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Development of thin-layer chromatographic method for determination of caffeine in black, green, and white tea

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RESEARCH ARTICLE

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ABSTRACT

Caffeine is naturally present in tea and coffee giving the pleasant and stimulant effect. Several different types of teas, black, green, and white teas bought in market were analysis for caffeine content. The boiled sample tea was filtered through filter paper. Lead(II) acetate was used to separate tannins from caffeine followed by filtration through filter paper with a black ribbon. The liquid-liquid extraction was carried out using dichloromethane (3×5 mL) and sodium sulfate as a drying agent. The TLC method was performed on Merck precoated silica gel plates 5×10 cm ($60F_{254}$, 200 µm) using either methanol or dichloromethane as solvents and the mobile phases were glacial acetic acid and ethyl acetate (95:5, v/v), while the second one was consisted of ethyl acetate and ethanol (80:20, v/v), respectfully. The R_f values were 0.36 and 0.86 for the first and the second mobile phase, respectively, in comparison to the standard caffeine. The values for pH of boiled sample teas were in the range from 4.85 to 5.80. The most abundant tea sample for caffeine was determined in green tea bought in the grocery store for health nutrition (2.04 %). The yield for tea samples from green market, white tea and two tea black samples were 0.06, 0.71, 0.07, and 0.05%, respectively. The developed TLC method can be used for determination of caffeine content in tea samples.

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1. Introduction

One of the most popular beverages for centuries is tea, primarily due to the aroma, pleasant taste, and stimulant effects. It is a complex mixture containing a variety of substances such as tannins and caffeine [1]. Purine is the basic structure of caffeine and it is present in more than 60 plants. Caffeine is extensively used in nonalcoholic beverages such as coffee or tea, energy drinks, and cola, which make the drinks addictive and also is present in chocolates. Caffeine is also present in a large number of products which are used as the counter pain relievers, headache remedies, and antihistamines. In recent years, caffeine has received increasing attention in the food and pharmaceutical industries, due to its pharmacological properties which comprise stimulation of the central nervous system [2] and muscle relaxant properties, for heart, renal, gastrointestinal and respiratory systems [3-6].

The chemical formula of caffeine is $C_8H_{10}N_4O_2$, its chemical name is 1,3,7-trimethylxanthine (Figure 1), and caffeine is an intensely bitter white powder. It can be found as odorless, white, fleecy masses, glistening needles of powder. Its molecular weight is 194.19 g/mol, the melting point is 236 °C, point at which caffeine sublimes is 178 °C at atmospheric pressure, pH is 6.9 (1 % solution), specific gravity is 1.2, volatility is 0.5%, vapor pressure is 760 mm Hg at 178 °C, solubility in water is 2.17 % [7]. Caffeine can be obtained by synthetic methods and by extraction from different plants. Synthetically, caffeine can be obtained from chloro acetic acid or uric acid [8]. Furthermore, they can be based on extraction from the filtrates of water-plant systems by means of solvents. The extraction processes depend upon the plants and solvents selected [8].

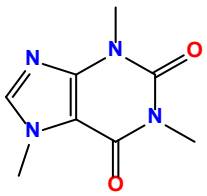

Figure 1. Structural formula of caffeine.

The purpose of this study was examination green, black, and white tea for caffeine content. Tea produced from the leaves of *Camellia sinensis* (L.) are classified according to the processing used into three different subtypes: green tea, black

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tea, and white tea [9]. The chemical composition of green tea is complex: proteins (15-20 % dry weight) whose enzymes constitute an important fraction; carbohydrates (5-7 % dry weight) such as cellulose, pectin, glucose, fructose, and sucrose; minerals, trace elements (5 % dry weight) such as calcium, magnesium, chromium, manganese, iron, copper, zinc, molybdenum, selenium, sodium, phosphorus, cobalt, strontium, nickel, potassium, fluorine, and aluminum, trace amounts of lipids, amino acids (1-4 % dry weight) such as theanine or 5-*N*-ethylglutamine, glutamic acid, tryptophan, glycine, serine, aspartic acid, tyrosine, valine, leucine, threonine, arginine, and lysine; and volatile compounds such as aldehydes, alcohols, esters, lactones, hydrocarbons, *etc*. [10,11].

