

Effect of long-term storage on degradation of 21 phenolic compounds in green tea

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The storage resistance of 21 selected phenolic compounds present in ten samples of commercially available green tea was monitored using the HPLC/MS/MS over a 16 weeks period using multivariate statistical analysis to compare the different types of packaging. The HPLC/MS/MS method with the computer-aided analysis enabled the fast evaluation of the long-time storage resistance of valuable compounds present in green tea, yielding useful information to customers about their degradation, which affected the sensory properties of the green tea, such as taste and colour. An explanatory data analysis provided an initial visualization of the multivariate data matrix while a factor analysis, hierarchical cluster analysis and discriminant analysis enabled the classification of the green tea samples into clusters according to the storage resistance of the compounds of interest. A significant reduction in the content of all monitored compounds was indicated after 3 weeks of storage, which was also found to be dependent on the type of packaging used. The samples in a plastic box were more stable during long-time storage in comparison to the samples in a paper box.

Keywords: Green tea; Long-term storage; Phenolic compounds; Packages of green tea; HPLC/MS/MS

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Introduction

Tea is a popular beverage consumed worldwide and it is normally produced from the leaves of two cultivated tea plants, *Camellia sinensis* (L.) O. Kuntze var. *sinensis* and *C. sinensis* var. *assamica* (Masters) Kitamura (Theaceae). Based on the processing procedures, it can be generally divided as green tea (nonfermented), oolong tea (semifermented), black tea (fully fermented by polyphenol oxidase), and Pu-Erh tea (postfermented by microbes) [1]. The chemical composition of green tea depends on several factors: genetic strain, climatic conditions, soil, growth altitude, and horticultural practices, the plucking season, sorting (grading) of the leaves, processing, storage, etc. [2,3] The leaves of green tea contain many polyphenolic compounds, including flavonoids, phenolic acids, and leucoanthocyanins which together with caffeine and theanine account for up to 40 % of the dry weight of green tea leaves [4]. Polyphenols are powerful antioxidants and free radical scavengers and approximately 60–80 % of the polyphenols in green tea are flavan-3-ols, commonly known as catechins [5]. These compounds contained in green tea occur in a higher concentration than those in black and oolong tea because no fermentation process takes place during the preparation of green tea [6]. During fermentation, the catechins are oxidized into theaflavin and, as a result, their content in black and oolong teas is reduced [7]. The main catechins are epigallocatechin-3-O-gallate (EGCG), epigallocatechin (EGC), epicatechin-gallate (ECG) and epicatechin (EC) [8,9]. They are highly unstable in solution and degrade through oxidative and epimerization processes [7,10]. Many studies have been performed being focused on monitoring of the degradation of EGCG in green tea infusions [11–14]. In general, EGCG is highly susceptible to degradation and its stability is pH and temperature dependent. The stability is higher at lower temperatures (4 to –20 °C), lower pH values (3.5 to 7.4) and in the presence of ascorbic acid (0.25 %, w/w) [15].

In powdered form, EGCG degrades at a lower rate, likely as a result of limited molecular mobility, and it also has an increased concentration in the solid phase [16]. Generally, powder systems are more stable and catechin degradation is slower than in aqueous solutions [17], being also dependent on the temperature and humidity during storage, with temperature as the dominant factor [18].

Different multivariate data computer-aided treatments can help to deal with the flood of chromatographic data and there are two major categories of the often used classification techniques – unsupervised and supervised [19,20]. Unsupervised classification is used for outcomes based on the software analysis of data without the user providing sample classes. The computer-aided methods determine which samples are related and thereby can be grouped into classes [21]. Factor analysis (FA) and hierarchical cluster analysis (HCA) are the two most common unsupervised recognition methods widely used in biological, chemical, food, and other applications [22–27].

The aim of this work was to develop a rapid method for the assessment of the degradation of compounds that carry health benefits in green tea in terms of the long-time storage of the tea in domestic conditions when the teabags had been stored in a cupboard in the original bags for 16 weeks. Pure green teas without any other flavour in different types of packages, paper or plastic material, were purchased in local markets in Czech Republic and then examined. The degradation of phenolic compounds was monitored approximately every three weeks and all of the results were evaluated by multivariate data analysis in order to classify each of the green teas according to its storage resistance.

