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COMPARATIVE ANALYSIS OF THE CAFFEINE CONTENT OF TEA DETERMINED WITH HPLC-MS/MS USING DIFFERENT EXTRACTION METHODS

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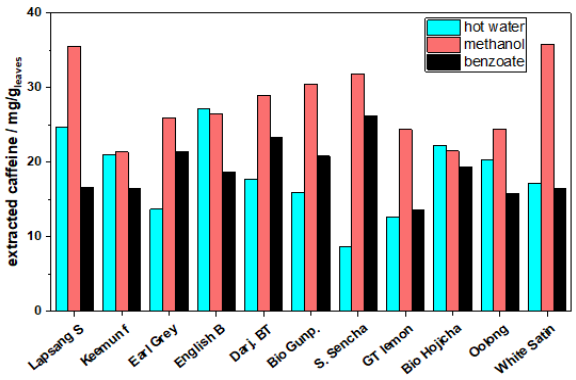
The effectiveness of three different caffeine extraction methods were used on commercially available tea types in Romania were compared. An HPLC-MS/MS system was used to perform the separation of the caffeine from matrix components, and the quantitative analysis. To improve the precision of the analysis, pentoxifylline was used as a novel internal standard in the process. The simple hot water extraction was compared to an organic solvent and a sodium benzoate-based eco-friendly method. Although all three methods extracted a significant amount of caffeine, the methanol-based process generally showed the best results, where extracted caffeine content varied between 21 and 36 mg/g of dried tea leaves. It was also concluded that the amount of extracted caffeine can differ greatly between methods with no clear tendency in their effectiveness, pointing to a significant effect from the physical characteristics of the tea leaves such as size, granulation and packaging style.



INTRODUCTION

Tea is the most consumed beverage in the world. It is brewed from the leaves and leaf buds of the evergreen shrub *Camellia sinensis.* The climate and annual variables (temperature fluctuations, precipitation, etc.) of the cultivation site have a significant effect on the flavor and aspect of the final brew.[1] Nevertheless, the aroma profile of the brew is mostly determined by the preparation method of the tea leaves used for the brew.[2] As such, the degree of fermentation determines whether the leaves become white, green, oolong or black tea.[3] The main types of tea are also used to produce flavored teas, such as Earl Grey which is black tea flavored with bergamot,[4] and specialty teas, such as Hojicha, which is roasted green tea.[5]

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Due to the presence of multiple bioactive compounds, tea consumption can have beneficial effects on the cardiovascular system,[6,7] as well as an anti-aging effect from the significant antioxidant content in the beverage.[8,9] Besides the taste, the main reason for tea consumption is the effect of the caffeine present in the leaves. Caffeine is a central nervous system stimulant,[10] it acts as an adenosine receptor antagonist.[11] It can be consumed from different sources, such as coffee or tea.

The effect of caffeine on individuals can differ greatly.[12] Some people also have a sensitivity to caffeine which severely limits the amount of tea they can consume. Additionally, caffeine consumption in the evening or night can result in the disruption of the circadian rhythm, leading to short term insomnia.[11] Due to these facts, the extraction of caffeine from tea has great importance and decaffeinated tea is projected to have a significantly increased market in the future,[13] rivaling that of naturally caffeine-free products.

Caffeine has a very high solubility in hot water, however, this is severely reduced at room temperature. Consequently, caffeine is usually extracted using organic solvents or CO_2.[14] While these methods are highly effective, the use of organic solvents is a negative aspect as they can be harmful to nature, while supercritical carbon dioxide extraction has a higher cost due to the need for specialized equipment. In this context, other methods should also be considered to develop an eco-friendly, cost-efficient extraction process.[14]

Sodium benzoate can form a stable complex with caffeine[15] and thus, could be used for caffeine extraction.

The goal of this work is twofold. On one hand it focuses on comparing the effectiveness of different caffeine extraction methods on solid tea samples that are widely available in the region. On the other hand, our goal was to validate the use of pentoxifylline as a novel internal standard during analytical process.

