Botanical Drug", now included into the 2012 European Guideline for the Management of Anogenital Warts [2]. Tea contains many compounds, especially polyphenols, and several studies show that these compounds reduce the risk of a variety of diseases [3]. Tea polyphenols include groups of compounds of different chemical structure and also possessing variable biological properties [4]. In general, fresh green tea leaves contain 36 % polyphenols, among which catechins prevail. Pharmacological properties of tea are due primarily to methylxanthines and catechins of which EGCG is the predominant in green tea leaves (48–55 % of total polyphenols) [5]. Differences in tea leaf constituents are due to many factors. It was stated that essential factors are: tea variety, collecting season, leaf age, climate, geochemical background of soil and cultivation practices, drying conditions as well as manufacturing processes [6, 7, 8, 9, 10]. Depending on the process, teas are classified into four major types: the ‘non-fermented’ white and green teas, produced by drying and steaming the fresh leaves; the ‘semi-fermented’ oolong tea, produced when the fresh leaves are subjected to a partial fermentation stage before drying; the ‘fermented’ black tea, obtained by a post-harvest fermentation stage before drying and steaming. The fermentation of tea leaves induces oxidation of catechins which are converted into a complex mixture of oxidation products [11, 12].

Many published papers deal with the overall chemical aspects, concerning the chemical composition of extracts and derived-tea foods [13], and extensive investigations have been published about the main factors affecting the chemical composition and the activity of different types of tea and tea-based products [14, 15, 16], also by use of non-conventional approaches [17, 18]. However, morphological studies of commercial tea in loose leaf form are lacking. The quality assurance of raw plant material is of great importance and should be consistent with the intended purpose of the final product [19]. For instance, since 2006, guidelines issued by the Italian Ministry of Health require botanical control from companies that transform plants to be used as food supplements. However, this requirement is not supported by reference standard data from official publications or scientific literature.

In the present work, the different macro- and micromorphology of teas in loose form commonly available on the Italian market were identified and described. Moreover, the content of the characteristic compounds were evaluated and coupled with the morphological data in order to identify discriminating indicators useful in preliminary analyses.