Materials and methods

Chemicals and solvents

The standards of studied phenolic compounds (kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, gallic acid, isoquercitrin, rutin, cryptochlorogenic acid, neochlorogenic acid, chlorogenic acid, epicatechin, catechin, epicatechin gallate, catechin gallate, galocatechin gallate, epigallocatechin gallate, galocatechin, epigallocatechin), methanol, and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Deionized water was prepared using a Milli-Q purification system (Merck Millipore, Darmstadt, Germany).

Sample preparation

Ten green tea samples (Table 1) were used for quantitative analysis in order to monitor the effect of the storage on the content of selected phenolic compounds in the tea. The different types of paper or plastic material packages were used. The extracts were prepared from one tea bag of approximately 2 g of green tea using 200 mL of hot water at a temperature of 80 °C for 4 min. This process was undertaken from previous work, where the effect of temperature on the stability of green tea infusions had been studied [28]. The exact amount of green tea in an individual tea bag was weighted before extraction. The extracts were cooled down to the laboratory temperature, filtrated through a 0.45 µm filter and analysed using the optimized HPLC/MS/MS method in four replicates ($n = 4$). The amount of phenolic compounds was expressed in milligrams of the individual compound in one gram of the green tea sample (mg g^{-1}). The intra-day precision was examined by measuring ten extracts prepared from the selected green tea in one day. The individual extracts were measured four times using the HPLC/MS/MS method ($n = 40$). The process was repeated after two days for extra-day precision. The RSD [%] was not more than 4.8 % in the case of all studied compounds for intra-day precision and was not more than 14.5 % for extra-day precision. To monitor

the storage effect, the extracts were prepared immediately after opening the tea box (denoted W0) and then after 3 (denoted W3), 7 (W7), 10 (W10) and 16 (W16) weeks. The tea bags were stored in the original boxes in a cupboard at laboratory temperature to simulate domestic storage conditions and thereby yield an objective view of the storage resistance of common green teas. The object codes describing the green tea samples are comprised of sample number (S 1,2 ...10) and the storage time in weeks (W0, 3, 7, 10 and 16). W0 corresponds to the analysis of an infusion of green tea prepared instantly after the opening of the tea box.

Table 1 The list of studied green tea samples

No	Sample	Package	Country of origin
1	Apotheke Bio	Double-chamber bags packed in paper packets and paper box	China
2	Jemča	Square/pillow bags in paper box	unknown
3	Saga	Round bags in plastic box	mixture
4	Pickwick	Double-chamber bags packed in paper packets and paper box	China
5	Dukat	Double-chamber bags packed in plastic packets and paper box	China
6	Dukat	Round bags in plastic box	China, Indonesia
7	Tesco	Double-chamber bags in paper box	Vietnam
8	Ahmad	Double-chamber bags in paper box	unknown
9	Teekanné	Double-chamber bags in paper packet and paper box	China
10	Loyd	Round bags in paper box	China

HPLC/MS/MS analysis

Chromatographic and mass spectrometric conditions for the separation of the phenolic compounds in green tea were adopted from our previous work [28]. HPLC system equipped with an LC-20AD binary gradient pump, a DGU-20A degassing unit, a SIL-20A autosampler (all Shimadzu, Kyoto, Japan) and an LCO 102 Single thermostat column (Ecom, Prague, Czech Republic) was coupled to a QTRAP 4500 mass spectrometer (AB SCIEX, Framingham, MA, USA). The reversed-phase separation of the phenolic compounds was performed with an Ascentis Express C18 (50 mm × 2.1 mm i.d. and 2 µm particle size) at 40 °C. The mobile phase consisted of 0.1 % (v/v) formic acid in water (A) and 0.1 % (v/v) formic acid in methanol (B). Gradient program was from 20 % to 70 % B in 2.5 min and the flow rate was 0.5 mL min⁻¹. The phenolic compounds were separated under these conditions in 2.5 min. This short separation is crucial for the exact

determination of the phenolic compounds content due to their degradation during staying of infusion. The quantification was carried out using mass spectrometry in multiple reaction monitoring (MRM) mode.

