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Fast gradient HPLC/MS separation of phenolics in green tea to monitor their degradation

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ABSTRACT

The degradation of catechins and other phenolics in green tea infusions were monitored using fast HPLC/MS separation. The final separation was performed within 2.5 min using Ascentis Express C18 column (50 mm × 2.1 mm i.d.) packed with 2 μm porous shell particles. Degradation was studied in relation to the temperature of water (70, 80, 90 °C) and the standing time of the infusion (up to 6 h). Along with chromatographic separation, the antioxidant properties of the infusions were monitored using two spectrophotometric methods. During staying of green tea infusion, the degradation of some catechins probably to gallic acid was observed. Finally, the influence of tea bag storage on antioxidant properties of green tea was evaluated. Rapid degradation of antioxidants after 3 weeks was observed. The principal component analysis, factor analysis and discriminant analysis were used for the statistical evaluation of obtained experimental data.

1. Introduction

Tea is consumed throughout the world and is, after water, the most popular beverage. It has been shown that a fresh tea leaf is unusually rich in the flavonoid group of polyphenols known as catechins (Graham, 1992). The main catechins in green tea are epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG) and epicatechin (EC). Tea catechins undergo many chemical changes during the course of manufacturing and brewing processes (Wang & Helliwell, 2000).

Heat treatments deactivate enzymes in the tea leaves. Tea leaves are also subject to subsequent rolling and drying processes. All steps are designed to achieve optimal oxidation of the tea catechins and produce tea products with good flavor and color (Wang & Ho, 2009). The composition of tea also varies according to the type of tea, season, age of leaves, climate, and horticultural practices (Jeszka-Skowron, Krawczyk, & Zgola-Grzeskowiak, 2015).

It is well known that the green tea has considerable health benefits for humans. The main catechin, EGCG, contributes to green tea's beneficial therapeutic effects and functions as a powerful antioxidant, anti-angiogenic, anti-inflammatory and an anti-tumor agent. The EGCG is found to be the most potent substance against bacteria, viruses, and fungi may be a potential agent for prophylaxis of mastitis (Chen et al., 2015). Green tea catechins are potent inhibitors of enzymes for carbohydrate digestion and influence the digestibility of starch. The fortification of green tea catechins in bread products may significantly contribute to glycaemic response reduction (Goh et al., 2015). There are numerous studies suggesting that the EGCG may be helpful in body weight reduction so green tea may be useful in preventing obesity and its complications, and thus act as a so-called functional food. Active substances in green tea, either catechins or EGCG-caffeine mixture, have a positive effect on weight loss and weight maintenance after a period of weight loss (Lisowska, Stawinska-Witoszynska, Bajerska, Krzyzanowska, & Walkowiak, 2015).

Individual catechins undergo epimerization at high temperatures. This epimerization takes place more easily in tap water than in purified water (Wang & Helliwell, 2000). The complexity of the ions in tap water and the different pH between tap and purified water are thought to be the main reasons for the different conversion rates of individual catechins. Stability studies of catechins in green tea strong infusion have shown that epimerization can be observed during the prolonged storage. Therefore, it is thought that, not only temperature (Spacil, Novakova, & Solich, 2010), but also the leaching time influences the epimerisation of catechins in green tea infusion (Wang & Helliwell, 2000). Tea epicatechin derivatives are susceptible not only to epimerisation but also to thermal degradation (Xu, Leung, Huang, & Chen, 2003), though it

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may be found in other studies that the epimerization reaction induced by heat treatment would not significantly alter the antioxidant activity, absorption and metabolism of tea polyphenols in canned and bottled tea drinks (Xu, Yeung, Chang, Huang, & Chen, 2004).

The analysis of catechins in biological matrices is typically performed using liquid chromatography coupled to a spectrophotometric detector or to a mass spectrometer (MS). The major drawback of most published HPLC methods is a long chromatographic run of about 20–45 min (Fernandez, Pablos, Martin, & Gonzalez, 2002; Jin, Ma, Ma, Yao, & Chen, 2014; Masukawa et al., 2006; Peng, Song, Shi, Li, & Ye, 2008; Svoboda, Vlckova, & Novakova, 2015; Yang, Ye, Xu, & Jiang, 2007; Yao et al., 2004; Zuo, Chen, & Deng, 2002). An alternative to improve HPLC separation efficiency and speed without reducing particle size is the use of superficially porous particles, also called core–shells (Fontana, Antoniolli, & Bottini, 2016). These are typically composed of a 1.9 μm solid core enclosed by a 0.35–0.5 μm porous shell (dp = 2.6–2.7 μm), providing reduced band broadening and outstanding efficiency, while preserving sufficient particle size to allow for acceptable operation pressure (Fontana et al., 2016; Jandera, Hájek, & Staňková, 2015). For these reasons, the new technology columns have been already successfully applied to the analysis of various compounds in several foods (Chocholous, Vackova, Sramkova, Satinsky, & Solich, 2013; Kaufmann & Widmer, 2013; Preti, Antonelli, Bernacchia, & Vinci, 2015).

This work focuses on the study of degradation of catechin and other phenolic compounds in green tea using fast gradient HPLC/MS analysis. The degradation was studied during 6 h of tea infusion after leaching. Further, the influence of the storage of the tea bag on the content of phenolic compounds was monitored. The results were compared with antioxidant capacity and total phenolic content measured spectrophotometrically. Multivariate data analysis was applied to classify substances in green tea.

2. Materials and method

2.1. Chemicals

Green tea samples were purchased from local supermarkets or drugstores in the town of Pardubice (Czech Republic). The standards of studied phenolic compounds (catechin, epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate, gallocatechin, gallocatechin-3-gallate, catechin-3-gallate, gallic acid, chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, rutin, isoquercitrin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside and hyperoside) were purchased from Sigma Aldrich, USA. Deionized water was prepared by Milli-Q purification system (Merck Millipore, Germany). Methanol and formic acid were purchased from Sigma Aldrich, USA. ABTS and 2 M Folin-Ciocalteu reagent (both Sigma Aldrich, USA), $K_2S_2O_8$ (Laborchemie Apolda, Germany) and Na_2CO_3 (Lach-ner, Neratovice) were used to measure antioxidant capacity.

