



Ecofriendly HPLC method for caffeine in dietary supplement determination using ethanol–water mobile phase and PFP-column

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ABSTRACT

Caffeine is a natural alkaloid recognized as an active central nervous system stimulant. This substance can be found in beverages, medicines, and dietary supplements, and its concentration must be controlled to minimize the side effects of overdose. High-performance liquid chromatography is a routine method for caffeine determination that employs the octadecylsilyl stationary phase (SiO₂-C18) in combination with methanol and acetonitrile solvents. Unlike the others, we propose applying pentafluorophenyl (SiO₂-PFP) stationary phase and water–ethanol mobile phase as an environmentally benign solvent alternative. Due to the additional interactions with the pentafluorophenyl ring of the stationary phase, the separation of caffeine from other compounds, such as theophylline and theobromine, can be significantly improved. Caffeine retained better by 1.5–2 times compared to the C18 column. Chromatographic column discovery F5 (4,6 × 250 mm, 5 μm particle size) was thermostatted at 40°C and gradient elution from 20% to 60% ethanol was used for chromatography. The method was validated for two dietary supplements' specificity and showed linearity in the range of 5–15 mcg/ml, precision of not more than 2.0%, and accuracy of not more than 1.0%.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is a natural alkaloid used as a component of beverages, medicines, and dietary supplements. It has been shown that caffeine has a behavioral and mental effect on humans and animals, similar to the impact of typical psychomotor stimulants (amphetamine, cocaine) (Garrett and Griffiths, 1997), as well as increased blood pressure in doses in caffeinated beverages and medicines (McMullen *et al.*, 2011). Caffeine can cause weight loss (Tabrizi *et al.*, 2019), which is often included in dietary supplements for weight loss. Controlling and labeling the caffeine content of dietary supplements are necessary to reduce the health risks for consumers of such products. Since weight loss products can also contain vitamins, plant extracts, and excipients, increasing the selectivity of the caffeine determination

method is an important task. In addition, modern methods must be safe for the environment and analysts.

A large number of methods, including high-performance liquid chromatography (HPLC) (Fajara and Susanti, 2017; Gliszczynska-Świgło and Rybicka, 2015; Rahim *et al.*, 2014; Srdjenovic *et al.*, 2008; Tzanavaras and Themelis, 2007; Zuo *et al.*, 2002), gas chromatography (Sereshti and Samadi, 2014), capillary electrophoresis (Regan and Shakalisava, 2005), ultraviolet (UV) spectroscopy (Belay *et al.*, 2008), and electrochemical method (Amare and Admassie, 2012), are used for assay of the caffeine content of coffee, tea, and caffeinated beverages.

Capillary electrophoresis allows rapid separation of caffeine from its counterparts. Still, the usual relative SD of the peak areas of successive injections is 3%–4% (Regan and Shakalisava, 2005), which significantly impairs the metrological characteristics of the method.

Direct UV spectrophotometry is a nonselective method; thus, the determination of caffeine involves using time-consuming sample preparation using toxic solvents—dichloromethane or chloroform (Belay *et al.*, 2008).

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As seen from the inspection of methods used for caffeine assay, the HPLC method is most often used. Almost all such methods use octadecylsilyl stationary phase (SiO₂-C18) in combination with traditional reversed-phase HPLC solvents—methanol and acetonitrile (Coura *et al.*, 2021; Fajara and Susanti, 2017; Fekry *et al.*, 2022; Gliszczyńska-Świągło and Rybicka, 2015; Palur *et al.*, 2020; Rahim *et al.*, 2014; Srdjenovic *et al.*, 2008; Tzanavaras and Themelis, 2007; Zuo *et al.*, 2002). This means that these methods do not meet the requirements for environmental safety because of toxic solvents. Ethanol is one of the safest solvents to use in liquid chromatography (Plotka *et al.*, 2013); however, it has certain limitations due to the higher system pressure and optical transparency of ethanol in the detection range of 190–220 nm (Yabré *et al.*, 2018).

This study aims to develop an environmental friendly, selective method for determining caffeine in dietary supplements for weight loss, and establish chromatographic parameters for separating caffeine from other components of nutritional supplements for weight loss.

MATERIALS AND METHODS

The following items were used in this study.

