

Validation of sibutramine and phenolphthalein determination by HPLC-PDA in natural dietary supplements for body-weight reduction

^{1,*}Nguyen, T.N.V., ¹Nguyen, H.K.N., ¹Nguyen, T.N.M., ¹Duong, T.N., ¹Pham, T.T.L. and ²Nguyen, T.K.

¹College of Pharmacy, Can Tho University of Medicine and Pharmacy, 179 Nguyen Van Cu Street, Can Tho City, Vietnam

²College of Medicine, Can Tho University of Medicine and Pharmacy, 179 Nguyen Van Cu Street, An Khanh Ward, Ninh Kieu District, Can Tho City, Vietnam

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Abstract

The worldwide demand for natural dietary supplements for body-weight reduction is increasing, because they are commonly believed to be safe. However, the abundance and diversity of available dietary supplements impede their quality control, potentially posing multiple health risks. A simple and cost-effective procedure involving high-performance liquid chromatography photodiode array detector (HPLC-PDA) and homogeneous liquid-liquid extraction (LLE) of samples were applied to analyze simultaneously sibutramine and phenolphthalein in natural dietary supplements used for body-weight reduction. The recoveries obtained ranged from 91.87% to 96.46%. The limits of detection and quantification were 0.33 µg/mL and 1 µg/mL, respectively. The phenolphthalein and sibutramine contents were in the range of 0.009-5.601 mg/tablet and 0.005-4.409 mg/tablet, respectively. The results showed that the proposed HPLC-PDA method was precise and accurate. It was simple to apply to identify and quantify these and other adulterants in natural dietary supplements.

1. Introduction

Dietary supplements for body-weight reduction are attractive to many customers seeking solutions to problems of being overweight and obese. The use of natural products as an alternative to obesity treatment has increased, which has led to a growing market for herbal remedies worldwide (Sargin, 2021). Because these products have “natural herbal components”, they are regarded as harmless and effective methods of obesity treatment (Ernst, 2002; Qaid *et al.*, 2021). However, their safety, efficacy, and quality control are not verified and adulterated herbal products are manufactured illegally. Health officials worldwide are extremely concerned about the potential health problems that might result from formulations of natural dietary supplements for body-weight reduction that have been contaminated or adulterated with synthetic materials and prescription medicine analogs (Khazan *et al.*, 2014).

Tucker *et al.* (2018) reported that, between 2007 and 2016, the US Food and Drug Administration (FDA) identified 776 adulterated dietary supplements, and 146 companies were held accountable. The majority of these items were advertised for weight loss (40.9%), muscle

building (11.9%), and sexual enhancement. Several contained more than one banned substance, the most common for body-weight reduction being sibutramine (84.9%), and phenolphthalein (23.7%). Sibutramine and phenolphthalein had been removed from the US market by the FDA in 2010 and 1999, respectively (Tucker *et al.*, 2018). In the United States, United Kingdom, European Union, Australia, Canada, and certain Asian nations, sibutramine was taken off the market after research revealed its link to an increased risk of cardiovascular events, heart failure, and sudden death (Rodriguez-Guerra *et al.*, 2021; Khan, 2021; Alruwaily *et al.*, 2022). Phenolphthalein is an effective ingredient in laxatives, aids in the treatment of constipation, breaks down fat, and assists in the removal of toxins via the digestive system; its side effects include intestinal cancer, enteritis, miscarriage, bleeding, and chronic constipation and congestion (Meng *et al.*, 2021; Suryoprabowo *et al.*, 2021; Adewuyi *et al.*, 2022). Quality control of phenolphthalein and sibutramine in natural body-weight reduction products is extremely necessary.

Worldwide, numerous analytical techniques have

*Corresponding author.

Email: ntnvan@ctump.edu.vn; nguyenthingocvanct@gmail.com

provided separate or simultaneous determinations of sibutramine and phenolphthalein, for example, capillary electrophoresis (CE), gas chromatography coupled mass spectrometry (GC-MS), liquid chromatography coupled mass spectrometry (LC-MS/MS), ultra-high pressure liquid chromatography (UPLC), high-performance liquid chromatography (HPLC) (Khazan *et al.*, 2014; Wang, Man, Shu *et al.*, 2016; Alabdoolo *et al.*, 2017; Zhang *et al.*, 2021; Pratiwi *et al.*, 2021; Lee *et al.*, 2022). The CE method is a modern, selective and accurate analytical technique with a fast analysis time. The GC-MS is a modern analytical method, with high efficiency, high resolution and sensitivity; it also saves solvents. However, the equipment is expensive, and the complicated sample extraction procedure is not yet popular in laboratories in Vietnam; therefore, its application is limited. Among the analytical techniques that have been applied, HPLC is the most common; applied routinely, it is a highly accurate technique.

