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Scientific paper

Analytical Method Development and Validation with Greenness Assessment of HPLC-DAD Method to Determine Methylxanthines

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Abstract

In this study, a simple, and eco-friendly high-performance liquid chromatography-diode array detection (HPLC-DAD) method was developed and validated for analyzing methylxanthines, including theobromine (TB), theophylline (TF), and caffeine (CF). This method demonstrated excellent performance using an isocratic mobile phase of water (85%) and ethanol (15%) at 0.1 mL min^{-1}, 25 °C in a core–shell Kinetex® C18 column. The method was linear (1–100 μg mL^{-1}), exhibiting high precision and accuracy with relative standard deviations below 2.67% and recoveries ranging from 98.15% to 108.88%. The detection and quantification limits ranged from 0.19–0.26 μg mL^{-1} and 0.64–0.87 μg mL^{-1}, respectively. We applied this method to analyze powdered lemon-flavored beverages enriched with black or green tea extracts. CF was detected exceeding 1.62 g kg^{-1}. The proposed method showed excellent greenness, evidenced by the Analytical Eco-Scale, Analytical GREEnness metric (AGREE) calculator, and GAPI.

Keywords: Green HPLC, methylxanthines, ethanol, core–shell particles, ecological metrics.

1. Introduction

Currently, the main objectives of chemistry are to develop and use cost-effective methodologies that respect the environment and analysts. In this sense, Green Chemistry (GC) has emerged with the intention of reducing the use of hazardous reagents, eliminating or decreasing the generation of toxic wastes, and introducing renewable resources.[1] Based on the principles of GC, various authors in analytical laboratories have created the concept of Green Analytical Chemistry (GAC).[2,3] The principal purposes of GAC are to achieve the reduction of reagents, wastes, and energy consumption; to guarantee safety for operators; and, on the other hand, to promote the use of automated and miniaturized processes with minimal sample treatment, and the development of multianalyte determinations.[4]

Within GAC and with respect to liquid chromatography (LC), efforts have focused on reducing and replacing toxic solvents and additives in the mobile phase, miniaturizing samples, and miniaturizing columns, and developing new technologies for stationary phases or packaging materials.[5,6] Most mobile phases in reversed-phase liquid chromatography (RP-LC) consist of a mixture of water (often with additives to adjust pH and ionic strength) and organic solvents, such as acetonitrile (ACN) and methanol (MeOH). However, ACN and MeOH are toxic to the environment and adversely affect human health, making waste disposal costly.[7–9]

One option to make LC greener is to replace traditional solvents with less hazardous ones, such as ethanol (EtOH). Compared to ACN and MeOH, EtOH is less toxic and more environmentally friendly due to its bio-sourcing. Additionally, EtOH has a lower vapor pressure, which leads to less evaporation and, consequently, lower amounts of solvent inhaled by analysts.[10,11] In recent years, ethanol, as a component of mobile phases, has been used in the analysis of various pharmaceutical products,[12–14] antioxidants,[15] and even pesticides,[16] among other compounds.

Another way to make LC eco-friendly and minimize solvent consumption is by modifying column parameters, such as reducing the column length, decreasing the internal diameter, reducing the particle size of the packed material, and/or replacing the type of packed material.[17,18] These changes represent a compromise between analysis time, solvent consumption, and the care and performance of the LC system. Depending on the parameters, desired effects such as reduced solvent consumption and improved chromatographic efficiency, or unwanted effects such as low resolution and increased back pressure, can be generated in the analysis.[19,20]

An interesting alternative to columns packed with fully porous particles is the use of a solid silica particle core covered with layers of porous silica (core–shell particles).[21] The presence of core–shell particles (superficially porous) reduces peak broadening due to longitudinal diffusion and dramatically improves column permeability due to fast mass transfer, which translates into less equilibrium time and total analysis time, and therefore a reduction in solvent use. Furthermore, these columns can be used in conventional LC systems.[22,23]

On the other hand, the methylxanthines caffeine, theobromine, and theophylline are alkaloids from plant secondary metabolism and are naturally present in coffee beans, cocoa seeds, and tea leaves; therefore, they are also found in foods and beverages made from them.[24,25] The consumption of methylxanthines is widespread worldwide. Various pharmacological activities have been attributed to these compounds. Caffeine stimulates the central nervous system, skeletal muscles, and respiratory system, and can induce addiction and anxiety. Theobromine and theophylline are used as bronchodilators, vasodilators, and mild muscle relaxants.[26] Despite this, data on the content of methylxanthines in commercially available foods and beverages in Mexico are scarce or nonexistent.

High consumption of foods containing methylxanthines can increase the daily intake of these stimulant substances and consequently amplify their desired or undesired effects on the human body. Therefore, we believe it is important to report the concentrations of methylxanthines on the nutritional labels of food products to facilitate better decision-making when consuming these foods and beverages. Although the number of published papers on green chromatography approaches is constantly increasing, these methods have not been widely implemented for routine analyses. Hence, this study aimed to develop and validate an eco-friendly high-performance liquid chromatography (HPLC) method for determining methylxanthines in powders used to prepare beverages enriched with black or green tea extracts. The method was designed using strategies aligned with the principles of GAC, and its green characteristics were evaluated using assessment tools such as the Analytical Eco-Scale, AGREE calculator, and GAPI.

2. Experimental

2. 1. Chemicals and Reagents

The standards of methylxanthines: caffeine (CF, Reagent Plus®), theobromine (TB, 99%), and theophylline (TF, 99%), were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC-grade ethanol was obtained from J. T. Baker (Phillipsburg, NJ, USA). Deionized water was sourced using a Pure Lab UHQ II Elga water purification system from Veolia Water (Paris, FRA).

2. 2. Preparation of Standard and Working Solutions

Individual stock solutions of methylxanthines were prepared by dissolving an appropriate amount of each compound to achieve concentrations of 500.00 μg mL^{-1} for CF and TF in ethanol, while TB was prepared at 200.00 μg mL^{-1} in deionized water. All stock solutions were stored at 4 °C in the dark until use. Working standard solutions were prepared in a mixture of deionized water and EtOH at a ratio of 85:15.

2. 3. Instrument and Chromatographic Conditions

HPLC analysis was performed using a Waters Alliance 2695 separation module (Waters Corporation, Milford, MA, USA) equipped with a quaternary pump, autosampler, column oven, and a Waters 2998 photodiode array detector (DAD). This system was controlled using Empower 3 software for data collection and acquisition. The target compounds were separated on a Kinetex® C18 reversed-phase column (150 × 3 mm, 2.6 μm) from Phenomenex (Torrance, CA, USA). The optimal mobile phase was composed of deionized water and ethanol, with isocratic elution profiles of 85% and 15%, respectively. The mobile phase was filtered using a vacuum pump filtration system with a 0.45 μm Nylon membrane from Merck Millipore (Burlington, MA, USA) and degassed in an ultrasonic bath, Bransonic 3510, from Branson Ultrasonics (Brookfield, CT, USA) daily before use. The flow rate was set at 0.1 mL min^{-1}, the injection volume was 3 μL, and the column temperature was maintained at 25 °C. The detection wavelength was 273 nm for all analytes. Analytical runs lasted 20 min, and all injections were performed in triplicate.