To determine the caffeine content of a beverage meant for human consumption and as a reference, the first method used was a simple extraction with hot water. The second method was based on methanol, an organic solvent in which caffeine shows good solubility.[16] The third method tested was an eco-friendly technique using sodium benzoate solution, which omits any organic solvents.

The separation of the caffeine from other species present in the tea extracts[17] and the quantitative analysis were realized using an HPLC-MS/MS system. Pentoxifylline was used as an internal standard to improve precision due to its similarity to the caffeine molecule, suggesting a similar fragmentation in mass spectrometry.[18]

The novelty of the work consists of the sodium benzoate-based method for caffeine extraction, as well as the comparison between the three different extraction processes. Additionally, to the best of our knowledge, no other works were published where pentoxifylline was used as an internal standard to analyze tea extracts using an HPLC-MS/MS system.

It should also be noted that all the studied tea types can be acquired in Roumania, so the study also has significance for the local consumers.

RESULTS AND DISCUSSION

HPLC-MS/MS measurement parameters

In the case of the hot water extraction, HPLC measurements (Fig. 1) show a retention time of 0.491 ± 0.006 min for caffeine. This was increased for pentoxifylline to 0.743 ± 0.017. In MS, the ratio between the quantifier ion and the qualifier ion signals (*i.e.* the area ratio of their respective signals expressed in percentages) was 25.799 ± 0.478 for caffeine and 41.438 ± 0.236 for pentoxifylline, respectively. These small deviations point to a good reproducibility of the measurement. The HPLC-MS/MS calibration curve for hot water extraction (Fig. 1D) obtained from the caffeine standards can be described using the following second-degree polynomial equation with $R^2 = 0.9994$:

$$\textbf{signal ratio} = -0.0097 \times c^2 + 0.5055 \times c + 0.1735.$$

Similarly, in the case of the second calibration (Fig. 1E), used to determine the caffeine content of analytical samples obtained from methanol and benzoate-based extractions, respectively, a retention time of 0.442 ± 0.00049 min was noted for caffeine and 0.633 ± 0.00155 min for pentoxifylline. The quantifier ion and the qualifier ion signal ratio was 21.97 ± 0.146 for caffeine and 39.120 ± 0.118 for pentoxifylline, respectively, signifying a good measurement reproducibility and instrument stability. The calibration curve can be described by the following equation with $R^2 = 0.9992$:

$$\textbf{signal ratio} = -0.00257 \times c^2 + 0.4239 \times c + 0.0233.$$

It should be noted that a first-degree polynomial fit was also tested, however, the R^2 values were

lower in this case, 0.9957 and 0.9992, respectively, as such, the second-degree polynomial fit was used for calibration. Similarly the sum of squared errors was lower for the second degree fitting than the first degree fitting, namely, 0.0086 and 0.0105 compared to 0.0516 and 0.014, respectively.