In the present study, we developed an easy and cost-effective high-performance liquid chromatography photodiode array detector (HPLC-PDA) method for simultaneous analysis of sibutramine and phenolphthalein in natural dietary supplements for body-weight reduction. The analytes were extracted by homogeneous LLE, which extracts solutes from a homogeneous phase into a water-immiscible phase by a change in temperature, ionic strength, or pH of the solution (Wang, Hu, Du *et al.*, 2016; Ma *et al.*, 2021; Hammad *et al.*, 2022). The procedures were preliminarily validated and used for analysis of some commercial natural dietary supplements for body-weight reduction to ensure the quality of the products, thereby protecting the health of consumers.

2. Materials and methods

2.1 Chemicals and reagents

All chemicals and reagents used in the experiments had analytical-grade purity. Methanol, acetonitrile, and HPLC-grade water were purchased from Merck (Germany). Ammonium acetate, formic acid, and ammonia solution (25%), magnesium sulfate anhydrous, sodium chloride were purchased from Fisher (USA). The 1-(4-Chlorophenyl)-N,N-dimethyl- α -(2-methylpropyl) cyclobutanemethanamine hydrochloride monohydrate (SB, Hydrochloride Monohydrate) reference standard (assigned purity 99.9%) was supplied by LGC Standard. 3,3-Bis (4-hydroxyphenyl)-1(3H)-isobenzofuranone (PP) reference standard was purchased from HBC Standard (assigned purity 99.8%).

2.2 Standard solutions and sample preparation

Individual standard stock solutions of sibutramine

and phenolphthalein (1000 $\mu\text{g/mL}$) in methanol were prepared. Working standard solutions were prepared daily by diluting stock solutions in methanol at a concentration of 400 $\mu\text{g/mL}$. The standard stock and working solutions were protected from light and stored at 4°C.

To prepare sample solutions, homogenized 0.5 g powders obtained by directly emptying capsules or grinding tablets were sonicated with a standard mixture of 100 ng/g and 5 mL of methanol solvent. It was ultrasonicated for 10 mins, then centrifugated at 4000 \times g for 5 mins at 35°C. The extraction was repeated twice. The solvent was evaporated in nitrogen gas.

2.3 Optimization of sample preparation

2.3.1 Extraction procedure

Ultrasound-assisted extraction (UAE) has several advantages including high extraction efficiency, good reproducibility, low solvent consumption, low cost, environmental friendliness, laboratory availability, and ease of scale-up for industrial applications. The UAE procedure was optimized with regard to the solvent (ethanol, methanol, acetonitrile) (Aceves-Hernández *et al.*, 2014; Guan *et al.*, 2019), solid-liquid ratio (1:5, 1:10, 1:15, 1:20 g/mL), extraction time (5, 10, 15, and 20 min), and sonication time (once, twice, and thrice). The sum of the phenolphthalein and sibutramine peak areas was used to evaluate the extraction efficiency.

2.3.2 Clean-up procedure

For effective solvent extraction, aqueous ammonia was examined at different concentrations (0.1%, 0.3%, 0.5%, 0.7%, 0.9% and 1%). Effectiveness was evaluated by peak area of phenolphthalein, sibutramine, and impurities in the matrix when spiking the sample.

Homogeneous LLE was applied for clean-up of the sample matrix. The sample was washed with 2 mL acetonitrile, and 2 mL 0.1% NH_4OH and 2 g NaCl were added. The upper layer was anhydrous with 2 g magnesium sulfate anhydrous. The eluted solvent was evaporated and reconstituted with 200 μL of acetonitrile and 300 μL of 0.2% formic acid. Non-polar impurities in the extract were removed by shaking with 500 μL n-hexane. The extract was then filtered through a 0.22- μm PTFE filter, and transferred into a vial.