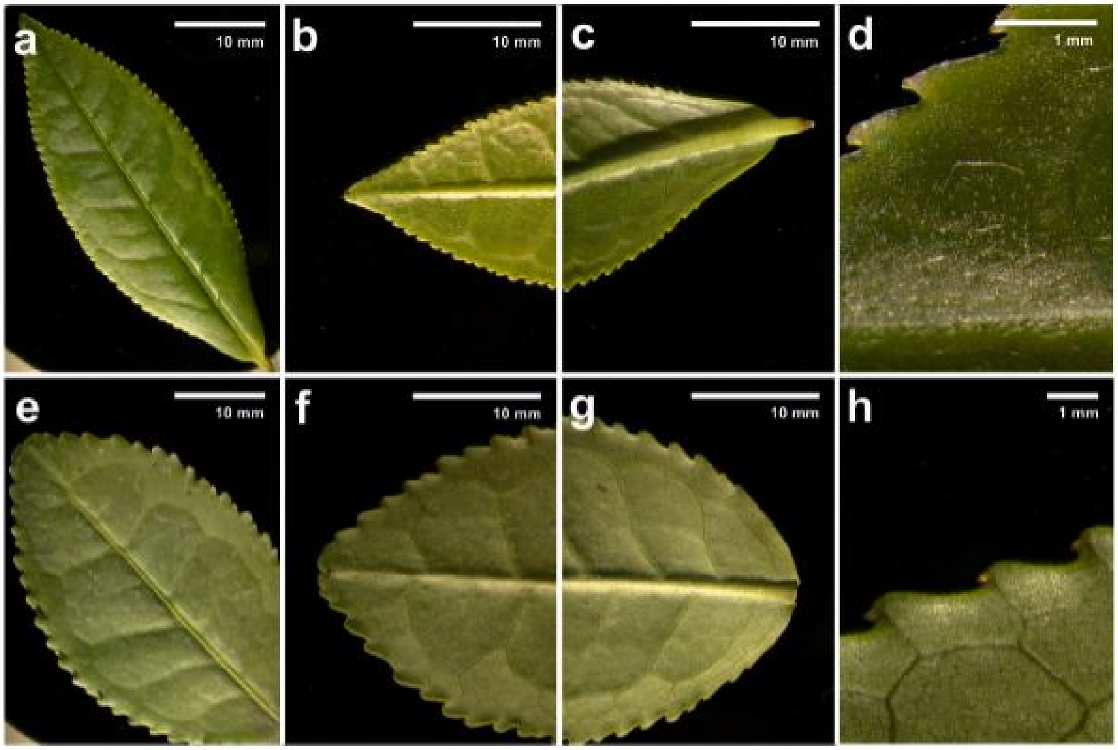



**Fig 1:** Fresh leaves of *Camellia sinensis* var. *sinensis*: **a-d** Young leaves; **e-h** Old leaves

## 2. Materials and methods

### 2.1 Plant material

Fresh leaves collected from two plants of *Camellia sinensis* var. *sinensis* growing in Botanical Garden of University of Padova; 18 types of tea in loose leaf form available in the Italian market: 6 white (W1-W6), 6 green (GG1-GG3 and GB1-GB3), 3 oolong (O1-O3) and 3 black (B1-B3) teas. Voucher specimens (No. H0025162, H0028904-921) were deposited in the Botanical Garden of Padua (Italy).

### 2.2 Macro- and Micromorphological Analyses

Intact fresh and dried leaves and leaves rehydrated by immersion in a hydroglyceric solution (1:1) over 12-24 h, were directly observed under a stereomicroscope (Leica MZF 16). Representative samples of each tea (2 g) were observed under the stereomicroscope in order to quantify buds, and to evaluate the ratio between leaves with and without hairs (HL/GL ratio). The same samples were observed after rehydration to detect and quantify the different marginal glands; the ratio between light and dark or detached glands was calculated (LG/DG ratio). The samples were analyzed in triplicate.

Fresh leaves were fixed overnight at 4 °C in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.9) and post-fixed for 2 h in 1 % osmium tetroxide in the same buffer at 4 °C. For light microscopy, the specimens were processed as described by Rascio *et al*. [20].

For the evaluation of the sclereid number, fragments of rehydrated leaves, 5 x 5 mm in size, were excised on side of midrib and near to basal margin. They were placed in a test-tube containing NaOH 1 N solution and heated on water-bath for 5-10 min. The softened material were washed with water and transferred to a slide. The fragments were broken applying a gentle rotation pressure on slide cover glass and then examined under a microscope in order to calculate the total number of sclereids. Samples were analyzed in quadruplicates.

### 2.3 Chemicals

Methanol and phosphoric acid (analytical grade), and acetonitrile (HPLC grade) from Carlo Erba. Ultrapure water from a Milli-Q system (Millipore). (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG) from Sigma-Aldrich; gallic acid (GA), caffeine (CAF), theobromine (TB), theophylline (TP) from Fluka.

### 2.4 Sample preparation and HPLC analysis
Tea samples (0.5 g) were conveniently powdered and exhaustively extracted with aliquots of 50 % aqueous methanol at 60 °C. The extracts were combined, filtered through 0.22 μm syringe filter. The extractions were performed in triplicates. The extracts, conveniently diluted, were analyzed by HPLC: ChromQuest pump P4000, photodiode array detector UV6000 (Thermoseparation, San Josè, CA, USA); Gemini RP-18 column 250 x 4.60 mm, 5 μm, equipped with a Gemini RP-18 guard column 4.0 x 3.0 mm (Phenomenex, Torrance, CA, USA). The data were recorded and processed using ChromQuest Chromatography Workstation. The separation was achieved with a reverse phase analytical column. The mobile phase consisted of phosphoric acid in water, pH 3 (A) and acetonitrile (B): 0-5 min A:B 95:5 v/v, 5-6 min from 95:5 to 87:13, 6-26 min 87:13, 26-27 min from 87:13 to 73:27, 27-40 min 73:27, 40-46 min from 73:27 to 0:100, 46-55 min 0:100; flow rate 1 ml/min; injection volume 20 μl. UV spectra were recorded in the 200–400 nm range; chromatograms were acquired at 273 nm. Peaks were assigned by spiking the samples with standard compounds, and by comparison of the UV spectra and retention times. Quantification of the compounds was based on peak area using external standards. Standard curves were created by preparing appropriate dilution series of each standard.

### 2.5 Data analyses
Data were subjected to analysis of variance (ANOVA) and comparison among means was determined according to Tukey's test. Principal component analysis was used to establish the statistical discrimination among samples. The statistical tests were generated with GraphPad Prism and XLSTAT.