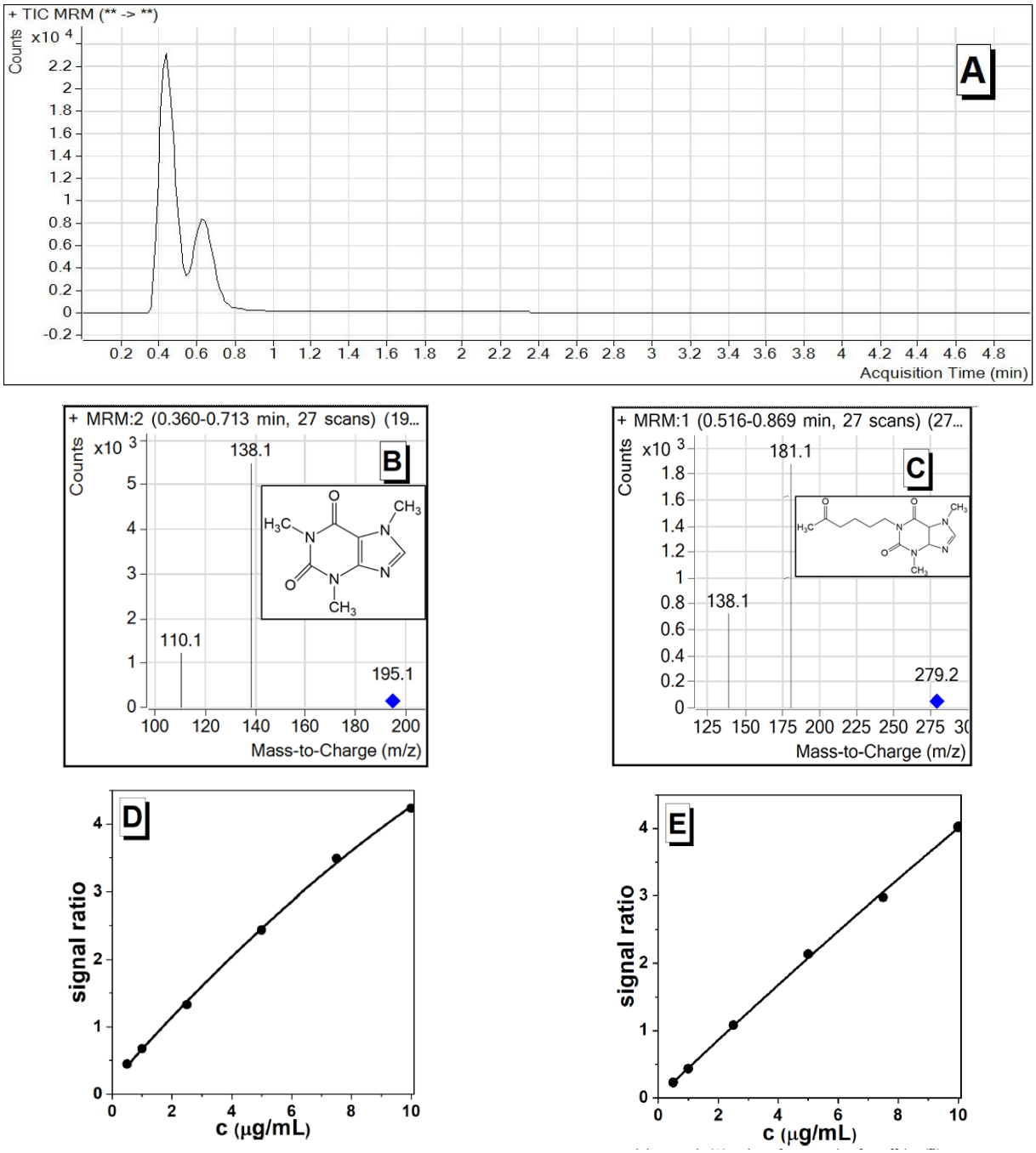

Fig. 1 – Example of a chromatogram obtained for a caffeine-containing sample (**A**) and MS fragmentation for caffeine (**B**) and pentoxifylline (**C**). MS calibration curve used for the quantitative analysis of caffeine-containing samples obtained with hot water extraction (**D**) and methanol/benzoate-based extractions (**E**)

Analytical features

The limit of detection (LOD) was calculated as the ratio of three times the standard deviation of the blank sample with respect to the slope of the calibration curves. The average value of 1.28 ng/mL proved to be significantly lower than the caffeine content of any studied sample. In addition, the average signal to noise ratio was 59.8 during the analysis. The precision of the method was assessed through the relative standard deviation (RSD) calculated for three successive determinations using the same sample. The

average RSD value was 1.76% points to a good reproducibility of the measurement.

Comparative analysis of different extraction methods

The extracted amount of caffeine in the case of black tea samples (Fig. 2 and Table 1) varies between tea types, but also between different extraction methods used for the same type of tea. In all cases, the methanol-based extraction shows the highest amounts of caffeine. It should also be noted that the hot water extraction is quite effective as it yielded basically the same amount of caffeine for both Earl Grey and Lapsang Souchong teas as extraction with the organic solvent.

