Vol. 13, No. 3, 2022
ISSN 2233-4203/ e-ISSN 2093-8950
ARTICLE www.msletters.org | Mass Spectrometry Letters

Simultaneous Determination of 80 Unapproved Compounds using HPLC and LC-MS/MS in Dietary Supplements

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Received June 29, 2022, Revised September 17, 2022, Accepted September 20, 2022
First published on the web September 30, 2022; DOI: 10.5478/MSL.2022.13.2.58

Abstract : We developed analytical methods using high performance chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-MS/MS) for the simultaneous determination of 80 unapproved compounds in dietary supplements. The target compounds for analysis were unapproved ingredients (e.g., pharmaceuticals) that have potential adverse effects on consumers owing to accidental misuse, overuse, and interaction with other medication in dietary supplement. Two analytical methods were tested to identify the optimal validation results according to AOAC guideline. As a result, limit of quantification (LOQ) was 0.14–0.5 μg mL^{-1}; linearity (r^2) was ≥ 0.99; accuracy (expressed as recovery) was 78.9–114%; precision (relative standard deviation) was ≤ 4.28% in the HPLC method. In the LC-MS/MS method, LOQ was 0.01–2 ng mL^{-1}, linearity (r^2) was ≥ 0.98, accuracy was 71.7–119%; precision was ≤ 12.5%. The developed methods were applied to 51 dietary supplements collected from 2019 to 2021 through MFDS alert system. Based on our previous monitoring study, major compounds were icariin, sibutramine, yohimbine, sildenafil, tadalafil, sennosides (A, B), cascarosides (A, B, C, D), and phenolphthalein. In this study, we re-analyzed samples of detected compounds, and evaluated the statistical difference using Bland-Altman analysis to compare two analytical approaches between HPLC and LC-MS/MS. These results showed a good agreement between two methods that can be used to monitor the unapproved ingredients in dietary supplements. The developed two methods are complementarily suitable for monitoring the adulteration of 80 unapproved compounds in dietary supplements.

Keywords : illicit compounds, dietary supplements, unapproved ingredients, HPLC, LC-MS/MS

Introduction

Lately, dietary supplements containing unapproved (hidden, undeclared, and unauthorized) ingredients (e.g., pharmaceuticals) that could be unsafe have been largely sold in global market. These unapproved compounds have potential adverse health effects on consumers owing to accidental misuse, overuse, interaction with other medications, underlying health conditions, or other pharmaceuticals within the supplements.[1] The consumers seeking optimum health or well-being have inflated the opportunities for exposure to unapproved ingredients compared to those in the past owing to the widespread distribution of illegal dietary supplements. Therefore, continuous monitoring study needs to be proceeded in order to protect consumer against illegal dietary supplements.

Due to the potential health risks associated with unapproved ingredients, regulatory authorities of each country have warning alert systems and exchange information in adulterated foods and dietary supplements.[1,2] Currently, the Korean 'Ministry of Food and Drug Safety (MFDS)' takes charge of safety management of adulterated dietary supplements according to the 'Food Sanitation Act' and 'Custom Law'. In these regulations, illegal compounds are defined as pharmaceutical ingredients and erectile dysfunction drugs, anti-obesity drugs, anti-diabetes, etc., and their analogues. These compounds should not be detected in food and dietary supplements and foods containing these compounds should not be sold, manufactured and imported. Illegal dietary supplements containing these compounds are monitored and blocked through MFDS alert system.[1]

Due to the increase in the adulteration of illegal compounds in dietary supplements, several analytical methods have been previously reported using high-performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/

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MS).[3–14] The multi-class analytical method using LC-ESI-MS/MS with MRM (multiple reaction monitoring) mode is the compound-specific method by the MS fragmentation patterns. LC-MS/MS method has been applied for reliable quantitation and confirmation of illicit compounds and suitable for simultaneously detecting rapidly target compounds, while HPLC assay is suitable for screening a wide variety of hidden compounds.[7,15,16] Thus, both analytical instruments can be complementarily used for monitoring of unapproved compounds.

In this study, we selected 80 illicit compounds, including erectile dysfunction drugs, anti-obesity drugs, anti-diabetes, anti-thyroid drugs, anti-psychotic drugs, laxatives, and botanical ingredients. Next, we developed high-performance liquid chromatography-photodiode array detector (HPLC-PDA) and LC-MS/MS analytical methods for simultaneous determination of 80 illicit compounds in dietary supplements. Furthermore, we reviewed these methods suitable for monitoring and detecting illicit compounds in adulterated dietary supplements.