## 3. Results and Discussion
### 3.1 Morphological observations
The stereomicroscope observation of fresh leaves from Botanical Garden underlined some typical characteristics of tea leaves (Figure 1). Leaf blade was symmetric, longley oblong or oblong in shape, abaxially pale green and glabrous or pubescent, adaxially dark green, shiny, and glabrous. Leaves base was cuneate or rounded and apex was short acuminate or obtusely acuminate. The shape, as well as the size and color, was closely related to the leaf age and its stage of growth. The margin spacing was regular. Unicellular covering trichomes occurred on the abaxial side of the young leaves and frequently more abundant on the midrib than on the margin. These trichomes were absent in the old leaves (Figure 1b,f). The marginal configuration was spherulate bearing a small-clavate glandular apical termination. In the young leaves these glands were light-colored and gelatinous while in the old ones were dark, leathery and easily detached (Figure 1d,h). Taking into account these basic features we have analyzed white, green, oolong and black teas in loose leaf form purchased from the Italian market and used for infusion preparation. The observation and comparison were made on the basis of bud number, ratio between leaves with and without hairs (HL/GL ratio), and ratio between light and dark or detached marginal glands (LG/DG ratio).

White tea is increasingly widespread but currently there is no general accepted definition of white tea and very little international agreement [21]. However, it is commonly accepted that white teas are made only from the unopened buds and one or two immature leaves that are plucked and dried with minimal processing [22]. The six studied commercial samples of white tea appeared to be very similar under stereomicroscope observation. The leaves were rolled in small pieces in green color and the buds were easily detectable and abundant, around 18 % of the total material (w/w, mean value). The leaves and mainly the unopened buds were thickly covered by fine silvery-white hairs (Figure 2 a1-d1). Figure 2 e1 showed a bud with a very young leaf after rehydration. Small-clavate glandular hairs on the margin of the rehydrated leaf were mostly light-colored and gelatinous (LG/DG ratio 9). The distinctive veinlet network and the abundant hairs on the midrib were also evident (Figure 2 f1). The abundance of buds, the high mean HL/GL ratio (about 10) and the absence of dusty elements showed a good quality of the examined samples.

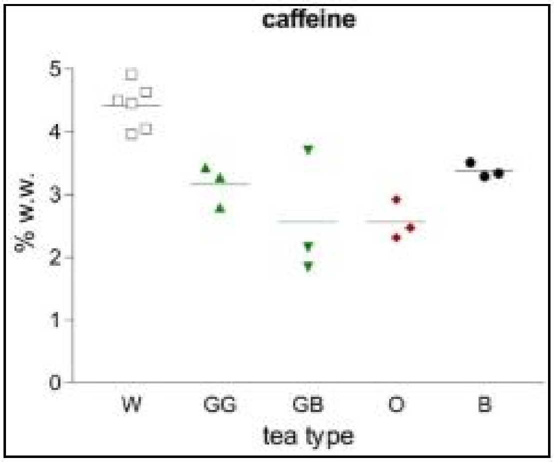
Nowadays, green tea is became very popular also due to its use in producing medicinal and dietary supplements besides in preparing infusions. The common feature of green teas is that the leaves are never subjected to condition for causing fermentation. However, the several variations in processing, along with the time harvesting and the environmental and cultural conditions have led to many different types of green tea. We analyzed two common types of green tea: the gun powdered (3 subtypes) and bancha (3 subtypes). The leaves constituting the three gunpowder teas were rolled into small pellets with dark grayish-green color and smooth surface (Figure 2 a2). The buds were present in fragments and in very low abundance (0.66 %). The pellet leaf shape made very difficult the observation of any other characters, then a long hydration time (24 h) has been needed to obtain a complete distension of the leaves (Figure 2 b2,c2). Bancha is a Japanese green tea, which is harvested as a third or fourth flush between summer and autumn and can include also leaf fragments and twigs. Under stereomicroscope observation the dried leaves appeared irregularly rolled to form tubular or flattened structures with green to yellowish color along with small leaf fragments, and buds were never detected (Figure 2 d2,e2). Both in gunpowder and bancha samples the rehydrated leaves showed the distinctive margin, with the two gland types (LG/DG ratio 1 and 0.25 respectively). Although young leaves with hairs have been detected in all the samples of the two tea types (HL/GL ratio 0.1), in the majority, the leaves didn’t show any hairs confirming their old age (Figure 2 c2,f2).