Objects

Pills XLS DUO Slim & Shape, batch A814 (XLS)

Ingredients: cocoa butter, 100 mg; green tea, 100 mg; apple, 50 mg; grapefruit, 50 mg; inulin, 50 mg; artichoke, 50 mg; pineapple, 25 mg; parsley, 20 mg; fennel, 10 mg; blackcurrant, 10 mg; excipients.

Instant coffee drink, Light Energy Drive, batch 11120 coffee light (CL)

Ingredients: organic instant coffee, 3,580 mg; L-carnitine tartrate, 68 mg; green tea extract, 68 mg; garcinia extract, 68 mg; pineapple extract, 68 mg; guarana extract, 68 mg; senna extract, 46, 8 mg; vitamin premix [vitamin A (beta-carotene), 4,400 IU; vitamin C, 1,332 mg; biotin, 4 mg; vitamin B1, 1,332 mg; vitamin B2, 1,332 mg; vitamin B6, 2,668 mg; vitamin B12, 5.2 mcg; nicotinic acid, 13.2 mg].

Certified reference materials

Caffeine produced by Supelco, cat. number: PHR1009; theophylline produced by Supelco, cat. number: PHR1023; theobromine, produced by Supelco, cat. number: 42993.

Reagents

Water for chromatography, R obtained from installation Simplicity UV, Millipore, USA; ethanol 96% (V/V) Lux, manufacturer—State Enterprise “UKRSPYRT,” Ukraine.

Equipment

High-performance chromatograph AZURA UHPLC manufactured by KNAUER (Germany), which consists of pump AZURA P 6.1L, autosampler AZURA 3,950, column thermostat AZURA CT 2.1, and diode array detector AZURA Detector DAD 6.1L. Chromatograms and UV spectra were processed using Open LAB CDS EZCrom Edition software.

Balances

Mettler Toledo XS204, permissible load, 220 g, discreteness, 0.1 mg. Centrifuge: SIGMA, Universal 320 R, Germany. Rotor 1620A, a radius of 99 mm. Ultrasonic cleaner: Daihan, Wuc-A010H, Korea.

Discovery HS C18 250*4,6 5 µm and Discovery F5 250*4,6 5 µm columns were used as a stationary phase in the study.

- Chromatography conditions were as follows:
- Channel A: water for chromatography, R
- Channel B: ethanol 96% (V/V)
- Column thermostat temperature: 40°C
- Wavelength: 272 nm
- Mobile phase flow rate: 1,0 ml/minute
- Injection volume: 20 µl

Preparation of solutions

Sample averaging and average weight determination were as follows: instant coffee drink Light Energy Drive: 10 sachets; pills XLS DUO Slim & Shape: 10 crushed pills.

Preparation of a solution for determining the suitability of a chromatographic system

Samples of caffeine, theobromine, and theophylline in about 10 mg were placed in a volumetric flask of 500 ml and made up to volume with water.

Preparation of caffeine solutions for determining the linearity of the method

Solutions with five following concentrations were prepared: 5, 8, 10; 12; 15 mcg/ml in water.

Preparation of test sample solutions for the study of precision

To analyze a sample of the test object CL, six samples with a nominal concentration of about 10 mcg/ml. Averaged samples of about 1.2 g were placed in 100 ml flasks, and 70 ml of distilled water was heated to 80°C. Selected samples were filtered through a PTFE(L) membrane filter with a pore size of 0.45 µm and a diameter of 25 mm.

To analyze a sample of the second test object XLS, six samples with a nominal concentration of 10 mcg/ml were prepared. Averaged samples of about 0.5 g were placed in 100 ml flasks; 5 ml of 0.1 M hydrochloric acid solution, 5 ml of ethanol 96% (V/V), and 20 ml of distilled water heated to 80°C were added. Flasks were placed in an ultrasonic cleaner for 30 minutes, after which the solutions were cooled and made up to volume. Afterward, a 5:50 dilution was carried out in 50 ml flasks. Selected samples were filtered through a PTFE(L) membrane filter with a pore size of 0.45 µm and a diameter of 25 mm.

Preparation of test sample solutions to verify the accuracy of the method

Six solutions of both CL and XLS were prepared in the same way for precision. The samples were centrifuged at 5,000 rpm. The supernatant was removed; sample preparation was carried out with the solid residue in the same way as that for

the corresponding samples. The samples were then analyzed for caffeine content.