2. 4. Analytical Performance

To verify the suitability of the chromatographic system for target molecules, a system suitability test was performed by triplicate injections of standard solutions of CF, TF, and TB (50.00 μg mL^{-1} in 85% water – 15% ethanol) were analyzed under the optimal chromatographic conditions. Then, the relative standard deviation (*%RSD*) of the

data obtained from different parameters such as retention time (t_R), resolution (R_s), retention factor (k), and number of theoretical plates (N) of the methylxanthines were calculated.

The proposed method was validated to demonstrate its suitability for selected applications by evaluating parameters such as linearity, selectivity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ), following the recommendations of the EURACHEM[27] and ICH[28] guidelines. To evaluate linearity, external standard calibration curves were constructed by analyzing standard solutions containing CF, TF, and TB at concentrations of 1.00, 5.00, 25.00, 50.00, 75.00, and 100.00 μg mL^{-1}, by triplicate. Standard solutions were prepared using a mobile phase composed of deionized water and ethanol at a ratio of 85:15. Linear regression analysis was performed, the equations of the lines were obtained, and the correlation and determination coefficients were calculated.

Selectivity was assessed by investigating potential endogenous interference in the mobile phase, samples, and blank samples at the retention times of the chromatographic peaks of the target analytes. The chromatograms of the blank samples, samples, and standards were compared by matching the retention times and UV spectra of the chromatographic peaks, which were obtained using a diode array detector (DAD).

Precision was estimated by conducting triplicate analyses of blank samples spiked at known low, medium, and high concentrations. The blank samples were spiked to 2.00, 40.00, and 80.00 μg mL^{-1} for the three target analytes. The *%RSD* of the sample concentrations was used as a measure of precision.

In the first step, the system accuracy was evaluated using a back-calculation process for each standard on the calibration curve with a linear equation. The correlation between nominal known concentrations of the standards and calculated concentrations was assessed through regression analysis. In another step, recovery assays were conducted for the target analytes by spiking blank samples with standards at the same concentration levels that were evaluated for precision. Therefore, the method accuracy is expressed in terms of the recovery percentage (*%R*).

The LOD and LOQ values for each compound were determined at signal-to-noise (*S/N*) ratios of 3 and 10, respectively.

To assess robustness, standard solutions of CF, TF, and TB (50 μg mL^{-1} in 85% water – 15% ethanol) were prepared. A Design of Experiments (DoE) approach was implemented using a Plackett-Burman model without central points or replicates, and factors of design corresponding to small changes in the chromatographic conditions, including the organic solvent proportion in the mobile phase (± 2%), the flow rate (± 0.01 mL min^{-1}), and the column temperature (+27 °C) were evaluated.

2. 5. Method Applicability and Sample Preparation

To evaluate the applicability of the proposed method, four commercially available samples from Monterrey, Nuevo León, Mexico were selected. The samples included two types of powder used to prepare lemon-flavored beverages enriched with black tea extract (BRAND 1 BT and BRAND 2 BT) and two types of powder used to prepare lemon-flavored beverages enriched with green tea extract (BRAND 1 GT and BRAND 2 GT). A powder for preparing lemon-flavored beverages without black or green tea extract was used as a BLANK SAMPLE. For each sample, 0.2 g was weighed and diluted to 10 mL using the mobile phase. The samples were then stirred in a vortex for one minute and, before injection into the HPLC system, were filtered using a 0.2 mm nylon acrodisc from PALL (Port Washington, NY, USA) and placed in amber glass vials. All analyses were performed in triplicate.

2. 6. Greenness Evaluation of the Developed Method

To assess the greenness profile of the developed HPLC method, three different GAC tools were used to assist in the qualitative and quantitative evaluation of the method's environmental friendliness. The Analytical Eco-Scale (AES), Green Analytical Procedure Index (GAPI), and Analytical GREEnness metric (AGREE) were utilized.

3. Results and Discussion

3. 1. Optimization of Chromatographic Separation

Numerous high-performance liquid chromatography (HPLC) methods have been developed to analyze methylxanthines in various matrices. Most of these methods use harmful solvents and chemical substances, and a significant amount of time is invested.[25,29,30] To develop the simplest, fastest, and most environmentally friendly method, we decided to use only mixtures of ethanol and water as the mobile phase components. We utilized a column with core–shell particle technology for the separation of the target compounds. By adhering to GAC principles, the use of ethanol in RP-HPLC minimizes negative environmental impacts and safety issues for analysts.[11] Furthermore, the core–shell particle column allows for a shorter run time with reduced solvent usage.[21] Aquel et al.[29] and Baek et al.,[30] report the analysis of methylxanthines by HPLC in a time of 30 min and 25 min, respectively, which are superior to the reported in this work, and also, with the generation of large volumes of waste per analytical run > 15 mL, in contrast with the generated on this work of only 2 mL. On the other hand, other published

papers report the analysis of the same molecules in shorter times between 1.7 min and 15 min, however, in all cases the residues generated were higher than those generated in the present work.[25,31–33] The target compounds, theobromine, theophylline, and caffeine are structurally very similar and have similar octanol-water partition coefficients (Log P): –0.7, –0.2, and –0.1, respectively.[34] Therefore, it was necessary to select the best composition of the mobile phase for their separation.

To confirm the retention times of the target molecules, injections of each standard were made at a concentration of 10 μg mL^{-1} under all test conditions. In all experiments, the three molecules were monitored at 273 nm because the DAD detector showed that this wavelength provided the greatest response for the three molecules.

Initially, a mobile phase composed of 30% ethanol and 70% water at a flow rate of 0.2 mL min^{-1} at 25 °C was chosen; however, this led to a considerable increase in the back pressure of the HPLC system. Subsequently, the flow rate was reduced to 0.15 mL min^{-1}, but an adequate reduction in back pressure was not achieved. The high back pressure may be due to one of the main disadvantages of using ethanol in HPLC: its high viscosity, even in mixtures with water, which can increase the back pressure in the chromatographic system.[7,35] Additionally, this aligns with reports from some authors that the back pressure generated by the core–shell column is slightly higher than that of totally porous particle-packed columns.[36,37]

It is known that mobile phase viscosity generally decreases with increasing column temperature, resulting in a reduction in back pressure.[38] However, from the start of the experiments, we chose to operate at 25 °C to minimize electrical energy consumption in the HPLC apparatus, in line with GAC principles which emphasize energy savings.[39]

Therefore, the flow rate was set to 0.1 mL min^{-1} to manage the back pressure limit of the conventional HPLC system from the laboratory equipment while minimizing solvent waste. Another drawback of using ethanol in HPLC is baseline drift during gradient elution.[40] Given these factors, we decided to work exclusively in the isocratic separation mode.

