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Research article

HPLC-DAD method for simultaneous determination of gallic acid, catechins, and methylxanthines and its application in quantitative analysis and origin identification of green tea

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ABSTRACT

The high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD) was optimized for the simultaneous determination of 11 compounds, belonging to polyphenols (gallic acid and seven catechins) and methylxanthines (caffeine, theobromine, and theophylline). The results obtained for all the validation parameters of the HPLC-DAD method showed that the method is sensitive enough for routine analysis with basic chromatographic equipment, thus it has a significant potential to be highly applicable in common laboratory practice. The method was used in the analysis of 60 green tea infusions originating from four tea-producing countries. The dataset contributes to enhancing current data on green tea. The analysis of green tea extracts revealed significant differences depending on the origin of the samples. Linear Discriminant Analysis (LDA) was applied to test the accuracy of identification of the origin of the tea samples, based on the chemical composition of tea with a focus on polyphenolic compounds and methylxanthines analysed in this study. Based on cross-validation results, the model showed 93.75 % accuracy in the classification of green tea originating from Japan, China (Mainland), China (Taiwan) and South Korea.

Chemical compounds studied in this article:

Abbreviations: **BAGI**, Blue applicability grade index; **C**, (+)-catechin; **CAF**, caffeine; **CG**, (+)-catechin-3-gallate; **CRM**, certified reference material; **DW**, dry weight; **EC**, (-)-epicatechin; **ECG**, (-)-epicatechin-3-gallate; **EGCG**, (-)-epigallocatechin-3-gallate; **ESI TIC**, electrospray ionisation total ion current chromatogram; **GA**, gallic acid; **GC**, (-)-gallocatechin; **GCG**, (-)-gallocatechin-3-gallate; **HPLC-DAD**, high performance liquid chromatography coupled with diode array detector; **LDA**, linear discriminant analysis; **LOD**, limit of detection; **LOQ**, limit of quantification; **MS**, mass spectrometry; N, number of measurements; **ND**, not detected; **PCA**, principal component analysis; **PLS-DA**, partial least squares discriminant analysis; $\mathbf{R^2}$, coefficient of determination; **RSD**, residual standard deviation; **TBM**, theobromine; **TEP**, theophylline; **tR**, retention time.

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Gallic acid (PubChem CID: 370; CAS: 149-91-7)
Caffeine (PubChem CID: 2519; CAS: 58-08-2)
Theobromine (PubChem CID: 5429; CAS: 83-67-0)
Theophylline (PubChem CID: 2153; CAS: 58-55-9)
(+)-Catechin (PubChem CID: 9064; CAS: 154-23-4)
(-)-Catechin-3-gallate (PubChem CID: 5276454; CAS: 130405-40-2)
(-)-Epicatechin (PubChem CID: 72276; CAS: 490-46-0)
(-)-Epicatechin-3-gallate (PubChem CID: 107905; CAS: 1257-08-5)
(-)-Epigallocatechin-3-gallate (PubChem CID: 65064; CAS: 989-51-5)
(-)-Gallocatechin (PubChem CID: 9882981; CAS: 3371-27-5)
(-)-Gallocatechin-3-gallate (PubChem CID: 199472; CAS: 4233-96-9)

1. Introduction

Green tea, *Camellia sinesis*, (L.) Kuntze, as the most widespread beverage in the world, possesses many desirable properties [1], which are also the result of the presence of secondary metabolites from the groups of polyphenols and methylxanthine alkaloids. Catechins, the dominant polyphenols in green tea, constitute 12–30 % of dry tea leaves [1,2]. They are synthesised through phenylpropanoid and flavonoid pathways and concentrate in the central vacuoles of mesophyll cells of leaf tissues [3–5], and their concentration changes during plant growth and declines with the age of the leaf [4]. Notable catechins include (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epicatechin (EC) [1], however, Cardoso et al. [6] identified even more compounds. The role of catechins is in the defence of tea plants against abiotic stress, microbial diseases, fungal infections, and some insects [7]. Catechins, with their immunomodulatory properties [1,8], contribute to cancer prevention, oxidative stress-related ailments, and exhibit anti-radiation and anti-aging effects [9–11]. EGCG, the most abundant green tea catechin, demonstrates the highest biological activity [12,13].

Methylxanthines, like caffeine (1,3,7-trimethylxanthine; CAF), theobromine (3,7-dimethylxanthine; TBM), and theophylline (1,3-dimethylxanthine; TEP), are prevalent psychoactive substances included in daily diet [14–16]. These alkaloids are found in common beverages like tea, coffee, cocoa, chocolate, soft drinks, energy drinks, and stimulatory supplements such as guarana [17,18]. Methylxanthines, similarly to other secondary metabolites may contribute to the resistance of tea plants against abiotic stress and pathogens, exhibiting pesticidal properties, insect toxicity, and inhibition effects on microbes and fungi [7,19]. The relatively low toxicity of methylxanthines, along with their numerous attributed beneficial effects over time, has garnered significant attention and provided ground for extensive scientific research [16]. Widely used in therapeutics, methylxanthines stimulate the central nervous system, enhance cardiac activity, and offer benefits in hypertension, cardiovascular diseases, bronchial muscle tone reduction, and diuresis [16,20]. Caffeine, theophylline, and theobromine have distinct effects, with caffeine being a potent central nervous system stimulant, theophylline acting as a bronchodilator or diuretic, and theobromine being used for treating cerebral vasospasm [20–22].

Various techniques, including spectrophotometry, spectrofluorimetry, Fourier transform-infrared spectrophotometry, gas chromatography-mass spectrometry, thin-layer chromatography, micellar electrokinetic chromatography, and capillary electrophoresis, are employed for qualitative and quantitative analyses of methylxanthines and polyphenols. High-performance liquid chromatography (HPLC), particularly coupled with diode array detectors (DAD), is widely used for methylxanthine determination in diverse matrices, as demonstrated by Komes et al. [22], Zuo et al. [23], and Aqel et al. [14]. Liquid chromatography coupled with mass spectrometry (LC-MS) has also been applied for plant samples, including tea analysis [14,24]. Datasets of chemical components of tea samples are used not only to evaluate the quality of the product but can also be processed to provide information on the origin of the samples, plant varieties, growth altitude, and climatic conditions [25].