The calibration solution of the phenolic compounds analysed was prepared by sequential dilution of the stock methanol solution (1 mg L⁻¹ for chlorogenic acid derivatives and 10 mg L⁻¹ for other compounds) by 20 % (v/v) methanol. The concentration range of the calibration was 2.5–170 µg L⁻¹ for derivatives of chlorogenic acid and 25–1700 µg L⁻¹ for other compounds.

Multivariate data analysis (MDA)

Data obtained according to the process defined above were analysed using different pattern recognition techniques, including an exploratory analysis of the multivariate data (EDA), factor analysis (FA), hierarchical cluster analysis (CLU), and discriminant analysis (DA) [29]. Visual analysis of the multivariate data can help in dealing with a large quantity of information when the user is directly involved in the data analysis process. The glyphs are the first graphical entities that convey one or more aspects of data value via attributes, such as the shape, size, and position. In a set of multivariate data with more than two variables, it is often useful to view the scatterplots of the correlation matrix of each pair of variables. This plot is symmetric about its diagonal and enables a row and column to be visually scanned for examining any correlation of one variable against all the others. If all of the correlations are small, let us say less than 0.3, there is no need for a factor analysis. With factor analysis, one can first identify the separate dimensions of the structure and then determine the extent into which each variable is explained by its dimension. The discriminant analysis separates objects into one of two or more alternative groups on the basis of a set of measurements. The groups are known to be distinct, and each individual belongs to only one of them. Discriminant analysis provides a classification of objects into groups where the respective ranking is known; at least, for the sample from which the classification equations are being derived.

All data analyses were performed using the software STATISTICA 12 (StatSoft, Prague, Czech Republic).

Results and Discussions

Explanatory data analysis (EDA)

The influence of long-time storage of green tea on the stability of the 21 selected phenolic compounds was monitored over a period of 16 weeks. The amount of each individual compound determined in the infusions prepared from sample 8

(Ahmad) instantly after opening the tea box and after 16 weeks is depicted in Fig. 1. From this figure, it is apparent that the content of all of the compounds is approximately 25 times lower in the infusion prepared 16 weeks after the opening of the tea box and with the highest degradation observed in the case of EGCG.

Principal component analysis (PCA) was used to distinguish the green tea samples according their storage resistance under common domestic conditions. The studied system can essentially be described by two components (PC1 71.1 % and PC2 11.6 %); while a third component is located close to an eigenvalue of 1 and its importance is lower (PC3 5.3 %). A very small effect of GCG (short vector) and similarity of many variables (their vectors are close to each other) is evident from the plot of principal component loadings (data not shown). Consequently, some of the insignificant variables were removed to simplify the data matrix and only compounds showing a high impact on the description of the system have been chosen to explain the variability of the data. These selected variables were catechins (EGC, EGCG, ECG + CG, EC), chlorogenic acid (chlA), quercetin-3-O-glycosylrutinoside (QglyRut), 5-coumaroylquinic acid (5-couA), gallic acid (galA), kaempferol-3-O-glycosylrutinoside (KgalRut), and galloylquinic acid (galQ). All these compounds were used as variables in all the methods employing multivariate data analysis.