2.2. Instruments and conditions

2.2.1. HPLC/MS analysis

The HPLC system equipped with a LC-20AD binary gradient pump, a DGU-20 degassing unit A, a SIL-20A autosampler (all Shimadzu, Kyoto, Japan) and a LCO 102 Single thermostat column (Ecom, Prague) was coupled to a QTRAP 4500 mass spectrometer (AB SCIEX, USA). The reversed-phase separation of phenolic compounds was performed on 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% formic acid in water (A) and 0.1% formic acid in methanol (B) using a gradient program from 20% to 70% B for 2.5 min. The flow rate was 0.5 ml/min and injection volume 0.5 μl. The conditions of mass spectrometry analysis in negative-ion mode were as follows: Curtain gas: 20 psi, Temperature: 500 °C, IonSource gas1: 50 psi, IonSource gas 2: 50 psi.

2.2.2. Spectrophotometric techniques

The antioxidant activity and total phenolic content were measured using the UV/VIS spectrophotometer Helios Epsilon (Thermo Scientific, Denmark). The experiments determining antioxidant capacity carried out in this work were repeated five times for each sample ($n = 5$).

Total polyphenol contents (TPC): The working solution was prepared from a 2 M Folin-Ciocalteu reagent in ratio 1:9 with water (Singleton, 1985). 1 ml of working solution was mixed with 1 ml of deionized water and 100 μL of five-times diluted tea extracts (or water in case of blank sample). After 5 min, 1 ml of 7.5% (w/v) aqueous solution of sodium carbonate (Na_2CO_3) were added and mixed well. The absorbance at 750 nm was measured against water blank after 30 min. The results were expressed as milligrams of gallic acid equivalents per gram of tea. The calibration solutions were prepared by diluting of gallic acid in water. Amount of gallic acid added to the reaction agent was in the range of 0.002–0.029 mg and the parameters of calibration equation were $y = 32.13\ (0.16) \times +0.0438\ (0.003)$, $R^2 = 0.9989$.

ABTS method: The preparation of ABTS radical cation was adopted from the literature (Re et al., 1999) with a slight modification. 5 ml of ABTS (c = 3.6 mmol/L) was mixed with 100 μl of $K_2S_2O_8$ (c = 0.064 mol/L), the solution was left for 12–16 h in the dark and then it was diluted approximately 40 times to achieve an absorbance of about 0.8. 3 mL of this working solution was mixed with 30 μL of five-times diluted tea extracts and the decrease of absorbance after 10 min of reaction (in dark) was monitored at a wavelength of 734 nm. Antioxidant activity was expressed as equivalent amount of standard Trolox using calibration curve. The calibration solutions were prepared by diluting of Trolox in methanol. Amount of standard Trolox added to the reaction agent was in the range of 0.003–0.069 μmol and the parameters of calibration equation were $y = 1373.2\ (3.2) \times +0.027\ (0.12)$, $R^2 = 0.9998$. The intercept was not significant in this case.

The calibration data for the ABTS and TPC methods were measured at nine concentration levels, each level was repeated five times ($n = 5$). The calibration data were fitted using the least square linear regression in the QCEXPERT 2.9 statistical program (TriloByte). Jackknife residuals and Pregibon, Williams and L-R graph were used to identify influential points. The significance of intercept of regression straight-lines was tested using Student's *t*-test.

2.3. Sample preparation

For our study, ten green tea samples (1 - Apotheke Bio, 2- Jemča, 3 - Saga, 4 - Pickwick, 5 - Dukat, angular bag, 6 - Dukat, circular bag, 7 - Tesco, 8 - Ahmed, 9 - Teekanné, 10 - Loyd) were bought in local markets (Table S1). The extracts were prepared from one tea bag (usually about 2 g) using 200 ml of hot water at temperatures of 70 °C, 80 °C and 90 °C. The amount of green tea in tea bag was weighted and the results were recalculated to 1 g of sample. The leaching time was four minutes. The extracts were cooled to the laboratory temperature, filtrated through a 0.45 μm filter, diluted and analyzed at one time using spectrophotometric methods and HPLC/MS. Dilution of samples was 5 times for spectrophotometric techniques and 20 times for HPLC/MS measurements.

The calibration solutions of analyzed phenolic compounds were prepared by the sequential dilution of storage methanol solution (c = 1 mg/L for derivatives of chlorogenic acid and 10 mg/L for other compounds) by the mobile phase at the initial gradient com-

position (20% methanol). The concentration range of the calibration solution was 2.5–170 μg/L for derivatives of chlorogenic acid and 25–1700 μg/L for other compounds.

2.4. Statistical multivariate data treatment

A principal component analysis, factor analysis and discriminant analysis were performed to investigate the influence of different factors on degradation of phenolic compounds in green tea. The statistical software STATISTICA 12 (StatSoft, Inc.) was used for that purpose.

2.4.1. Principal component analysis

The objective of principal component analysis (PCA) is the representation of the objects in the new principal component (PC) coordinate space which performs a transformation into a more relevant co-ordinate system which lies directly in the centre of the data swarm of objects, and a dimensionality reduction using only the few principal components which reflect the structure in the data. The specific goals of PCA are to summarize patterns of correlations among observed variables, to reduce a large number of observed variables to a smaller number of latent variables, to provide an operational definition for an underlying process by using observed variables. Steps in PCA include selecting and measuring a set of variables, preparing the correlation matrix, extracting a set of principal components or components from the correlation matrix, determining the number of principal components, probably rotation of factors to increase interpretability, and, finally, interpreting the results.

Map of variables provides a projection view of the inter-variable relationships and shows how much each variable contributes to each PC. Correlations or components loadings are read directly from these graphs by projecting perpendicular lines from each variable point to each of the component axes. One of the primary goals of PCA and the motivation behind extraction, is to discover the minimum number of component axes needed to reliably position variables. A second major goal and motivation is to discover the meaning of the components that underline response to observed variables. Components loadings are the correlation of each variable and the component. Variables with a high degree of systematic variation typically have large absolute variances, and consequently large loadings. Variables of little importance lie near origin. Variables close to each other, situated out towards the periphery of the loading plots, covary strongly, proportionally to the degree distanced from the PC-origin. If the variables lie on the same side of the origin, they have a positive correlation. If they lie on opposite sides of the origin, they are negatively correlated. Loadings which are at 90 degrees to each other through the origin are independent. Loadings close to a PC axis are significant only to that PC.

