Original articles

A Single-Run HPLC–PDA Method for Simultaneous Screening of Sibutramine, Phenolphthalein, Caffeine and Furosemide in Weight-Loss products

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Abstract

Background: A high-performance liquid chromatography method with photodiode-array detection (HPLC-PDA) was developed and validated for the simultaneous quantification of caffeine (CAF), furosemide (FUR), phenolphthalein (PHE), and sibutramine (SIB) in herbal weight-loss products.

Materials and Methods: The method employed methanol for sample extraction and utilized a C18 column with a phosphate buffer-acetonitrile gradient.

Results: Validation according to ICH and AOAC guidelines confirmed excellent linearity (10 - 150 µg/mL, $R^2 \geq 0.991$), accuracy (spike recovery: 95.89-103.08%), and precision (intra- and inter-day RSD ≤ 2.66%). The limits of detection and quantification were sufficiently low to detect these adulterants at trace levels. Application to 30 commercial products from Vietnam revealed that 20.0% (6 samples) were adulterated with four synthetic drugs, primarily with SIB alone or in combination with PHE.

Conclusion: These findings underscore the need for enhanced regulatory oversight of the weight-loss supplement market. Thus, this method represents a robust, sustainable, and readily applicable solution for quality control laboratories.

Keywords: *sibutramine; phenolphthalein; furosemide; caffeine; HPLC; adulteration; weight-loss products*

1. INTRODUCTION

Obesity is a major social and public health problem because it affects millions of overweight individuals worldwide. Instead of exercising and adopting a reasonable diet, many people turn to herbal preparations advertised to support weight-loss aids with the hope of losing weight quickly but still safely because the products are of herbal origin. However, many studies have shown that several pharmaceutical chemicals such as sibutramine, phenolphthalein, caffeine, and furosemide, have been illicitly mixed into these herbal preparations [1-6]. In 2010, sibutramine was banned by the US Food and Drug Administration (FDA) and health authorities around the world (including Vietnam) due to clear evidence of increasing the risk of serious cardiovascular events such as heart attack and stroke [5]. In addition, some studies found that sibutramine was mixed with phenolphthalein because phenolphthalein is easy to use and has the effect of stimulating the intestinal mucosa, increasing intestinal motility, helping to push stool out quickly; effectively reducing weight [7]. When using a medicinal product such as weight-loss tea, if user quickly feels alert, excited, and less tired immediately, they may believe that the product is effective. Caffeine is the substance responsible for this immediate sensation, deceiving users regarding the true effectiveness of the other herbal ingredients [8]. In order to achieve rapid weight loss, furosemide, a potent loop diuretic, is also sometimes mixed in herbal products to deceive customers and increase profits [3].

The methods for detecting the four active ingredients mainly belong to two groups: chromatographic methods and spectroscopic methods [1-6]. Among of them, HPLC method is public and suitable for many laboratories. Therefore, this study was conducted to develop the single-run HPLC-PDA method for simultaneous screening of sibutramine, phenolphthalein, caffeine and furosemide in herbal products, specifically application of this method for the analysis of four substances adulterated in weight-loss products in Vietnam.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Reference standards of caffeine (CAF), furosemide (FUR), phenolphthalein (PHE), and sibutramine hydrochloride (SIB) were of certified purity (≥ 98%). Specifically, caffeine (control No. C0521099) and furosemide (control No. C0222128) were obtained from the National Institute of Drug Quality Control (NIDQC), Vietnam; phenolphthalein (lot No. C15045607) from Shanghai Macklin Biochemical Technology Co., Ltd. (China); and sibutramine hydrochloride (lot No. 3-AMR-153-1) from Toronto Research Chemicals Inc. (Canada). HPLC-grade acetonitrile and methanol, potassium dihydrogen phosphate (KH_2PO_4), hydrochloric acid, and diethyl ether were used as received.

2.2. HPLC analysis

Chromatographic analyses were performed on a Shimadzu LC-20A system equipped with an autosampler and a photodiode-array (PDA) detector, using InertSustain™ C18 (4.6 × 250 mm, 5 µm) and Agilent Eclipse C18 (4.6 × 150 mm, 5 µm) as the analytical column.