Calculation of capacity factor (k) and efficiency (N)

N and k determined for isocratic elution and linear gradient elution experiments were calculated the same way, using the formula prescribed in Ph.Eur.2.2.46.

RESULTS AND DISCUSSION

Development of the method

Selection of chromatographic column and mobile phase

When ethanol-water mixtures are used as mobile phases, SiO_2 -C18 is usually used as a corresponding stationary phase. It is known that the retention of compounds on SiO_2 -C18 depends on the level of dispersion interactions between the adsorbate and the adsorbent. In the case of polar compounds, this retention significantly deteriorates. To improve the retention of compounds of hydrophilic nature, stationary phases with bonded groups of alternative selectivity may be used. In particular, in this study, the SiO_2 -PFP stationary phase was tested for the retention and separation of purine alkaloids—caffeine, theophylline, and theobromine.

Two commercially available stationary phases Discovery F5 250*4,6 5 μm and Discovery HS C18 250*4,6 5 μm were compared on the caffeine retention with the mobile phases

of acetonitrile-water, ethanol-water mixtures in different ratios, as shown in Figure 1.

It was found that there is no significant difference in caffeine retention factors in both stationary phases when using acetonitrile, Figure 1a and 6. In contrast, when using ethanol, the caffeine retention factor (k) on SiO_2 -PFP significantly exceeds that of SiO_2 -C18. This can be explained by the fact that ethanol, unlike acetonitrile, does not deactivate alternative interactions, namely, the π - π interaction of the pentafluorophenyl (PFP) group with π -electrons of the analyte (Thevenon-Emeric *et al.*, 1991) and the dipole-dipole interaction of the molecule dipole and the dipole of the PFP group. The difference between ethanol and acetonitrile for reversed-phase HPLC lies in the absence of π -electrons in the ethanol molecule and a much smaller dipole-dipole moment of this solvent.

Increased caffeine retention factor while using PFP stationary phase combined with ethanol-water mobile phase can increase the selectivity of separation of purine alkaloids, which are characterized by the high dipole moment of a molecule and the presence of π -electrons.

Selection of chromatographic column temperature

One of the disadvantages of using ethanol as a solvent in mobile phases for chromatography is that its mixtures with water give viscous solutions, which causes increased pressure in the chromatographic system. For example, a water-ethanol 96% 50:50 mixture causes a pressure of 364 bar at a flow of 1.0 ml/