2.4. Chromatographic conditions

Method development, quantification, and validation studies were performed on a Shimadzu (LC 20AD), detector DAD SPD-M20A. Chromatographic separation was achieved using a Restek C_{18} column (250 mm \times 4.6 mm I.D, 5 μm) at ambient temperature. The mobile

phases of the isocratic and gradient elution programs are shown in Table 1. The mobile phase consisted of acetonitrile containing 0.1% formic acid (A), methanol (B) and methanol–water (40:60) containing 0.2% ammonium acetate (C). The injection volume was 20 μ L, and all chromatographic separations were performed at a flow rate of 1.0 mL/min. The analytes were identified at a wavelength of 225 nm.

Table 1. The mobile phases survey of the isocratic elution program and gradient elution program.

No.	Mobile phases
<i>Isocratic elution program</i>	
1	Methanol– Water
2	KH ₂ PO ₄ buffer 0.05M pH 4 – Acetonitrile
3	KH ₂ PO ₄ buffer 0.05M pH 4.5 – Acetonitrile
4	KH ₂ PO ₄ buffer 0.05M pH 5 – Acetonitrile
5	KH ₂ PO ₄ buffer 0.05M pH 5 – Acetonitrile
<i>Gradient elution program</i>	
	A: Formic acid 0.1%/ Acetonitrile
6	C: Acetate ammonium 0.2% + formic acid 0.1%/ Water B: Methanol
7	C: Acetate ammonium 0.2% + Formic acid 0.1%/ Water A: Formic acid 0.1%/ Acetonitrile
8	B: Methanol C: Acetate ammonium 0.2%/ Methanol – Water (40:60) A: Acid formic 0.1%/ACN
9	B: Methanol C: Acetate ammonium 0.2%/ Methanol – Water (40:60)

3. Results and discussion

3.1 Optimization of sample preparation

3.1.1 Extraction method

The results of the optimization of the sample preparation procedure are shown in Figure 1. Based on the solubility of the analytes and referring to some related references (Aceves-Hernández *et al.*, 2014; Guan *et al.*, 2019), three extraction solvents were selected: methanol, ethanol, and acetonitrile. The results showed that the extraction efficiency of phenolphthalein and sibutramine from the sample matrix was highest with methanol solvent (Figure 1a). Therefore, methanol was selected as the extraction solvent. The effect of solvent volume on extraction yields was investigated using methanol as solvent from 2.5 to 10 mL. Figure 1b illustrates that both analytes were significantly affected by the volume of extraction solvent. As a result, the volume of solvent extraction selected was 5 mL.

For ultrasonic-assisted extraction, the time factor has a strong influence on extraction efficiency. Figure 2c shows the effect of time on the yields of sibutramine and phenolphthalein using 5 mL methanol as an extraction solvent. The yields of both analytes reached the maximum value at 10 mins, and then decreased sharply. Therefore, a time of 10 mins was preferred as ultrasonic time. The data shown in Figure 1d illustrate that the ideal number of extractions was two in order to extract more than 99% of the analytes from the matrices. The best result of extraction experiments involved ultrasonic-assisted extraction procedure to yield 0.5 g of the sample, which was extracted twice with 5 mL methanol each time for 10 mins.