2.3. Samples and sample preparations

A representative placebo matrix used during method development was a weight-loss support capsule (Slimtosen) composed of five botanicals: *Nelumbo nucifera, Alisma orientale, Garcinia cambogia, Gynostemma pentaphyllum,* and *Cassia obtusifolia*. This placebo matrix was employed for specificity and recovery experiments and to prepare matrix-matched standards.

For application, 30 marketed products were obtained from the Vietnamese market: capsules (n = 24), tablets (n = 1), powders (n = 3), and tea bags (n = 2). Samples were labelled VNB01-VNB30.

Samples were homogenized, and an amount equivalent to one-half of the labeled dose was weighed into a 50-mL polypropylene (Falcon®) tube. Twenty-five milliliters of *n*-hexane acidified with HCl was added and the tube shaken for 5 min; after phase separation, the hexane layer was discarded and the residual pellet air-dried. The dry residue was then extracted with 25.0 mL absolute methanol by vortex-mixing for 5 min followed by sonication for 10 min. The extract was centrifuged at 6000 rpm for 5 min, filtered through a 0.45 µm membrane, diluted to within the calibration range, and injected for HPLC analysis.

2.4. Method validation

Method validation followed ICH and AOAC single-laboratory guidance [9]. The following characteristics were assessed: system suitability, specificity, linearity, accuracy, precision, and detection/quantification limits.

System suitability: A mixed standard containing CAF, FUR, PHE, and SIB was injected six times under the final HPLC conditions. Retention time stability, theoretical plates, tailing factor, and peak-area %RSD were recorded to confirm proper system performance.

Specificity: Chromatograms were acquired for blank matrices (capsule VNB13 and tea TB31 with no target actives), single-component standards, the mixed standard, and matrix-spiked samples. Peak identity was confirmed by retention time and agreement of PDA spectra with those of reference standards.

Linearity: Calibration was established with single-component standards across a working range suitable for herbal matrices (nominally 10-150 µg/mL per analyte). Least-squares regression was used for evaluation.

Accuracy: Trueness was evaluated by spike-recovery at three levels (low, medium, high). For each level, three independent samples were fortified, processed, and analyzed. Percent recovery and %RSD were calculated for each analyte and level.

Precision: Repeatability (intra-day) was determined from the three spike levels analyzed in triplicate within one day and expressed as %RSD. Intermediate precision (inter-day) was determined by repeating the same protocol on a second day and reporting %RSD across days.

Limits of detection (LOD) and limits of quantification (LOQ): LOD was defined as the lowest concentration that can be detected but not reliably quantified. LOQ was defined as the lowest concentration that can be quantified with acceptable precision. Both were estimated from signal-to-noise ratios in matrix-matched conditions, using S/N ≈ 3:1 for LOD and S/N ≈ 10:1 for LOQ.

2.5. Statistical analysis

Mean, standard deviation (SD), relative standard deviation (RSD), regression equation, correlation coefficient (R^2), variance, and recovery were calculated during data processing and method validation using Microsoft Excel 2016.

3. RESULTS

3.1. Optimization of HPLC conditions

Two reversed-phase columns were compared, Agilent Eclipse C18 (4.6 × 150 mm, 5 µm) and InertSustain™ C18 (4.6 × 250 mm, 5 µm), together with two detectors, RF-20A and PDA SPD-M20A. The

InertSustain™ C18 column provided better resolution and peak symmetry for phenolphthalein and sibutramine while maintaining acceptable efficiency for caffeine and furosemide. The PDA detector was selected because it provided adequate sensitivity at 225 nm and enabled spectral confirmation of peak identity. The mobile phases consisted of acetonitrile (phase A) and potassium dihydrogen phosphate buffer at pH 3.2 (phase B). Flow rates between 0.6 and 1.0 mL/min were investigated. A flow of 0.8 mL/min offered a good compromise between resolution, analysis time, and system back-pressure. The final gradient program for phase A was: 0-1 min 20%, 1-3 min 40%, 3-11 min 50%, 11-12 min 50%, 12-13 min 20%, and 13-28 min 20% for re-equilibration. The injection volume was 20 µL, the column temperature was 40 °C and the detection wavelength was 225 nm.