Several assays were conducted, and the fraction of ethanol in the mobile phase had the most significant effect on the separation of methylxanthines. Retention of the molecules investigated decreased as the ethanol fraction in the mobile phase increased. The analytes were not retained in the 30% ethanol solution. Theobromine was not retained when 20% ethanol was used, whereas theophylline and caffeine had retention times of 10.13 min and 11.66 min, respectively. To improve the retention of the target molecules, we tested 15% ethanol. Under these conditions, all the molecules were retained and showed satisfactory

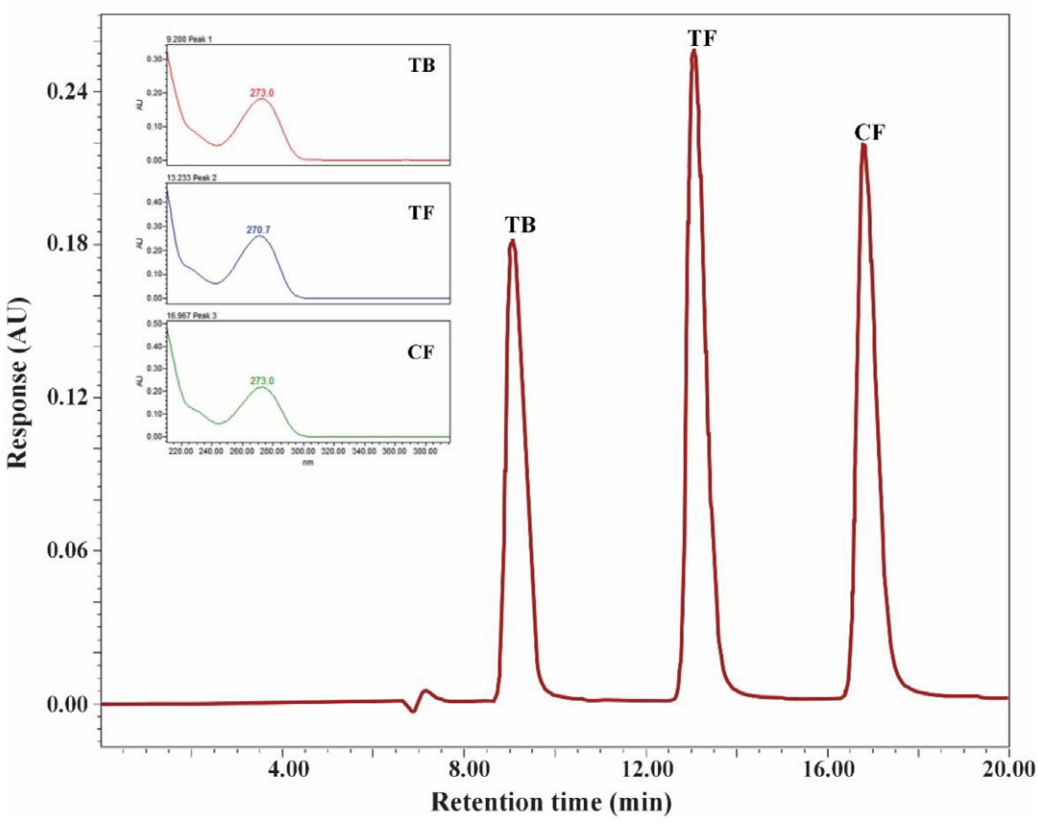


Fig. 1. HPLC-DAD chromatogram and respective UV spectrum obtained for the compounds of methylxanthines standard mixture (50 μg mL^{-1}, in 85% water – 15% ethanol) on Kinetex® C18 column (150 × 3 mm, 2.6 μm), with a mobile phase of 85% water – 15% ethanol, flow of 0.1 mL min^{-1}, at 25 °C, and 272 nm as wavelength detection.

separation: theobromine had a retention time of 9.23 ± 0.08 min, theophylline 13.20 ± 0.05 min, and caffeine 17.05 ± 0.04 min. The peak resolution was 2.55 between theobromine and theophylline, and 2.38 between theophylline and caffeine.

This study was conducted at a low flow rate, achieving relatively high analyte retention times. However, Assassi et al. reported that the strategy of increasing the flow rate in a core–shell column with a particle size under 3 µm to obtain rapid analysis with constant efficiency was not successful with ethanol as an organic modifier, since the use of ethanol increases the C term (mass transfer) of the Van-Deemter equation, regardless of the stationary phase geometry.[41] Therefore, the combination of the core–shell column with small particles (2.6 µm) and working at a low flow rate in a conventional HPLC instrument allowed us to achieve an adequate performance of the developed method with minimal solvent consumption.

A chromatogram obtained under the selected conditions is shown in Fig. 1. As depicted, caffeine, with the highest log P value, had the longest retention time, while early eluting analytes had lower log P values. The chromatographic behavior of the analytes corresponds to their polarity and interactions on a reversed-phase column (C18).[42-44] The chromatographic conditions ensured good separation between methylxanthines, achieving a total run time of 20 min and low solvent consumption. Fig. 1 shows a representative chromatogram obtained for a standard methylxanthine solution under optimal conditions and the DAD spectrum corresponding to each compound.

Additionally, system suitability under the optimized conditions was assessed (Table 1). Statistical data for different parameters such as retention time (t_R), resolution (R_s), retention factor (k), and the number of theoretical plates (N) of the target molecules were calculated. As shown in Table 1, the performance parameters of the analytical method performed well. The *% RSD* for t_R of the three analytes was <1.0%, resolution values were greater than 2, retention factors (k) ranged from 0.30 to 1.41, and the number of theoretical plates was more than 600. Good chromatographic separation was achieved for the three compounds, with resolution values greater than 2. Theobromine (TB) showed low retention (k = 0.31 ± 0.01), which can be explained by its being the most polar molecule or having the lowest log P value. The other analytes showed values of k in the range of 0.84 ± 0.02 to 1.37 ± 0.04, indicating stronger retention and suggesting a greater number of interactions between these molecules and the column stationary phase. Good efficiencies were obtained for the investigated methylxanthines, with N values greater than 1000, except for TB (N = 608 ± 51).

3. 2. Analytical Performance

The performance of the proposed method using the Kinetex® C18 column was evaluated, and results are summarized in Table 2. For quantitative analysis, linearity, precision, accuracy, and limits of detection and quantification were assessed.

A linear regression analysis was performed, and the results, summarized in Table 2, showed a good relationship between the chromatographic peak areas and the entire range of concentrations evaluated, with correlation (R) and determination coefficients (R^2) greater than 0.99. Therefore, the developed method was linear for all the analytes.

Precision was determined in terms of repeatability (intra-day) on the same day and reproducibility (inter-day)

Table 1. System suitability of the proposed method.

Analyte	Retention time (t_R) (min ± *SD*)	*%RSD* of t_R	R_s (mean ± *SD*)	k (mean ± *SD*)	N (mean ± *SD*)
Theobromine	9.23 ± 0.08	0.50	_	0.31 ± 0.01	608 ± 51
Theophylline	13.20 ± 0.05	0.47	2.55 ± 0.03	0.84 ± 0.02	1149 ± 82
Caffeine	17.05 ± 0.04	0.38	2.38 ± 0.06	1.37 ± 0.04	1786 ± 122

Rs = chromatographic resolution. k = retention factor. N = number of theoretical plates. *SD* = standard deviation of three determinations. *%RSD*, relative standard deviation of three determinations.