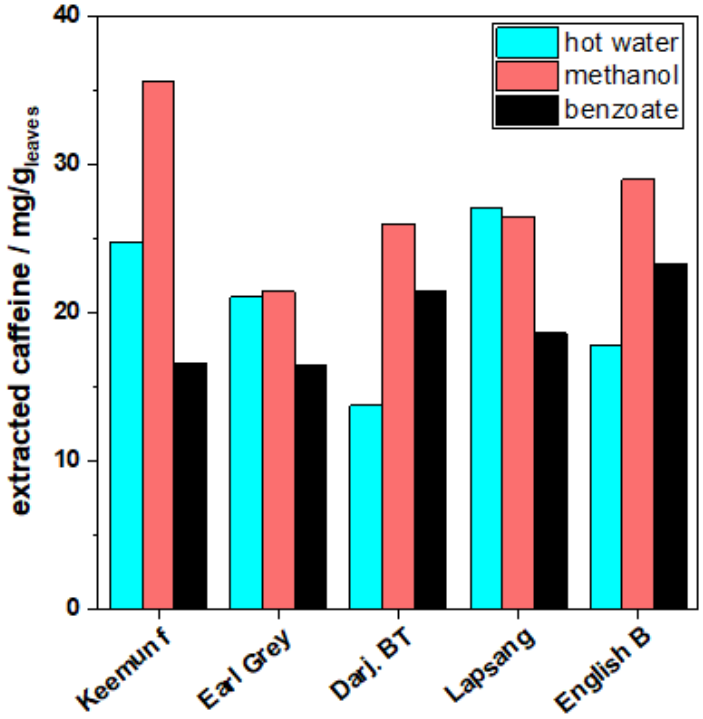

Fig. 2 – Comparative analysis of the caffeine content of black tea extracts, extracted with three different methods.

Table 1

Caffeine content of black tea extracts expressed in mg/g solids

	Tea type				
Extraction method	Keemun f.	Earl Grey	Darj. BT	Lapsang	English B
Hot water	24.73	21.04	13.70	27.09	17.74
Methanol	35.55	21.37	25.92	26.42	28.97
Benzoate	16.56	16.44	21.37	18.61	23.28

When comparing green tea samples (Fig. 3 and Table 2), it can be noted that, similarly to the case of black teas, the methanol-based extraction proved to be most effective, however, in this case, the hot water extraction in much less efficient for 3 of the 5 samples (Gunpowder, Green Tea Lemon and Sencha) than either the sodium benzoate or the methanol extraction. One possible explanation would be the fact that due to no oxidation, and a shorter processing time, green tea leaves are generally more integral, and thus have a smaller surface area, which can affect the results in the case of the shorter hot water brewing process. In contrast, for a longer extraction process (*i.e.* methanol and benzoate), the leaves have time to open up and swell, increasing surface area, and resulting in higher yields. The high amount of caffeine extracted with methanol compared to hot water from the white tea also points to this conclusion.

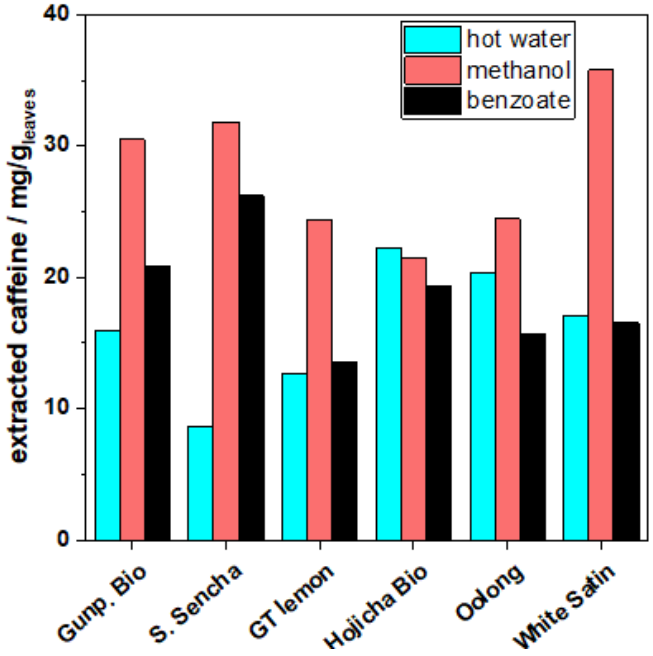

Fig. 3 – Comparative analysis of the caffeine content of green, oolong and white tea samples extracted with three different methods.