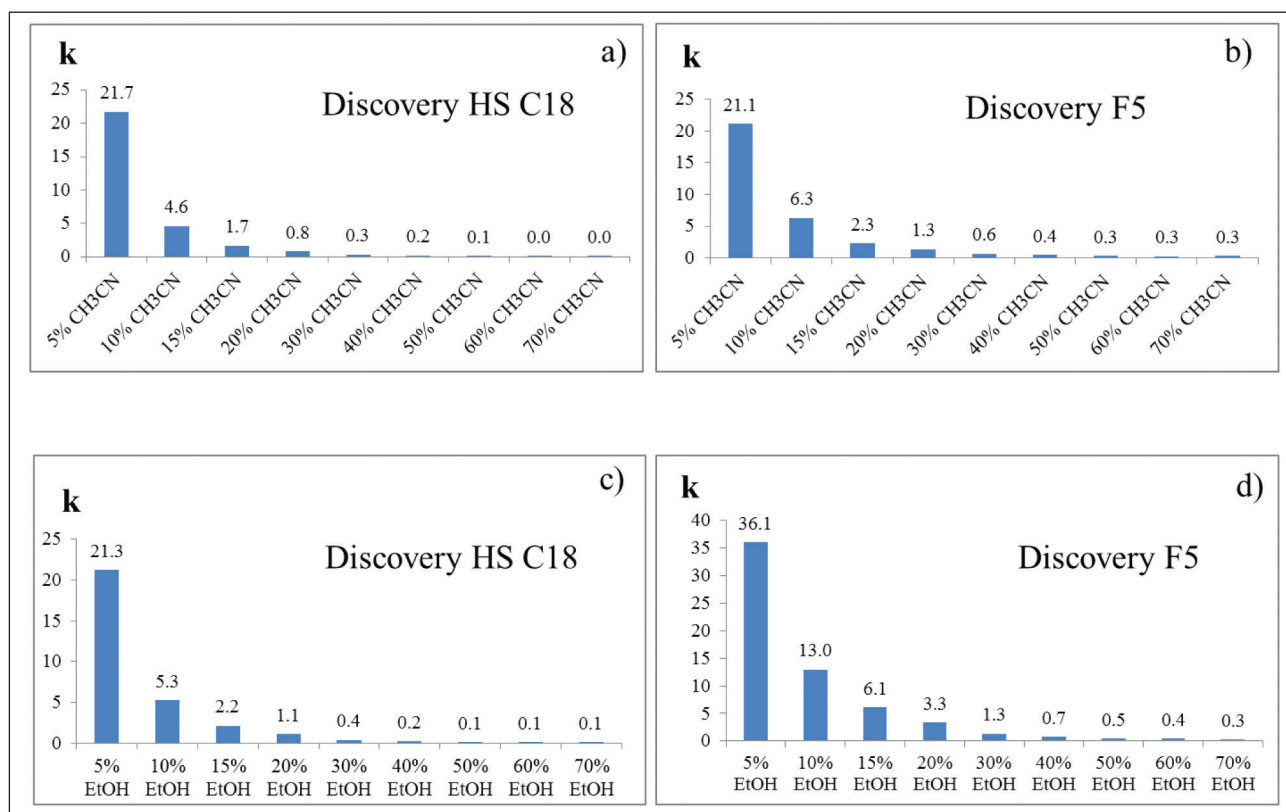


Figure 1. Diagrams of the retention factor based on the composition of the mobile phase, both stationary phase columns. (a) Caffeine retention factor on Discovery HS C18 depending on the content of acetonitrile in mobile phase. (b) Caffeine retention factor on Discovery PFP depending on the content of acetonitrile in mobile phase. (c) Caffeine retention factor on Discovery HS C18 depending on the content of ethanol in mobile phase. (d) Caffeine retention factor on Discovery PFP depending on the content of ethanol in mobile phase.

minute on a chromatographic column Discovery F5 250 × 4.6 (5 μm), as shown in Figure 2.

One of the ways to reduce the pressure in the chromatographic system is to increase the temperature of the chromatographic column (Li and Carr, 1997). The influence of temperature on pressure is established in Figure 3.

Increasing the temperature of the chromatographic column solves the problem of high pressure when using mobile phases based on water–ethanol mixtures. A temperature of 40°C was chosen for chromatography, given that the pressure is significantly reduced at this temperature, and the use of higher temperatures can reduce the service life of the chromatographic column.

Selection of detection wavelength

Detection at the maximum of caffeine absorption in the selected mobile phase is chosen at 272 nm, which coincides with the data from references (Belay *et al.*, 2008).

Selection of gradient program

Gradient elution for samples with a complex matrix is almost mandatory, as it is necessary to wash all matrix

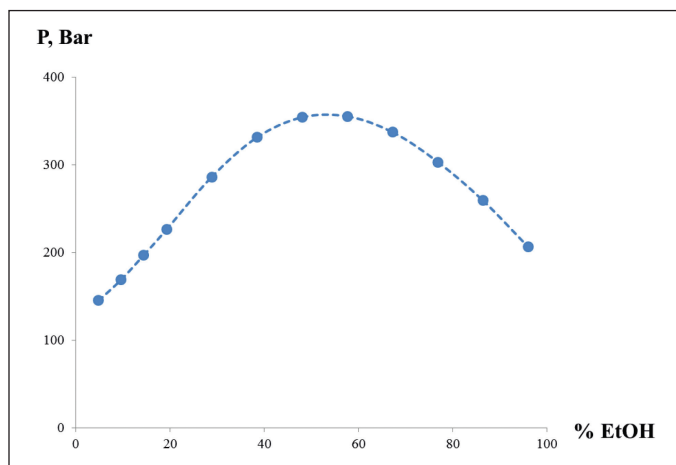


Figure 2. Relation between the pressure in the chromatographic system and the ethanol–water ratio in the mobile phase.