Green tea contains polyphenols, these compounds may account for up to 30 % of the dry weight. Most of the green tea polyphenols are flavanols, commonly known as catechins. Products derived from green tea are mainly extracts of green tea in liquid or powder form that vary in the proportion of polyphenols (45-90 %) and caffeine content (0.4-10 %) [10]. The amount of catechins in the tea can be affected by the way how leaves are harvested, how the leaves are processed, and how the tea is prepared. In addition, the leaves are grown (geographically) and the growing conditions affect catechin amounts. Polyphenols are quickly oxidized after harvesting due to the enzyme polyphenol oxidase. To prevent the loss of the polyphenols, green tea leaves are heated rapidly to inactivate polyphenol oxidase [12].

Green tea is produced without fermentation and thus oxidation of the polyphenolic components is prevented [9]. Black tea leaves are dried, then rolled and crushed, which promotes oxidation, which means that the manufacture is carried out by fermentation ensuring a high degree of enzymatically catalyzed aerobic oxidation of the polyphenols followed by a series of chemical condensations [9]. Therefore, black tea has far fewer active catechins than green tea [11]. White tea is produced with minimal fermentation from new buds and young leaves, which are harvested only once a year in early spring [9], protected from sunlight to avoid the degradation of its polyphenols [13].

"In average expected composition of black tea solid extract includes: catechins (10-12%), flavonols (6-8%), the flavins (3-6%) besides this, the arubigins (12-18%), phenolic acids (10-12%), amino acids (13-15%), methylxanthines (8-11%), carbohydrates (15%), proteins (1%), and minerals (10%). The most important flavonols in black tea are myricetin, quercetin, kaempferol, and ruthin, similarly as in green tea. Black tea also contains phenolic acids, caffeine (about one-third of the amount typical for coffee), and amino acids including theanine (5-*N*-ethyl-glutamine) which occurs only in the tea leaves" [14]. As we mentioned above that black tea is enriched with theaflavin, which has good antioxidant properties. Besides this, theaflavin-enriched black tea extract has also shown anti-stress effects in a number of randomized crossover studies. Black tea polyphenols exert an anti-stress and antidepressant action against chronic stressors in mice through a direct alteration of brain monoaminergic responses and antioxidant status [15].

The aim of this study was to determine the content of caffeine in some of the tea brands available in North Macedonia. Caffeine itself may have a wide range of biological activities, and by doing a simple modification on the basic ring of the caffeine, we can get a better opportunity for the development of others methods for caffeine. The method is focus to introduce an easy to handle method for the determination of caffeine content in five samples of tea. The present tannins were eliminated using a solution of lead (II) acetate. A simple, rapid, and precise thin-layer chromatography (TLC) method was developed to determine caffeine in tea.

2. Experimental

2.1. Sample collection

Samples of tea were bought from the local market, green tea from the grocery store for health nutrition (1) and green market (2), white tea from local market (3), black tea from the green market (4) from 2010 and (5) from 2020. The exception for the white tea which was in filter bag, the others were in bulk. Standard of caffeine (98.5 %) and lead(II) acetate were purchased from Acros Organics (USA), ethyl acetate (99.8 %), dichloromethane (99.8 %), and methanol were from Fluka (Switzerland), ethanol (70 %) and sodium sulfate from Alkaloid AD (Republic of North Macedonia), and glacial acetic acid was bought from Merck (Germany). All reagents were with analytical purity.

2.2. Determination of pH

The pH values of extracted samples of tea were determined using pH-meter (Benchtop Biobase 210, pH/mV meter, PR China). All measurements were obtained from triplicate analyses.

2.3. Extraction and isolation

Each tea samples (25±0.0001 g) were weighted and transferred to laboratory beakers (1000 mL) and on a hot plate, in the period of 1 hour and 30 minutes all samples of tea were boiled in distilled water (500 mL). The filtration was performed in order to separate the solid parts of tea with filter paper up to 100 mL. 5 mL of lead(II) acetate 10 % solution was added to the filtrates. In the process, tannins were separated and were on the bottom. A black ribbon (589/1) filter paper (Germany) was used where the filtrates had green color. The extraction was done using liquid-liquid extraction with dichloromethane (3×5 mL) in a separatory funnel. Sodium sulfate was used as a drying agent. Beakers were left at 25 °C over night where the solvent was evaporated.

2.4. Thin layer chromatography

The next day, the obtained caffeine was weighted for each sample and the analysis of caffeine was carried out using thin-layer chromatography (TLC). The samples of one grain were dissolved either in 1 mL methanol for the first mobile phase and in 1 mL dichloromethane for the second one. The Merck pre-coated silicagel plate 5×10 cm ($60F_{254}$, 200 μm) was used in the TLC analysis and then the plates were visualized at 254 nm using UV lamp from Analytikjena (Germany). A comparison study with two types of mobile phase solvent systems were applied to analysis, the first was consisted of glacial acetic acid and ethyl acetate (95:5, v/v), while the second one was consisted of ethyl acetate and ethanol (80:20, v/v). The obtained results were done in triplicate analyses.