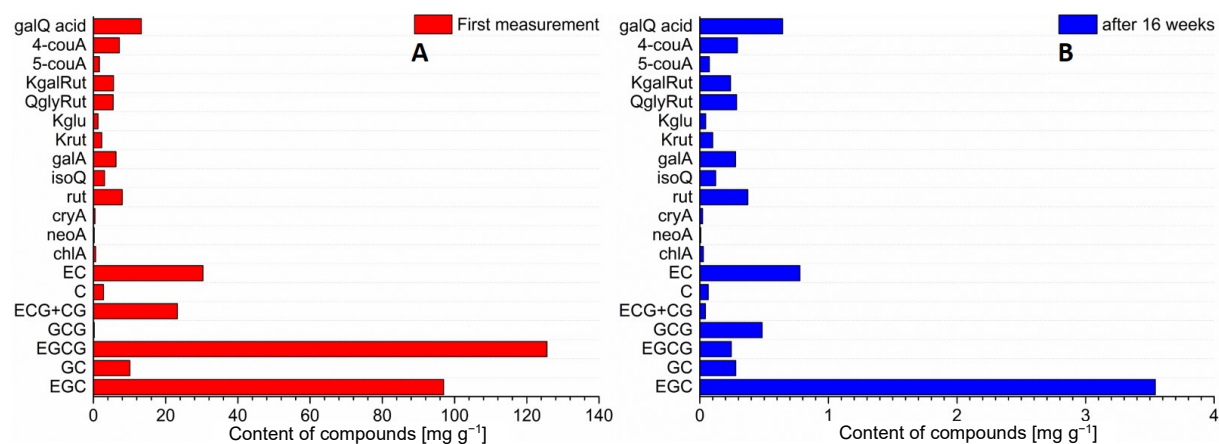


Fig. 1 The content (in mg g⁻¹) of the compounds monitored in green tea infusions prepared from sample 8 (Ahmad) after opening of the tea box (A) and after 16 weeks of storage (B)

galQ: galloylquinic acid, 4-couA: 4-coumaroylquinic acid, 5-couA: 5-coumaroylquinic acid, KgalRut: kaempferol-3-O-glycosylrutinoside, QglyRut: quercetin-3-O-glycosylrutinoside, Kglu: kaempferol-3-O-glucoside, Krut: kaempferol-3-O-rutinoside, GalA: gallic acid, isoQ: isoquercitrin, rut: rutin, cryA: cryptochlorogenic acid, neoA: neochlorogenic acid, chlA: chlorogenic acid, EC: epicatechin, C: catechin, ECG+CG: epicatechin gallate + catechin gallate, GCG: galocatechin gallate, EGCG: epigallocatechin gallate, GC: galocatechin, EGC: epigallocatechin.

The matrix graph (Fig. 2) illustrates the weakest and the strongest correlation of a pair of characters, with the matrix being symmetrical according to the diagonal. Positive correlations observed for all the compounds with a p -value ≤ 0.05 are highlighted in the table in Fig. 2. Major differences were identified in the pairs EGC – 5-couA ($r = 0.958$), QglyRut – KgalRut ($r = 0.947$), EGC – EC ($r = 0.938$) and EGC – galA ($r = 0.935$). The main dissimilarities occurred for the pairs ECG+CG – EC ($r = 0.502$), EGC – ECG+CG ($r = 0.426$), chlA – KgalRut ($r = 0.415$), and ECG+CG – galA ($r = 0.413$). There are also insignificant correlations between ECG+CG – QglyRut ($r = 0.095$), ECG+CG – KgalRut ($r = 0.124$) and ECG+CG – 5couA ($r = 0.320$).