Materials and methods

Standards and reagents

All chemical standards and reagents were HPLC-grade. Two psychotropic compounds [β-methylphenylethylamine (BMPEA), 95.0%; and fenfluramine hydrochloride, 99.2%] were taken from the MFDS according to the Narcotics Control Act. Most of the target compounds including acetaminotadalafil, acetil acid, acetylvardenafil, aminotadalafil, avanafil, benzylsildenafil, carbodenafil, chlorodenafil, chloropretadalafil, cinnamyldenafil, *cis*-cyclopentyltadalafil, *trans*-cyclopentyltadalafil cyclopentynafil, demethylhongdenafil, demethyltadalafil, descarbonsildenafil, desmethylpiperazinylpropoxysildenafil, desulfonylchlorosildenafil, desulfovardenafil, dichlorodenafil, dimethylsildenafil, dimethylthiosildenafil, dithiopropylcarbodenafil, gendenafil, homosildenafil, homotadalafil, hongdenafil, hydroxychlorodenafil, hydroxyhomosildenafil, hydroxyhongdenafil, hydroxythiohomosildenafil, hydroxyvardenafil, imidazosagatriazinone, isopropylnortadalafil, methylhydroxyhomosildenafil, nitrodenafil, norneosildenafil, norneovardenafil, N-octylnortadalafil, oxohongdenafil, piperidinohongdenafil, propoxyphenylthioaildenafil, propoxyphenylthiohomosildenafil, propoxyphenylthiohydroxyhomosildenafil, propoxyphenylthiosildenafil, pseudovardenafil, thiohomosildenafil, thiosildenafil, thioquinapiperifil, udenafil, vardenafil, xanthoanthrafil, cascarosides (A, B, C, D), chlorosibutramine, chlorosipentramine, desmehtylsibutramine, didesmethylsibutramine, icaritin, and N-nitrosofenfluramine were synthesized or isolated by the MFDS. Commercial standard compounds [mirodenafil, sildenafil citrate, tadalafil, yohimbine hydrochloride, rauwolscine hydrochloride (α-yohimbine), icariin, ephedrine hydrochloride, fluoxetine, glibenclamide, gliclazide, glimepiride, glipizide, levothyroxine (T4), liothyronine (T3), orlistat, phenolphthalein, β-phenylethylamine hydrochloride (β-PEA), sennosides (A, B), and sibutramine] were purchased from Sigma-Aldrich (St. Louis, MO, USA), Cayman Chemical (Ann Arbor, MI, USA), Pfizer (New York, USA), Eli Lilly and company (Indianapolis, IN, USA), SK Chemical (Gyeonggi-do, Korea) and Hanmi (Seoul, Korea). HPLC-grade water, acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Sodium-1-hexane sulfonate was obtained from Tokyo Chemical Industry (Tokyo, Japan); phosphoric acid was purchased from Wako (Osaka, Japan); and formic acid (≥ 95%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The polytetrafluoroethylene (PTFE) membrane filters (0.22 μm pore size) were obtained by Teknokroma (Barcelona, Spain).

Preparation of standard solutions

The stock standard solutions were individually produced at 1,000 μg mL^{-1} by weighing and dissolving each standard in methanol. The mixed working solutions were prepared by performing a dilution of aliquots of the stock solutions with methanol. For calibration curve and validation, working solutions were diluted to obtain seven serial dilutions that cover the range of 0.5–50 μg mL^{-1} (0.5, 1, 2, 5, 10, 20, and 50 μg mL^{-1}) for HPLC; and six serial dilutions that cover the range of 1–20 ng mL^{-1} (1, 2, 5, 8, 10, and 20 ng mL^{-1}) for LC-MS/MS. All solutions were stored at 4°C in amber vials.

Sample preparation

A total of 51 samples advertised as sexual enhancement, weight-loss, muscular strengthening were collected between 2019 and 2021 through the MFDS alert system that monitors unapproved ingredients in dietary supplement. The sample types were capsule, tablet, powder, and liquid. The inner powder of capsule form was homogenized after removing a shell of capsule. Tablet form was grinded into powder using a mortar and pestle. In brief, homogenized sample weighed at 1 g was transferred into a 50 mL conical tube. The sample was mixed with 15 mL of water for 1 min. Then, 25 mL of methanol was added to sample solution and the solution was sonicated for 10 min. After the solution was set to the final volume up to 50 mL, the supernatant was filtered through a 0.22 μm PTFE syringe filter. Final extract of the sample was directly applied or appropriately diluted with 70% methanol before injecting into HPLC or LC-MS/MS.

HPLC-PDA analysis

A Shiseido Nanospace S1-2 HPLC system (Osaka soda Co., Ltd., Tokyo, Japan) accompanied with a PDA detector and a Capcell Pak C_{18} column (MG II, 4.6 × 250 mm, 5.0 μm) was used. The UV detection was performed at 210 nm, 220 nm and 291 nm. The oven temperature was held at 40°C. The injection volume was 5 μL and the flow rate was 1.2 mL min^{-1}. The binary mobile phase consisted of a 0.5 mM sodium-1-hexane sulfonate in 0.1% (*v*/*v*) phosphoric acid solution (A) and 95% acetonitrile (B). The

gradient elution program was as follows: 0–6 min (A 85%, B 15%); 6–21 min (A 70%, B 30%); 21–31 min(A 60%, B 40%); 31–35 min (A 60%, B 40%); 35–43 min (A 0%, B 100%); 43–50 min (A 0%, B 100%); 50–52 min (A 85%, B 15%); and 52–60 min (A 85%, B 15%).