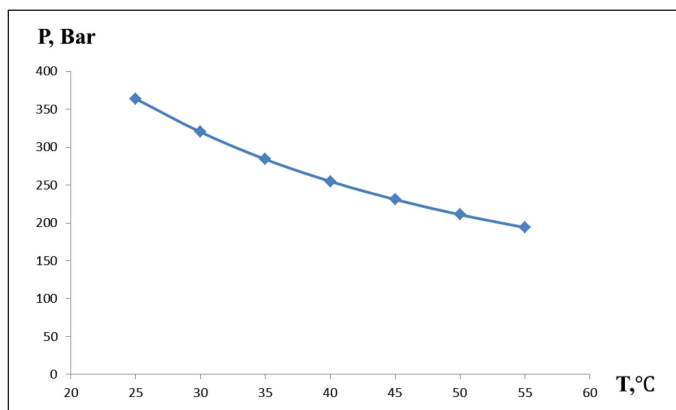


Figure 3. Pressure versus temperature; water–ethanol 50:50 mixture used as mobile phase.

components out of the stationary phase to ensure reproducible chromatography of successive injections. For this, the chromatographic characteristics of the caffeine peak were tested under chromatography conditions 1 and 2 (Tables 1 and 2), which differ in the rate of ethanol content increase in the mobile phase (4%/minute and 8%/minute, respectively).

Given that under chromatography conditions 1, the retention factor is higher, and the efficiency of the chromatographic column is higher (Table 3), the separation ability of chromatography conditions 1 will be potentially higher.

Suitability of the chromatographic system

The suitability of the chromatographic system was checked before the validation work. The results are shown in Table 4. Peaks of all structural analogs are sufficiently separated from each other, as seen on the chromatogram in Figure 4.

According to the results of the calculations, this method is suitable, as all of the above conditions are met.

Table 1. Gradient program 1.

Time, minutes	Channel A, %	Channel B, %
0	80	20
10	40	60
10	80	20
15	80	20

Table 2. Gradient program 2.

Time, minutes	Channel A, %	Channel B, %
0	80	20
10	0	100
10	80	20
15	80	20

Table 3. Chromatographic parameters of caffeine peak during chromatography with different gradients.

	RT, minute	k	N, number of theoretical plates
Chromatography conditions 1	6.467	1.6	34,448
Chromatography conditions 2	4.25	0.7	25,627

Table 4. The results of checking the suitability of the chromatographic system.

Parameter	Requirement	Output	Result
RSD, % of peak areas (5 inj.)	Not less than 1.5%	0.47	Pass
RSD, % of retention times (5 inj.)	Not less than 0.5%	0.07	Pass
Column efficiency (N)	Not less than 8,000	33,410	Pass
Resolution (R _s)	Not less than 3.0	6.1	Pass
Caffeine/theophylline			
Asymmetry factor (A _s)	0.8–1.5	1.18	Pass

Validation

Specificity

To confirm the specificity of the method, chromatography of a standard sample of caffeine with a concentration of 10 mcg/ml, test sample solutions, and solvent (mobile phase) was performed; the spectral purity of caffeine peaks was determined.

Specificity was based on the fulfillment of such conditions:

1. The retention time and the UV spectrum of the standard sample chromatogram peak coincide with the test sample solutions. Obtained chromatograms are shown in Figures 5–7.

The relative deviation of the retention time for the test samples was calculated. For CL, $\Delta = 0.01\%$, and for XLS, $\Delta =$

0.38%. UV spectra are similar and have a maximum of about 272 nm. The spectral purity of the peaks exceeds 99.9%.

2. No peaks on the solvent chromatogram could interfere with the assay or identification of caffeine, as shown in Figure 8.
3. Separation from structural analogs (theophylline and theobromine) is shown in Figure 4. All substances are reliably separated from each other.

Linearity

The linearity of the method was examined in the concentration range from 5.0 to 15.0 mcg/ml. Measurements were performed for five solutions of different concentrations. As can be seen from the data in Table 5, the proposed method satisfies all criteria; the method is linear in the studied range of 5.0–15.0 mcg/ml of caffeine concentrations.