Application of fingerprinting to determine the geographical origin of tea has been studied using various chemical components [26] such as stable isotopes [27], mineral elements [28], secondary metabolites [29], non-targeted metabolomics [30], and spectral and sensorial fingerprinting technologies [26]. The data are further processed, especially by chemometric analysis, which has proven to be a valuable method for identifying tea categories. Discrimination of a region of origin is also a great challenge especially for the tea leaves from the same category (fresh unprocessed leaves) within smaller regions of planting [31]. The most preferred methods for processing large amounts of data obtained are principal component analysis (PCA) and linear discriminant analysis (LDA) [26]. Results for the LDA analysis are usually expressed in prediction ability and correct classification range. Using the profile of 39 mineral elements analysed by the stepwise linear discriminant analysis model resulted in a prediction ability of 88.3 % and correct classification rates for the geographical region of teas of 96.0 % [32]. Shevchuk et al. [25] used Partial Least Squares Discriminant Analysis (PLS-DA) on black tea based on liquid chromatography–mass spectrometry data and showed that origin-related PLS-DA explained variance ranged from 36 to 42 %. The application of the most important feature diagram increased the explained variance to 54 %. Near-infrared hyperspectral imaging combined with chemometrics was applied for the differentiation of green tea origin [33]. The PLS-DA discrimination accuracy was 96.25 % for the calibration set and 92.5 % for the prediction set. Multidimensional statistical methods have shown themselves to be a powerful practical tool suitable for the classification of samples [34,35].

The present study aimed at the development and evaluation of an HPLC method for the simultaneous determination of eleven biologically active compounds belonging to the groups of alkaloids – methylxanthines and phenolic compounds – catechins and gallic acid in tea samples. The method was developed after study of previously published works by El-Shahawi et al. [36], Svoboda et al. [37], and more analytes were included; thus, it was modified and optimized, followed by validation with the main aim of providing a reliable tool for incorporation in ordinary laboratory practice with applications for tea infusions and herbal extracts to provide a

relatively cheap analysis that is valuable for the use in common analytical practice in many laboratories. The practicality and applicability of the method were also evaluated by applying the Blue applicability grade index (BAGI).

Since the tea samples were of different geographic origins, different sampling periods with an impact on developmental stages, and were differently processed, this experimental setting allowed us to study levels of individual methylxanthines, catechins and gallic acid as a function of the tea processing and botanical origin of the samples. Examination of these biologically active substances is important from the viewpoint of medical purposes and the quality of raw material.

2. Materials and methods

2.1. Chemicals

The caffeine and catechines mix standard (as Green Tea Catechine Mix – a certified reference material (CRM)) – a mixture of 8 compounds: (+)-catechin (C); caffeine (CAF); (−)-catechin-3-gallate (CG); (−)-epicatechin (EC); (−)-epicatechin-3-gallate (ECG); (−)-epigallocatechin-3-gallate (EGCG); (−)-gallocatechin (GC); and (−)-gallocatechin-3-gallate (GCG) was purchased from Lambda Life Inc., Bratislava, Slovakia (producer: Cerriliant Corp., RR, Texas, USA). Single-component standards - gallic acid (GA), theobromine (TBM) and theophylline (TEP) were purchased from Lambda Life Inc. Bratislava, Slovakia (producer: Sigma-Aldrich Chemie GmbH, Steiheim, Germany). Acetonitrile (HPLC gradient grade), methanol (HPLC grade) and phosphoric acid (ACS grade) were purchased from Lambda Life Inc., Bratislava, Slovakia (producer: Sigma-Aldrich Chemie GmbH, Steiheim, Germany). Ultrapure water (18.2 mΩ cm^{-1}, 25 °C) was prepared in a Simplicity 185 purification system (Millipore SAS, Molsheim, France) and was used in sample preparation steps and analysis, as well. Individual green tea catechin mix standard compounds, analysed with the HPLC-DAD method, were also confirmed by mass spectrometry (MS) spectra, which are included in supplementary materials (Fig. 1S). Formulas of analysed compounds are visualized in Fig. 2S.

2.2. Tea samples

Samples of 60 different green teas were obtained from Tea House of Good People, Ltd. (Nitra, Slovakia). Tea samples are sumarised in Table 1.

2.3. Sample preparation

Hot water extracts of tea were prepared by pouring 1.000 ± 0.001 g of the tea with 100 mL of 85 °C ultrapure water, based on recommendations in the study of Svoboda et al. [37] and Horžić et al. [38], and with respect to the recommendation of tea houses on standard tea preparation (2 g of tea is recommended to be prepared in 200 mL of hot water with steeping 5–8 min). Thus, the ratio of 1g of tea to 100 mL of hot water was maintained. The mixture was stirred properly in the glass beakers and samples were extracted for 5 min. During the extraction, the samples were manually mixed twice. This kind of preparation of tea infusion is normally used by consumers. Filtration was performed with Munktell filter paper no. 390 (Munktell & Filtrak, Bärenstein, Germany) into 20 mL glass vials. Approximately 5 mL of extracts were also filtered through the Q-Max RR syringe filters (0.22 μm PVDF, diameter: 25 mm; Frisenette ApS, Denmark) to the amber vials (2 mL, Agilent Technologies, Santa Clara, CA, USA) and used for determination of methylxanthines and polyphenols by HPLC method. Until the start of the analysis, the samples were kept in the dark at 4 °C in the rack of the autosampler unit. The analysis was performed on the same day as sample preparation. The moisture content of tea samples was determined by the moisture analyser KERN DAB 100-3 (Kern & Sohn GmbH, Balingen, Germany). The tea infusions were further analysed by the HPLC-DAD method described below.

2.4. HPLC-DAD system

High-performance liquid chromatography was used for the determination of methylxanthines and polyphenols in samples of tea, using Agilent Infinity 1260 Agilent Technologies GmbH (Agilent Technologies GmbH, Waldbronn, Germany) equipped with a quaternary pump, autosampler and Peltier cooler, column thermostat and DAD detector. The separation was realized on LiChroCART 250-4 Purospher STAR, RP-18 end-capped column (250 mm × 4 mm × 5 μm; Merck KGaA, Darmstadt, Germany). The mobile phases were as follows: acetonitrile (A) and 0.1 % H_3PO_4 in ultrapure water (v/v) (B). Gradient elution was as follows: 0–1 min isocratic elution (20 % A + 80 % B), 1–5 min linear gradient elution (25 % A + 75 % B), 5–15 min linear gradient elution (30 % A + 70 % B), 15–25 min linear gradient elution (40 % A + 60 % B). Post-run equilibration was 3 min. The flow rate was 1 mL min^{-1} and the injection volume

Table 1
Characterization of green tea samples.