Plotting the components scores gives information how the objects are related to one another. The objects located far away from the origin can be extremes, or even outliers. Objects located in clear groups are similar to each other. The layout of the overall object structure in score plots must be interpreted. The length of the arrows reflects the variances of the corresponding variable, and the angles between them indicate the size of their correlations, small angles corresponding to high correlations. The relative positions of objects indicate similarities and differences.

2.4.2. Factor analysis

Factor analysis (FA), like PCA, attempts to explain a set of multivariate data using a smaller number of dimensions, factors. While PCA is merely a transformation of the data and no assumptions are made about the form of the covariance matrix from which the data comes, FA supposes that the data comes from the well-defined model, where the underlying factors satisfy the assumptions. If these assumptions are not met, then FA may give spurious results. In FA the emphasis is on a transformation from the underlying factors to the observed variables whereas in PCA the emphasis is on a transformation from the observed variables to the principal components. FA tries to explain the covariances or correlations of the observed variables by means of a few common factors. PCA is primarily concerned with explaining the variance of the observed variables. Substantial changes in all factors can be observed in FA if the number of factors is changed.

2.4.3. Discriminant analysis

Discriminant analysis techniques are used to classify individuals 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 one of them. These techniques can also be used to identify which variables contribute to making the classification. The investigator has one set of multivariate observations, the training sample, for which group membership is known with certainty apriori, and a second set, the test sample, consisting of observations for which group is unknown and which have to be assigned to one of the known groups as accurately as possible. The basic purpose of discriminant analysis is to estimate the relationship between a single nonmetric (categorical) dependent variable and a set of metric independent variables. Discriminant analysis is capable of handling either two or more groups. Groups are spaced along the various discriminant functions according to their centroids. Discriminant functions form axes and the centroids of the groups are plotted along the axes. If there is a big difference between the centroid of one group and the centroid of another along a discriminant function axis, the discriminant function separates the two groups. Many groups can be plotted along a single axis.

3. Results and discussion

3.1. HPLC/MS separation

Four columns packed with the porous shell octadecyl silicagel stationary phase differing in length, diameter and particle size (Kinetex C18 – 5 cm × 2.1 mm, 1.3 μm, Ascentis Express C18 – 15 cm × 3.0 mm, 2.7 μm, Ascentis Express C18 – 10 cm × 2.1 mm, 2.7 μm, Ascentis Express C18 – 5 cm × 2.1 mm, 2 μm) were used to achieve the separation of all phenolics in very short time. Further, gradient profile, organic solvent (methanol or acetonitrile), amount of formic acid (0.1% or 0.3%) and temperature (30 and 40 °C) were tested to obtain the separation of all catechins in a very short time with an emphasis on the resolution of individual isomers, which have the same molar mass and fragmentation behavior. A short separation is crucial for monitoring the degradation of catechins during staying of the infusion. Flow rate was set in the range 0.4–0.8 mL/min depending on the column. The Kinetex column was not possible to use with our HPLC system. Due to very high pressure, small flow rate had to be used and high peak broadening were observed. Further, two Ascentis Express columns with particle size 2.6 μm differed in length and diameter were chosen. On these two columns the separation of all isomers of catechins in reasonable resolution were performed in time higher than 8 min. This time was not satisfactory for this study, therefore, the column Ascentis Express C18 – 5 cm × 2.1 mm, packed with 2 μm porous shell particles was chosen for separation. At first experiments, the time of elution was about 5 min. Many gradient profiles with acetonitril or methanol as elution solvents were tested to obtain very fast separation of all studied compounds. In our case, the best resolution of catechin isomers was observed with

linear gradient 20–70% of methanol in water in 2.5 min. The influence of formic acid amount was negligible, therefore, only 0.1% of HCOOH was selected to safe the column. The flow rate was set with respect of pressure limit of HPLC system. Shorter separation with same resolution and lower pressure was achieved with temperature 40 °C. Overall conditions are written in the Experimental part. The separation of 24 green tea phenolic compounds (Fig. 1A) was performed using those conditions in 2.5 min, with catechins separated during 2 min. This separation is shorter than in the case of UHPLC separation recently published (Spacil et al., 2010), however, the classic HPLC system can be used. After chromatographic separation the mass spectrometric conditions were optimized. The quantification of studied compounds was performed using multiple reaction monitoring (MRM) mode. The parameters important for MRM transitions, declustering potential, collision energy and collision cell exit potential, were optimized for all compounds using direct infusion of individual standards (Table 1). When standards were not available, the MRM transition parameters were obtained by direct infusion of real samples.

3.2. Monitoring of catechin degradation in green tea

The quantity of individual phenolic compounds was calculated based on the calibration equations for the available standards. The calibration data were measured at eight concentration levels, each level four times ($n = 4$). The linearity of calibration curves was checked by inspecting plots of residuals while the significance of intercept of regression straight-lines was tested using Student's t-test. The coefficient of determination (Table 1) was for all compounds higher than 0.99, demonstrating high linearity. The limits of detection (LOD) calculated as the concentration yielding signal-to-noise ratio S/N = 3 were for most of the compounds below 20 μg/L (Table 1). The accuracy and precision was checked by measuring calibration solutions at two concentration levels for ten times. For that purpose the concentration corresponding