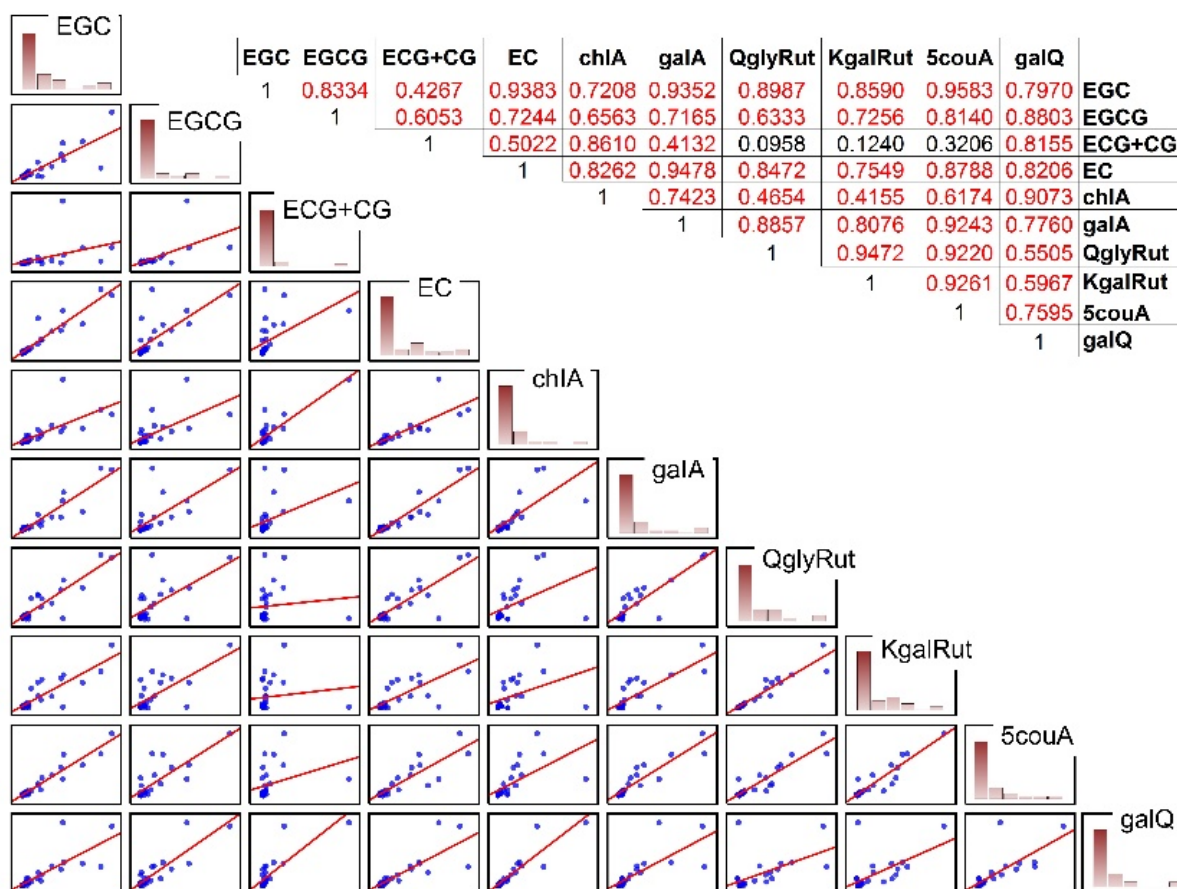


Fig. 2 The scatterplot of the correlation matrix and correlation table ($p \leq 0.05$)

Factor analysis

Factor analysis allows us the visualization of the variability among the green tea samples. Only measurements performed instantly after opening the tea box (W0) and then after 3 and 16 weeks were used for the factor analysis for the sake of clarity of the data, because only insignificant differences between measurements after 7, 10 a 16 weeks could be observed.

The variables KgalRut and QglyRut suit the factor 1, because they are located close to value 1 on the axis of factor 1 (Fig. 3a). The content of these compounds in the individual samples strongly depends on the storage time of the sample; therefore, the factor 1 is connected to the speed of degradation. The variables ECG+CG (Fig. 3a) are useful for the factor 2 description because they are located closed to value 1 on the axis of the factor 2. Catechins have high antioxidant properties; therefore, the factor 2 was connected to the antioxidant capacity of the samples.