2.5. HPLC analysis of caffeine

The chromatographic separation was conducted on a S5ODS1 C-18 column (100 mm length × 4.6 mm i.d., 5 μm particle size) using a mixture of methanol and water (90:10, v/v) isocratic elution and Breeze2 Software at 278 nm with Waters 2998 Photodiode Array Detector, flow rate 1 mL/min, 10 μL of sample. All analyses were repeated three times.

3. Results and discussion

In the period of 1 hour and 30 minutes is expected the main content of the tea samples to pass from the samples through the

Table 1. Determination of the yield of caffeine and pH values of extracted tea samples

Sample [1]	Yield of caffeine ±SD (%) [2]	RSD (%) [2]	pH value of extracted tea samples ±SD [2]	RSD (%) [2]
1	2.04±0.006	0.28	5.80±0.01	0.17
2	0.06±0.006	0.95	5.19±0.01	0.19
3	0.71±0.006	0.81	5.49±0.02	0.28
4	0.07±0.006	0.82	5.51±0.04	0.65
5	0.05±0.006	1.14	4.85±0.01	0.21

[1] Green tea from the grocery store for health nutrition (1) and green market (2), white tea from local market (3), black tea from the green market (4) from 2010 and (5) from 2020.
[2] Mean value (n=3) ± Standard deviation (SD); Relative standard deviation (RSD).

Table 2. Determination of R_f values for extracted and isolated caffeine in tea samples.

Sample [1]	R_f ±SD [2] Glacial acetic acid and ethyl acetate, 95:5, v/v	RSD (%)[2]	R_f±SD [2] Ethyl acetate and ethanol, 80:20, v/v	RSD (%) [2]
1	0.36±0.0023	0.64	0.86±0.0057	0.67
2	0.36±0.0029	0.79	0.86±0.0015	0.17
3	0.36±0.0028	0.25	0.86±0.0026	0.31
4	0.36±0.0027	0.73	0.86±0.0027	0.32
5	0.36±0.0026	0.26	0.86±0.0023	0.26
Standard of caffeine	0.36±0.0029	0.79	0.86±0.0026	0.31

[1] Green tea from the grocery store for health nutrition (1) and green market (2), white tea from local market (3), black tea from the green market (4) from 2010 and (5) from 2020.
[2] Mean value (n=3) ± Standard deviation (SD); Relative standard deviation (RSD).

laboratory beakers where hot water is present. The filtration was performed at hot, at first using a simple filter paper. The addition of a solution of lead(II) acetate allowed tannins to be separated in a form of a white precipitate at the bottom of the beaker. The filtration process was an important step in the analysis of caffeine content in the samples of tea. Although at the beginning the trials were with the use of Whatman (42) filter paper, and with white ribbon filter paper (589/1), and the filter paper with blue ribbon (589/1), satisfactory results were obtained using filter paper with black ribbon (589/1). The time-consuming was not too long and the filtrates had the characteristic color of green with the use of filter paper with black ribbon. The caffeine content and determined pH value of each analyzed tea sample were given in Table 1.

It was found that the content of caffeine was higher in the samples of tea obtained from the green tea (1). In the comparison between the tea sample from green tea (2) and the black tea samples (4,5), it was found that the results of yield were not significantly different. The obtained results for the yield of caffeine were 0.06, 0.07, and 0.05 % for green sample tea (2), and two types of black tea (4,5), respectively. In the comparison of two types of black tea from different years, the caffeine content was slightly differed between 0.07 and 0.05 %. This difference can be associated with the origin of tea, environmental conditions, and harvest time and as the most important factor can be the age of the tea leaves and the processes involved in the production of the plant material [5]. The caffeine content in white tea (0.71 %) was higher related to the black tea samples.

According to the results from the scientific paper [16] the content of caffeine in "Darjeeling Green tea" is about 2.92 % while in the scientific paper [17] the yield of caffeine in the green tea is 0.65 % which are slightly different from the results that we have obtained in our paper work, the yield of caffeine is 2.04 % in the green tea from the grocery store for health nutrition and in the tea from the green market is 0.06 %. The results obtained from Ashihara and Kubota (1986) found that caffeine biosynthesis is the most active in young tea leaves and buds [18]. The displayed results confirmed that the caffeine content present in our samples can be as a result of many factors such as the tea is from different places, different growing and climatic conditions, soil type, genetic and environmental variability, harvest time and processes involved in the production of tea etc. [19]. In our results is confirmed the highest caffeine content in green tea from the grocery store for health nutrition, and black tea from the green market (4) from 2010 and (5) from 2020, the most processed tea type, contains the lowest caffeine content. In results obtained by Komes *et al.* (2009), it was found that the method with lead(II) acetate exhibited significantly low results in comparison with the rest of the methods that were applied for the determination of caffeine [5].