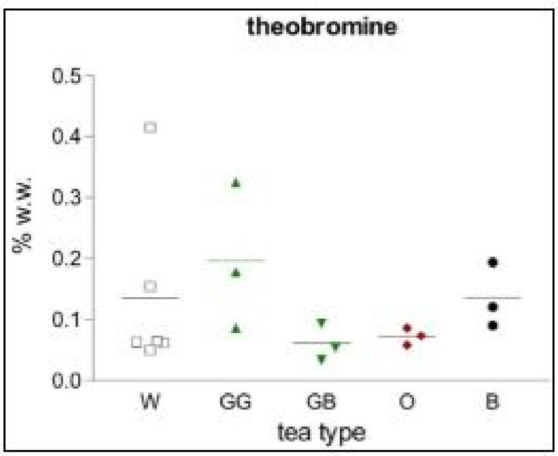
Oolong is a partially fermented tea and can be produced with young green shoots. Oolong tea accounts only for about 2 % of worldwide tea consumption and some fine oolong varieties are sold at a premium price in the market [16, 23]. Under the stereomicroscope the three observed samples appeared to be brown-reddish in color and constituted by leaves rolled to form tubular structures and by fragment more or less different in size. Only few fragments of buds have been detected in the samples (about 0.8 %). On the whole, only few leaves showed hairs on the lamina suggesting that mature leaves were present to a higher extent than young leaves (HL/GL ratio 0.3) (Figure 2 a3,b3). The rehydrated leaves showed the distinctive margin, with the two gland types (LG/DG ratio 0.4). In sample O3 some rehydrated leaves showed quite different margin: the basal side of the tooth shape appeared to be slightly concave rather than straight-convex, with more protruding glandular tooth apex (Figure 2 c3).

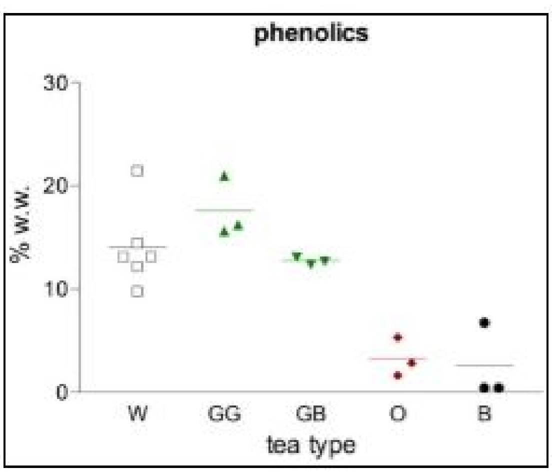
Black tea is the most common type of tea outside of Asia being the most versatile in usage. The full fermentation process gives the characteristic dark color easily detectable to the naked eye and under stereomicroscope observation (Figure 2 d3). The leaves appeared to be irregularly rolled to form longitudinally shrivelled tubes. Although in a low extent some buds have been found (2.5 %), as well as young leaves with hairs (HL/GL ratio 0.9). Twigs and little fragments have been detected but never dusty fragments (Figure 2 e3). The rehydration of the plant material didn't show entire leaves but only leaf fragments in which the typical margin and the hairs on the abaxial lamina can be easily recognized (LG/DG ratio 0.7) (Figure 2 f3).


**Fig 2:** White tea: **a1-c1** Tea in loose form with buds and leaves; **d1** Silvery-white hairs on the bud; **e1** Rehydrated bud; **f1** Leaf margin. Green tea: **a2-c2** Gunpowder; **d2-f2** Bancha. Oolong tea: **a3-c3.** Black tea: **d3-f3**. Leaf sclereids: **a4** Mesophyllum with sclereids; **b4-c4** Rhizosclereid; **d4** Astrosclereid; **e4** Palosclereid; **f4** T-Y shaped sclereid