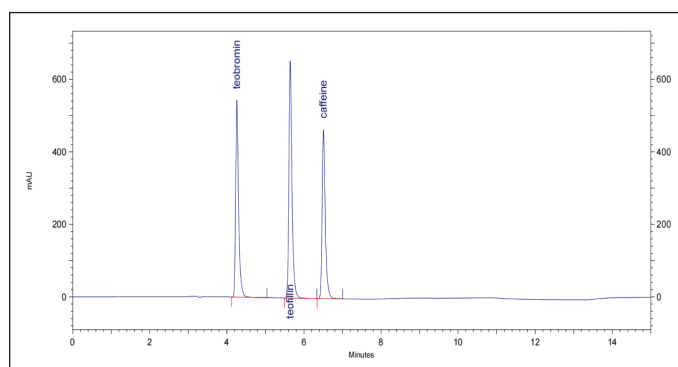


Figure 4. Chromatogram of the solution is used to check the suitability of the chromatographic system.

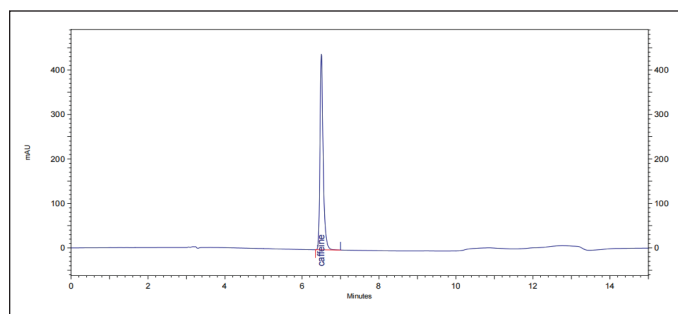


Figure 5. Chromatogram of a standard sample of caffeine with a concentration of 10 mcg/ml.

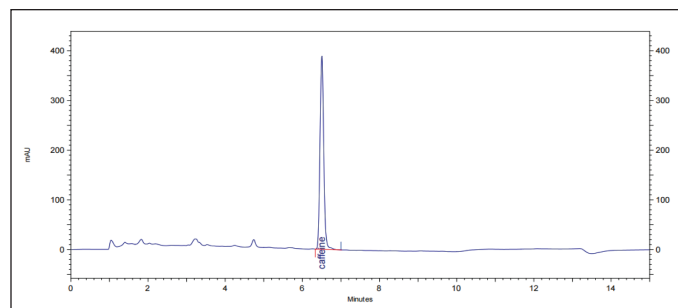


Figure 6. Chromatogram of the solution of the test sample CL.

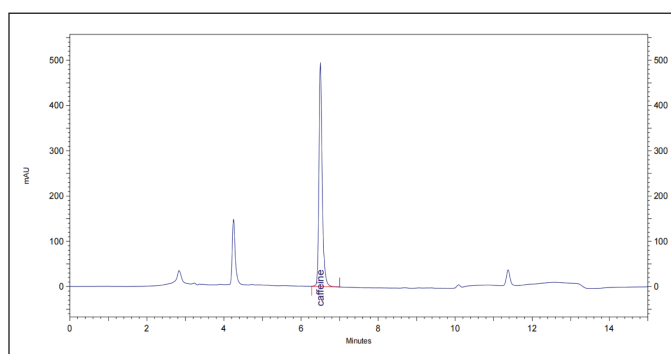


Figure 7. Chromatogram of the solution of the test sample XLS.

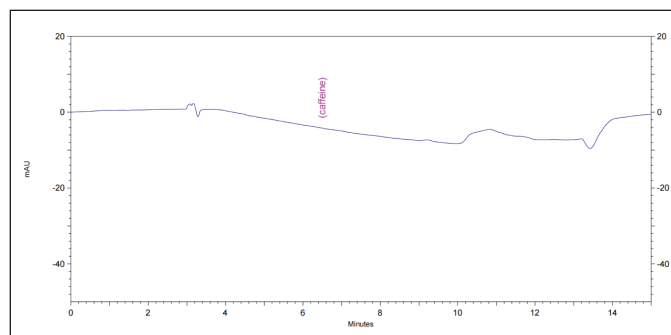


Figure 8. Chromatogram of the solvent (water).

Table 5. The results of the linearity study.

Parameter	Parameter value	Requirement	Result
y-intercept	70.0	$\leq 2 \cdot SD$	Pass
SD of y-intercept	56.7		
y-intercept/signal of St with nominal concentration, %	2.6	≤ 3.2	Pass
Slope	259.1		
SD of slope	5.4		
Coefficient of regression	0.9990	≥ 0.998	Pass

Precision (repeatability and intermediate precision)

1. Verification of precision for the studied object CL

Two series of measurements were performed by two analysts using the same method on different days to examine precision. In each series, six test solutions were used.