3.2. Assay method validation

3.2.1. System suitability

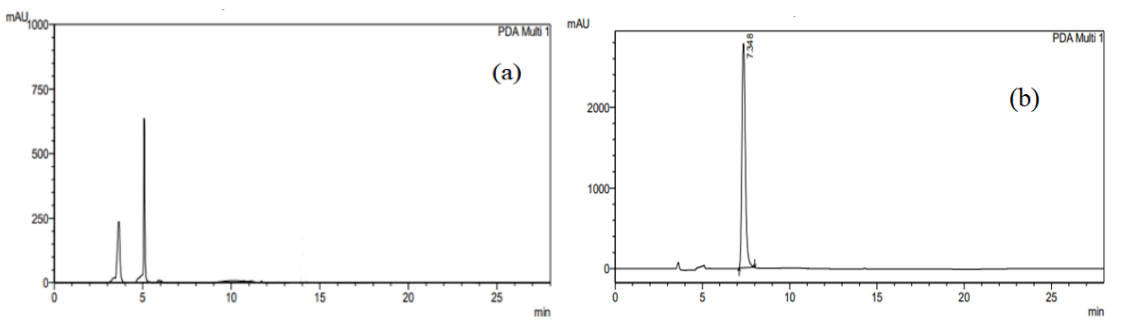
Six consecutive injections of the mixed standard (CAF, FUR, PHE, SIB; 25 µg/mL each) were performed under the finalized HPLC conditions to verify system suitability. Retention-time (R_t) stability, plate number (N), tailing factor (T_f), and peak area %RSD were evaluated against preset limits (area %RSD ≤ 2%), as shown in Table 1. System suitability satisfied expected criteria (stable retention, N > 2000, T_f ≤ 2, area RSD ≤ 2%), indicating an adequate state of control for simultaneous quantification. Thus, all analytes met the acceptance criteria, indicating that the system operated within control and that subsequent analytical batches are comparable and valid.

Table 1. System suitability study of the chromatographic system

	Parameters	CAF	FUR	PHE	SIB
RSD%	R_t	0.215	0.208	0.220	0.139
	Peak area	0.561	0.743	0.675	0.814
	T_f	0.835	0.548	0.400	0.435
	N	0.642	0.256	0.760	0.430

3.2.2. Specificity

The specificity of the method was high when comparing retention time, peak purity and UV spectrum. Specificity was supported by blank matrices free of interferences at the analyte windows and by concordant UV spectra for standards and matrix-spiked samples. Firstly, distinct peaks for CAF, FUR, PHE, and SIB of each single-component standard in Figure 1.b – 1.e, appeared at retention times of 7.35 min, 15.28 min, 17.87 min, and 18.51 min, respectively. In the chromatograms of the blank matrices of Figure 1.a, no peaks were observed at these corresponding retention windows. In the multi-component standard of Figure 1.f, four peaks occurred at the same retention times as in the single-component standards, they also occurred at the same retention times as on the mixed standard spiked into matrix of Figure 1.g, Secondly, the purities of all peak in Figure 1.a - 1.g were higher than 0.9990, which means that the peaks were completely separated. Thirdly, the UV spectra of all four analytes in matrix-spiked samples matched those of the corresponding reference standards, in Figure 1a – 1.d. Thus, these observations demonstrate high specificity of the method for detecting CAF, FUR, PHE, and SIB when admixed in the surveyed herbal weight-loss preparations.