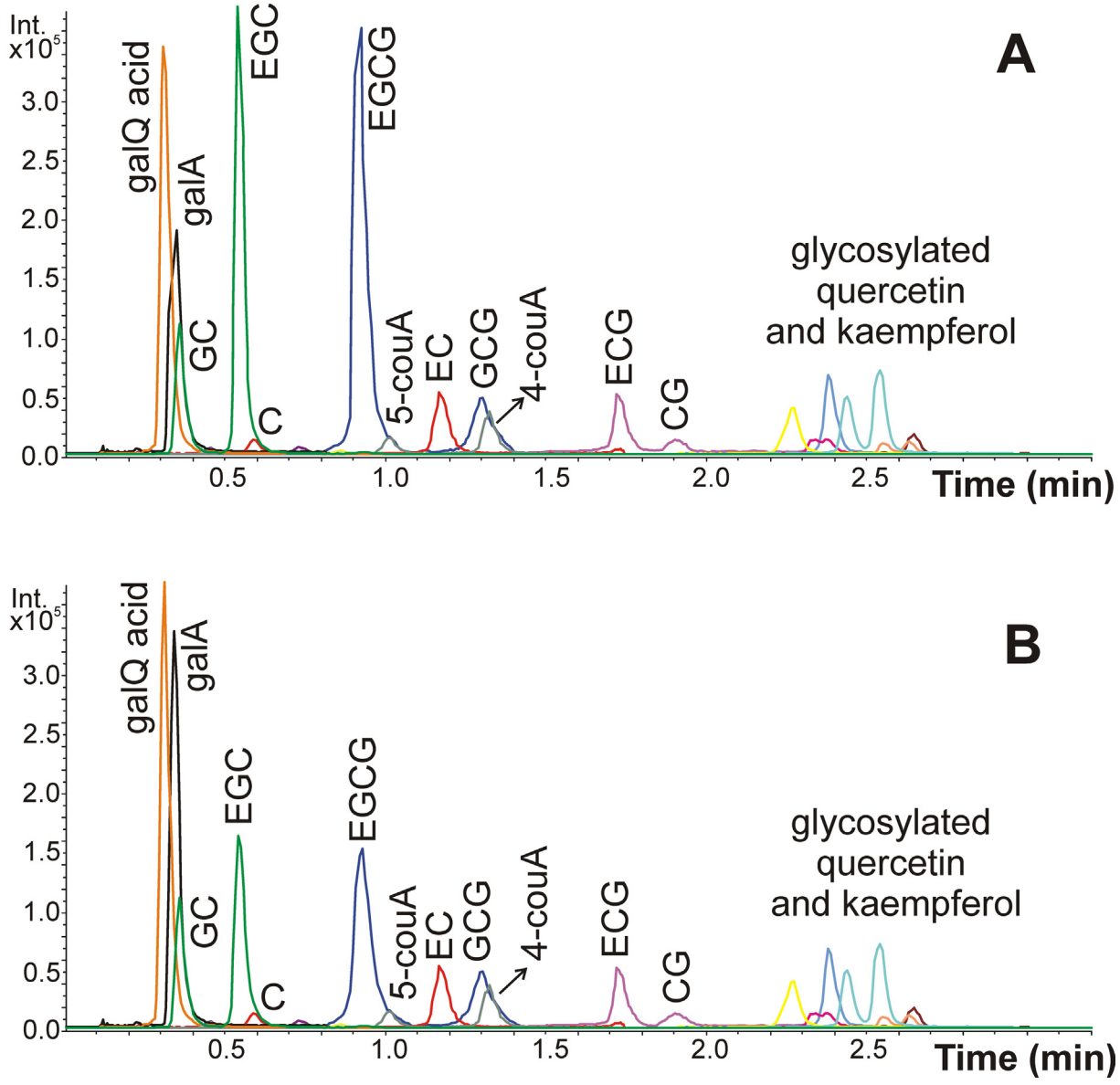

Fig. 1. Optimized chromatographic separation of studied phenolic compounds. Separation of phenolics in sample 5 (Dukát) immediately after leaching (A). The degradation of the EGC and EGCG monitored after 6 h (B). Ascentis Express C18 (5 cm × 2,1 mm × 2 μm), 20 %–70% of methanol in water in 2.5 min (+0,1% of formic acid in both solvents), T = 40 °C, F_m = 0,5 ml/min, injection 0,5 μl.

Table 1
Parameters of MRM transitions, declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP), optimized for individual compounds. Limit of detection (LOD) and coefficient of determination (R^2).

Compounds	t_R (min)	MRM transition	DP (V)	CE (V)	CXP (V)	LOD [ng/ml]	R^2
Galloylquinic acid (galQ acid)	0.31	343/191	−75	−28	−7	–[a]	–[a]
Gallic acid (galA)	0.34	169/125	−45	−22	−10	0.05	0.997
Gallocatechin (GC)	0.36	305/125	−95	−30	−9	0.06	0.999
Neochlorogenic acid (neoA)	0.45	353/179	−75	−28	−5	2.00	0.997
Epigallocatechin (EGC)	0.55	305/125	−95	−30	−9	0.08	0.998
Catechin (C)	0.59	289/245	−105	−22	−9	0.01	0.997
Chlorogenic acid (chlA)	0.73	353/191	−75	−26	−7	0.13	0.996
Cryptochlorogenic acid (cryA)	0.85	353/173	−90	−24	−7	0.60	0.996
Epigallocatechin gallate (EGCG)	0.92	457/169	−95	−24	−5	0.44	0.998
5-coumaroylguinic acid (5-couA)	1.00	337/173	−55	−20	−15	–[b]	–[b]
Epicatechin (EC)	1.17	289/245	−5	−22	−9	0.08	0.997
Gallocatechin gallate (GCG)	1.29	457/169	−95	−24	−5	0.47	0.996
4-coumaroylquinic acid (4-couA)	1.32	337/173	−55	−20	−15	–[b]	–[b]
Epicatechin gallate (ECG)	1.73	441/169	−100	−28	−5	0.10	0.998
Catechin gallate (CG)	1.91	441/169	−100	−28	−5	0.50	0.996
Quercetin-3-*O*-glycosylrutinoside (QglyRut)	2.27	771/300	−195	−62	−7	–[c]	–[c]
Isoquercitrin (IsoQ)	2.34	463/300	−135	−38	−9	0.07	0.998
Hyperoside (Hyp)	2.37	463/300	−135	−38	−9	–[c]	–[c]
Rutin (rut)	2.38	609/300	−165	−50	−11	0.14	0.998
Kaempferol-3-*O*-galactosylrutinoside (KgalRut)	2.44	755/285	−185	−52	−9	–[d]	–[d]
Kaempferol-3-*O*-glucosylrutinoside (KgluRut)	2.54	755/285	−185	−52	−9	–[d]	–[d]
Kaempferol-3-*O*-galactoside (Kgal)	2.56	447/285	−120	−38	−9	–[d]	–[d]
Kaempferol-3-*O*-glucoside (Kglu)	2.63	447/285	−120	−38	−9	0.17	0.997
Kaempferol-3-*O*-rutinoside (Krut)	2.64	593/285	−160	−44	−9	0.11	0.999

[a] Gallic acid was used for quantification;
[n] Chlorogenic acid was used for quantification;
[c] Isoquercitrin was used for quantification;
[d] Kaempferol-3-*O*-glucoside was used for quantification.