Region	Origin	Form	Number (Representatives)
China (Mainland)	Original	loose	10
South Korea	Original	loose	7
Japan	Original	loose	40
Taiwan	Original	loose	3

was 3 μL. The column thermostat was set at 30 °C. The data acquisition was obtained at the wavelength set at 280 nm. Scanning of the spectrum was performed in the range of 210–400 nm. The range of wavelengths was used to extract the spectra of analytes and thus for identification of analytes in tea samples based also on comparison of spectra of pure standards and the analytes in the tea. The spectral data obtained in this way were processed using the Agilent OpenLab ChemStation software for LC 3D Systems.

2.5. Standard solutions

From the methanolic stock solution of compounds, standard solutions were prepared for calibration by dilution of stock solution with an initial composition of mobile phase mixture. The concentration ranges of individual compounds are specified in Table 1S. The solutions were stored at 4 °C and used within one week.

2.6. Statistical analysis

Basic characterization and evaluation (summarisation, mean, median, standard deviation, minimum and maximum values) were performed by use of descriptive statistics in Microsoft Office Excel 365 for iOS and Addinsoft 2022. Evaluation of BAGI was based on the work of Manousi et al. [39]. XLSTAT statistical and data analysis solution (New York, USA) was applied for analysis of possibilities to discriminate the geographical origin of the green tea *C. sinensis*. Multidimensional statistics represented by the LDA method were used for the samples from four regions of origin to create a model.

Table 2
Intermediate precision of the concentration and retention time of individual methylxanthines and polyphenols.

Intraday precision of the retention time and concentration of methylxanthines and polyphenols in standard solution and tea sample extract									
Compound	Standard solution					Tea sample - Green needles (China, Mainland)			
	Concentration calculated	[a]Retention time		[a]Concentration measured		[a]Retention time		[a]Concentration	
	mg L^{-1}	min	RSD	mg L^{-1}	RSD	min	RSD	mg g^{-1}	RSD
GA	9.16	3.97	0.02	9.25	0.13	3.99	0.02	0.54	0.34
GC	12.6	4.61	0.02	12.8	1.58	4.67	0.03	2.14	2.64
TBM	7.74	5.39	0.03	7.81	0.67	5.45	0.02	1.14	0.37
TEP	6.70	8.39	0.01	6.75	0.24	8.59	0.03	0.36	0.16
C	12.6	9.05	0.02	12.7	0.48	9.09	0.02	2.74	0.54
CAF	12.6	11.2	0.16	12.7	0.27	11.2	0.02	22.5	0.14
EGCG	12.6	12.1	0.01	12.8	0.89	12.1	0.03	20.0	0.44
EC	12.6	13.2	0.20	12.7	1.61	13.2	0.20	7.27	0.48
GCG	12.6	14.4	0.18	12.7	1.54	14.5	0.02	1.58	1.73
ECG	12.6	16.6	0.15	12.7	1.40	16.6	0.03	11.1	0.24
CG	12.6	18.1	0.02	12.7	0.24	18.1	0.03	0.11	2.80
Interday precision of the retention time and concentration of methylxanthines and polyphenols in standard solution and tea sample extract									
Compound	**Standard solution**					**Tea sample - Green needles (China, Mainland)**			
	Concentration calculated	[b]Retention time		[b]Concentration measured		[b]Retention time		[b]Concentration	
	mg L^{-1}	min	RSD	mg L^{-1}	RSD	min	RSD	mg g^{-1}	RSD
GA	9.16	3.96	0.15	9.25	0.10	3.99	0.15	0.54	0.55
GC	12.6	4.62	0.22	12.8	1.26	4.67	0.02	2.13	2.19
TBM	7.74	5.41	0.01	7.80	0.73	5.45	0.02	1.14	0.35
TEP	6.70	8.40	0.19	6.76	0.21	8.59	0.02	0.36	0.14
C	12.6	9.05	0.24	12.9	1.46	9.06	0.02	2.74	0.58
CAF	12.6	11.2	0.16	12.7	0.26	11.2	0.02	22.5	0.14
EGCG	12.6	12.1	0.19	12.8	0.89	12.1	0.03	20.1	0.40
EC	12.6	13.2	0.20	12.7	1.30	13.2	0.19	7.26	0.49
GCG	12.6	14.4	0.18	12.7	1.55	14.5	0.02	1.58	1.44
ECG	12.6	16.6	0.14	12.7	1.20	16.6	0.03	11.1	0.19
CG	12.6	18.1	0.15	12.7	0.38	18.1	0.03	0.11	1.93

Note: GA: gallic acid; GC: (−)-gallocatechin; TBM: theobromine; TEP: theophylline; C: (+)-catechin; CAF: caffeine; EGCG: (−)-epigallocatechin-3-gallate; EC: (−)-epicatechin; GCG: (−)-gallocatechin-3-gallate; ECG: (−)-epicatechin-3-gallate; CG: (−)-catechin-3-gallate.

[a] concentration of individual compounds in the standard solution, data presents mean values calculated from six repeated measurements within one day.

[b] concentration of individual compounds in the standard solution; data presents mean values calculated from twelve repeated measurements within three days.

3. Results and discussion

3.1. Method validation

Validation of analytical methods is currently an essential procedure coupled with the method development and testing, it is a confirmation that the particular requirements for a specific intended use are fulfilled, and the results of analysis are verified for the reliability of the method Validation parameters are repeatability, accuracy, linearity, limit of detection, limit of quantification and uncertainty of the methods [40,41]. A validation was carried out to verify the reliability of the method developed. Several validation parameters were evaluated to assert that the HPLC method had performances compatible with those required for routine analysis of methylxanthines and polyphenols in plant material, especially tea samples.

Repeatability and intermediate precision were measured on standard solutions and the tea infusion and were expressed by RSD (%) of repeated measurements within one and three days of analysis. Linearity was determined for individual compounds within the concentration range and expressed by the coefficient of determination (R^2). Results for repeatability, intermediate precision, linearity, limits of detection and quantification, and recovery are summarized in Tables 2 and 3.

3.1.1. Repeatability and intermediate precision

Repeatability (evaluated as intra- and interday precision) of the method was tested by six replicate injections of both the standard solution (composed of 12.6 mg L^{-1} individual catechins and caffeine, 9.16 mg L^{-1} gallic acid, 7.74 mg L^{-1} theobromine and 6.70 mg L^{-1} theophylline) and a green tea (Green needles, China (Mainland)) sample extract. The responses measured on each chromatogram were the retention time of peaks of individual compounds and the corresponding peak area. Table 2 shows intra- and interday variations of retention times and peak area, expressed in RSD (%). Variation in retention times was very low with RSD values ranging from 0.01 to 0.20 % for intraday measurements of standard solution and RSD values for the concentration of individual compounds ranging from 0.13 % to 1.61 %. In the tea infusion, intraday RSD values of retention time were below 0.20 % and the concentration did not exceed 2.64 %. We observed a slight delay in retention times (between 0.00 and 0.18 s) of tea extracts compared to the standard solution. The slight shift was probably due to the matrix effect of tea extract.