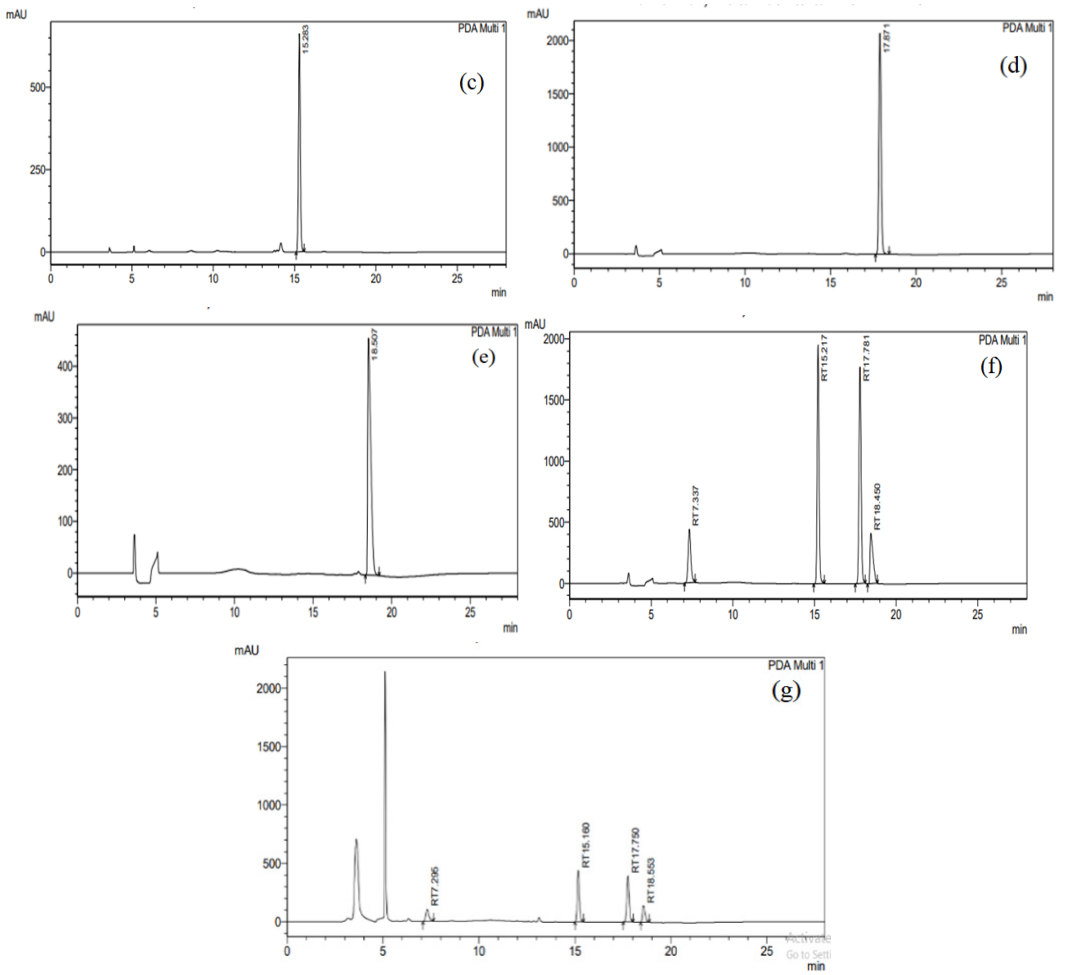


Figure 1. Chromatograms for specificity assessment: blank matrix (a); single-analyte standards: caffeine (b), furosemide (c), phenolphthalein (d), sibutramine (e); mixed standard (f); mixed standard spiked into matrix (g).

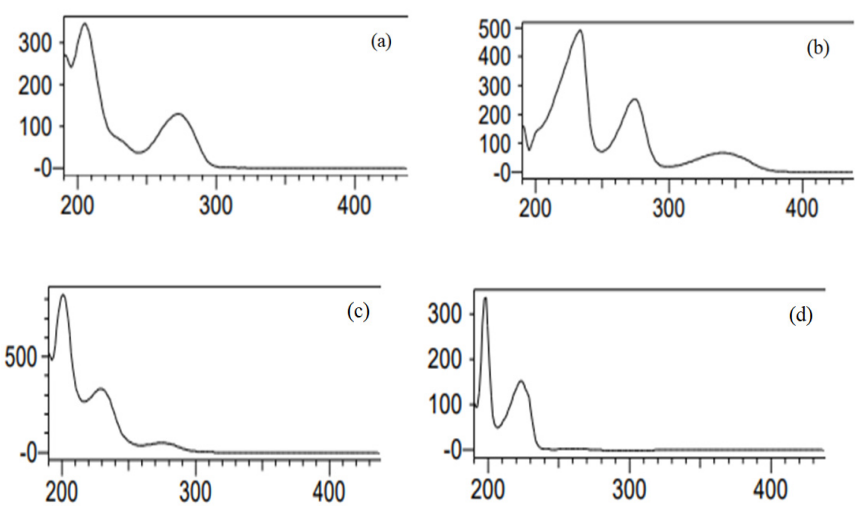


Figure 2. UV spectra of caffeine (a), furosemide (b), phenolphthalein (c), and sibutramine (d)