Table 2. Validation parameters of the proposed method for quantitative analysis of methylxanthines.

Analyte	Linear range[a] (µg mL^{-1})	Equation	R	R^2	LOD[c] (µg mL^{-1})	LOQ[d] (µg mL^{-1})
Theobromine	1–100[b]	y = 100566x – 2572	0.999	0.999	0.19	0.64
Theophylline	1–100[b]	y = 97521x – 48787	0.999	0.999	0.20	0.68
Caffeine	1–100[b]	y = 85841x – 26973	0.999	0.999	0.26	0.87

[a] = triplicate analysis for each set of standards at 273 nm. [b] = analysis of 18 standards for each analyte. [c] = signal-to-noise (*S/N*) ratio of 3. [d] = signal-to-noise (*S/N*) ratio of 10.

Table 3. Precision assessment of the proposed method for analysis of methylxanthines.

Analyte	Spiked concentration (μg mL^{-1})	Intra-day[a]		Inter-day[b]	
		Calculated concentration (mean ± *SD*[c])	*%RSD*[d]	Calculated concentration (mean ± *SD*[c])	*%RSD*[d]
Theobromine	2.00	2.04 ± 0.04	1.84	2.11 ± 0.06	2.67
Theophylline		2.07 ± 0.03	1.40	2.13 ± 0.03	1.46
Caffeine		2.12 ± 0.03	1.26	2.14 ± 0.03	1.62
Theobromine	40.00	39.75 ± 0.42	1.06	40.30 ± 0.59	1.45
Theophylline		39.85 ± 0.36	0.89	40.04 ± 0.52	1.31
Caffeine		39.91 ± 0.29	0.68	40.07 ± 0.42	1.04
Theobromine	80.00	79.70 ± 1.37	1.72	79.91 ± 1.56	1.95
Theophylline		80.15 ± 0.69	0.87	80.10 ± 0.84	1.05
Caffeine		80.12 ± 0.42	0.53	80.28 ± 0.49	0.61

[a] = within the same day. [b] = three consecutive days. [c] = *SD*, standard deviation of three determinations. [d] = *%RSD*, relative standard deviation of three determinations.

over three consecutive days by analyzing samples spiked with all analytes. Table 3 presents the *%RSD* for each analyte concentration in the blank samples spiked at 2.00, 40.00, and 80.00 μg mL^{-1}. The highest *%RSD* values were obtained for inter-day precision, with values between 0.61and 2.67%, whereas for intra-day precision, they were lower, ranging from 0.53 to 1.84%. In both cases, the highest values were observed at the lowest concentrations. Theobromine exhibited the greatest variation. However, for all experiments, the %*RSD* values were less than 3%, which indicates adequate precision.

As an approximation for the system accuracy, the concentration of calibration standards was back-calculated using the linear equation, and the correlation between these calculated concentrations and the actual ones was evaluated through regression analysis.[45] According to this model, the system demonstrated acceptable accuracy, with all analytes showing values of > 0.99 and *slope* values close to 1.[46] In the same context, the method accuracy was evaluated by triplicate analysis of methylxanthines in spiked blank samples at 2.00, 40.00, and 80.00 μg mL^{-1}, under the same conditions for precision. As shown in Table 4, recoveries ranged from 98.15 to 108.88% for the three analytes.

In the bibliography, we find the AOAC official method 980.14 for determining theobromine and caffeine in cacao products in multiple steps.[47] The method of this paper has been developed for theobromine, theophylline, and caffeine determination in powder enriched with black or green tea extracts for preparing beverages. Therefore, it was decided to compare the proposed method with some reported in the literature for the simultaneous analysis of the three target molecules in samples like this work in terms of accuracy and precision. Aquel et al.,[29] mention that they achieved good extraction efficiencies for the three analytes with recovery percentage values between 82.40 and 96.60%, while intra-day and inter-day precision ranged from 0.08 to 3.05%. In the same sense, Baek et al.[30] reports that all measurements of the same methylxanthines achieved an acceptable accuracy range with recovery percentage values of 91.82–104.72% and precision (inter-day and intra-day) between 0.16 and 4.12%. Therefore, our work presents accuracy and precision comparable to other reports in the literature.

The detection and quantification limits were determined under optimal chromatographic conditions. Table 2 lists the LOD and LOQ values for each compound. The found values were in the range of 0.19 to 0.26 μg mL^{-1} for

Table 4. Accuracy assessment of the proposed method for analysis of methylxanthines.

Analyte	Correlation for back-calculated *vs* real concentration of standards		Recovery assays	
	Slope	R^2	Spiked concentration (μg mL^{-1})	*%R*[a]
Theobromine	1	0.999	2.00	100.08–108.88
			40.00	98.75–102.43
			80.00	98.15–103.26
Theophylline	1	0.999	2.00	102.22–108.23
			40.00	98.62–100.36
			80.00	98.92–100.98
Caffeine	1	0.999	2.00	104.37–108.03
			40.00	99.12–101.34
			80.00	99.69–100.90

[a] = recovery percentage of three determinations.

detection limits and between 0.64 and 0.87 μg mL^{-1} for quantification limits.

The selectivity of the developed method was evaluated using DAD. This detector can provide absorbance and spectral data that can be used for the quantification and identification of chromatographic peaks.[48] To find and choose the most suitable wavelength for the analysis of target molecules, different wavelengths were tested (211–400 nm) with standard solutions in the mobile phase (85:15), water, and ethanol, respectively. A wavelength of 273 nm was selected as the optimal wavelength for the analyses because it provided the highest chromatographic peak area at the same standard concentration and the characteristic UV spectrum of each target molecule. Chromatograms of the standard solutions, blank samples, and spiked samples of all analytes were compared. The identity of the chromatographic peak was confirmed by the retention times and the UV spectrum at 273 nm. As can be seen in Fig. 2, the obtained results show that no interference between the analytes and matrix samples was observed for methylxanthines. Consequently, the proposed method can be applied to sample analyses.[30,49]

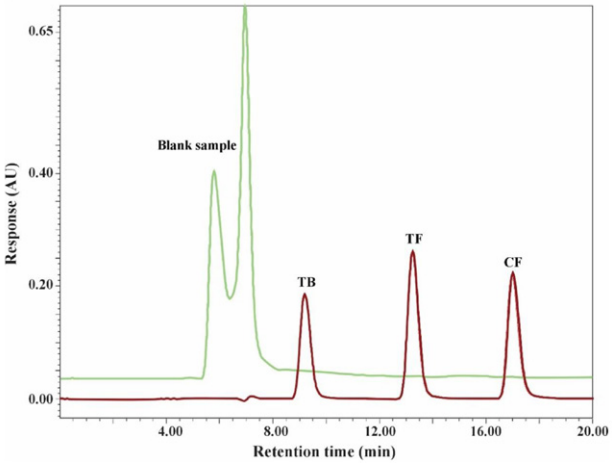


Fig. 2. Representative chromatograms for the methylxanthines mixture at 50 μg mL^{-1} (red line) and a blank sample (green line) without adding methylxanthines, both under optimal conditions.