Evaluation of interday precision showed that the variability of retention times for standard and tea extract did not exceed 0.24 % RSD. In the case of measured concentration, 1 the variability was 1.55 % RSD in the standard solution and 2.19 % RSD in the concentration of individual compounds on dry weight basis. We observed a slight delay in retention times (between 0.00 and 0.18 s) of tea extracts compared to the standard solution. The slight shift may have been due to the matrix effect of tea extract. The method also showed very good intraday and interday precision based on RSD values for retention times and concentration of standard and tea extract, calculated from repeated measurements over three days.

Compared to validation characteristics for methylxanthines in the work of Chorti et al. [42], we achieved lower RSD values for repeated measurements of concentrations. Wangkarn et al. [43] determined intraday and interday precision as RSD% for 9 analytes (catechins, GA and CAF) in the range of RSD 0.58–1.28 % for the retention time and 0.48–1.33 % for the peak area, showing the highest variability in retention time of C and peak area for GCG and EC that is similar to the results in our study.

3.1.2. Linearity, limit of detection, limit of quantification and recovery

Overall results for linearity, limits of detection (LOD), limits of quantification (LOQ) and recovery are shown in Table 3. Linearity was evaluated within the defined interval of concentration range for individual compounds. To determine linearity, the coefficient of determination (R^2) was used, calculated from three individual measurements of each standard concentration level. The method provided high R^2 values for individual compounds ranging from 0.9991 (EC) to 0.9999 for TEP and CG. In general, the calibration ranges covered adequately the variability in the amounts of individual polyphenols and methylxanthines tea samples, however during

Table 3
Linearity, limits of detection, and limits of quantification for individual methylxantines and polyphenols.

Compound	Linearity	LOD	LOQ	LOD	LOQ	Recovery
	R^2	mg L^{-1}	mg L^{-1}	mg g^{-1}	mg g^{-1}	%
GA	0.9998	0.08	0.23	0.008	0.023	95.3
GC	0.9995	0.23	0.69	0.023	0.069	96.6
TBM	0.9998	0.23	0.70	0.023	0.070	94.5
TEP	0.9999	0.18	0.55	0.018	0.055	98.8
C	0.9994	0.28	0.84	0.028	0.084	100.5
CAF	0.9998	0.18	0.54	0.018	0.054	101.2
EGCG	0.9998	0.20	0.61	0.020	0.061	99.1
EC	0.9991	0.29	0.88	0.029	0.088	96.6
GCG	0.9998	0.24	0.71	0.024	0.071	102.4
ECG	0.9998	0.20	0.59	0.020	0.059	99.0
CG	0.9999	0.18	0.54	0.018	0.054	109.9

Note: LOD (limit of detection) and LOQ (limit of qualification) mean values calculated from three dependences of peak area and concentrations within three days; recovery calculated from nine repeated measurements of tea sample (Green needles, China (Mainland)) unspiked and spiked with standards on the concentration levels: 4.58 mg L^{-1} (GA); 6.30 mg L^{-1} (GC, C, CAF, EGCG, EC, GCG, ECG, CG); 3.87 mg L^{-1} (TBM); 3.35 mg L^{-1} (TEP).

the application of the method we observed some cases, where dilution was needed to avoid exceeding the calibration range.

Calibration dependence was used for the calculation of the LOD and LOQ based on the upper limit approach [40]. Both LOD and LOQ were calculated in concentration for water infusion and expressed also for the dry tea material. The lowest LOD and LOQ were achieved for the GA and the highest for the EC but in general, the method is able to reliably detect selected compounds on the level 0.20–0.30 mg L^{-1} (Table 3) which makes it very suitable for the common use for the determination of the analytes in tea infusions and similar matrices with basic chromatographic equipment.

Previously published works on the determination of polyphenols and methylxanthines with simple HPLC equipment covered usually a smaller number of simultaneously analysed analytes with higher LOD and LOQ values, compared to our study. The LOD limits are around 91 % lower than those reported by Fernando & Soysa [44] for GA, CAF, EC and EGCG. Baek et al. [45] determined LOD and LOQ for CAF by HPLC on the concentration levels that present 47 % of concentrations in our validation results. The mentioned method was developed and validated only for selected methylxanthines. Development of the method for determination of seven catechins, GA and CAF published by Wangkarn et al. [43] showed LOQ (mg L^{-1}) ranging from 0.67 (CAF) to 2.18 (EC) were higher (from 19.4 to 89.1 %) than results obtained for the same compounds in our study, but the linearity was similar to our results.

The method was tested on recovery with use of spiking method. Calculation of recovery was done from tea infusions diluted with water and the same tea infusion spiked with a mixture of standards resulting in the same volume as diluted tea infusion. We used standard mixture with spiking concentration levels 4.58 mg L^{-1} for GA, 6.30 mg L^{-1} for GC, C, CAF, EGCG, EC, GCG, ECG, and CG, 3.87 mg L^{-1} for TBM, and 3.35 mg L^{-1} for TEP. These concentrations were expected to present differences between the unspiked and spiked infusions. Recovery (%) was calculated as a the ratio of the difference of concentration between spiked and unspiked sample measured, and expected (added) concentration of standards. The result was multiplied by 100. Our results showed mean recovery based on 9 measurements of unspiked and spiked samples between 94.5 % (TBM) and 109.9 % (CG).

3.1.3. Selectivity of the method

Selectivity was acceptable, as the resolution, observed between the peaks of individual compounds analysed was higher than 1.5. Fig. 1 shows typical chromatograms of the compounds analysed in the standard mixture (A) and the extracts (B).

Based on the method validation parameters, the results proved that the method is very efficient for the quantitative determination of eleven principal compounds in tea infusions and presents a simple and adequate approach in routine practice.

3.1.4. Practicality and applicability of the method

The application of the HPLC-DAD method in routine practice is preferred over LC-MS in the case of analytes, which are commonly present in the samples in detectable amounts and, as it is cost-effective, requires less expertise, less time-consuming and simple, moreover allows accurate quantification of compounds in UV-VIS spectra.