Table 2

Caffeine content of green, oolong and white tea extracts, expressed in mg/g solids

	Tea type					
Extraction method	Gunp. Bio	S. Sencha	GT Lemon	Hojicha Bio	Oolong	White Satin
Hot water	15.94	8.67	12.65	22.24	20.35	17.12
Methanol	30.48	31.83	24.37	21.48	24.43	35.79
Benzoate	20.80	26.21	13.59	19.34	15.72	16.53

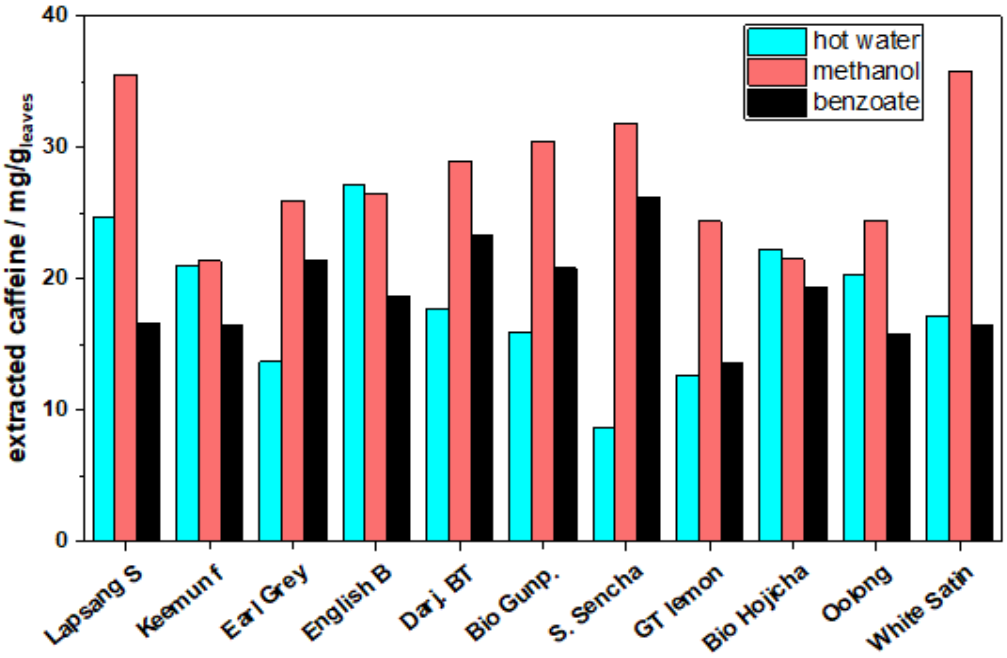

Fig. 4 – Comparative analysis of the caffeine content of black, green, oolong, and white tea samples extracted with three different methods.

When the results for black, green, oolong and white tea are compiled (Fig. 4), it can be observed, that the maximum amount of caffeine extracted from all tea types is similar. In contrast, when comparing results for hot water extraction, as expected, black tea yields higher amounts of caffeine with a medium extracted quantity of 20.86 mg/g compared to 14.88 mg/g in green teas. This difference of *ca.* 25% is similar to what other studies observed for the difference in mean caffeine amount[19] between black and green teas. It should be noted, however, that the amount of caffeine can deviate significantly compared to the mean amount in the case of individual tea types.

EXPERIMENTAL

Materials

The caffeine reference standard and the sodium benzoate were purchased from Merck, the pentoxifylline secondary reference standard from AK scientific. Methanol, acetonitrile, formic acid, HPLC grade water were purchased from VWR. All reagents were of analytical grade and were used without any further purification steps.