80% of LOQ and concentration at half of calibration curve was used. The relative standard deviations (RSD) did not exceed 5%. The repeatability was checked by measuring 10 infusions prepared from selected sample. The RSD was below 3%.

Ten green tea samples (Table S1) were used for quantitative analysis and to monitor the degradation of catechins and other phenolic compounds in green tea. The degradation was monitored every hour for six hours of infusion prepared by pouring 70 °C, 80 °C and 90 °C hot water on a tea bag. The representation of phenolic compounds was similar in all samples. They differed in amount of individual phenolics, especially in catechin contents. The significant decrease of EGC and EGCG content including their epiforms and conversely significant increase of gallic acid (galA, Fig. 1B) was observed in infusions measured after 6 h. The degradation was monitored every hour during 6 h. This degradation was observed even after one hour of infusion staying. Further, the influence of the time of tea bag storage on the content of catechins was measured every 3 weeks over the course of 14 weeks. The tea bags were stored in the box in which they were bought. In almost all cases, the tea bags were packed in paper boxes; only Saga tea (3) was packed in a plastic box. The content of catechins rapidly decreased during the storage of tea bags even after 3 weeks of storage (Fig. 2). This data are in agreement with previously published data (Friedman, Levin, Lee, & Kozukue, 2009), where the stability was tested during 6 months storage. The highest decrease was observed in content EGCG (28%) and ECG (51%) in two months. In our study only 5% of catechins were presented in green tea after 14 weeks of storage. The highest decrease was observed in case of EGCG and EGC, followed by EC and ECG.

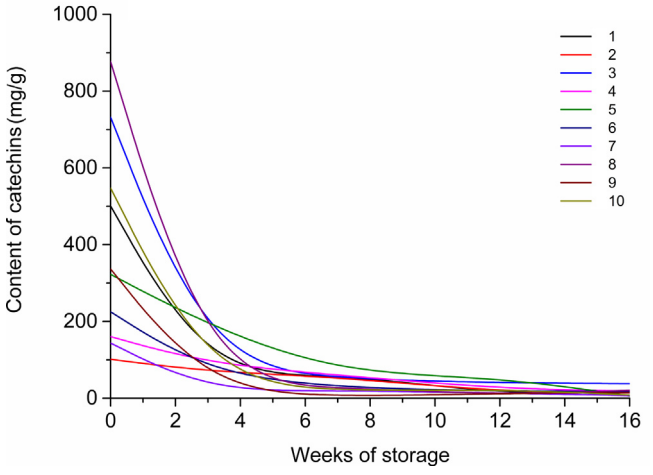

Fig. 2. The influence of storage on the content of catechins in green tea samples 1–10 measured using HPLC/MS technique.

3.3. Correlation of spectrophotometric methods

The samples measured by HPLC/MS were at the same time measured using spectrophotometric techniques (ABTS and TPC, Fig. S1), which are described in the experimental part. In addition, pure standards of phenolic compounds were measured to obtain information on the antioxidant activity of all individual compounds.

First, the results obtained using both spectrophotometric techniques were compared and the correlation between the antioxidant capacity and the total phenolic content was constructed for infusions measured immediately after leaching, which were prepared by water differing in temperature (Fig. S2A). A high correlation coefficient ($r = 0.9671$, $p < 0.001$) was observed between these spectrophotometric methods and the statistical significance was proven.

Next, the antioxidant capacity of green teas was calculated from the HPLC/MS data and the antioxidant activity of individual standards. These calculated antioxidant capacities were correlated with the total phenolic content measured spectrophotometrically

(Fig. S2B) with a significant correlation coefficient of value $r = 0.8549$ ($p < 0.001$). This correlation shows good agreement between the chromatographic and spectrophotometric results. Therefore, spectrophotometric methods could be used for fast screening the antioxidant capacity of individual green tea infusions. However, only total antioxidant properties of the sample can be measured using spectrophotometric techniques. Information on the composition of individual samples is missing. Consequently, the antioxidant capacity and total phenolic content were the same for infusions measured over 6 h. This is probably caused by the antioxidant properties of the degradation products of catechins (i.e. gallic acid).

3.4. Multivariate data analysis

The multivariate data treatment was used to investigate the influence of water temperature and time of staying of infusion on degradation of phenolic compounds in green tea.

The multivariate data matrix consisted of a series of 14 selected *variables*, i.e. phenolic compounds EGC, GC,. . ., galoylQ, in columns being monitored on a number of 208 *objects* in rows (denoted 1A0, 1A1, . . ., 10C6) where the object codes describe an actual type of tea (1, 2, . . ., 10), temperature of infusion (A means 90 °C, B means 80 °C, C means 70 °C), and time of staying of infusion in hours (0, . . ., 6).

Exploratory analysis of the multivariate data matrix (14 × 208) made a visualization and visual data analysis which helped to deal with the flood of information. The correlation matrix and the correlation diagram (Fig. S3A) prove that there exists quite a strong correlation among the variables of multivariate data matrix. The Box-and-whisker plot (Fig. S3B) examined sufficient variability of data.

ANOVA was applied to investigate the effect of the various factors such as type of green tea (1, . . ., 10), temperature (A, B, C) and time (0, . . ., 6) on the variability of data and to determine which part of the variation in a population is due to systematic reasons, called factors, and which is due to random effects.

3.4.1. Plot of the component loadings – Map of variables

The principal component extraction yields a solution in which observed variables are vectors that run from the origin to the point indicated by the coordinate system (Fig. S4). The principal components (PC) serve as axes for the system. In this study, there are three PC (Fig. S4A), therefore the space has three axes or three dimensions and describes 82.95% of the total data variance. The loading plot provides a projection view of the inter-variable relationships and shows how much each variable contributes to each PC. Variables with a high degree of systematic variation typically have large absolute variances, and consequently large loadings. Since this analysis is based on correlations, the largest variable-PC correlation that can occur is equal to 1.0. The closer a variable on this plot is located to the unit circle, the better its representation by the current coordinate system.

When assessing importance, it is imperative also to consider the proportions of the total explained variance along each PC. If on Fig. S4B the PC1 (GaloylQ, ChlA, Krut) explains 55.46% and PC2 (QglyRut a 4couQ) only 18.05%, then the variables with large loadings in PC1 are much more important than those with large loadings in PC2 – in fact 3 times as important.