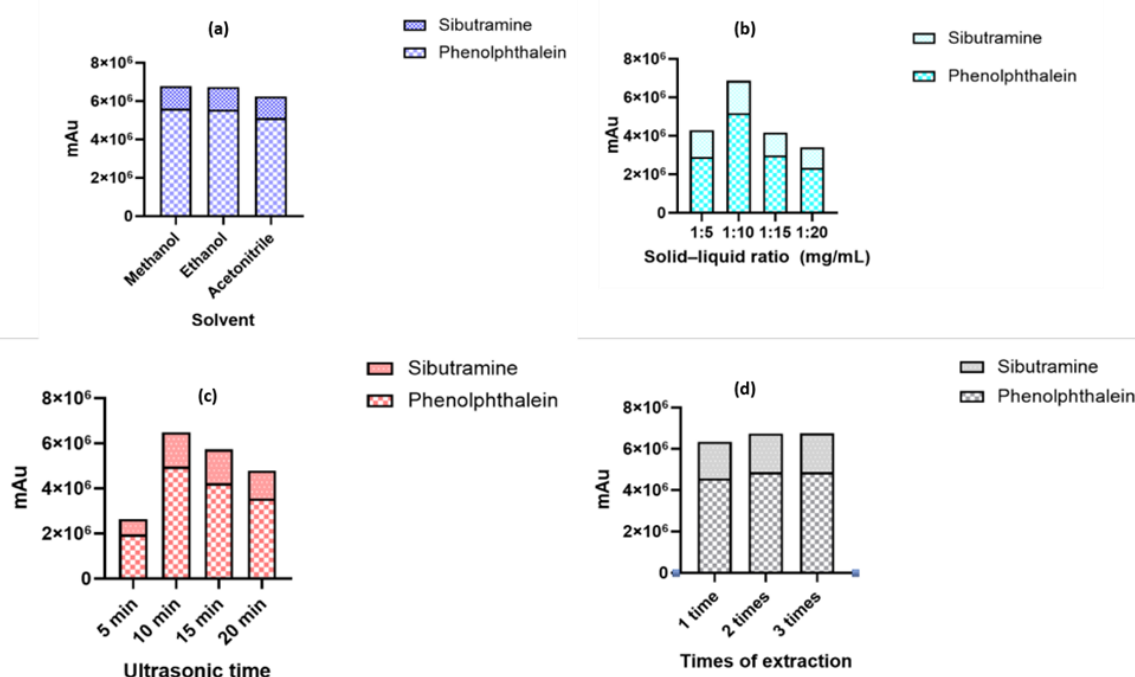


Figure 1. Results of the optimization of the sample preparation procedure (The peak area (mAu) was measured at 225nm detection wavelength).

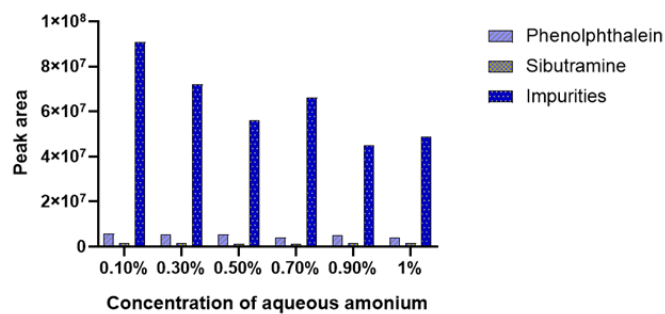


Figure 2. Peak area of analyte and impurities in different concentrations of aqueous ammonium (NH_4OH).

3.1.2 Clean-up procedure

The selected extraction solvent was a strong soluble solvent; in addition to effectively extracting the analytes from the sample matrix, it removed large amounts of impurities, especially polar impurities. This required a suitable method to be developed to reduce the impurities signal while still retaining the majority of the analyte. Literature search helped us identify a two-stage impurity removal process: Homogeneous LLE to remove polar impurities and n-hexane solvent to remove less polar impurities (Wang *et al.*, 2016; Duong *et al.*, 2022).

The selection of an appropriate extraction solvent is one of the most important parameters for the establishment of an efficient homogeneous LLE technique. The extraction solvent must fulfill several criteria such as high extraction capability of the analytes, low density compared to water, low solubility in water, good chromatographic behavior, and formation of a homogeneous solution with water by pH adjustment (Farajzadeh *et al.*, 2016). On this basis, aqueous ammonia was selected. As shown in Figure 3, NH_4OH was used at different concentrations (0.1%, 0.3%, 0.5%, 0.7%, 0.9% and 1%).

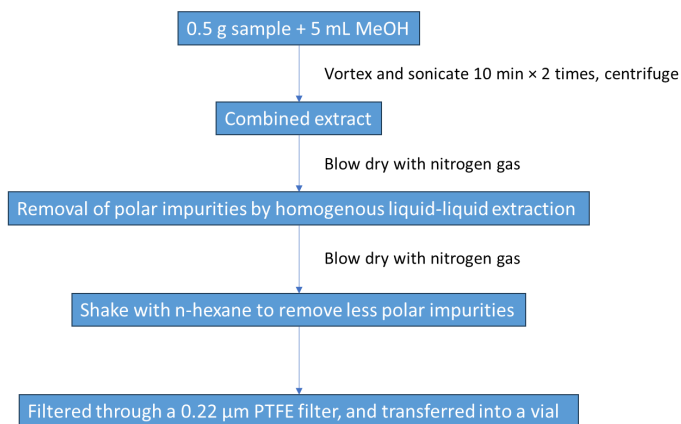


Figure 3. Procedure of sample preparation.