LC-MS/MS analysis

A Waters AQUITY ultra-pressure liquid chromatography (UPLC) equipped with Waters Xevo TQ-S (Waters, Milford, MA, USA) and AQUITY UPLC® HSS C_{18} column (2.1 × 150 mm, 1.8 μm) was used. The column temperature was held at 40°C. The injection volume was 5 μL. The binary mobile phases were 0.1% (*v*/*v*) formic acid in water (A) and 0.1% (*v*/*v*) formic acid in acetonitrile (B). The flow rate was 0.3 mL min^{-1}. The mobile phase gradient was as follows: 0–2 min (A 95%, B 5%); 2–19 min (A 30%, B 70%), 19–20.1 min (A 0%, B 100%); 20.1–21.9 min (A 0%, B 100%); 21.9–22 min (A 95%, B 5%); and 22–25 min (A 95%, B 5%). The mass spectrometer was operated in electrospray ionization (ESI) positive or negative mode, and data acquisition was practiced in the MRM mode using the MassLynx v4.1 software (Waters, Milford, MA, USA). The source settings were as follows: capillary voltage of 3.5 kV and -2.8 kV in ESI positive mode and negative mode, respectively; source temperature of 150°C; desolvation temperature of 600°C; cone nitrogen gas flow rate of 60 L h^{-1}; and desolvation gas flow rate of 650 L h^{-1}. Collision-induced dissociation was performed using argon as the collision gas at a pressure of 4×10^{-3} mbar in the collision cell. The $[M + H]^+$ and $[M - H]^-$ ions were selected as precursor ions; and the two or three intense product ions were used as product ions. The most intense ion was selected for quantification, and the other ions for confirmation.

Method validation

The validation on analytical method was carried out in accordance with AOAC guideline (AOAC, 2002). The validation parameters were linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision. Blank samples were confirmed to be free of the target analytes. The linearity was gained from the correlation coefficient (r^2) of the calibration curve. The calibration curves were based on a spiked standard calibration. The LOD and LOQ were calculated by measuring the equation, $3 \times \sigma/s$ and $10 \times \sigma/s$ of the standard calibration curve, respectively. The accuracy (recovery, %) and precision [relative standard deviation (RSD), %] were determined through the analysis of spiked blank sample in five replicates for three different concentrations. The intra-day tests were analyzed on single day by performing five replicates at each level, whereas the inter-day tests were performed once a day during 3 days at each level.

HPLC-PDA and LC-MS/MS method comparison

To compare HPLC-PDA and LC-MS/MS methods, concentrations of detected compounds were determined in 51 dietary supplements by the two different analytical approaches. For method comparison, the linear correlation of the relationship between the two methods was assessed by the Passing and Bablok regression analysis.[17] The results were also compared by means of Bland-Altman plot.[18] Statistical analysis was carried out using MedCalc (Windows) version 20.1 (MedCalc Software, Ostend, Belgium)

Results and Discussion

Optimization of HPLC parameter

HPLC-PDA was used for screening and confirming the presence of 80 illicit compounds in dietary supplements. We optimized the basic chromatographic conditions and HPLC parameters, including mobile phase and detection wavelength, after testing the different conditions that affect HPLC analysis.[19–21] The parameters offered the best separation, apparent peak shape, and maximum signal. The chromatographic division of target compounds was also optimized by adjusting the retention time of each compound. Figure 1 represents HPLC chromatograms of 80 illicit compounds.

Using an organic mobile phase with lower absorbance and a proper buffer solution results in less noise and fewer ghost peaks for baseline in reversed-phase chromatography and UV detection, leading to high-sensitivity analysis. The elution capacity is also higher when aqueous and organic solvents are mixed together.[22,23] The separation of target compounds was achieved using 0.5 mM sodium-1-hexane sulfonate in 0.1% (*v*/*v*) phosphoric acid solution and 95% acetonitrile as organic phase on a C_{18} column. The proportion of the mobile phase components was optimized by adding variety to mobile phases and analyzing repeatedly the standard mixture. The optimized gradient condition enabled good peak resolution and narrow retention time within 50 min. When we scanned the standard working solutions for 80 illicit compounds in the range from 190 to 400 nm using HPLC-PDA detection method for adulteration provided by the "Food code", target compounds were integrated at UV wavelengths 210 nm, 220 nm, and 291 nm. A total of 60 peaks were detected at the wavelength of 291 nm, while 12 peaks and 8 peaks were separated at 220 nm and 210 nm, respectively. Moreover, the sensitivity of certain compounds decreased when applying these method. In order to achieve the optimum sensitivity with HPLC-PDA, we improved analyzing wavelengths by adjusting from 195 nm to 210 nm for orlistat, and from 291 nm to 210 nm for phenolphthalein, which represent good response and maximum peak intensities.