3.2.3. Linearity

Calibration with single-component standards demonstrated linear response over 10-150 µg/mL for all analytes. The calibration equation of CAF was $y = 39551x - 142811$ ($R^2 = 0.992$); FUR: $y = 131489x - 203355$ ($R^2 = 0.995$; PHE: $y = 124694x - 614569$ ($R^2 = 0.991$); SIB: $y = 55355x - 65107$ ($R^2 = 0.996$). All Correlation coefficients exceeded 0.99 and residual plots showed no curvature, indicating sustained linearity suitable for external calibration.

3.2.4. Accuracy

Accuracy was assessed by spike-recovery at three concentration levels (low, medium, high), each level prepared in triplicate and processed. The results are shown in Table 2. Mean recoveries across analytes ranged from 95.8% to 103.08%, indicating minimal systematic bias after extraction and clean-up. These results demonstrate that the trueness of the method meets AOAC single-laboratory acceptance criteria.

Table 2. Accuracy (spike-recovery) assessment

Spike added (µg)	% Mean recovery			
	CAF	FUR	PHE	SIB
625	101.74	95.89	103.08	98.35
1875	98.24	97.15	98.22	100.37
3750	103.04	97.92	96.27	101.63

3.2.5. Precision

Repeatability (intra-day) at three spike levels gave a maximum %RSD of 2.66%, and intermediate precision (inter-day) assessed on a subsequent day gave a maximum %RSD of 2.61%. Both values are within the AOAC criterion (RSD ≤ 3.7%) for the tested concentration range. The details are shown in Table 3.

Table 3. Precision assessment

		Conc. (µg/ml)	CAF	FUR	PHE	SIB
RSD%	Intra-day	25	0.74	2.66	0.33	0.79
		75	0.75	2.03	0.47	1.85
		150	0.37	0.11	1.45	2.58
	Inter-day	25	1.31	0.21	0.56	2.55
		75	2.61	1.24	1.40	0.72
		150	2.28	0.84	2.19	0.58

3.2.6. Limits of detection and quantification

Detection capability was estimated from signal-to-noise ratios in matrix-matched conditions, using approximately 3:1 for LOD and 10:1 for LOQ. The resulting LOD/LOQ pairs (µg/mL) were 0.75/2.25 for CAF, 0.25/0.75 for FUR, 0.042/0.125 for PHE, and 0.125/0.375 for SIB.

3.3. Application to marketed products

A total of 30 herbal weight-loss products were collected from the Vietnamese market across four dosage forms: capsules (n = 24), tablets (n = 1), powders (n = 3), and tea bags (n = 2). The validated HPLC method was applied to these samples to screen for CAF, FUR, PHE, and SIB. Six products tested positive for at least one target analyte, corresponding to a 20.0% positivity rate. Co-adulteration was common; the most frequent pattern was SIB and PHE in 2/30 products (6.67%), SIB and CAF occurred in 1/30. Quantitatively, SIB was detected in 5/30 products with per-dose amounts ranging from 7.66 to 21.40 mg. PHE was detected in 2/30 products with 8.26 to 12.48 mg per dose. FUR appeared in 1/30 at 32.44 mg per dose, and CAF in 1/30 at 71.04 mg per dose. Notably, five SIB-positive samples exceeded the historical 10-15 mg/day maximum adult dose in use before sibutramine withdrawal, underscoring a significant consumer safety concern [5]. Detailed per-sample results, including both mg g^{-1} and mg per labeled dose, are reported in Table 5 (e.g., VNB06, VNB09, VNB21, VNB22, VNB23, VNB30). For example, the chromatograms of sample VNB23 in Figure 3.a contained two peaks at the retention of SIB and PHE. Both peaks had purities greater than 0.99, and their UV spectra overlaid well with those of the corresponding standards, confirming the presence of SIB and PHE (Figure 3.a). These findings show that a sizeable fraction of marketed products contain undeclared pharmaceuticals at pharmacologically meaningful levels, and they support the routine use of the present HPLC protocol for market surveillance and enforcement.