Adding NH_4OH to the acetonitrile extract caused an acid-base reaction and the target analytes were separated into the acetonitrile phase. At 0.1% concentration, the

peak areas of analyte and impurities were both high. Because the analytical matrix is from natural products, it may contain many polar phytochemicals such as flavonoids, glucosides, and alkaloids. At a concentration of 0.1%, these polar impurities produce the highest peak area. Increasing the concentration of NH_4OH changes the pH, converting these polar impurities such as alkaloids to bases, which enter the acetonitrile phase with the two analytes; therefore, the peak area of the impurities decreases. In addition, we found that with increasing NH_4OH concentration, the peak signals of phenolphthalein and sibutramine tended to decrease. Therefore, after considering the amount of analyte obtained and the amount of impurities, we selected 0.1% NH_4OH to optimize the homogeneous extraction solvent. The sample preparation procedure is shown in Figure 3.

3.2 Orientation of chromatographic conditions

Identification of phenolphthalein and sibutramine was based on their retention times (t_R), UV-Vis spectra, and co-injection with standards. From previous research analyzing phenolphthalein and sibutramine by HPLC-PDA (Ancuceanu *et al.*, 2013; Hammadi *et al.*, 2014; Yun *et al.*, 2018), the wavelengths from 210 to 250 nm were selected. The analytes gave good signals at 225 nm, which was selected as the detection wavelength for the procedure.

Based on the chemical nature and solubility of the two analytes, the mobile phase was selected to survey the following components: acetonitrile, methanol, phosphate buffer, and ammonium acetate buffer. When using the methanol-water mobile phase, the retention time of the analytes was relatively long and the effective elution of methanol was low, so we changed the mobile phase to acetonitrile. Suneetha *et al.* (2011) and Patil *et al.* (2009) surveyed isocratic elution programs with mobile phase systems including phosphate buffers and organic solvents. Using phosphate buffer to adjust to acidic pH, sibutramine is more polar to shorten the retention time. To prolong the retention time for the two substances, phosphate buffer was increased by 10% in KH_2PO_4 0.05 M pH 5 - acetonitrile (60:40), and the peaks were separated well. However, this mobile phase system showed the peak drift phenomenon, with different retention times for the two main peaks between days. At the same time, the phosphate buffer ratio was quite high, and it was easy to precipitate with acetonitrile. The use of phosphate buffer in the chromatography system without proper wash may lead to salt deposition and system corrosion, resulting in errors in analysis results.

Therefore, the procedure of Hammadi *et al.* (2014) was adopted. Ahmed *et al.* (2019) and Nasr *et al.* (2017), used ammonium acetate buffer with a gradient elution

program instead of phosphate buffer. Formic acid was added to bring the pH of the mobile phase to about 3, and ammonium acetate buffer to dissociate ions; the sibutramine dissociates into N^+ which will bond with CH_3COO^- of ammonium acetate salt. In addition, the SiO^- radical of the silica gel returns to the undissociated $SiOH$ form at pH 3, which improves the peak tailing phenomenon because of the decreasing secondary association of SiO^- with the N^+ radical of sibutramine (Snyder *et al.*, 2010). In addition, an unstable baseline leads to difficulty in signal recognition of the two analytes. Therefore, the ammonium acetate buffer phase was added to the methanol-water mixture (40:60) to reduce the difference in optical refractive properties of the mobile phase to make the baseline more stable. Because of the problem of an unbalanced baseline, a gradient program incorporating three solvent channels should be investigated. After adjusting for time in the elution program, a mobile phase consisting of acetonitrile/0.1% formic acid (A), methanol (B) and methanol-water (40:60)/0.2% ammonium acetate (C) was selected.

3.3 Method validation

3.3.1 System suitability

Six duplicate injections of a mixed standard solution (10 $\mu\text{g/mL}$) were used to verify the system stability, and the theoretical plate number (N), resolution (R_s), symmetry factor (A_s), and repeatability (relative standard deviation (RSD) of residence time (RT) and peak areas) of the analytes were calculated. All analytes had RSD values for RT and peak areas less than 2.0%. As a result, the suggested approach satisfied this condition (Table 2).