Optimization of LC-MS/MS parameter

Simultaneous LC-MS/MS method was developed for identifying 80 target compounds in dietary supplements (Figure 2). We adjusted the LC parameters, and optimized the chromatographic separation on a basis of previous

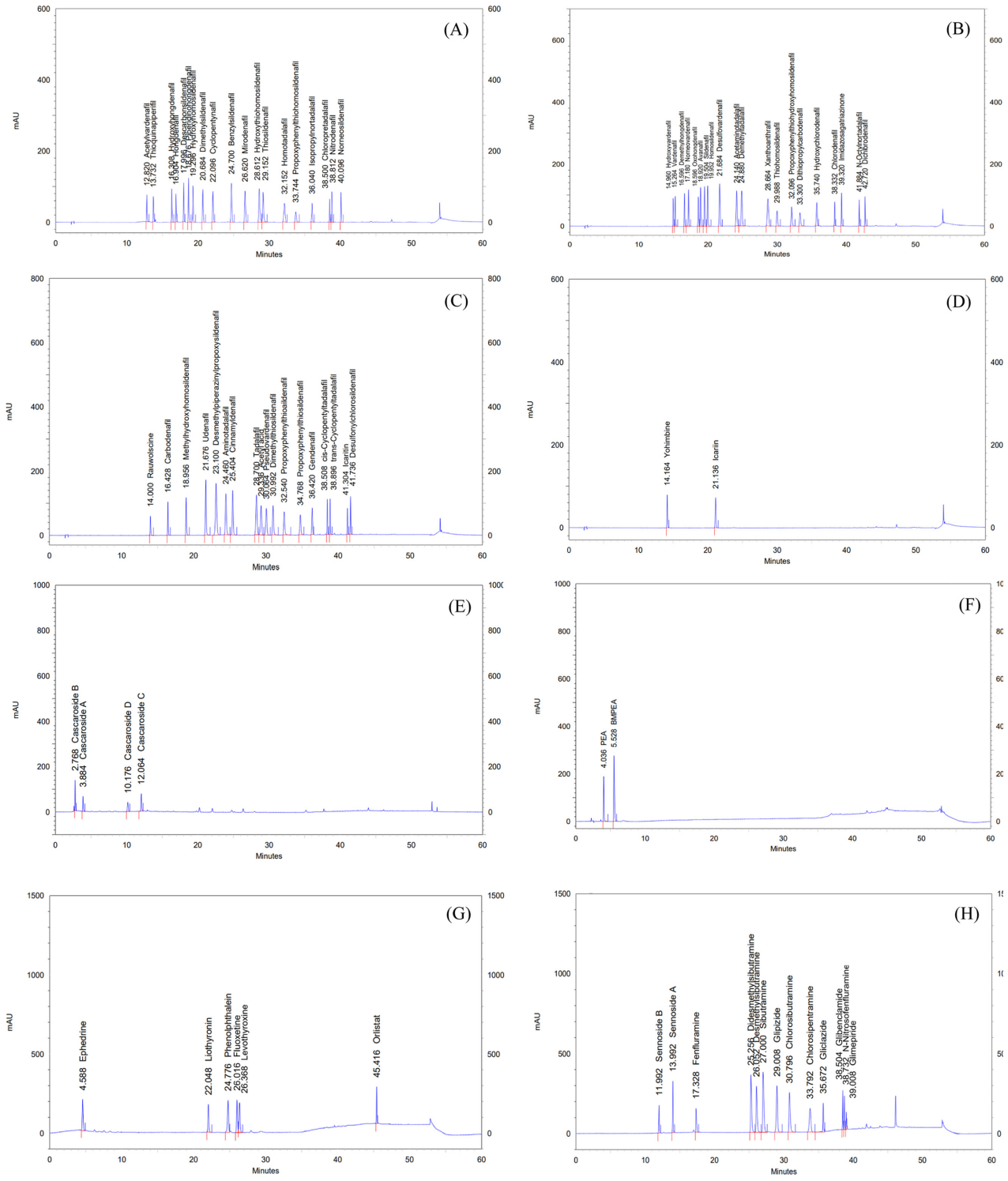

Figure 1. HPLC Chromatograms of 80 unapproved compounds: (A) 19 compounds were detected at 291 nm; (B) 20 compounds were detected at 291 nm; (C) 18 compounds were detected at 291 nm; (D) 2 compounds (yohimbine and icariin) were detected at 291 nm; (E) 4 compounds (cascaroside A, cascaroside B, cascaroside C, and cascaroside D) were detected at 291 nm; (F) 2 compounds (β-PEA and BMPEA) were detected at 210 nm; (G) 6 compounds (ephedrine, liothyronin, phenolphthalein, fluoxetine, levothyroxine and orlistat) were detected at 210 nm; 13 compounds (sennoside A, sennoside B, fenfluramine, didesmethylsibutramine, desmethylsibutramine, sibutramine, glipizide, chlorosibutramie, chlorosipentramine, gliclazide, glibenclamide, N-nitrosofenfluramine, and glimepiride) were detected at 220 nm.