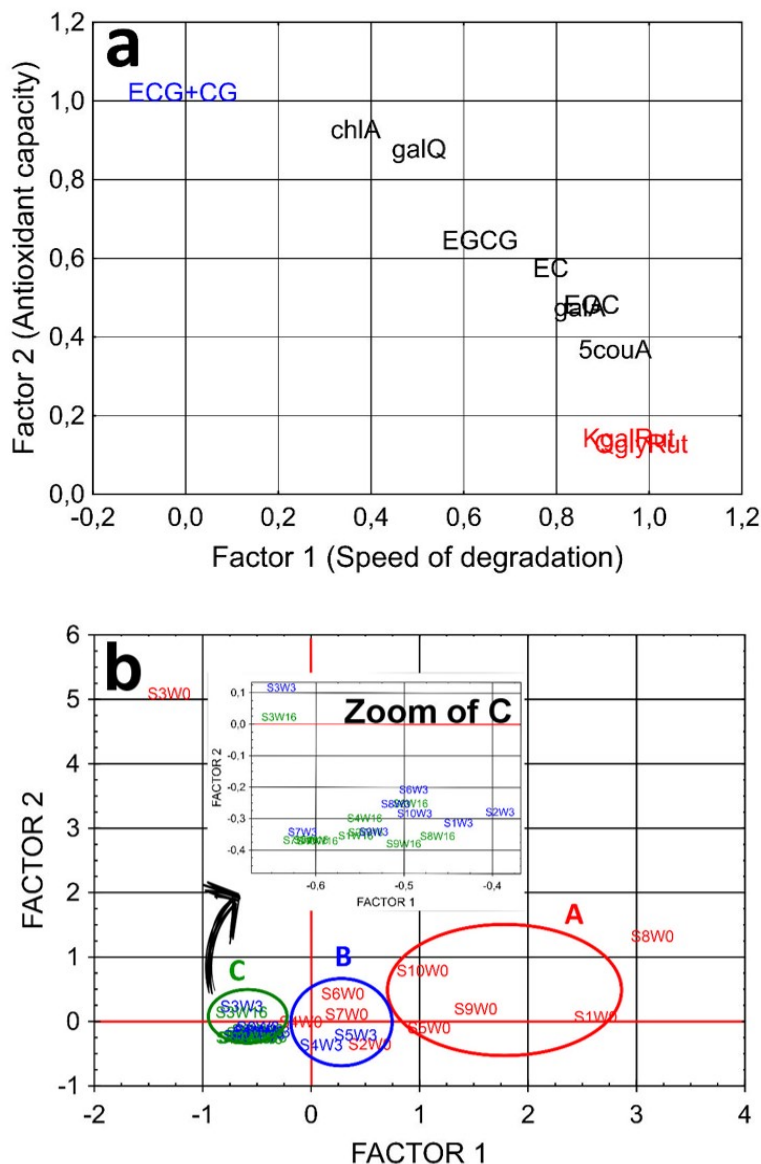


Fig. 3 (a) The factor loadings plot after normalized varimax rotation demonstrates a similarity of variables,
 (b) The factor scatterplot indicates four clusters of samples of green tea: A, B, C and outlier S3W0.

Using FA, the samples of green tea were grouped into three clusters A–C (Fig. 3b), with clusters A and B including the samples measured at the beginning of the study instantly after opening the tea box (W0). Sample 5, measured after 3 weeks (S5W3), was located in cluster B probably due to its low degradation during the first three weeks. The rest of the samples measured after 3–16 weeks are located in cluster C, where significant degradation of the studied compounds was observed. These samples are located close to each other because the content of the monitored compounds had decreased rapidly after only 3 weeks. Sample 3, measured after the opening of the tea box (S3W0), was located far from the other samples because of only very small amounts of variables KgalRut and QglyRut and, on the other hand, a very high amount of catechins, therefore a high antioxidant power was observed.

The largest decrease in the content of catechins, EGCG (28 %) and ECG (51 %), was also observed after two months in other study [30]. However, that study was focused only on the stability of the catechins in green tea samples. Rapid degradation of the EGCG and ECG, by more than 97 %, was further observed in the study, where the degradation of amorphous EGCG, ECG, EGC and GCG powder was studied over a period of 40 days [16].

Hierarchical cluster analyses

Ward's method was used for cluster building in hierarchical cluster analysis and the distance between the clusters was computed using an Euclidean method. The dendrogram of the variables in Fig. 4a separates 10 variables into four clusters (A–D), whereas the dendrogram of the green tea samples in Fig. 4b classifies the samples into four main clusters, marked by the letters A–D, and two outliers, according to the speed of degradation of all of the monitored compounds, having confirmed also the FA results.