Light micrograph of a leaf transversal section showed bifacial structure and unilayered epidermis. In the mesophyllum thickness numerous sclereids were present (Figure 2 a4). Zhang *et al*. [24] have demonstrated that foliar sclereids in the section *Thea* are relatively stable and their diversity and regularity are of some taxonomic value. In *C. sinensis* var. *sinensis* they detected filiform sclereids, palosclereids, rhizosclereids and ramiform sclereids. On the other hand, Rajanna and Ramakrishnan [25] reported only the presence of astrosclereids in *C. sinensis* var. *sinensis*. In order to evaluate the possible value of the sclereids in assessing the quality control of different tea types, we evaluated the types and the density of sclereids, it being known that these cells are numerous only in older leaves. Except white tea, in which any sclereids have never been detected, in all the analysed tea samples have been found at least three types of sclereids which could be clearly assimilated to rhizosclereids (Figure 2 b4,c4), astrosclereids/ramiform sclereids (Figure 2 d4), and to a lesser degree palosclereids (Figure 2 e4). Moreover, a fourth type of sclereids with intermediate characteristics between T and Y-shaped sclereids has been frequently found (Figure 2 f4). These data should confirm that white tea is mainly constituted by young leaves usually lacking of sclerenchymatic elements. On the contrary, green, oolong and black teas showed a great variability in sclereid number. Because of the non-homogeneous characteristics of these teas (shape, fragment size) and therefore the lower precision in selecting and preparing a representative sample to analyze, these quantitative data didn't show any additional useful information in assessing the sample quality.



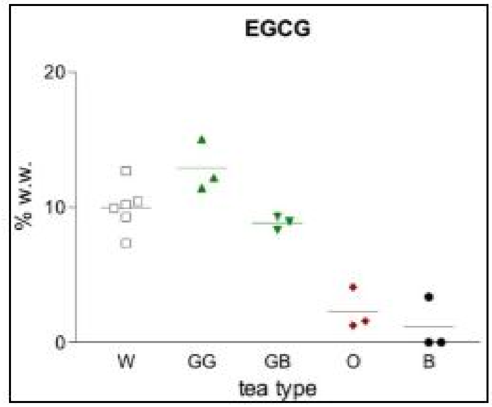




**Fig 3:** Caffeine, theobromine, phenolics and EGCG contents in the different tea types and subtypes; bars depict mean content values

### 3.2 Chemical analysis

The polyphenol and methylxanthine contents have been compared in the 4 tea types from different commercial sources. The methylxanthine and polyphenol contents are provided in Figure 3 and Figure 4. Results show inter- and intra-variations in tea types and subtypes. Caffeine and theobromine were found in all the samples, whereas theophylline has never been detected, probably due to levels too low to allow its detection, as already reported [26]. Caffeine content was found to be higher than theobromine in all the samples and white teas had the highest ($p < 0.001$) mean value (4.42 g/100 g dry weight). In the other teas the mean caffeine levels were in the range from 2.56 to 3.38 g/100 g dry weight, and the differences were not statistically significant ($p > 0.05$). By looking at each tea type, a rather low variability could be observed in caffeine value ranges in white (3.96-4.92 g/100 g dry weight), green gunpowder (2.79-3.43 g/100 g dry weight), oolong (2.31-2.92 g/100 g dry weight) and black tea (3.29-3.51 g/100 g dry weight). On the contrary, a higher variability in caffeine content was observed in the three green bancha teas (1.84-3.7 g/100 g dry weight). Our results confirm that white tea is the richest of caffeine as already reported [21, 27], and underline the impropriety of misleading generalizations about the caffeine content of the different tea types. Theobromine mean content (around 0.12 g/100 g dry weight) was comparable ($p > 0.05$) in different tea types; however the analysis revealed strikingly different amounts among tea subtypes within the white, gunpowder and black tea (values range from 0.05 to 0.41, from 0.09 to 0.32 and from 0.09 to 0.19 g/100 g dry weight respectively). As formerly reported by some authors, theobromine distribution showed much greater variability than that of caffeine [28, 29], and there is not a characteristic trend of caffeine/theobromine levels across different subtypes of tea.

The mean amounts of phenolics (around 15 g/100 g dry weight) were comparable ($p > 0.05$) in white and green types and significantly higher ($p < 0.001$) than in oolong and black types (around 2.9 g/100 g dry weight). The highest content was found in the samples W6 and GG3 ($p < 0.05$). EGCG was the major catechin in all the analyzed teas (range from 55 to 95 % of total phenolics), save B1 and B2, in which only gallic acid was detected. These results are in accordance with the range of values obtained in previous studies, confirming that semifermented and fermented teas contain lower catechins than white and green teas

[21]. Although previous studies have reported that green teas were the richest source of phenolics [30], our results have shown that there are no statistically significant differences ($p > 0.05$) between green and white tea. However, as frequently pointed out in literature, several factors could affect the relative amounts of compounds in tea types like geographical origin, seasonal climate, as well as processing and storage conditions [31].