The method's robustness was evaluated by altering the following factors: organic solvent proportion in the mobile phase, the flow rate, and the column temperature. These factor effects were studied through the retention time and chromatographic peak areas obtained for each analyte. Then, with the data obtained the coefficient plots were constructed on the Modde 13.1 program from Umetrics and are presented in Fig. 3. From regression coefficient plots for TB, TF, and CF, the method proved to be non-robust regarding retention times when organic solvent proportion in the mobile phase ranged from 83% to 87% or when flow rates varied between 0.09 mL min^{-1} and 0.11 mL min^{-1}. In contrast, the method was robust to temperature changes within the range of 25–27 °C. According to the results, an increase in the mobile phase proportion and a decrease in the flow rate significantly extended the retention times. Concerning peak areas, none of the evaluated factors affected the response for TB and CF. However, for TF, the peak area was influenced by flow rate; a decrease in flow rate tended to increase the peak area. For reliable use of this method, it is critical to note that the ethanol proportion and elution flow rate are key parameters in the run analytical. Given that, deviating from the established conditions would significantly impact the identification and quantification of the analytes.

3. 3. Method Applicability

The proposed method was applied to the analysis of methylxanthines in four different samples, analyzed in triplicate. Two samples were powders used to prepare lemon-flavored beverages enriched with black tea extract (BRAND 1 BT and BRAND 2 BT), and two were powders for beverages enriched with green tea extract (BRAND 1 GT and BRAND 2 GT), which are commercially available in supermarkets and commonly consumed in Mexico. The contents of caffeine, theobromine, and theophylline obtained by the HPLC-DAD method in these samples are shown in Table 5, and representative chromatograms are displayed in Fig. 4. Each resulting chromatographic peak was verified based on its retention time and UV spectrum,

Table 5. Methylxanthines contents in powdered lemon-flavored beverages enriched with black or green tea extract.

Sample	Analyte					
	Theobromine		Theophylline		Caffeine	
	Mean (g kg^{-1} ± *SD*[a])	*%RSD*[b]	Mean (g kg^{-1} ± *SD*[a])	*%RSD*[b]	Mean (g kg^{-1}± *SD*[a])	*%RSD*[b]
BRAND 1 BT	0.13 ± 0.01	3.23	ND	ND	3.55 ± 0.12	3.42
BRAND 2 BT	0.22 ± 0.01	5.17	0.05 ± 0.02	5.48	2.46 ± 0.13	5.30
BRAND 1 GT	0.19 ± 0.02	5.07	0.19 ± 0.01	5.21	3.14 ± 0.17	5.51
BRAND 2 GT	ND	ND	0.11 ± 0.01	5.28	1.62 ± 0.09	5.75

BRAND 1 BT = Sample of powdered lemon-flavored beverage enriched with black tea extract from brand 1.
BRAND 2 BT = Sample of powdered lemon-flavored beverage enriched with black tea extract from brand 2.
BRAND 1 GT = Sample of powdered lemon-flavored beverage enriched with green tea extract from brand 1.
BRAND 2 GT = Sample of powdered lemon-flavored beverage enriched with green tea extract from brand 2.
[a] = *SD*, standard deviation of three determinations
[b] = *%RSD*, relative standard deviation of three determinations.

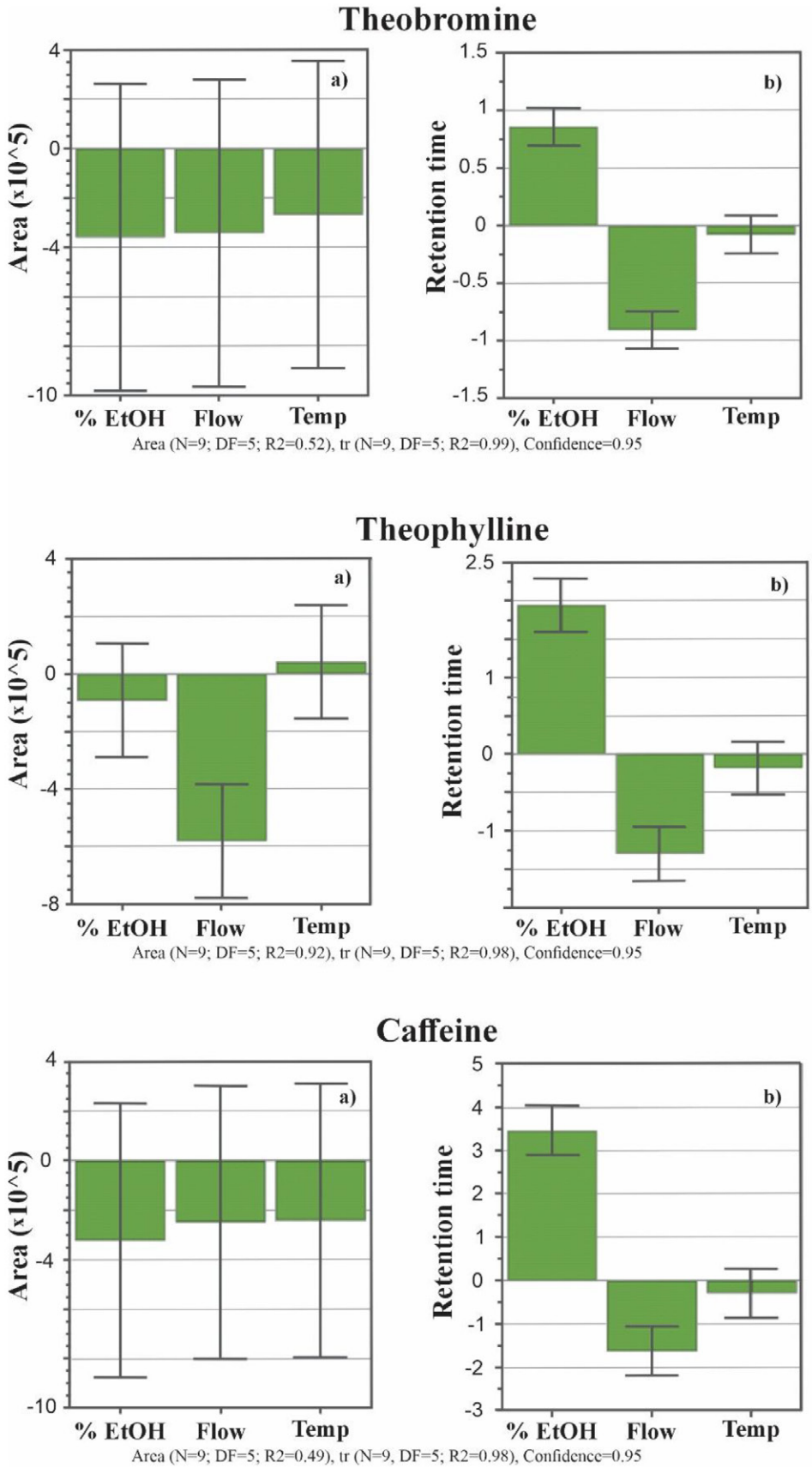


Fig. 3. Coefficient plots about robustness evaluation: the effects of the organic solvent proportion in the mobile phase (%EtOH), the flow rate (Flow), and the column temperature (Temp) of the proposed method on a) the area and b) the retention time from TB, TF, and CF.

and these parameters were compared with the standard chromatogram of each compound.