Assessment of practicality and applicability was proposed as the BAGI index [39], evaluating the main attributes of the method – the type of analysis; the number of simultaneously analysed analytes; the analytical technique and instrumentation required; the simultaneous sample preparation; the sample preparation; the number of samples per hour (sample preparation and analysis time); the reagents and materials; requirement for preconcentration; the automation degree; and the amount of sample. The HPLC-DAD method

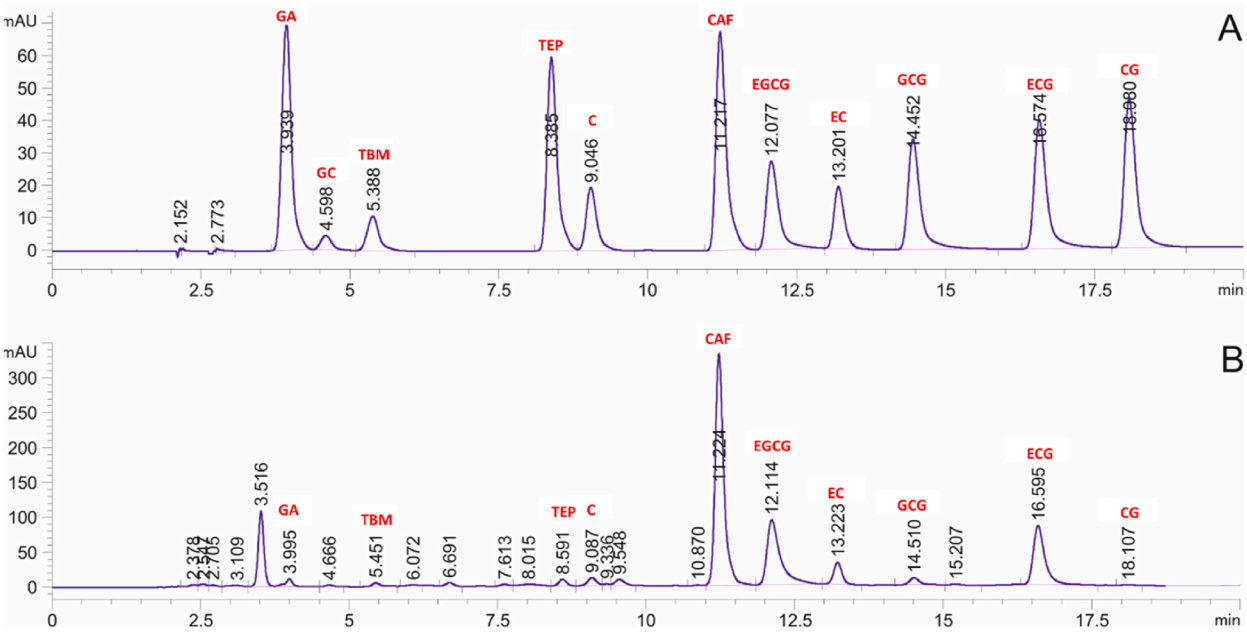

Fig. 1. HPLC-DAD chromatograms of standards of methylxanthines and polyphenols measured at 280 nm; A: Standard mixture, B: Tea sample.

is considered quantitative, simultaneously analysing 11 compounds, with a simple sample preparation by hot water extraction without the need for pre-concentration, with a total time of analysis of 28 min (23 min separation + 5 min post run). The method requires common reagents and materials, it is semi-automated and requires 1 g of tea sample for the analysis. Giving points to the attributes (Table 2S), we obtained a total of 80 points for the BAGI score that is assigned by Manousi et al. [39] to the method with good applicability.

3.2. Content of gallic acid, catechins and methylxanthines in green tea from different regions

The determination of eleven compounds was performed in green tea infusions by the validated method, described above. The HPLC method allowed the separation, qualitative analysis, and quantification of three methylxanthines (TBM, TEP and CAF), seven catechins (C, GC, EC, EGCG, GCG, ECG, and CG), along with GA, which is present in esterified catechins in tea. The composition of green teas, grouped according to their country of origin, investigated in this study is summarized in Tables 4 and 5.

Based on the results, the concentrations of catechins and GA decreased in the following order according to the following trend: EGCG > EC > ECG > C > GC > GCG > GA > CG. In most samples, catechins EGCG, EC and ECG were the most represented, making them the dominant compounds in green tea infusions. However, various surveys on green tea do not confirm a strict order of abundance of selected catechins in water infusions. EGC and EGCG were observed as the most prominent polyphenols, and thus the major catechins extracted in green tea infusions [23,29,46,47].

Determination of individual catechins and GA showed that the analytes were found in a wide concentration range. Overall, the highest mean content of the total amount of catechins (55.6 mg g^{-1} DW) was calculated from the samples from China (Mainland). Tea samples in this study contained the total amount of catechins in agreement with the previously published results by Svoboda et al. [37].

GA had the lowest levels in the teas from China (Taiwan) (0.37 ± 0.11 mg g^{-1} DW). GA was analysed in green tea by Zuo et al. [23] and Koch et al. [29], and concentrations in the extracts were found in the range of 0.0018–0.148 mg mL^{-1}, depending on extraction efficiency.

Catechins from the group of *cis*-catechins (especially EGCG, EGC and ECG) served as quality markers of the green tea [48,49]. Our findings bring the determination of two of the polyphenols – EGCG and ECG to contribute to the evaluation of tea quality. EGCG as the primary catechin in the majority of analysed material ranged between 24.5 and 34.7 mg g^{-1} for the tea from China (Mainland), Japan, South Korea, and China (Taiwan). Several studies reported the predominance of EGCG in tea with similar levels to our research [4,46,47]. Lee et al. [48] studied different green teas depending on the plucking period and the concentration of EGCG was also dominant in catechins with mean concentrations of 104–112.86 mg g^{-1}, which is 3–4 times higher than in samples with dominant EGCG content in our study. The highest levels of EGCG were observed in the Deajak tea harvested in late May. Significantly lower content of EGCG was published by Zuo et al. [23] compared to our results.

EC was determined in the highest mean concentrations 6.97 ± 3.27 mg g^{-1} in China (Mainland). Perva-Uzunalić et al. [47] found EC in dry green tea leaves in the amount of 0.90 mg g^{-1}, similar levels were found in the green tea from China (Mainland), Japan and

Table 4
Concentration of selected polyphenols (gallic acid and catechins; expressed in mg g^{-1} DW) in the green tea.