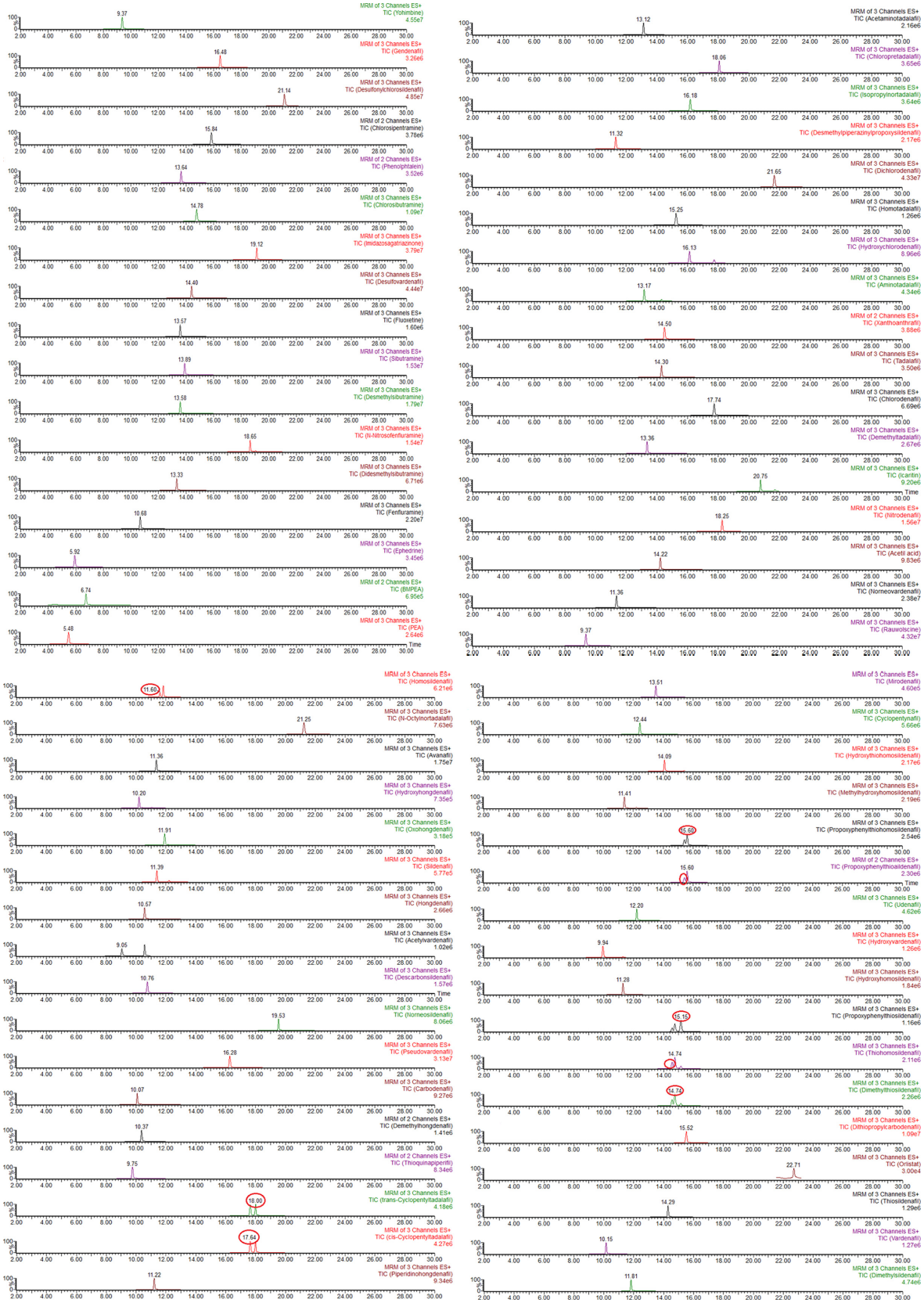

Figure 2. LC-MS/MS Chromatograms of 80 unapproved compounds.