Table 5. Quantified content in positive real samples

Sample ID	Detected analyte	Content (mg/g)	Content (mg/dose)
VNB06	CAF	4.44	71.04
	SIB	0.48	7.66
VNB09	PHE	5.50	8.26
	SIB	12.14	18.20
VNB21	SIB	35.53	14.21
VNB22	SIB	42.80	21.40
VNB23	PHE	24.96	12.48
	SIB	32.99	16.50
VNB30	FUR	64.88	32.44

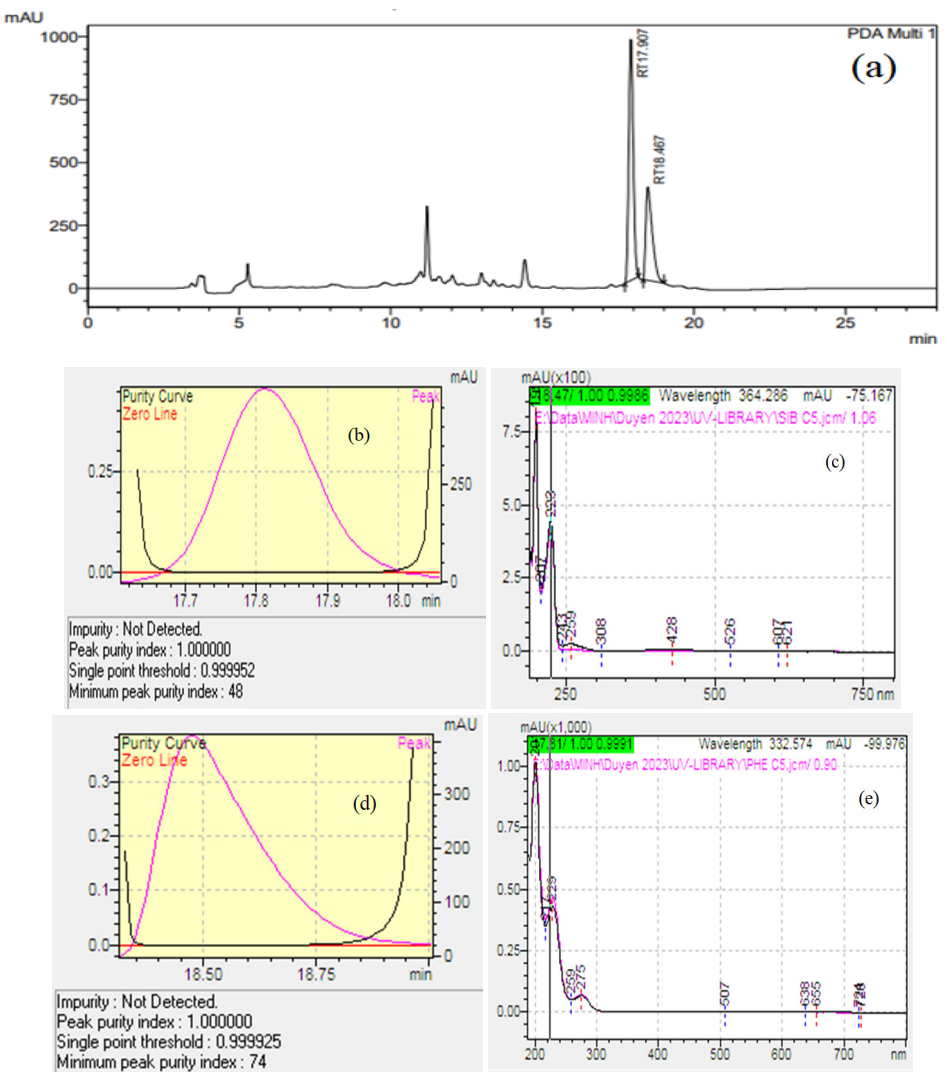


Figure 3. Chromatograms of certain positive samples: sample VNB23 (a); purity of the SIB peak (b); spectral overlay with the SIB standard for the SIB peak (c); purity of the PHE peak (d); and spectral overlay with the PHE standard for the PHE peak (e) of sample VNB23.