The pH values were in the range from 4.85 to 5.80. The obtained results showed that the medium is slightly acids. It was found that only for the black tea bought in 2020 the pH is the lowest in comparison to the other tea samples where the pH values were ranged between 5.19 and 5.80. According to the Bhattarai *et al.* (2019), the pH value of the green tea is 6.663 [17], while in our results the pH value for the green tea from the grocery store for health nutrition and green market is 5.80 and 5.19. In the scientific paper [1] the R_f value of the green tea with the mobile phase chloroform: acetone: methanol (1:1:1, v/v/v) is found to be 0.53. In the study paper [6], a TLC plate was developed using chloroform as the mobile phase and the R_f value was measured and it was found to be 0.63 in green and black tea and the retention factors (R_f) were given in Table 2. It was found that for the obtained results for the determination of caffeine content in samples of tea related to the standard of caffeine, it was a pure substance according to the determined R_f values in both types of mobile phases.

The R_f values were 0.36 and 0.86 depending on the use of the mobile phase, either the first one or the second one, respectively. The retention factor values also depended on the solvent where the samples were dissolved either methanol or dichloromethane. From the results of paper [16], the R_f value of the green tea is 0.55, this result is obtained when is used the mobile phase of ethyl acetate: acetic acid (19:1, v/v) with the distance travelled by the solvent and by the solute 4.5 cm and 2.5 cm.

3.1. High performance liquid chromatography (HPLC)

We used HPLC method to determine the retention time and the relative peak area of extracted caffeine from all the tea samples studied in this work. From the HPLC chromatograms that are shown in Figures 2-7 and from Table 3, we can conclude that the retention time of extracted caffeine from all the tea is the same with the retention time of the standard of caffeine, which confirms the identity of caffeine.

4. Conclusion

The main goal of our study was the isolation of caffeine from different tea. We were focused on filtration, extraction and isolation of caffeine from different tea and the content of isolated caffeine.

Table 3. Retention time for the standard of caffeine and different tea samples.

No	Sample	Retention time (min)
1	Green tea from the grocery store for health nutrition	2.40
2	Green tea from the green market	2.40
3	White tea from local market	2.40
4	Black tea from the green market from 2010	2.40
5	Black tea from the green market from 2020	2.40
-	Standard of caffeine	2.40

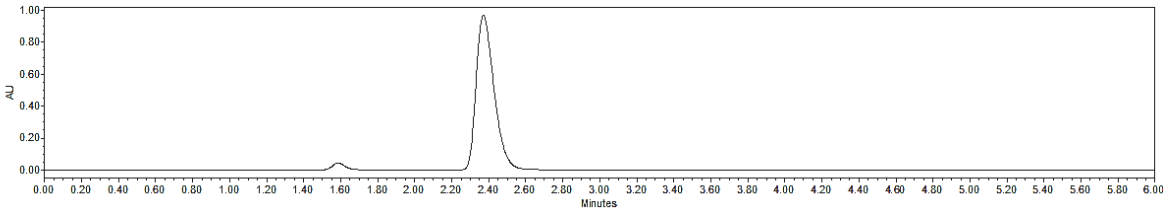

Figure 2. HPLC chromatogram of standard caffeine.

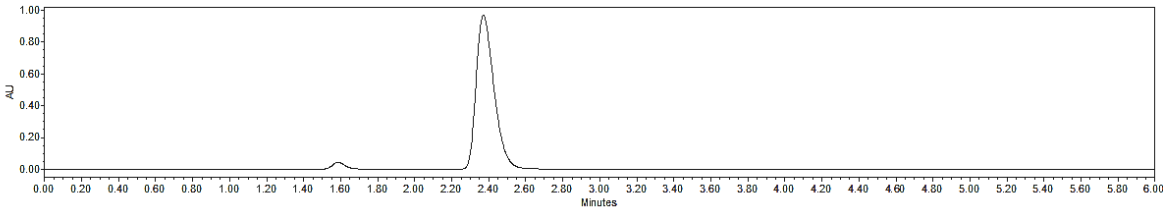

Figure 3. HPLC chromatogram of caffeine isolated from green tea from the grocery store for health nutrition.