3.3.2 Selectivity

The selectivity of the method indicates the ability to measure the analyte response in the presence of matrix interferences. The selectivity was evaluated by comparing the RT and UV absorption spectra of each component in standard solutions with those of the peaks obtained in the analyzed samples. As shown in Figure 4, the HPLC method could distinguish phenolphthalein and sibutramine from other components in the matrix. The peak purity of the two compounds was >99.9%, as

obtained from the spectrum overlaying the graphs of three-point purity detection.

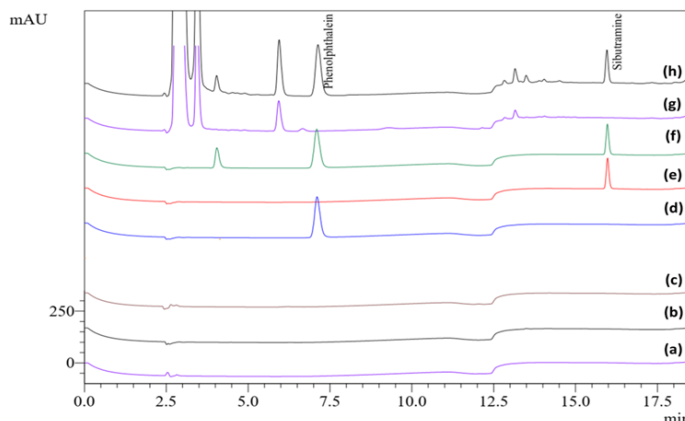


Figure 4. HPLC chromatograms. a: Mobile phase solvent, b: Solvent, c: Extraction solvent, d: Standard solution of phenolphthalein, e: Standard solution of Sibutramine, f: Mixed standards solution, g: Spiked sample, h: Standard addition.

3.3.3 Linearity, limit of detection, and limit of quantification

Linearity data for sibutramine and phenolphthalein are given in Table 3. The stock solutions were diluted and mixed in eight different concentrations ranging from 1.0 to 80 $\mu\text{g/mL}$ of sibutramine and phenolphthalein. Square correlation coefficients (R^2) were higher than 0.995 for sibutramine and phenolphthalein. The limits of detection (LOD) and limits of quantification (LOQ) of both substances were 0.33 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$, respectively.

3.3.4 Precision

The precision of the method was verified by evaluating the intra-day and inter-day precisions. The percentage RSD was selected as a measure of precision. The intra-day precision was examined by analyzing six samples of each substance in a single day, while the inter-day precision was determined by analyzing six samples each day for three days. The precision results shown in Table 3 indicate that the overall intra- and inter-day variations (%RSD) were below 6%, which conforms to AOAC guidelines (Association of Analytical Collaboration (AOAC) International, 2002)

Table 2. System suitability of the HPLC-PDA method.

		t_R (min)	S (mAU)	A_s	R_s	k'	N
Phenolphthalein	Mean	6.74	1206546	1.145	11.053	2.744	8056
	SD	0.004	1177	0.001	0.102	0.002	14.919
	RSD%	0.05	0.10	0.11	0.92	0.07	0.19
Sibutramine	Mean	15.94	442991	1.137	39.776	7.856	154326
	SD	0.012	5106	0.01	0.05	0.007	1365
	RSD%	0.08	1.15	0.84	0.13	0.09	0.88

t_R : Retention time, S: peak area, A_s : symmetry factor, R_s : resolution, k' : capacity factor, N: theoretical plate number.

Table 3. Precision, accuracy and calibration curve.

Substance	Calibration curve		Precision		Recovery (%)		
	Regression equation	R ²	Intra-day RSD (%) (n=6)	Inter-day RSD (%) (n=18)	Low-level (50%)	Mid-level (100%)	High-level (150%)
Phenolphthalein	y = 146066x - 122622	0.9989	1.48	2.46	93.55	96.46	93.97
Sibutramine	y = 41104x - 10455	0.9999	2.24	3.10	91.87	94.43	92.02

3.3.5 Accuracy

The accuracy of the method was evaluated by performing recovery studies. Low, medium, and high concentrations of the reference compounds were added to the blank samples and the spiked samples were added and quantified according to the method described above. The results indicated that the method exhibited good accuracy, with an overall recovery ranging from 91.87% to 96.46%.