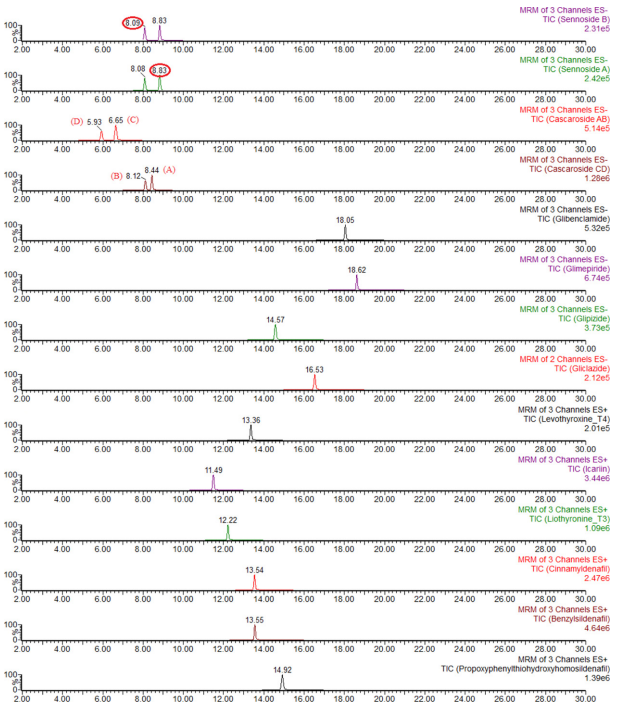

Figure 2. Continued.

study.[19] The MS signal intensity depends on multiple factor such as mobile phase pH and organic percentage, type and concentration of mobile phase electrolytes, and LC separation efficiency.[24]

Reversed-phase solvents (water, acetonitrile, methanol, etc.) are suitable for ESI application as they transfer ions from the liquid phase to the gas phase. They can support ions in solution, sensitivity results are better than normal-phase solvents (hexane, toluene, dichloromethane, etc.). In addition, adjusting pH is an effective strategy of facilitating analyte ionization. It can be good for making the analyte into charged form, and increasing a signal intensity. Ion pair reagent should be used, such as acidic additives (formic acid, acetic acid, etc.) and basic additives (ammonium formate, ammonium acetate, etc.).[24,25]

When we compared peak intensities using formic acid, ammonium formate, and ammonium acetate, formic acid increased MS signal intensities and offered a good peak separation (data not shown). Therefore, 0.1% formic acid was chosen to improve chromatographic resolution. As a result, the optimal separation of target compounds was achieved using 0.1% formic acid in aqueous mobile phase and 0.1% formic acid in acetonitrile as organic phase on a C_{18} column. The gradient elution condition was also optimized to yield good separation over 25 min by the variation of mobile phase with the repeated analysis of the standard solution.

The ideal MS/MS parameters was specifically set up for each compound with the evaluation of sensitivity and abundance. The determination was performed by the direct infusion of individual solution (100–500 ng mL^{-1}) using ESI positive or negative mode. We got the maximum of intensity for the fragment ions while adding cone voltages (10–70 V) and collision energies (5–60 eV). Both the singly protonated ($[M + H]^+$) and deprotonated ($[M - H]^-$) molecular ions were selected as the precursor ions for 74 compounds and 6 compounds, respectively. The MRM transitions were produced with the most abundant ion for quantification and the other ions for confirmation. As seen from Table 1, there are the optimized MRM conditions for 80 illicit compounds. When applying developed this method, the chromatographic condition provided the best peak shape, separation, and resolution. Furthermore, this method can be achieved simultaneous detection of 80 compounds with high quality separation despite a large number of target analytes.

Table 1. Optimized multiple reaction monitoring (MRM) conditions for 80 unapproved compounds.

No.	Compounds	Molecular weight (g/mol)	Ionization mode (ESI +/–)	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)
					204[1)]	60
1	Acetaminotadalafil	432.4	+	433.0	262	32
					311	14
					256	36
2	Acetil acid	356.4	+	357.0	300	32
					329	24
					127	28
3	Acetylvardenafil	466.6	+	467.1	**151**	50
					341	30
					204	58
4	Aminotadalafil	390.4	+	391.0	262	32
					269	12

Table 1. Continued.

No.	Compounds	Molecular weight (g/mol)	Ionization mode (ESI +/–)	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)
					155	48
5	Avanafil	484.0	+	484.0	233	34
					375	26
					91	34
6	Benzylsildenafil	550.7	+	551.1	134	36
					377	30
7	β-Methylphenylethylamine (BMPEA)	135.2	+	136.1	**91**	15
					119	10
					147	42
8	Carbodenafil	452.6	+	453.1	311	34
					339	22
9	Cascarosides (A, B, C, D)					
	Cascaroside A	580.1	–	579.1	268	52
					297	38
					459	20
	Cascaroside B	580.1	–	579.1	268	52
					297	38
					459	20
	Cascaroside C	563.8	–	563.2	251	64
					281	40
					443	24
	Cascaroside D	563.8	–	563.2	251	64
					281	40
					443	24
10	Chlorodenafil	388.8	+	389.0	**285**	32
					311	30
					361	24
11	Chloropretadalafil	426.9	+	426.9	**135**	18
					204	64
					274	32
12	Chlorosibutramine	314.3	+	314.2	**159**	30
					173	16
					187	16
13	Chlorosipentramine	328.3	+	328.2	159	28
					173	16
					187	16
14	Cinnamyldenafil	554.7	+	555.1	**117**	34
					355	26
					437	22
15	*cis*-Cyclopentyltadalafil	443.5	+	444.0	135	26
					169	38
					322	14
16	*trans*-Cyclopentyltadalafil	443.5	+	444.0	135	30
					169	42
					322	16

Table 1. Continued.