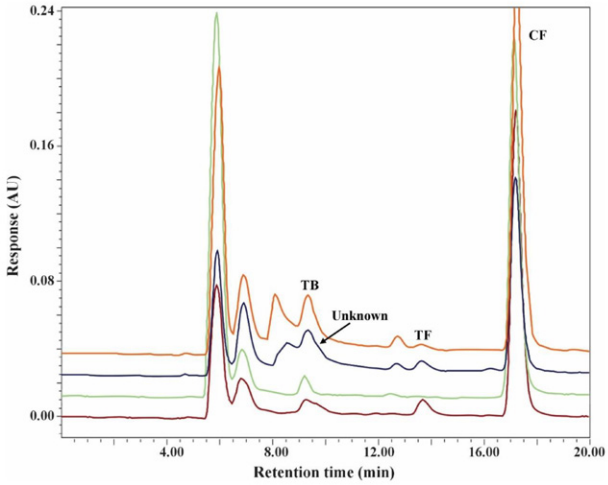


Fig. 4. Representative chromatograms of the different powdered samples were obtained under the optimal analysis conditions. Samples: BRAND 1 GT (red line), BRAND 1 BT (green line), BRAND 2 GT (blue line), and BRAND 2 BT (yellow line).

Caffeine was the predominant methylxanthine in all samples. The caffeine content in powder from BRAND 1 was similar in the two samples, with concentrations of 3.55 ± 0.12 g kg^{-1} and 3.14 ± 0.17 g kg^{-1} for the powders enriched with black tea extract and green tea extract, respectively. For BRAND 2 powders, the range was between 2.46 ± 0.13 g kg^{-1} and 1.62 ± 0.09 g kg^{-1} for those enriched with black tea extract and green tea extract, respectively.

The theobromine content in BRAND 1 powders was 0.13 ± 0.01 g kg^{-1} for the powder with black tea extract and 0.19 ± 0.02 g kg^{-1} for the powder with green tea extract. In BRAND 2, theobromine was only found in the powder with black tea extract, at a level of 0.22 ± 0.01 g kg^{-1}. Although a chromatographic peak corresponding to theobromine's retention time was found in the green tea extract powder from BRAND 2, its UV spectrum did not match that of the standard compound; therefore, the presence of this methylxanthine in the sample was ruled out.

Theophylline was detected in all powders, except for the powder enriched with black tea extract from BRAND 1. The powder enriched with green tea extract from BRAND 1 showed a theophylline concentration of 0.19 ± 0.01 g kg^{-1}. For BRAND 2 powders, the powder enriched with black tea extract had 0.05 ± 0.02 g kg^{-1}, while the one enriched with green tea extract had 0.11 ± 0.01 g kg^{-1}.

The powders from BRAND 1 contained caffeine and theobromine, while only the powder enriched with black tea extract lacked theophylline. The BRAND 2 powders contained caffeine and theophylline, but the powder enriched with green tea extract did not contain theobromine. These results are consistent with those of previous studies, which have reported that tea extracts contain varying amounts of methylxanthines, though caffeine is generally predominant.[25,29,30]

The content of methylxanthines in the samples varied, likely due to the diverse types of plant materials used in their preparation by different commercial brands, as each type of plant material can contain distinct chemical characteristics.

Therefore, based on the results obtained, we believe that enhanced quality control is necessary in the preparation of beverage powders, especially those enriched with pharmacologically active substances such as methylxanthines. These findings suggest that beverages prepared at home can increase the daily intake of stimulant substances like caffeine. Moreover, greater control over labeling is required for food products containing these substances to facilitate more informed consumption decisions.

2. 4. Greenness Evaluation

Evaluating the greenness of an analytical method is complex, as it involves multiple aspects and lacks definitive guidelines. Although initially focused on chemical synthesis procedures, the concept of GAC now extends to analytical chemists considering environmental, health, and safety issues during their activities, which are not easily quantifiable.[50] It is suggested that to truly characterize the greenness of an analytical process, one should consider the required materials (both quality and quantity), waste generation, energy consumption, analyst safety, pre-treatment, and the equipment's location relative to the research object. Additionally, it should be noted that not all analytical criteria carry the same weight; sometimes simplicity is prioritized, while in other cases, the reduction of reagents and waste is emphasized.[51–53] Therefore, in this study, we evaluated the proposed method using three scales.

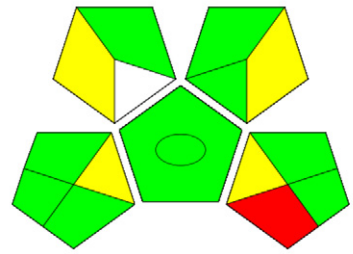
3. 4. 1. Analytical Eco-scale

Analytical Eco-Scale is a semi-quantitative methodology introduced in 2012 by Galuszka and collaborators to assess the "greenness" of an analytical method. This approach assigns penalty points to analytical processes that fall short of the ideals of green chemistry. The primary goal of this scale is to facilitate the comparison and selection of the most environmentally friendly alternative, whether evaluating a newly developed method or one that has been modified. The higher the score, the greener and more economical method is considered.[53] To calculate the Analytical Eco-scale for an analytical method, the following steps are considered: Step 1: Reactants, Step 2: Hazard, Step 3: Energy (Instrument used), Step 4: Occupational Hazard, and Step 5: Waste. An ideal ecological analysis is achieved when it scores 100; a score above 75 is considered excellent, 50 is acceptable, and below 50 is deemed inadequate. This method's evaluation according to the Analytical Eco-scale yielded 90 points, categorizing it as excellent. This

high score was due to the one solvent used, which is considered hazardous (two pictograms) but used in small quantities per analysis. Additionally, the method employs minimal sample size and a low flow rate, consequently generating low waste. Furthermore, no sample pre-treatment was required. The complete information about the greenness evaluation of the proposed method is presented in Table 6.

Table 6. Green evaluation of the proposed method.

Analytical Eco-Scale			AGREE	GAPI
Reagents				
Amount	< 10 mL (g)	1		
Hazard	Ethanol	3		
Instruments				
Energy	≤ 1.5 kWh per sample	1		
Occupational hazard	Analytical process hermetization	0		
Waste	< 1 mL (g)	1		
	No treatment	3		
	Total penalty points	9		
	Total score	**91**		



achieved because the developed methodology presented only one environmentally unfriendly aspect, related to the off-line positioning of the analytical equipment in relation to the sample. The sections considered moderately ecological were: 1 – the methodology being off-line regarding the sampling method; 3 – because the reagents used in the sample preparation process are not reusable; 8 – the number of analytes determined in a single run per hour, where three analytes can be analyzed per run and three runs can be conducted in an hour, though ideally, more analytes should be analyzed in a single run to minimize energy consumption; 9 – the use of HPLC equipment post-sample preparation for analysis, which consumes a moderate amount of electrical energy; and 10 – the reagents used, with the same issue highlighted by other scales, as solvents like ethanol cannot be avoided in LC techniques with reverse phase columns, which does not guarantee a completely safe procedure for the operator, however, the ethanol is greener than other solvents such as methanol or acetonitrile.