Samples 3 and 8, measured instantly after opening of the tea box, are outliers (S3W0, S8W0) because they contain a high amount of the monitored compounds in comparison to the rest of other samples. The remaining samples are arranged into four clusters mainly according to the time of storage with few outliers. The samples measured instantly after the opening of the tea box form clusters A and D, together with sample 5 (S5W3) measured after 3 weeks (cluster A). The content of the target compounds in sample 5, measured after 3 weeks of storage, is equivalent to that of samples 2, 4, 6 and 7 (S2W0, S4W0, S6W0, S7W0) measured immediately after the opening of the tea box, which was caused by a slow degradation process, probably because the tea bag in this sample was packed individually in plastic wrap. On the contrary, the samples 9 and 7 (S9W3, S7W3), measured after 3 weeks, are situated in cluster B together with the samples measured after 16 weeks because these samples underwent a rapid degradation within 3 week period. The smallest content of the monitored compounds occurred

after 16 weeks in all cases. However, the samples 3 and 6 (S3W16, S6W16) had the highest content of the monitored compounds after 16 weeks compared to the rest of the samples, probably again due to their plastic-type packaging. Therefore, these samples are included in cluster C where samples measured after 3 weeks are located. The plastic box plays an important role in the storage of green tea, probably because of the protection it offers against the relative humidity, which is one of the crucial parameters affecting the degradation of catechins [7,18]. The other monitored compounds could also be affected by the relative humidity and, therefore, slow degradation of all of the monitored compounds in the green tea was observed in the case of samples packed in a plastic box. Further, the better protection against of the environmental condition serves the plastic packet of individual tea bags (sample 5) in the comparison with the one plastic box, where all tea bags are placed (samples 3 and 6).

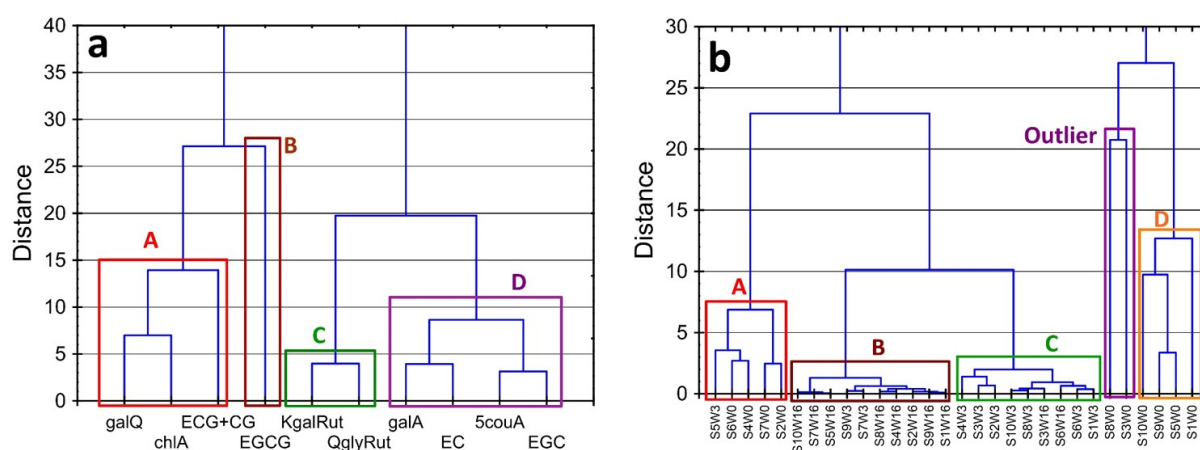


Fig. 4 Two dendrograms of the hierarchical clustering of variables and samples using Ward's method with an Euclidean distance of standardized data:

- (a) the dendrogram of 10 selected variables (compounds) found 3 clusters and one outlier
- (b) the dendrogram of green tea samples found 4 clusters and 2 outliers

Discriminant analysis

Discriminant analysis confirmed the results obtained using the two previous multivariate data analysis methods. The DA classified samples of green tea into 3 clusters based on the time of storage (see Fig. 5). The samples which were measured instantly after opening the tea box are grouped in cluster A, where a higher variability between the individual green tea samples is observed. Due to the fast degradation process, the samples of green tea become similar after 3 and 16 weeks, as seen in clusters B and C in Fig. 5.