As seen in Table 6, the relative standard deviation (RSD) of the analysis results from analysts one and two does not exceed the established criteria, indicating the good reproducibility of the results.

2. Verification of precision for the studied object XLS

As seen in Table 7, the RSD of the analysis results from analysts one and two does not exceed the established criteria, indicating the proper reproducibility of the results.

Stability of solutions over time

A solution of standard caffeine (Std) with a concentration of 10 mcg/ml and test solutions (CL, XLS) were injected into the chromatographic system immediately after preparation and after 1 day of being stored at 8°C. The criterion of the insignificance of change in concentration of the solutions is set at no more than 1.0%.

The analytical solutions were found to be stable for 1 day when stored at 8°C (Table 8).

Accuracy

The accuracy was determined by checking the complete extraction from the solid residue. For this, sample preparation for the assay was centrifuged, the supernatant was removed, and the residue was resampled the same way as the test solution.

The amount of caffeine in solid residue does not exceed 2.0% (Table 9). Therefore, the method can be considered correct, and one-step extraction is sufficient for accurate assay, significantly simplifying and speeding up sample preparation.

Table 6. Results of the verification of precision of the method for CL.

	Analyst 1	Analyst 2	Requirement	Result
Result, mg/sachet	165.0	168.5	Value difference being no more than 3.2%	Value difference of 2.1% Pass
RSD, %	0.65	2.00	No more than 2.0%	Pass

Table 7. Results of the verification of precision of the method for XLS.

	Analyst 1	Analyst 2	Requirement	Result
Result, mg/pill	20.4	20.5	Value difference being no more than 3.2%	Value difference of 0.5% Pass
RSD, %	0.25	0.72	No more than 2.0%	Pass

Table 8. Stability of analytical solutions.

	Δ , %	Result
Std	-0.06	Pass
CL	0.46	Pass
XLS	0.23	Pass

Table 9. The results of the accuracy study.

	% of the residue after the first extraction		% of the residue after the first extraction
XLS1	0.2	CL1	0.8
XLS2	0.2	CL2	0.8
XLS3	0.1	CL3	1.0
XLS4	0.3	CL4	0.7
XLS5	0.8	CL5	0.6
XLS6	0.4	CL6	0.9
Mean	0.4	Mean	0.8

Table 10. Robustness results.

	Flow rate, ml/minute	Pressure, bar	R_s	Assay for XLS
Discovery F5 250 × 4.6 (5 μ m)	1.0	210	6.1	20.4
Discovery F5 150 × 4.6 (5 μ m)	0.6	75	5.5	20.5

Robustness

The method's robustness in replacing the chromatographic column discovery F5 250*4.6 (5 μ m) with Discovery F5 150*4.6 (5 μ m) was tested. The change in the geometric parameters of the chromatographic column was taken into account by changing the flow rate from 1.0 to 0.6 ml/minute.

The assay results for XLS dietary supplements under these changes differed by 0.5%, which satisfies the requirements for intermediate precision, as shown in Table 10.

It should be noted that reducing the length of the chromatographic column can significantly reduce the pressure, whereas the separation between caffeine and theophylline changes insignificantly. This approach solves the problem of high pressure when using water-ethanol mixtures as a mobile phase without losing the separation capability of the chromatographic system.

CONCLUSION

The developed chromatographic method using SiO₂-PFP stationary phase and the water-ethanol mobile phase is an effective and reliable technique for determining caffeine in dietary supplements for weight loss. The method was validated for two dietary supplements, Light Energy Drive and XLS DUO Slim & Shape. The validation characteristics meet the acceptance criteria, which indicates that this method can be used to analyze multicomponent dietary supplements for caffeine content. Light Energy Drive contains a small amount of caffeine, namely, about 20 mg per single dose, whereas XLS DUO Slim & Shape contains about 165 mg in a single dose. The high caffeine content in this drug can endanger the patient's mental or physical health if taking 2-3 tablets at a time. Additionally, optimization direction was suggested by replacing the column with a 40% shorter length to decrease pressure build-up in the system. Overall, the proposed method provides a safe and environmental friendly approach to analyzing caffeine in dietary supplements.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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