Variables close to each other, situated towards the periphery of the loading plots, strongly covary, proportionally to the degree distanced from the PC-origin. Fig. S4B shows that, for example, the variables GCG and ChlA vs. QglyRut do not correlate at all, but a strong correlation is exhibited by variables ECG + CG with Krut and Rut, followed by the pair ChlA with GCG, or variables EGCG with GaloylQ and GC. On Fig. S4C variable EC and C correlate because they are epimers. Further, correlation is observed for variables EGCG, EGC, 4couQ and GCG (Fig. S4C). Variable 4couQ has the smallest significance. On Fig. S4D variables GC, GaloylQ and ChlA are close to the origin, therefore they have the smallest significance. If the variables lie on the same side of the origin, they covary in a positive sense, i.e. they have a positive correlation. If they lie on opposite sides of the origin, they are negatively correlated. Loadings which are at 90 degrees to each other through the origin, are independent, as e.g. Krut or ECG + CG, Rut, C *vs.* 4couQ, GC or EGCG, GaloylQ *vs.* QglyRut, etc.

3.4.2. Scatterplot of principal components scores – Map of objects

The scatterplot of principal components scores (PC-scores) is simply any two pair of PC-scores vectors plotted against each other (Fig. S5). The score vectors are only the "footprints" of the objects projected down onto the Principal Components. The most commonly used plot in multivariate data analysis is the PC-score vector for PCl versus the score for PC2. They are two dimensions, along which the data swarm exhibits the largest and the second largest variances (in this case 55.46% and 18.05%). In total their explain 73.51% of the total data variance, what should be satisfactory.

Objects close to the co-ordinate origin are the most typical, the most "average". The ones far away from the origin may be extremes, or even outliers, but they may also be legitimate end-members. Objects close to each other are similar. The infusions prepared by water 90 °C and 80 °C hot always forms a cluster which is far from infusions prepared by water 70 °C hot. At a lower temperature the extraction efficiency is lower, therefore the samples prepared at 70 °C have the lowest antioxidant capacity. Further, a greater influence of the type of tea and temperature has been found than the influence of degradation. This is understandable as the degradation is connected with Factor 3 and represents the smallest percentage of the data description.

The cluster of green teas located above left contains sample 3 (Saga) prepared at 90 °C and 80 °C. All data contained in this cluster were observed during 6 h in the same infusion of tea. Close to this cluster is another cluster of objects including the same sample (3, Saga) prepared at 70 °C. Data observed during 6 h in the same infusion of green tea are also located in this group. The next big cluster includes small clusters of samples of green tea. The cluster contains sample 8 (Ahmed) prepared at 90 °C and 80 °C, sample 6 (Dukat) prepared at 70 °C, sample 7 (Tesco) prepared at 90 °C and also sample 2 (Jemča) prepared at 90 °C. Other clusters of samples are similar, therefore, these clusters are close to each other.

The lowest content of 4couQ and QglyRut in comparison with other samples was detected in sample 3 prepared at 80 °C. The highest content of 4couQ and QglyRut were found in sample 8 prepared at 80 °C (Fig. S5C).

3.4.3. Factor analysis

Factor analysis defines the underlying structure in a data matrix. It addresses the problem of analyzing the structure of the inter-relationships, i.e., correlations, among a large number of variables by defining a set of common underlying latent variables known as factors. Factor loadings can be interpreted as the correlations between the factors and the variables. The first factor shows most of the highest loadings.

Fig. S6 brings factor loadings after varimax rotation. From this figure it is evident, that QglyRut is useful for the Factor 2 description. It was found that an amount of this compound is not changed within an interval of 6 h but it varies with the temperature of the poured water. Catechins ECG + CG, GCG, EGCG with high antioxidant capacity suit the Factor 1 description. Therefore, it was concluded that Factor 1 is represented with the antioxidant capacity while the temperature is connected with Factor 2. When looking at a 3D chart the noticeable decrease ECG and EGCG is shown

and, on the contrary, an increase of the gallic acid can be observed and the Factor 3 was named as the degradation activity.

Fig. 3B brings a cluster of sample 3 at 90 °C and 80 °C. Then the clusters of sample 3 at 70 °C, sample 7 at 80 °C and 90 °C, then sample 7 at 90 °C are seen. The clusters are located at the lower part of this figure. These types of green tea contain the smallest quantities of QglyRut and 4couQ which have been not changed within 6 h and their concentration was almost constant. The amount of these compounds is dependent on the water temperature. Cluster in the upper part of this figure corresponds to the sample 8 at 90 °C and 80 °C. This tea sample exhibits the highest concentration of compounds QglyRut and 4couQ.

Clusters of the right and bottom exhibit the highest antioxidant capacity, i.e. a cluster of sample 3 at 90 °C and 80 °C. Samples positioned right have increased antioxidant efficiency, and the samples placed close to the highest amount of substances QglyRut and 4-couQ are affected by the temperature of the water. When looking at a 3D chart, we can see the influence of Factor 3, which is the degree of degradation. Sample 7 prepared at 90 °C exhibits a high degree of degradation. The figure shows that over the course of 6 h the catechins to gallic acid were degraded.

3.4.4. Discriminant analysis

Discriminant loadings, sometimes referred to as structure correlations, measure the simple linear correlation between each independent variable and Fisher's linear discriminant function. Discriminant analysis separated all the samples of green tea into three clusters according to the temperature (Fig. 4). First, it was necessary to identify the content of the Root 1 and Root 2. The Root 1 corresponds to the degree of extraction and Root 2 corresponds to the time. Constructing the 3D graph in which the abscissa means

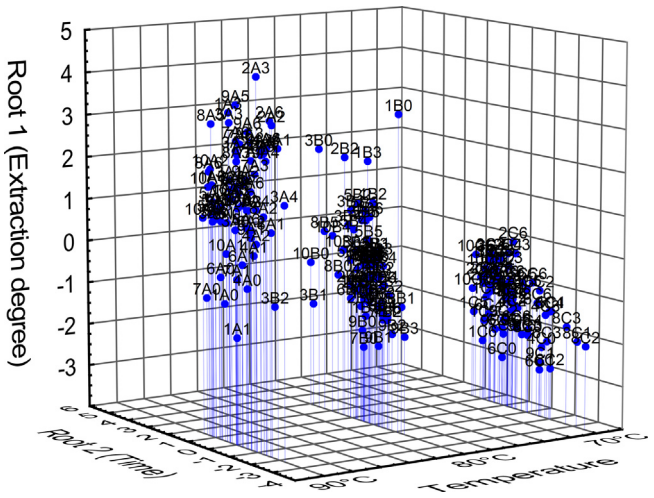

Fig. 4. The scatterplots of the linear discriminant scores: 3D presentation.