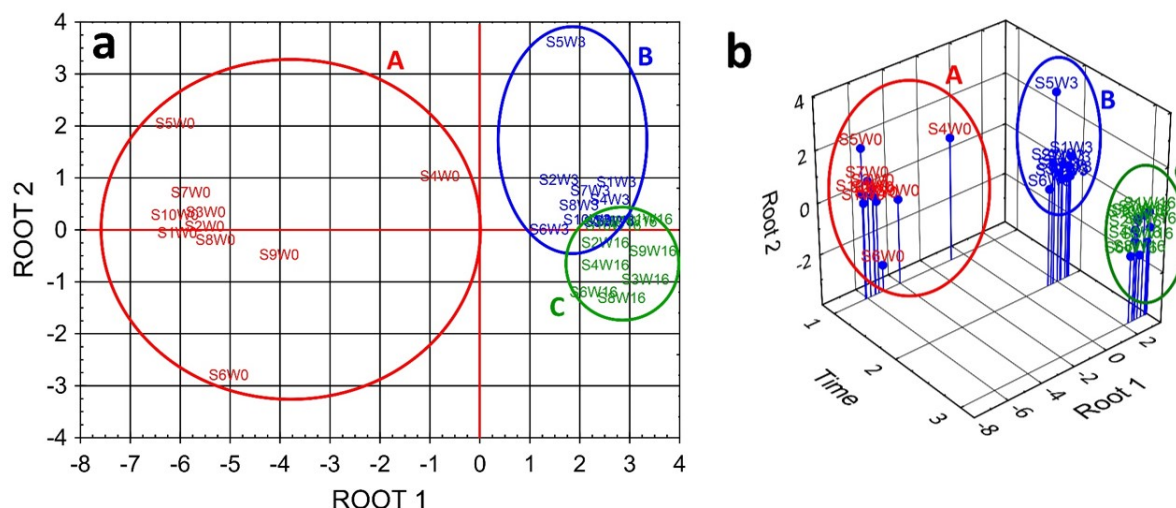


Fig. 5 Scatterplots of the linear discriminant scores give a visual impression of a quality of the Fisher linear discriminant functions classifying the data

The samples of green tea are divided into 3 clusters based on the storage time: (a) 2D plot projection, (b) 3D plot projection

Sample 5 (S5W3) deviates from cluster B because the degradation of the compounds for this sample was slow within the first 3 weeks. The degradation process can play significant role in sensory properties of green tea. In study by Wang et al. [31], it was found that the colour and taste of green tea has been changed during storage due to decreasing amount of catechins.

Conclusions

The storage resistance of 21 selected phenolic compounds in ten green tea samples that had varied in their packaging was indicated using four different methods of multivariate data analysis. All multivariable statistical methods used in this study divided the green tea samples into clusters according to their storage resistance over the period of 16 weeks. A significant variation between the samples measured immediately after opening of the tea box was observed using computer-aided methods. In particular, sample 3 (Saga) contained a high amount of compounds with antioxidant capacity and therefore is located far from the other samples. During storage, the packaging of the individual tea bags plays an important role. Green tea samples 3 (Saga), 5 (Dukat) and 6 (Dukat) are protected against the environmental conditions (e.g. humidity) by plastic packaging, therefore, their degradation occurred more slowly. On the other hand, the degradation of all of the other samples, which were packaged in paper boxes, is similar and more significant. The HPLC/MS/MS method with the multivariate statistical methods enables the fast evaluation of the long-time storage resistance

of valuable compounds present in green tea and yields useful information to customers about their degradation, affecting the sensory properties of the green tea, such as taste and colour. The right storage condition or right packaging of the green tea is important to keep the sensory properties of the product. As also found out, the plastic box has played an important role in the storage of green tea, probably because of the protection against the relative humidity, which is one of the crucial parameters affecting the degradation of catechins in tea.

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