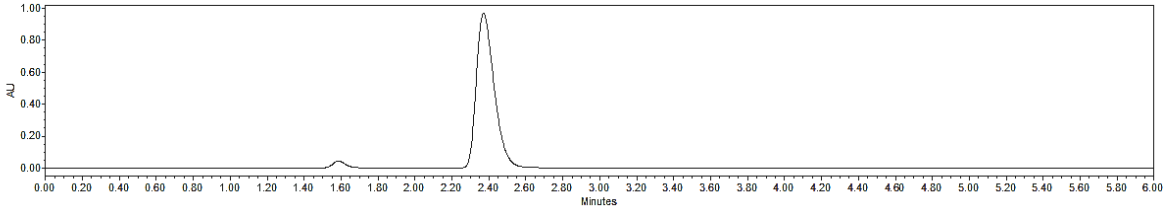

Figure 4. HPLC chromatogram of caffeine isolated from green tea from the green market.

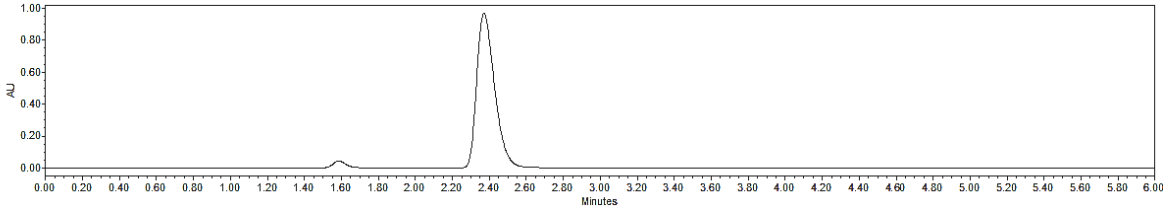

Figure 5. HPLC chromatogram of caffeine isolated from white tea from local market.

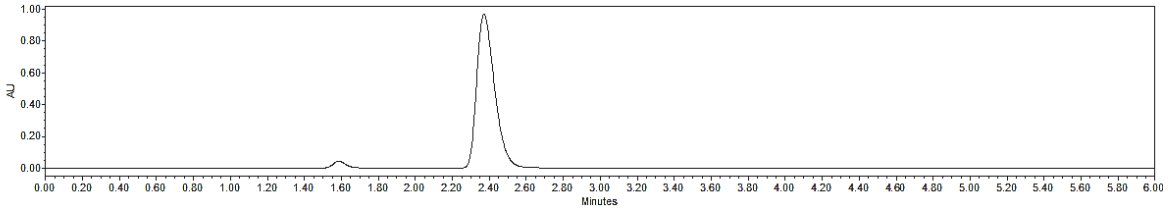

Figure 6. HPLC Chromatogram of caffeine isolated from black tea from the green market from 2010.

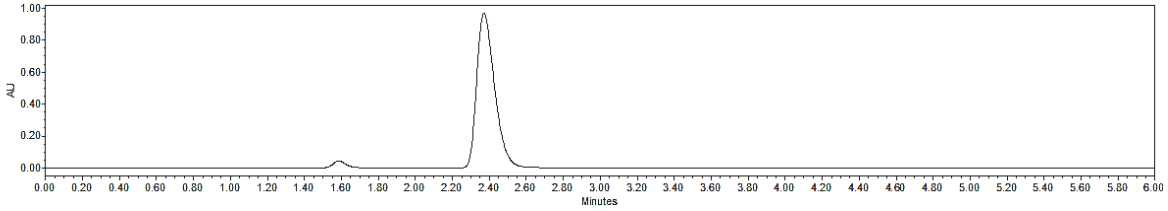

Figure 7. HPLC Chromatogram of caffeine isolated from black tea from the green market from 2020.

We used different techniques for identification of caffeine such as pH determination, TLC, and HPLC. The developed TLC method was successfully applied for the determination of caffeine in tea samples. The method is easy to handle and also can be used in the routine analysis for the determination of caffeine content in samples of green, black, and white tea. The percentage of caffeine depends on the tea type, which is attributed to their processing manner and condition of storing. All the results obtained were compared and perfectly matched with the result of standard of caffeine.

Disclosure statement

All work and the result presented in this study were done in Mother Teresa University-Skopje.
Conflict of interests: The authors declare that they have no conflict of interest.
Author contributions: All authors contributed equally to this work.
Ethical approval: All ethical guidelines have been adhered.
Sample availability: Samples of the compounds are available from the author.

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