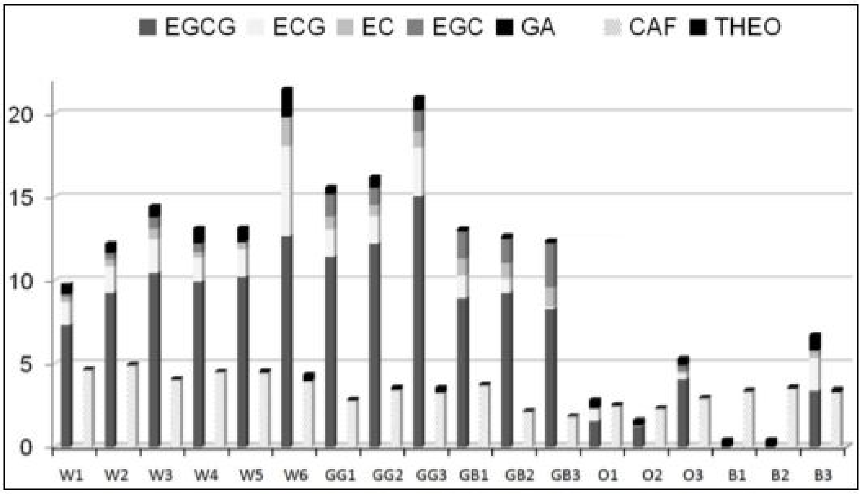



**Fig 4:** Phenolic and methylxanthine content expressed in g/100 g of dry weight in the different tea types and subtypes

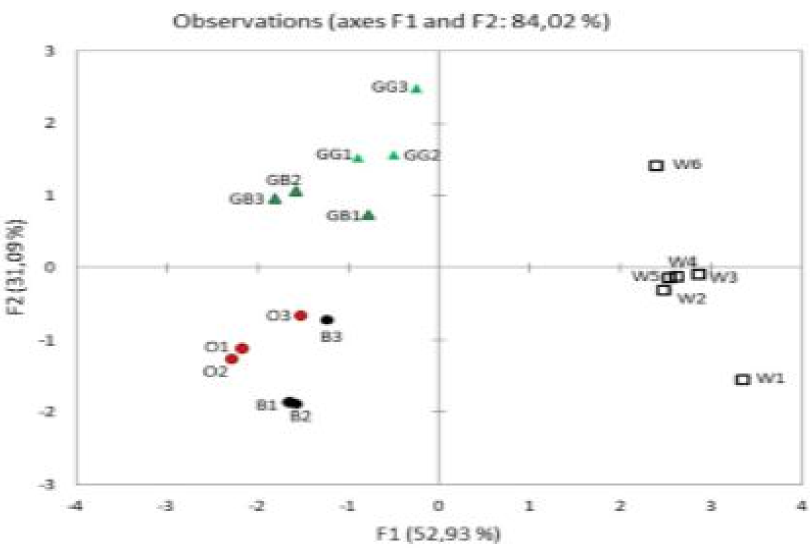



**Fig 5:** Principal component analysis of the different tea types and subtypes. F1: principal component 1; F2: principal component 2

In order to visualize the data trends and provide an evaluation of the discriminant efficiency of the morphological and chemical features with respect to different tea types, the data were subjected to principal component analysis. Two principal components were extracted explaining up to 84.02 % of the total variance. Figure 5 shows the corresponding scores plot. At a glance, a clear separation between the different tea types can be observed. A clustering of the scores of white teas occurs along the F1 axis at positive values, while the green, oolong and black tea scores clustered along the F2 axis. The green teas appear at the left upper side in two separate clusters, whereas the oolong and black types are situated at the left lower side of the plot, at negative values of F1. After examination of the variable loadings of F1, LG/DG ratio, bud abundance, caffeine and HL/GL ratio result the descriptors with more contribution.

**4. Conclusions**

This study demonstrated evidence of differences among macroscopic organoleptic features (color, shape, size), some macro- and microscopic characteristics (bud abundance, relative abundance of young and old leaves, and type of glands on leaf margin), and chemical constituents of different tea types. PCA allowed description of results with 84.02 % of variance establishing a clear grouping for each non-fermented tea, less evident in the two fermented tea types, oolong and black. The obtained data indicate that a simple morphological observation could be an efficient support to chemical analysis in the preliminary evaluation of samples of tea. This is of great importance to guarantee an high quality standard and to safeguard the consumers health.

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