3. 4. 2. Analytical GREEnness Metric Approach

The AGREE scale, developed by Gdańsk University of Technology, employs software that evaluates 12 parameters aligned with the 12 principles of GAC. Each principle was rated on a scale of 0–1.[51] The principles are as follows: Principle 1 emphasizes the use of direct analytical techniques to avoid sample treatment; Principle 2 aims for minimal sample size and number of samples; Principle 3 advocates for *in situ* measurements; Principle 4 promotes the integration of analytical processes and operations to save energy and reduce reagent usage; Principle 5 encourages the selection of automated and miniaturized methods; Principle 6 advises against derivatization; Principle 7 seeks to avoid generating large volumes of analytical waste and ensures proper waste management; Principle 8 favors multi-analyte or multi-parameter methods over those that analyze one analyte at a time; Principle 9 focuses on minimizing energy use; Principle 10 prefers reagents obtained from renewable sources; Principle 11 mandates the elimination or replacement of toxic reagents; Principle 12 emphasizes increasing operator safety. Each parameter was assigned a color indicating its environmental impact at three levels: low (green), medium (yellow), or high (red). This scale provides a visual ecological assessment of the method, making it easy and quick to identify the most environmentally friendly method for a specific analysis. The output is represented as a clock-like graph with the overall score and color representation in the center.

According to the AGREE scale, the evaluation method received a score of 0.73, indicating that it is considered environmentally friendly (Table 6). This rating was

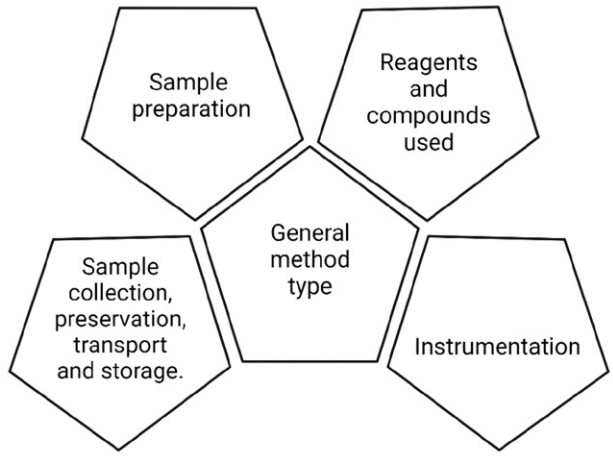


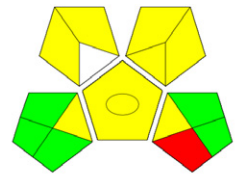
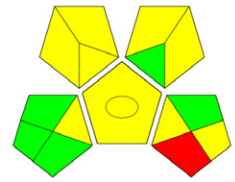
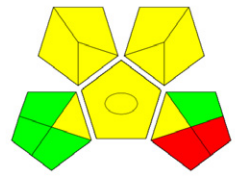
Fig. 5. Composition of Green Analytical Procedure Index pictogram and evaluation description of steps from analytical methodology.

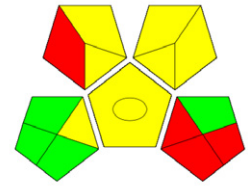
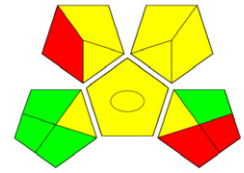
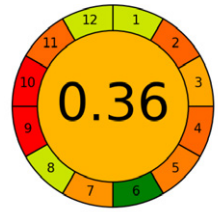
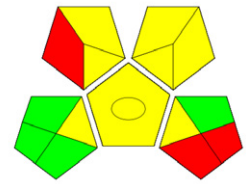
3. 4. 3. Green Analytical Procedure Index (GAPI)

GAPI is a qualitative scale for assessing the greenness of an analytical method based on the processes or steps involved.[52] It utilizes a pictogram to classify the greenness at each step of the analytical methodology, applying a color scale with three levels of evaluation for each stage. This scale features five pentagrams (Fig. 5), which can be used to assess and quantify the environmental impact of each methodological step, ranging from low (green, environmentally safe) to medium (yellow) to high (red, not eco-friendly).[54] GAPI primarily focuses on three aspects: sample preparation, reagents and solvents, and instrumentation. These categories are further divided into 15 subcategories.[55]

Evaluation of this method using GAPI software revealed that there was only one environmentally unfriendly condition related to sample collection, preservation, transportation, and storage (Table 6). This is because the sample was obtained from a shopping center rather than directly from a production line. During sample preparation, no significant issues were identified as no additional treatments were required; no extraction was performed (indicated in white), though solvents were used (indicat-

Table 7. Green comparison between reported methods for methylxanthines determination.

Method	Analytical Eco-Scale			AGREE	GAPI
Analyte: caffeine Sample: dietary suplements Method: UHPLC, C18 column, UV-Vis detector Mobile phase: ethanol: water in gradient elution (initial 10% ethanol with increase to 20% over 2.5 min) Flow 0.3 mL min^{-1} Injection 1µL Time: 10 min[31]	**Reagents**				
	Amount	< 10 mL (g)	2		
	Hazard	Ethanol	3		
	Instruments				
	Energy	UHPLC ≤ 0.1 kWh per sample	0		
		Sonicator ≤ 0.1 kWh per sample	0		
	Occupational hazard	Analytical process hermetization	0		
	Waste	< 1 mL (g)	1		
		No treatment	3		
		Total penalty points	9		
		Total score	**91**		
Analytes: caffeine and theobromine Sample: commercial teas Method: HPLC, C18 column, UV-Vis detector Mobile phase: ethanol:water (5% ethanol) Flow 1.4 mL min^{-1} Injection: 2 µL Time: 1.7 min[32]	**Reagents**				
	Amount	< 10 mL (g)	2		
	Hazard	Ethanol	3		
	Instruments				
	Energy	HPLC ≤ 1.5 kWh per sample	1		
		Hot-plate 1.5 kWh per sample	2		
	Occupational hazard	Analytical process hermetization	0		
	Waste	1–10 mL (g)	3		
		No treatment	3		
		Total penalty points	14		
		Total score	**86**		
Analytes: caffeine Sample: dietary suplements Method: UHPLC, SiO_2-PFP y C18 column, UV-Vis detector Mobile phase: ethanol:water (gradient from 20 to 100% of ethanol) Flow 1 mL min^{-1} Injection: 20 µL Time: 15 m0in[33]	**Reagents**				
	Amount	10–100 mL (g)	2		
	Hazard	Ethanol	3		
	Instruments				
	Energy	UHPLC ≤ 0.1 kWh per sample	0		
		Centrifugue ≤ 1.5 kWh per sample	1		
	Occupational hazard	Analytical process hermetization	0		
	Waste	1–10 mL (g)	3		
		No treatment	3		
		Total penalty points	12		
		Total score	**88**		