4. DISCUSSION

4.1. Analytical workflow and chromatographic conditions

The finalized HPLC setup (InertSustain™ C18, 4.6 × 250 mm, 5 µm; ACN/KH_2PO_4 pH 3.2; gradient elution; 0.8 mL min^{-1}; 20 µL; PDA at 225 nm) achieved baseline separation of CAF, FUR, PHE, and SIB with compact, symmetric peaks and appropriate retention. The organic modifier was ACN, whereas some authors reported MeOH as the organic phase [10], aligning with studies that favored ACN for its lower viscosity and robust elution [11, 12]. KH_2PO_4 was selected as the aqueous buffer system, mirroring [2] and differing from ammonium acetate [12], phosphoric acid [13], or formic acid [11]. PDA detection at 225 nm parallels the use of diode-array detectors in related work [6, 14], although other authors have employed FLD for different analytical purposes or analyte panels [11].

4.2. Sample preparation considerations

Given the complexity of capsule, tablet, powder, and tea matrices, a dedicated clean-up was introduced prior to extraction. Among 30 samples, there are 24 capsules so that the representative placebo matrix used during method development was a weight-loss support capsule (Slimtosen). It composed of five botanicals: *Nelumbo nucifera, Alisma orientale, Garcinia cambogia, Gynostemma pentaphyllum,* and *Cassia obtusifolia.* In contrast to many prior protocols that extracted directly into a polar solvent without a defatting step [2, 6, 22], *n*-hexane was used here to remove co-extractives, thereby stabilizing baseline and improving peak shape. For the extraction solvent, methanol (100%) produced higher overall recovery than ethanol (96%) or acetonitrile, consistent with the use of neat methanol in R. Ancuceanu and colleagues and in Saito's study [2, 6], while differing from methanol 70% [22]. A 10-min sonication proved sufficient to liberate analytes without excessive co-extraction observed at longer times, which aligns with the goal of maximizing recovery while controlling matrix effects. The resulting workflow, defatting with acidified % followed by a short methanol extraction reduced interferences and generated sharp, well-resolved chromatographic peaks.

4.3. Method validation

System suitability satisfied expected criteria (stable retention, N > 2000, Tf ≤ 2, area RSD ≤ 2%), indicating an adequate state of control for simultaneous quantification. Specificity was supported by blank matrices free of interferences at the analyte windows and by concordant UV spectra for standards and matrix-spiked samples. The calibration range of 10-150 µg/mL met quantitative needs and compares favorably with narrower ranges reported elsewhere, for example, 40 - 60 µg/mL for all four analytes in [29] and 25 - 50 µg/mL (PHE) or 40-90 µg/mL (SIB) in [2] though some authors have reported wider spans for selected targets (e.g., CAF 4-200 µg/mL; SIB 40-400 µg/mL) [6].

4.4. Market findings

Application to 30 marketed herbal weight-loss products revealed adulteration in 20.0% of samples, with frequent SIB and PHE co-occurrence. This prevalence is directionally consistent with international findings but varies by market and sampling frame: Saito observed SIB in 74% and PHE in 36% of 39 Chinese-origin products and also reported CAF in 20% [6], Cheng detected SIB in 24.16% of 120 samples [15], Moreira reported two FUR-positive products among 26 Brazilian herbal items [3], and Wang identified SIB in 10 of 22 supplements, three of which also contained PHE [16]. The per-dose quantities observed here, including instances exceeding historical therapeutic SIB dosing, mirror the pharmacologically meaningful levels cited across these reports and underscore the risk of unsupervised exposure to anorectics, laxatives, diuretics, and stimulants in products marketed as "herbal".

5. CONCLUSIONS

A single-run HPLC-PDA method was developed, optimized, and validated for the simultaneous determination of caffeine, furosemide, phenolphthalein, and sibutramine in herbal weight-loss matrices. The sample-preparation strategy and chromatographic conditions yielded clean separations and acceptable validation performance with respect to specificity, linearity, precision, trueness, and detection capability. When applied to 30 marketed products, the method revealed a substantive proportion of positives, including frequent co-adulteration and per-dose amounts at pharmacologically relevant levels. These results support the routine adoption of the protocol for market screening and reinforce the need for continued regulatory oversight of weight-loss supplements.

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Declaration of competing interest

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

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