Parameter	GA	GC	C	EGCG	EC	GCG	ECG	CG	Total
China (Mainland)									
N	40	40	40	40	40	40	40	40	
Mean	0.70	1.82	2.98	30.9	6.97	0.81	11.4	0.04	55.6
SD	0.14	1.27	1.76	6.37	3.27	0.46	7.78	0.11	
Median	0.66	2.22	2.70	29.4	6.97	0.63	8.62	ND	
Range	0.53–0.94	ND-3.20	1.05–6.10	19.4–43.4	2.25–14.4	0.41–2.13	4.17–29.9	ND-0.37	
Japan									
N	160	160	160	160	160	160	160	160	
Mean	0.41	1.47	1.23	32.2	6.93	0.61	4.62	0.22	47.7
SD	0.14	1.31	0.49	12.0	2.61	0.58	1.87	0.27	
Median	0.41	1.74	1.30	33.1	7.61	0.43	4.54	0.19	
Range	0.14–0.69	ND-3.90	ND-2.22	12.1–64.5	2.33–12.4	ND-4.59	1.56–9.90	ND-1.42	
South Korea									
N	28	28	28	28	28	28	28	28	
Mean	0.85	0.58	1.57	34.7	6.40	0.91	8.25	0.82	54.1
SD	0.35	0.94	0.21	5.17	1.22	0.80	2.32	0.73	
Median	0.90	0.00	1.54	36.5	6.60	0.65	9.62	1.21	
Range	0.34–1.43	ND-2.38	1.24–1.95	24.2–41.2	4.73–7.89	0.30–2.91	4.78–10.5	ND-1.60	
China (Taiwan)									
N	12	12	12	12	12	12	12	12	
Mean	0.37	1.14	1.16	24.5	3.68	0.57	2.85	ND	34.3
SD	0.11	0.84	0.52	8.40	0.81	0.21	1.09	ND	
Median	0.36	1.59	0.98	22.6	4.12	0.55	2.59	ND	
Range	0.23–0.50	ND-1.85	0.67–0.87	15.8–35.3	2.57–4.36	0.30–0.99	1.68–4.24	ND	

Note: GA: gallic acid; GC: (−)-gallocatechin; C: (+)-catechin; EGCG: (−)-epigallocatechin-3-gallate; EC: (−)-epicatechin; GCG: (−)-gallocatechin-3-gallate; ECG: (−)-epicatechin-3-gallate; CG: (−)-catechin-3-gallate; DW: dry weight; N: number of measurements; SD: standard deviation; ND: not detected.

Table 5
Concentration of selected methylxanthines (CAF, TBM and TEP; expressed in mg g^{-1} DW) in the green tea.

Parameter	TBM	TEP	CAF	Total
China (Mainland)				
N	40	40	40	
Mean	4.79	0.85	32.4	38.0
SD	3.10	0.23	6.26	
Median	3.50	0.87	32.3	
Range	0.91–10.7	0.46–1.17	21.7–41.3	
Japan				
N	160	160	160	
Mean	2.62	1.96	26.4	31.0
SD	1.50	0.77	7.14	
Median	2.58	2.08	27.8	
Range	0.52–6.20	0.64–3.46	8.23–38.1	
South Korea				
N	28	28	28	
Mean	5.27	1.24	24.5	31.0
SD	2.68	0.28	1.85	
Median	4.75	1.28	24.5	
Range	1.90–11.1	0.67–1.67	21.8–27.7	
China (Taiwan)				
N	12	12	12	
Mean	3.37	1.37	21.7	26.4
SD	0.95	0.30	4.11	
Median	3.95	1.54	23.1	
Range	2.08–4.10	0.97–1.61	16.3–25.6	

Note: TBM: theobromine; TEP: theophylline; CAF: caffeine; DW: dry weight; N: number of measurements; SD: standard deviation.

South Korea.

ECG was present at the lowest concentrations in the samples from Japan (1.56 mg g^{-1}) and the highest levels were reached in the China (Mainland) tea samples (29.9 mg g^{-1} DW). Similarly, the highest ECG concentrations were found in the China samples by Koch et al. [29], although analytes were measured in tea infusions. Lee et al. [48] found higher ECG concentrations (between 38.14 and 41.19 mg g^{-1} DW) compared to our results, but in the different harvesting periods, the concentrations were at a similar level. Lin et al. [46] and Perva-Uzunalić et al. [47] reported ECG reported ECG values that are in agreement with our results.

C was not detected in one sample from Japan, prepared from small leaves from spring harvesting. The highest concentration of C was determined in China (Mainland) tea (6.10 mg g^{-1}), similar to the studies by Koch et al. [29] and Lee et al. [48]. Mamati et al. [4] reported C concentration in the range of 0.20–0.90 mg g^{-1} similar to our observations.

EC was present in all tea samples, and the highest concentrations were observed in tea from China (Mainland) (maximum value 14.4 mg g^{-1}) and Japan (12.4 mg g^{-1}), showing a very wide range. Koch et al. [29] similarly observed maximum EC levels in samples from China and Japan. EC content was also in agreement with the levels reported by Lee et al. [48] and Perva-Uzunalić et al. [47].

GC was found between non-detectable concentration and 3.90 mg g^{-1} DW with the highest values in the samples from Japan. Lee et al. [48] found GC in the range from 2.28 to 3.65 mg g^{-1} DW which agrees with our findings. Mamati et al. [4] reported content of GC up to 20 mg g^{-1}, and its concentrations decreased from buds to mature leaves.

The content of CG was found in the samples from non-detectable amounts to 1.60 mg g^{-1} in the Japanese samples, similar to Mamati et al. [4].

GCG was observed to vary from non-detectable amounts to 4.59 mg g^{-1} and both minimum and maximum values of GCG were found in Japanese tea samples. Mamati et al. [4] reported similar concentrations of GCG (between 0.5 and 4.5 mg g^{-1}). Higher concentrations were reported by Lee et al. (2014) in the range of 5.61–6.76 mg g^{-1} DW.

CAF is a dominant methylxanthine in all tea samples. The order of methylxanthines in the tea samples was as follows CAF > TBM > TEP. The content of CAF ranged from 11.8 to 41.3 mg g^{-1} DW with the highest concentrations (over 30 mg g^{-1}) measured in samples from China (Mainland) and Japan. CAF concentration in green tea reported by Zuo et al. [23] ranged between 0.38 and 0.14 mg mL^{-1} with a higher ability to extract in acidic conditions. Lin et al. [46], Perva-Uzunalić et al. [47], and Aqel et al. [14] reported similar concentrations of CAF as were obtained in our study. Baek et al. [45] reported the mean contents of CAF, TBM and TEP in different teas as 5.56, 0.40 and 0.02 mg g^{-1}, respectively.