Method	Analytical Eco-Scale			AGREE	GAPI
Analytes: caffeine, theobromine and theophylline Sample: commercial teas Method: UHPLC, C18 column, MS detector Mobile phase: water:acetonitrile (90:10) with 0.1% formic acid Flow 0.5 mL min^{-1} Injection: 5 µL Time: 30 min[29]	**Reagents**				
	Amount	10–100 mL (g)	2		
	Hazard	Acetonitrile	3		
		Formic acid	6		
	Instruments				
	Energy	UHPLC/MS 1.5 kWh per sample	2		
		Hot-plate 1.5 kWh per sample	2		
	Occupational hazard	Analytical process hermetization	0		
	Waste	> 10 mL (g)	5		
		No treatment	3		
		Total penalty points	23		
		Total score	**77**		
Analytes: caffeine, theobromine and theophylline Sample: commercial teas Method: HPLC, C18 column, UV-Vis detector Mobile phase: water:acetic acid:methanol (79:1:20) Flow 0.9 mL min^{-1} Injection: 10 µL Time: 25 min[30]	**Reagents**				
	Amount	10–100 mL (g)	2		
	Hazard	Methanol	6		
		Acetic acid	4		
	Instruments				
	Energy	HPLC ≤ 1.5 kWh per sample	1		
		Hot-plate 1.5 kWh per sample	2		
	Occupational hazard	Analytical process hermetization	0		
	Waste	> 10 mL (g)	5		
		No treatment	3		
		Total penalty points	23		
		Total score	**77**		
Analytes: caffeine, theobromine and theophylline Sample: commercial beverages Method: HPLC, RP-8 column, UV-Vis detector Mobile phase: water-THFA 0.1%: acetonitrile (90:10) Flow 0.8 mL min^{-1} Injection: 10 µL Time: 12 min[25]	**Reagents**				
	Amount	10–100 mL (g)	2		
	Hazard	Tetrahydrofurane	5		
		Acetonitrile	3		
		Methanol	6		
		Chloroform	5		
	Instruments				
	Energy	HPLC ≤ 1.5 kWh per sample	1		
		Sonicator ≤ 0.1 kWh per sample	0		
		Hot-plate 1.5 kWh per sample	2		
		Rotary evaporator <1.5 kWh per sample	2		
	Occupational hazard	Analytical process hermetization	0		
	Waste	> 10 mL (g)	5		
		No treatment	3		
		Total penalty points	34		
		Total score	**66**		

ed in yellow). Only a very small volume of a moderately toxic reagent was used. The main issue is the unavoidable use of solvents such as ethanol, which, despite being used in small amounts, remains flammable (indicated by yellow). The instrumentation section is marked red because the solvents used are neither recycled nor treated and yellow due to the use of HPLC for determination. Overall, the chromatographic proposed method in this work was considered environmentally friendly (central pentagram).

2. 5. Green Comparison with Other Methods

The proposed methodology was compared in green terms with some previous studies for analysis of one or all three methylxanthines in various samples, utilizing either

HPLC or UHPLC. Table 7 presents a comparison of the results; the table shows the results from the greenest to the least green method.

The methodologies proposed by O. Kalisz et al.,[31] H. Shaaban et al.,[32] and O. Syrotchuk et al.[33] share a similar approach to the present study, focusing on the use of solvents with minimal environmental impact, minimizing the reagent usage and waste generation. Therefore, the green evaluations in these studies are comparable to the method proposed in the present work. However, several differences can be noted.

For instance, in the case of O. Kalisz's method,[31] which has the closest score to ours, the Analytical Eco-Scale suggests similar ratings due to using the same solvents in similar quantities with minimal sample treatment. However, the AGREE and GAPI scales reveal two main differences: Kalisz and collaborators employed UHPLC (resulting in a lower energy consumption penalty); but they did perform a sample treatment, use a higher flow rate, and quantify only one analyte, which led to more penalties.

Regarding H. Shaaban's work,[32] HPLC and ethanol were used, but the key difference in their methodology lies in employing a heating plate as part of the sample treatment. They also employed a much higher flow rate, and although the analyses took less time, this increased solvent consumption and waste generation. Additionally, they quantified only two analytes.

On the other hand, O. Syrotchuk and colleagues[33] used a UHPLC, which helped reduce their penalty scores. However, despite using ethanol, they are penalized because they used a significantly higher flow rate for a much longer duration concerning Shaaban's approach, this increased solvent consumption and waste production, and further, they quantified only one analyte.

In contrast with our work, the reported methodologies did not prioritize compliance with GAC standards, as seen in their sample preparation processes and solvent volumes used in their methodologies. This resulted in low scores on the proposed scales, making these methods less environmentally friendly. In summary, the most significant difference that makes our method greener is that it requires no prior sample treatment and uses the most eco-friendly solvents in minimal quantities while still achieving excellent performance for the three target analytes.

4. Conclusions

The developed method, using a mobile phase of water and ethanol at a low flow rate and a column with core–shell particles, allowed for good separation in a relatively short analysis time with minimal solvent consumption. The use of non-toxic organic solvents during the analysis generates little to no hazardous waste. Thus, one of the most important features of this method is its eco-friendliness, as demonstrated by the AES, GAPI, and AGREE techniques, which indicate the greenness of the proposed method. Compared with other reported methods, this method is greener due to the low amounts of non-toxic reagents used and minimal waste generation. Additionally, the method developed in this study exhibited good linearity, precision, and accuracy; therefore, the proposed procedure can be used as a reliable tool for routine detection or screening of methylxanthines in quality control of food products, beverages, herbal extracts, and other matrices. This study offers new opportunities to adopt green chromatographic methods for various applications.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Povzetek

V tej študiji je bila za analizo metilksantinov, vključno s teobrominom (TB), teofilinom (TF) in kofeinom (CF), razvita in validirana preprosta in okolju prijazna metoda na osnovi tekočinske kromatografije visoke ločljivosti in detektorja z nizom diod (HPLC-DAD). Ta metoda je pokazala odlično učinkovitost z uporabo kolone Kinetex® C18, ki je bila termostatirana na 25 °C, in izokratske elucije in sestavo mobilne faze voda-etanol (85:15) pri pretoku 0,1 mL min^{-1}. Metoda je bila linearna (1–100 μg mL^{-1}), izkazovala je visoko natančnost in točnost z relativnimi standardnimi odkloni pod 2,67 % oziroma izkoristki v razponu od 98.15 % do 108.88 %. Meje detekcije in kvantifikacije so se gibale med 0,19–0,26 μg mL^{-1} oziroma 0,64–0,87 μg mL^{-1}. To metodo smo uporabili za analizo pijač v prahu z okusom limone, obogatenih z izvlečki bodisi črnega bodisi zelenega čaja. Ugotovljeno je bilo, da kofein presega mejo 1,62 g kg^{-1}. Predlagana metoda je pokazala odlično zelenost, kar dokazujejo analitična ekološka lestvica, kalkulator analitične metrike GREENness (AGREE) in GAPI.