All types of tea were purchased locally in Romania. The comparative analysis includes tea leaves that have a different degree of fermentation. As such, black, green, oolong and white teas were studied. This includes 5 types of black tea, namely Earl Grey from Teavalley, Darjeeling Black Tea from Mount Himalaya Tea, Bio Lapsang Souchong and China Keemmun Finest from Demmers Teehaus, and English Breakfast from Lipton; 5 types of green tea, namely Spectacular Sencha from Lipton, Green Tea Lemon from Teavalley, and Hojicha Bio Green Tea and Bio Gunpowder from Demmers Teehaus. We also tested Formosa Dong Ding Green oolong tea and White Satin tea, both from Demmers Teehaus.

Sample preparation

Three different types of extraction were used. One method was based on conventional tea brewing techniques with hot water, the second method was based on an organic solvent (methanol) that extracts caffeine with high efficiency, while the third extraction method used a sodium benzoate solution. For the sake of a better comparison between the techniques, as well as to obtain data on beverages meant for consumption, the tea leaves were used as packaged, *i.e.* were not ground to improve extraction efficiency.

Initially, the caffeine was extracted from the dried tea leaves. Following stabilization, the resulting solutions will be named *initial extracts*. For the hot water extraction, 100 mL of boiled water was poured on 1 gram of tea leaves for 6 minutes, and mixed. A stable solution was obtained by adding 1000 µL of methanol to 1000 µL of the extract to prevent any precipitation of the extract. The eco-friendly extraction method using sodium benzoate was realized by pouring 25 mL of a 0.1 w/w% sodium benzoate solution on 0.1 g of tea leaves. The resulting mixture was sonicated for 60 minutes at 30ºC to extract the caffeine from the tea samples into the initial extracts. The methanol-based extraction was realized in a similar manner to the benzoate solution, however, in this case, 25 mL of 50 V/V% methanol was poured on 0.1 g of tea leaves.

The thus-prepared initial extracts were used to make analytical samples with a volume of 1000 µL for the HPLC-MS/MS analysis. For this, 10 µL of each extract was diluted by adding 25 µL of acetonitrile (which contained 10 V/V% formic acid to facilitate the ionization of the caffeine) and 965 µL of an aqueous acetonitrile solution to obtain a final volume of 1000 µL and a water:acetonitrile ratio of 3:1. The pentoxifylline internal standard was also added to the solution to reach a final concentration of 5 µg/mL in the 1000 µL analytical sample.

Parameters of the HPLC-MS/MS analysis

The analytical samples were studied using an Agilent 1200 series HPLC system coupled to an Agilent 6410B triple quad mass spectrometer.

This consisted of a reversed-phase liquid chromatography separation step using a Kinetex 2.6 µm C18-100 Å 50X2.10 mm column from Phenomenex, with a water:acetonitrile mixture (ratio of 3:1), also containing 0.1% formic acid to sustain the ionization, as the mobile phase; as well as a MS/MS step, utilizing an electron spray ion source operated in positive mode, at 350 ºC, a capillary voltage of 4 kV and a nitrogen flow of 12 L/min with a pressure of 40 psi. To obtain the necessary quantitative data, the spectrometer was used in multiple reaction monitoring (MRM) mode.

The MS setup contained three quadrupole units. The initial one only allowed the caffeine (m/z = 195.1) and pentoxifylline (m/z=279.1) pseudomolecular ions to pass. In the second quadrupole (collision cell), fragmentation occurs. The caffeine molecule yields two main fragments with m/z ratios of 138.1 and 110.1, respectively. The pentoxifylline yields fragments with 181.1 and 138.1, respectively. The fragment with the higher intensity is considered the quantifier ion, while the lower intensity one is considered the qualifier ion (Fig. 1). For the determination to be reproducible, the ratio between these two ions should be constant. At the third quadrupole unit, only selected fragments pass towards the detector.

The setup was calibrated using caffeine reference solutions with concentrations of 0.5; 1; 2.5; 5; 7.5 and 10 µg/mL.