the temperature, the y axis is time and the *z* axis is the extraction degree, the sample teas were classified according to the temperature of 90 °C, 80 °C and 70 °C. From Fig. 4 it is clear that with decreasing temperature the extraction efficiency of the selected compounds decreases. At 70 °C the extraction efficiency seems to be lowest. The concentration of selected compounds is highest for teas prepared at 90 °C. However, these infusions exhibited the most noticeable degradation of catechins within 6 h. For green tea samples prepared at 70 °C this degradation was not so marked. It may therefore be concluded that when the green tea is prepared

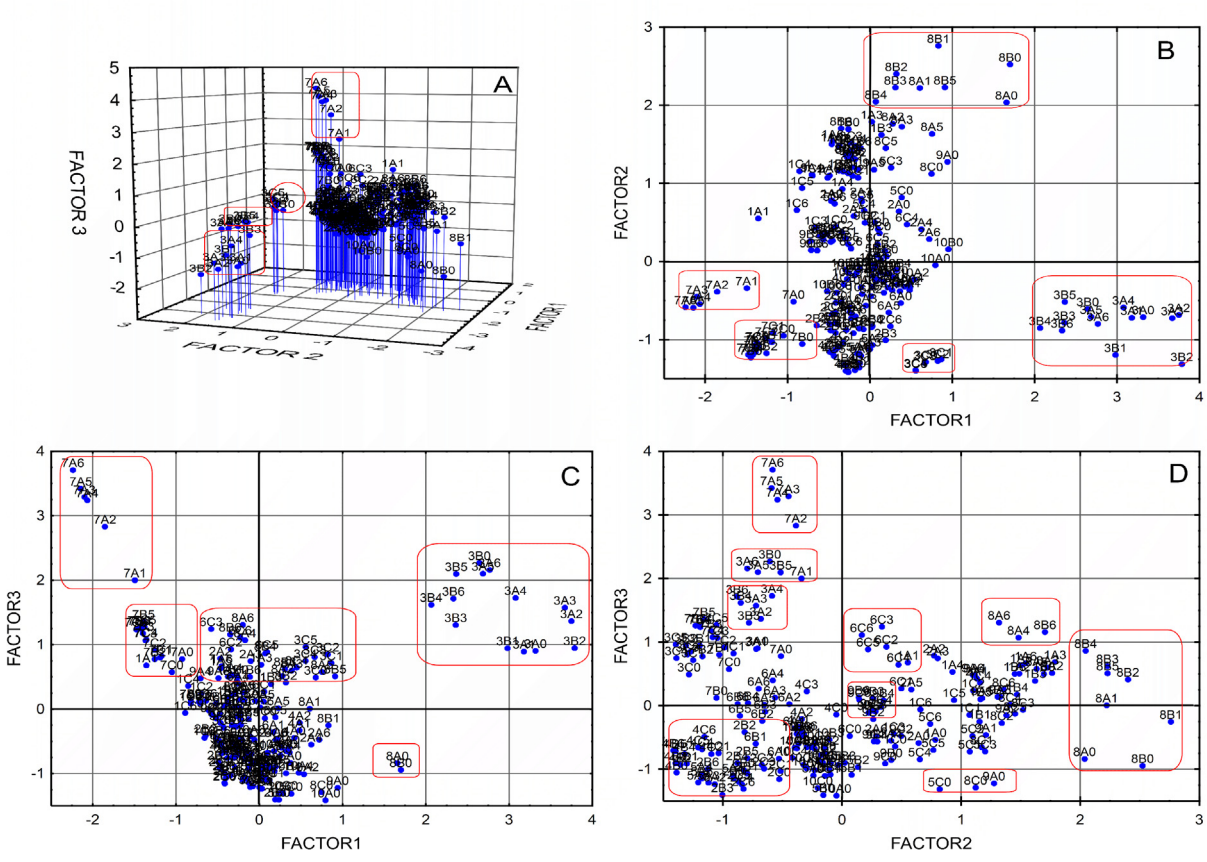

Fig. 3. The set of four scatterplots of factor scores after normalized varimax rotation demonstrates a similarity of objects studied which form clusters of similar properties: (A) 3D presentation, (B) Factor 1 – Factor 2 graph, (C) Factor 1 – Factor 3 graph, (D) Factor 2 – Factor 3 graph, (STATISTICA, StatSoft).