TBM content in our study ranged from 0.52 to 11.1 mg g^{-1} DW with very wide variation even in the samples from the same country. The highest concentrations of TBM (over 10 mg g^{-1} DW) were recorded in teas from China (Mainland) and South Korea. Concentrations of TBM were investigated by Aqel et al. [14] and Chorti et al. [42] and their results were in agreement with those lower concentrations (up to 3 mg g^{-1} DW) in our study.

TEP concentrations ranged from 0.46 (China, Mainland) to 3.46 mg g^{-1} DW (Japan). Aqel et al. [14] reported in commercial teas TEP from ND to 2.26 mg g^{-1} DW which is in agreement with our findings.

3.3. Impact of harvesting period regarding the developmental stage of tea leaves and concentration of tea chemical constituents

In the chemotaxonomy approach, secondary metabolites play an important role in investigating plant species and in the field of determination of plant species varieties or the origin of plants of the same species. The synthesis of these secondary metabolites is dependent on the genetically encoded ability of a specific taxonomic group to synthesize them. However, besides genetic prerequisites, the synthesis of secondary metabolites is also influenced by environmental conditions, including abiotic and biotic stress factors, which affect plant mechanisms in the sense of the formation of compounds that are used by plants for defense against these stress factors [50, 51].

Major bioactive constituents in tea are catechins, phenolic acids, flavonols, and alkaloids [35] belonging to secondary metabolites of tea. The levels of total catechins and polyphenols in leaves decreased with the age of tea leaves, but the alterations in specific catechins differed. A general reduction in CG and EGCG was confirmed, while EGC and ECG showed an increase [4]. Lee et al. [48] confirmed increasing content of EGCG and EGC in the order of plucking periods: Woojeon (late April)<Sejak (early May)<Joongjak (mid-May)<Daejak (late May). Consideration of the whole season of possible tea harvesting from early spring to autumn was investigated by Bhandari & Goswami [52]. The study showed a tendency for a decrease in concentration in fresh tea materials for CAF, EGC, EGCG and ECG.

Plotting the concentrations of individual polyphenols and methylxanthines in relation to the harvesting period (Fig. 3S), it is possible to observe some general trends in the production of secondary metabolites in tea material. In the case of methylxanthines, decreasing content of caffeine and theophylline and increasing content of theobromine were found in the approaching vegetation period. In the case of major catechins, represented by EGCG, EC and ECG, increasing concentrations were observed in EGCG and EC. ECG was found in highest concentrations in the early spring harvesting period in buds and small leaves, and in summer harvest in matured leaves. In the case of other polyphenols, represented by GA, GC, C, GCG and CG, a decreasing trend was found in C compared to the spring harvest, followed by other increases towards the summer harvest of mature leaves. The content of GA was found to increase as vegetation development approached. Considering the average content of GC and GCG, it is possible to see relatively balanced amounts of these catechins. CG was found in higher concentrations in the samples from the summer harvest. The mean contents of methylxanthines were, according to our data, decreasing in CAF and TEP, but in the case of theobromine, the trend was the opposite. The amount of caffeine found in tea plants fluctuates over time, influenced by climate elements like precipitation, daily average temperature, and humidity [19]. The contents of theanine, TBM, CAF, C, and GCG were found to be significantly decreased along with the period of tea leaf plucking [48].

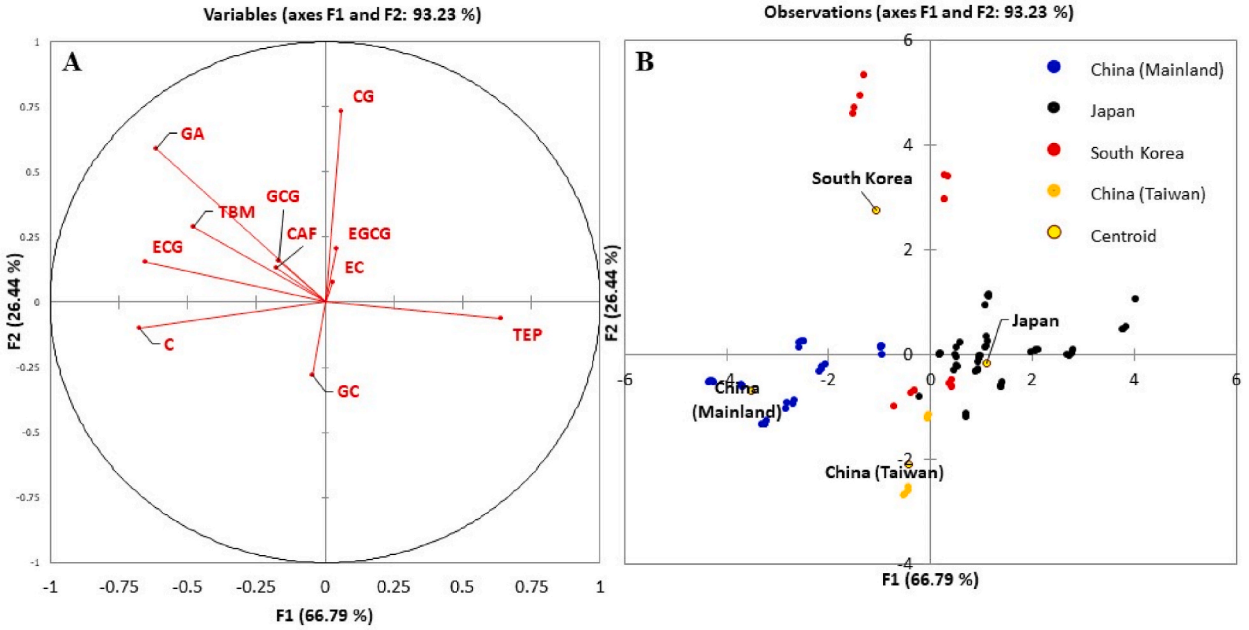

Fig. 2. Identification of geographical origin regarding the chemical composition of green tea by LDA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Note: A: two-dimensional linear discrimination analysis representing variables and their mutual correlation; GA: gallic acid; GC: (−)-gallocatechin; TBM: theobromine; TEP: theophylline; C: (+)-catechin; CAF: caffeine; EGCG: (−)-epigallocatechin-3-gallate; EC: (−)-epicatechin; GCG: (−)-gallocatechin-3-gallate; ECG: (−)-epicatechin-3-gallate; CG: (−)-catechin-3-gallate; B: LDA plot of identification of geographical origin regarding the chemical composition.