To improve precision, pentoxifylline was added to all calibrators and samples as an internal standard, to reach a final concentration of 5 µg/mL. It was chosen due to its similar structure to caffeine, resulting in similar behavior.

The 1000 µL analytical samples obtained from tea extraction were introduced into the system. An amount of 10 µL was injected for each measurement. The HPLC-MS/MS output values (retention time, fragment intensity ratio and calibration curve) were determined separately in the case of the hot water extraction and the methanol/benzoate extraction. Results were normalized to show the total obtained caffeine content for 1 gram of solid tea in all cases.

The flow of the mobile phase was 0.45 mL/min. The temperature during the chromatography step was 25°C. After the studied molecules passed through the HPLC column, the eluent flux was maintained for a further 5 minutes to remove any possible residue.

CONCLUSION

All three methods were effective in extracting significant amounts of caffeine from tea samples. The novel, sodium benzoate-based method was less effective than using an organic solvent. Using pentoxifylline as an internal standard proved to be a simple and efficient method when improving the precision of the quantitative analysis of caffeine with mass spectrometry.

REFERENCES

1. S. Ahmed, J. R. Stepp, C. Orians, T. Griffin, C. Matyas, A. Robbat, S. Cash, D. Xue, C. Long, U. Unachukwu, S. Buckley, D. Small, E. Kennelly, *Plos One*, **2014**, *9*, e109126.
2. C.-T. Ho, X. Zheng, S. Li, *Food Sci. Hum. Wellness*, **2015**, *4*, 9–27.
3. Y. Kim, K. L. Goodner, J.-D. Park, J. Choi, S. T. Talcott, *Food Chem.*, **2011**, *129*, 1331–1342.
4. A.-M. Orth, I. Poplaceam, O. Fastowski, K.-H. Engel, *Food Addit. Contam. A*, **2014**, *31*, 207–217.
5. H. Fujiki, *Chem. Rec.*, **2005**, *5*, 119–132.
6. N. Khan, H. Mukhtar, *Curr. Pharm. Des.*, **2013**, *19*, 6141–6147.
7. A. Deka, J. A. Vita, *Pharmacol. Res.*, **2011,** *64*, 136–145.
8. N. Muntean, G. Szabó, *Stud. U. Babes-Bol. Che.*, **2013**, *LVII*, 175–183.
9. Q. Li, J. Li, S. Liu, J. Huang, H. Lin, K. Wang, X. Cheng, Z. Liu, *Int. J. Mol. Sci.*, **2015**, *16*, 14007–14038.
10. K. Cakir, C. Erkmen, B. Uslu, N. G. Goger, *Rev. Roum. Chim.*, **2019**, *64*, 801–808.
11. J. V. Rétey; M. Adam, R. Khatami, U. F. O. Luhmann, H. H. Jung, W. Berger, H.-P. Landolt, *Clin. Pharmacol. Ther.*, **2007**, *81*, 692–698.
12. A Goldstein, R. Warren, S. Kaizer, *J. Pharmacol. Exp. Ther.*, **1965**, *149*, 156–159.
13. Report on the decaffeinated tea markert, *Fortune Business Insights*, **2022** (accessed 24.08.2022).
14. K. Ramalakshmi, B. Raghavan, *Crit. Rev. Food Sci.*, **1999**, *39*, 441–456.
15. T. Higuchi, D. A. Zuck, *J. Am. Pharm. Assoc. Sci.*, **1952**, *41*, 10–13
16. J. Zhong, N. Tang, B. Asadzadeh, W. Yan, *J. Chem. Eng. Data*, **2017**, *62*, 2570–2577.
17. S. P. J. N. Senanayake, *J. Funct. Foods*, **2013**, *5*, 1529–1541.
18. https://massbank.eu/, caffeine and pentoxifylline data records (accessed 24.08.2022).
19. C. Astill, M. R. Birch, C. Dacombe, P. G. Humphrey, P. T. Martin, *J. Agr. Food Chem.*, **2001**, *49*, 5340–5347.