No.	Compounds	Molecular weight (g/mol)	Ionization mode (ESI +/–)	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)
17	Cyclopentynafil	528.7	+	529.1	98	36
					283	42
					461	28
18	Demethylhongdenafil	452.6	+	453.1	**97**	30
					297	38
19	Demethyltadalafil	374.4	+	376.0	204	52
					254	12
					262	32
20	Descarbonsildenafil	462.6	+	463.0	**283**	38
					311	30
					418	26
21	Desmethylpiperazinylpropoxysildenafil	406.5	+	407.0	256	34
					336	36
					365	24
22	Desmethylsibutramine	265.8	+	266.2	**125**	26
					139	14
					153	12
23	Desulfonylchlorosildenafil	346.8	+	347.0	256	32
					290	32
					319	24
24	Desulfovardenafil	312.4	+	313.1	**151**	26
					256	30
					284	24
25	Dichlorodenafil	407.3	+	406.9	280	42
					363	34
					379	26
26	Didesmethylsibutramine	251.8	+	252.2	125	22
					139	10
					153	10
27	Dimethylsildenafil	488.6	+	489.1	**113**	30
					283	42
					311	32
28	Dimethylthiosildenafil	504.7	+	505.0	**113**	28
					299	38
					327	30
29	Dithiopropylcarbodenafil	498.7	+	499.1	179	54
					343	36
					371	24
30	Ephedrine	165.2	+	166.2	117	20
					133	20
					148	13
31	Fenfluramine	231.2	+	232.2	109	35
					159	15
					187	15

Table 1. Continued.

No.	Compounds	Molecular weight (g/mol)	Ionization mode (ESI +/–)	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)
32	Fluoxetine	309.3	+	310.1	44	10
					117	50
					148	10
33	Gendenafil	354.4	+	355.0	285	30
					298	30
					327	24
34	Glibenclamide	494.0	–	492.1	127	50
					170	32
					367	18
35	Gliclazide	323.4	–	322.1	106	42
					170	22
36	Glimepiride	490.6	–	489.3	**225**	34
					350	20
					364	28
37	Glipizide	445.5	–	444.1	154	54
					170	34
					319	20
38	Homosildenafil	488.6	+	489.1	99	32
					113	28
					283	40
39	Homotadalafil	403.4	+	404.0	169	34
					204	58
					282	14
40	Hongdenafil	466.6	+	467.1	**111**	32
					127	30
					166	50
41	Hydroxychlorodenafil	390.9	+	391.0	285	30
					313	32
					363	26
42	Hydroxyhomosildenafil	504.6	+	505.1	**99**	40
					112	30
					487	24
43	Hydroxyhongdenafil	482.6	+	483.1	**127**	30
					143	28
					297	42
44	Hydroxythiohomosildenafil	520.7	+	521.0	99	32
					129	30
					299	38
45	Hydroxyvardenafil	504.6	+	505.1	**151**	48
					299	38
					312	40
46	Icariin	676.6	+	677.3	313	58
					369	32
					531	16

Table 1. Continued.

No.	Compounds	Molecular weight (g/mol)	Ionization mode (ESI +/–)	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)
47	Icaritin	368.3	+	369.1	135	35
					187	40
					313	23
48	Imidazosagatriazinone	312.4	+	313.1	241	36
					256	30
					285	24
49	Isopropylnortadalafil	402.1	+	418.0	135	30
					204	64
					296	12
50	Levothyroxine (T4)	776.8	+	777.6	324	54
					351	46
					605	40
51	Liothyronine (T3)	650.9	+	651.7	**197**	68
					225	42
					479	34
52	Methylhydroxyhomosildenafil	518.0	+	519.1	**99**	40
					112	30
					129	30
53	Mirodenafil	531.7	+	532.1	268	50
					296	40
					312	36
54	Nitrodenafil	357.4	+	358.0	136	46
					284	32
					330	22
55	N-Nitrosofenfluramine	260.2	+	261.1	109	44
					159	22
					187	12
56	Norneosildenafil	459.6	+	460.1	136	68
					255	46
					283	38
57	Norneovardenafil	356.4	+	357.0	**151**	30
					300	30
					329	24
58	N-Octylnortadalafil	487.6	+	488.1	**204**	72
					338	26
					366	16
59	Orlistat	495.7	+	496.4	114	20
					160	12
					319	14
60	Oxohongdenafil	480.6	+	481.1	**166**	58
					297	44
					410	28
61	β-Phenylethylamine (β-PEA)	121.2	+	122.0	77	20
					79	20
					105	10

Table 1. Continued.