3.4. Method application

These results may be compared with those of Nasr *et al.* (2017) and Tran *et al.* (2015). Using HPLC for the simultaneous detection of caffeine and phenolphthalein in weight-reducing supplements, Nasr *et al.* (2017) found a linear relationship over concentrations of 10-100 µg/mL for both caffeine and phenolphthalein, with mean correlation coefficients of 1.000 and 0.999, respectively. Caffeine and phenolphthalein had LOQ values of 2.35 g/mL and 1.44 g/mL, respectively, while their LOD values were 0.77 g/mL and 0.47 g/mL, respectively. For both intra-day and inter-day accuracy, the percentage relative standard deviation was under 2%. For caffeine and phenolphthalein, the computed mean recovery values were 105.84% and 113.58%, respectively. Tran *et al.* (2015) analyzed multiple weight loss drugs in dietary supplements by LC-MS/MS. The method was validated at various fortification levels (250 and 1000 g/g) for 11 drug compounds. The overall recoveries averaged 93.8% with the majority of individual recoveries in the range of 76%–110%. The overall LOQ values for analytes averaged 10 ng/g. An overall indicator of linearity (R²) averaged at 0.9993 (0.0-250.0 ng/mL). The precision of five replicates of the 50 ng/mL level averaged 98.4%. Although the LOD and LOQ values in our method were lower than in the above-mentioned research, this can be explained by sensitivity differences in the technique in each laboratory, and the recoveries and inter-day and intra-day precision met the requirements according to AOAC guidelines.

Using the technique, 18 samples of commercially marketed dietary supplements for body-weight reduction were obtained and tested for sibutramine and phenolphthalein content. Eight of 18 samples (44.44%) tested contained phenolphthalein and 13 of 18 samples (72.22%) contained sibutramine, while only four were

below LOD (Table 4). The phenolphthalein and sibutramine contents were in the range of 0.009-5.601 mg/tablet and 0.005-4.409 mg/tablet, respectively. The content of sample UD2 was significant with both phenolphthalein and sibutramine at their highest concentration. The results showed higher levels of banned substances than in Alabdoolo *et al.* (2017), who found that 60% of dietary supplements for body-weight reduction contained sibutramine or phenolphthalein and 33% of samples contained both.

Table 4. Analysis results of Phenolphthalein and Sibutramine content in real samples.

No.	Sample	Phenolphthalein contents (mg/tablet*)	Sibutramine contents (mg/tablet*)
1	UD1	0.015	1.951
2	UD2	5.601	4.409
3	UD3	4.472	2.484
4	UD4	< LOD	0.008
5	UD5	< LOD	1.874
6	UD6	< LOD	0.567
7	UD7	< LOD	0.008
8	UD8	0.009	2.306
9	UD9	5.404	1.268
10	UD10	2.762	1.149
11	UD11	< LOD	0.005
12	UD12	0.041	< LOD
13	UD13	5.495	0.355
14	UD14	< LOD	< LOD
15	UD15	< LOD	0.833
16	UD16	< LOD	< LOD
17	UD17	< LOD	< LOD
18	UD18	< LOD	< LOD

*The weight of each tablet is 1000 mg

Pratiwi *et al.* (2021) showed that HPLC is still widely used for the detection of adulterated drugs because it has a high separation capacity utilizing a complex mixture, and can simultaneously detect multi-drug components. The use of HPLC can be combined with various detectors, such as UV and MS. HPLC-PDA techniques can provide rapid, sensitive, and reproducible analytical results, with a low sample requirement, high accuracy, and precision to determine compounds simultaneously.

4. Conclusion

In the present study, a cost-effective and reliable analytical method for natural dietary supplements for body-weight reduction, in which analytes were extracted by homogeneous liquid-liquid extraction using acetonitrile and aqueous ammonium 0.1% was developed. The method exhibited good linearity ($R^2 > 0.995$), with recoveries ranging from 91.87 to 96.46% and RSD from 1.48 to 4.11%, while the LOD and LOQ values were 0.33 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$, respectively. In analyses of commercially marketed natural dietary supplements, the method proved useful for simultaneous monitoring of sibutramine and phenolphthalein. It can be employed to ensure public safety by inspecting dietary supplements for body-weight reduction which may contain these illegal substances. The developed method has the advantages of simplicity and accuracy, and the analytical procedures developed can be easily expanded to encompass other contaminants.

Conflict of interest

The authors declare no conflict of interest.

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