3.4. Model of implications based on the chemical composition of green tea

Tea classification approaches, based on remarkably different content of secondary metabolites in combination with statistical methods, were proposed by Chen et al. [35]. GC, EGCG, and ECG were distinguished as pivotal biomarkers in effective tea classification. In this study, we applied use of the secondary metabolites like methylxanthine alkaloids and polyphenols (catechins and phenolic acids) as characteristics of different tea origin. ANOVA ($p \geq 0.05$) test was applied to find differences among the geographical origins in chemical composition.

The Wilks' Lambda test shows that the difference between the mean vectors of the groups is significant (F observed = 26.499; F critical = 1.45). The discriminant analysis revealed that 93.23 % of the total variation embodied in two variables could be effectively condensed into and explained by the first two factors, with eigenvalues of 3.07 (% of discrimination = 66.79) and 1.22 (% of discrimination = 26.44), respectively. The variable axes chart illustrates the correlation of the initial variables (chemical compounds in tea) with the two factors (Fig. 2A). Specifically, F1 correlates with CG, EGCG, EC, and GC, whereas F2 correlates with C, ECG, TBM, CAF, and TEP content (Fig. 2A). Classification functions were established to assign observations to four different countries: China (Mainland), Japan, South Korea, and China (Taiwan). Notably, for China (Mainland) samples, the most influential variables in the classification include GA with a positive coefficient of 12.08 and C with a positive coefficient of 4.87. For Japanese samples, GA and TEP stand out as significant variables with positive coefficients of 13.3 and 6.57, respectively. In the case of South Korean samples, GA had the most substantial impact with a positive coefficient of 29.4. For the samples from China (Taiwan), TEP emerges as a critical variable with a notably high positive coefficient of 18.4. These coefficients reflect the variables that play the most vital roles in classifying observations into their respective countries, providing insights into the discriminant analysis results.

Linear discriminant analysis (LDA) is an approach for classification and dimensionality reduction [53]. LDA was applied for the model of the implication of chemical composition regarding the geographic origin of green tea. The LDA map (Fig. 2B) depicts the observations on the distribution pattern of polyphenols and methylxanthines on the factor axes, derived from the original explanatory variables. Samples from Japan and China (Mainland) are highly correlated with dimension 1. For Japanese samples, high values of TEP are characteristic, along with lower values of compounds C, ECG, TBM, and GA. On the other hand, samples from China (Mainland) are characterized by high contents of C, ECG, TBM, and C, with simultaneously low content of TEP. Samples from South Korea and China (Taiwan) are both characterized by dimension 2, with South Korea having a high content of CG and GA, and simultaneously a low content of GC. In contrast, samples from China (Taiwan) are characterized by high GC content and simultaneously a low content of CG and GA.

Based on cross-validation results, the model showed the possibility of classification of green tea originating from Japan, China (Mainland and Taiwan) and South Korea. The analysis of green tea extracts revealed significant differences depending on the origin of samples, however some misclassifications were observed. The confusion matrix summarizes the percentage of well-classified observations, which is the ratio of the number of observations that have been well-classified over the total number of observations. Cross-validation (Table 6) showed misclassification of four samples from Japan within the samples from China (Mainland). Japanese teas were classified with 100 % correctness. Misclassified were also 8 teas from Japan and one from China (Taiwan) within the South Korean samples and two samples from Japan within the tea from China (Taiwan). In total, 93.75 % of the samples were classified correctly. Total results for the cross-validation: prior and posterior classification, membership probabilities, scores and squared distances are provided in supplementary data (Table 3S).

The results of discriminant analysis are presented in Table 6, focusing on the confusion matrix for estimation samples and cross-validation outcomes achieved by LDA. For the estimation sample, the table reveals that LDA demonstrated a 95 % accuracy in classifying cases, with particularly high accuracy for Japan (100 %) and China (Taiwan) (100 %). However, South Korea's classification accuracy was slightly lower at 71.43 %. In the cross-validation results, the overall accuracy was 93.75 %, indicating the model's robustness. Notably, Japan maintained a perfect accuracy rate of 100 %, while China (Taiwan) achieved 83.33 % accuracy. South Korea's classification accuracy in cross-validation was 67.86 %, which was slightly lower compared to the estimation sample. These

Table 6
Confusion matrix for estimation samples and the cross-validation results achieved by LDA.

Confusion matrix for the estimation sample						
from \ to	China (Mainland)	Japan	South Korea	China (Taiwan)	Total	% correct
China (Mainland)	36	4	0	0	40	90.00 %
Japan	0	160	0	0	160	100.0 %
South Korea	0	7	20	1	28	71.43 %
China (Taiwan)	0	0	0	12	12	100.0 %
Total	36	171	20	13	240	95.00 %
Confusion matrix for the cross-validation results						
from \ to	China (Mainland)	Japan	South Korea	China (Taiwan)	Total	% correct
China (Mainland)	36	4	0	0	40	90.00 %
Japan	0	160	0	0	160	100.0 %
South Korea	0	8	19	1	28	67.86 %
China (Taiwan)	0	2	0	10	12	83.33 %
Total	36	174	19	11	240	93.75 %

findings highlight the effectiveness of LDA in discriminating between these countries in the dataset, with some variations in classification accuracy across the classes.

4. Conclusions

A method of high-performance liquid chromatography coupled with diode-array detection has been developed and tested for simultaneous determination of eleven analytes, namely gallic acid, (+)-catechin, (−)-catechin-3-gallate, (−)-epicatechin, (−)-epicatechin-3-gallate, (−)-epigallocatechin-3-gallate, (−)-gallocatechin, (−)-gallocatechin-3-gallate, caffeine, theobromine and theophylline, belonging to the groups of polyphenols and methylxanthines with application on tea infusion analyses. Based on the tested validation parameters the method has been confirmed as highly sensitive, reproducible and accurate, allowing the determination of the analytes in tea infusions at the concentration levels of sub-mg mL^{-1}. The method is characterized by a simple sample preparation and the advantage is that it could be used to analyse nearly any type of plant material with potentially occurring catechins, gallic acid and methylxanthines. Regarding the application of the method, it can be concluded that the method can be easily applied in analysis with basic chromatographic equipment thus presenting its advantage in routine laboratory practice.

Determination of chemical composition based on commonly occurring catechins, gallic acid and methylxanthines in green tea from different tea-producing countries was used for the investigation of differences among the samples regarding their origin. The variability of individual chemicals should be taken into account in the identification of origin.

Ethical approval and consent to participate

Not applicable.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Silvia Jakabová: Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Július Árvay:** Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Marek Šnirc:** Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Jana Lakatošová:** Investigation, Data curation. **Alena Ondejčíková:** Writing – review & editing, Conceptualization. **Jozef Golian:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35819.

Appendix A. Supplementary data

Supplementary data to this article can be found online at.

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