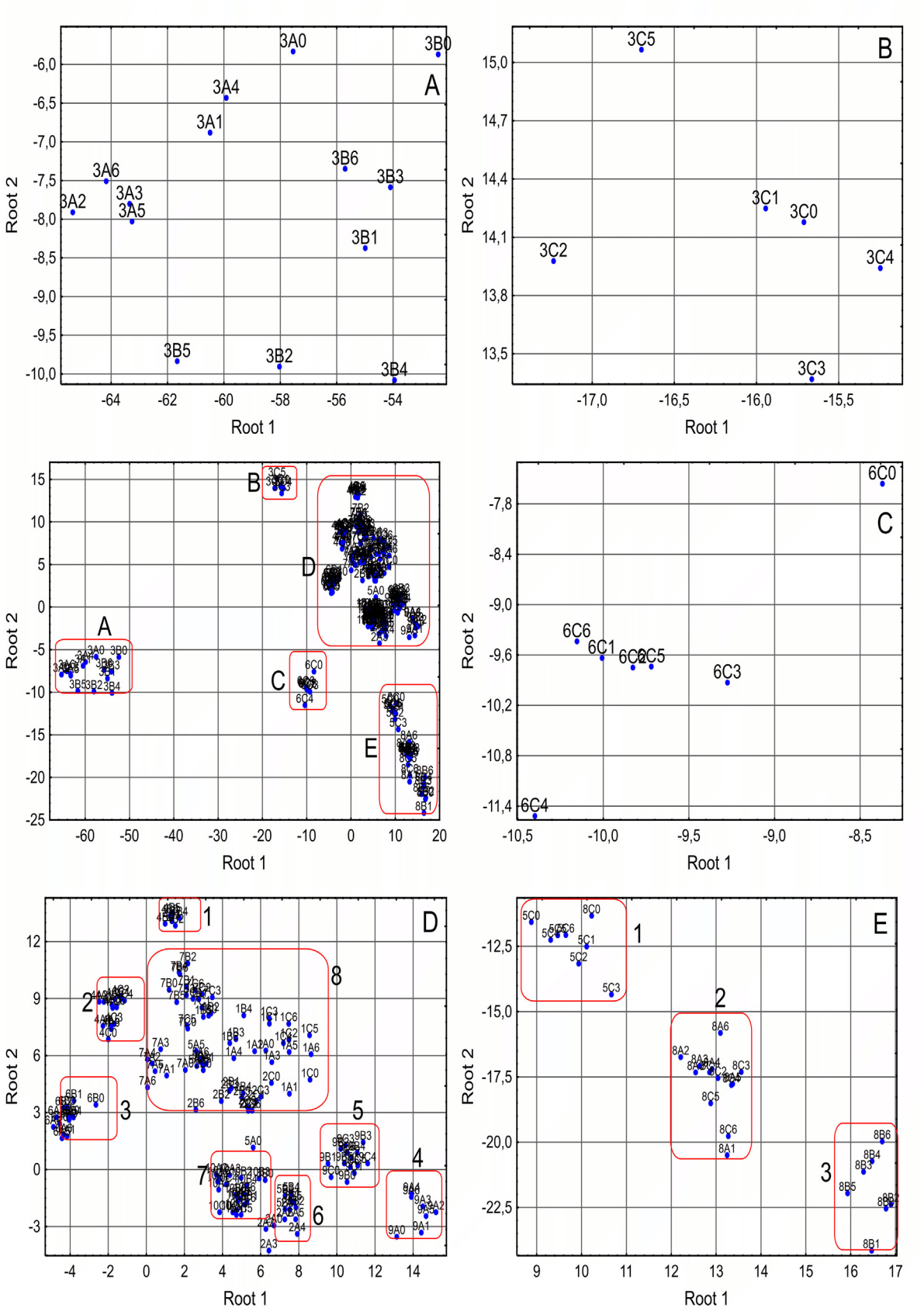

Fig. 5. The set of six scatterplots of the linear discriminant scores gives a visual impression of how well the Fisher linear discriminant functions classify the data: Root 1 – Root 2 graph, (A) Zoom of cluster A, (B) Zoom of cluster B, (C) Zoom of cluster C, (D) Zoom of cluster D, (STATISTICA, StatSoft).

at a lower temperature, the efficiency of the extraction is not as high but it is possible to drink this tea for a longer time during the day. In such a green tea prepared at lower temperatures the healthy compounds during 6 h are not degraded. Root 1 can be connected with the extraction efficiency and antioxidant effectiveness.

Discriminant analysis divided tea samples into clusters according to the type of tea and temperature (Fig. 5). This figure clearly shows five clusters in separate zoom figures denoted A, B, C, D and E. These clusters are displayed here at higher zoom resolution. The cluster A and B conceals sample 3, which was prepared at 90 °C, 80 °C (A) and 70 °C (B). This green tea was packed in a plastic bag and these bags have a round shape. This tea is not of a single variety but contains a mixture of several kinds of unspecified green tea. This sample exhibited one of the highest antioxidant capacities and the highest concentration of EGCG and GCG. Cluster C conceals sample 6, which was prepared at 70 °C. This tea is a mixture of Chinese and Indonesia green tea. Cluster D contains several clusters that are very close to each other and are similar. They can distinguish eight small clusters numbered 1–8: sample 4 at 80 °C (cluster 1), sample 4 at 90 °C and 70 °C (cluster 2), sample 6 at 90 °C and 70 °C (cluster 3), sample 9 at 90 °C (cluster 4), sample 9 at 80 °C and 70 °C (cluster 5), sample 5 at 80 °C and sample 2 at 90 °C (cluster 6), sample 10 at 90 °C, 80 °C and 70 °C (cluster 7) and sample 1 at 90 °C, 80 °C, 70 °C, sample 7 at 90 °C, 80 °C and 70 °C and sample 2 at 80 °C and 70 °C (cluster 8). All these green teas came from China, with a few exceptions such as sample 6, which consists of Chinese and Indonesian tea, sample 7, which comes from Vietnam and sample 2 for which the origin is not available. Sample 1 comes from China, but it is an organic bio-green tea. This tea sample exhibited the highest concentration of QglyRut at 70 °C. This sample tea was packed in a cardboard box and individual bags were packed separately in transparent plastic packaging. Cluster E contains clusters numbered 1–3, sample 5 prepared at 70 °C (cluster 1), sample 8 prepared at 90 °C and 70 °C (cluster 2) and sample 8 prepared at 80 °C (cluster 3). This tea of sample 8 was produced in the UK, but an actual kind of green tea was not available. The tea was packed in cardboard packaging and individual tea bags were packed in HDPE packaging. This sample exhibited the high antioxidant activity and the high content of EGC substances. The tea of sample 5 comes from China and the cluster of objects is close to the largest cluster D.

4. Conclusion

The fast gradient separation of green tea phenolic compounds taking less than 3 min using classical HPLC system was optimized in this work. This fast chromatographic separation was used to monitor degradation of catechins and other phenolics in green tea infusions prepared by 90 °C, 80 °C and 70 °C hot water. The degradation was monitored for 6 h after leaching. During this time the decrease of catechins, mainly EGC and EGCG, occured, but on the contrary an increasing of gallic acid content was observed. The multivariate statistics treatment of the data, principal components analysis, discriminant analysis and factor analysis, confirmed this statement. Further, the influence of temperature on degradation was monitored. It was found that the samples of green tea prepared at 70 °C contain a lower concentration of health beneficial substances in comparison with the samples prepared at 90 °C. However, the degradation of these compounds is significantly higher that in case of samples prepared at a lower temperature. The influence of the storage of tea bags on catechin content was also monitored. During the few weeks of tea bag storage, the content of important catechins rapidly decreases. Together with an HPLC/MS analysis, the antioxidant capacity and total polyphenol content were measured using spectrophotometric techniques. The satisfactory correlation of these techniques were observed. However, the spectrophotometric techniques did not expose the degradation of catechins during staying of infusion probably due to significant antioxidant properties of degradation products.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.05.133.

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