No.	Compounds	Molecular weight (g/mol)	Ionization mode (ESI +/–)	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)
62	Phenolphthalein	318.3	+	319.1	141	42
					197	30
					225	22
63	Piperidinohongdenafil	437.5	+	438.1	**98**	28
					166	48
					297	36
64	Propoxyphenylthioaildenafil	518.7	+	519.0	113	30
					299	34
65	Propoxyphenylthiohomosildenafil	518.7	+	519.1	**113**	28
					299	36
					327	32
66	Propoxyphenylthiohydroxyhomosildenafil	534.7	+	535.0	**99**	40
					129	32
					299	36
67	Propoxyphenylthiosildenafil	504.7	+	505.0	**299**	36
					313	32
					329	34
68	Pseudovardenafil	459.6	+	460.0	110	76
					151	42
					312	38
69	Sennosides (A, B)					
	Sennoside A	862.7	–	861.2	224	40
					386	36
					699	28
	Sennoside B	862.7	–	861.2	224	40
					386	36
					699	28
70	Sibutramine	279.9	+	280.2	**125**	22
					139	14
					153	14
71	Sildenafil	474.6	+	475.0	**100**	26
					283	40
					311	28
72	Tadalafil	389.4	+	390.0	135	24
					169	34
					268	12
73	Thiohomosildenafil	504.7	+	505.0	**113**	28
					299	38
					327	32
74	Thioquinapiperifil	448.6	+	449.0	186	38
					204	26
75	Thiosildenafil	490.6	+	491.0	**299**	38
					327	30
					341	30
76	Udenafil	516.7	+	517.1	**112**	34
					283	42
					325	36

Table 1. Continued.

No.	Compounds	Molecular weight (g/mol)	Ionization mode (ESI +/–)	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)
77	Vardenafil	488.6	+	489.1	**151**	48
					312	36
					376	34
78	Xanthoanthrafil	389.4	+	390.0	107	50
					151	14
79	Yohimbine	354.4	+	355.2	117	40
					144	32
					212	22
80	Rauwolscine (α-Yohimbine)	354.4	+	355.2	117	40
					144	32
					212	22

1) Values in underline denote quantification ion.

Method validation

Table 2 summarized linearity, LOQ, accuracy and precision for target compounds in dietary supplements using HPLC. The calibration curves were made by seven-point calibrations of standards in blank matrices at 0.5–50 μg mL^{-1}. The correlation coefficients (r^2) were above 0.99 in all compounds. The LOQs were 0.14–0.50 μg mL^{-1}. The accuracy and precision were evaluated in spiked blank samples at three target concentrations of 2, 10, and 20 μg mL^{-1}. The accuracy (expressed as recovery) was in ranges of 78.9–114% for intra-day, and 83.9–109% for inter-day. The precision (expressed as RSD) was below 4.28% for intra-day, and below 2.21% for inter-day.

Table 3 describes linearity, LOQ, accuracy and precision for target compounds in dietary supplements using LC-MS/MS. The calibration curves were made by six-point standard calibration in blank solid matrices at 1–20 ng mL^{-1}. All correlation coefficients (r^2) were higher than 0.98, which showed a good linear relationship. The LOQs were ranged from 0.01 to 2 ng mL^{-1} in solid-type blank samples. The accuracy and precision were evaluated in spiked blank samples at concentrations of 1, 5, and 10 ng mL^{-1}. The LOD was below 1 ng mL^{-1}. The target testing level of three compounds (cascarosides, sennosides, and β-PEA) were 10, 50, and 100 ng mL^{-1} due to the lower sensitivity. The accuracy (expressed as recovery) was in ranges of 71.7–119% for intra-day, and 78.3–114% for inter-day. The precision (expressed as RSD) below 12.5% for intra-day, and below 12% for inter-day. These analytical methods showed satisfactory values for all method validation parameters (linearity, LOD, LOQ, accuracy, and precision) according to the requirements of AOAC guidelines.

Table 2. Linearity, limit of quantification (LOQ), accuracy, and precision of 80 unapproved compounds using HPLC-PDA.

No	Compounds	Linearity (r^2)	LOQ (μg mL^{-1})	Spiked concentration (μg mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
1	Acetaminotadalafil	0.999	0.34	2	92.7	89.3	0.62	0.34
				10	99.7	98.6	0.40	0.32
				20	102	97.3	0.64	0.35
2	Acetil acid	0.999	0.33	2	93.7	95.3	0.47	0.32
				10	104	105	0.12	1.21
				20	100	105	0.65	0.53
3	Acetylvardenafil	1.000	0.36	2	92.4	89.3	0.14	0.18
				10	98.7	101	0.29	0.31
				20	97.9	98.9	2.26	0.97
4	Aminotadalafil	0.999	0.29	2	92.2	90.7	0.12	0.25
				10	104	100	0.13	1.30
				20	101	105	0.80	0.42
5	Avanafil	0.999	0.35	2	99.3	93.9	0.52	0.96
				10	101	98.4	0.98	0.67
				20	103	98.2	0.57	0.47

Table 2. Continued.

No	Compounds	Linearity (r^2)	LOQ (μg mL^{-1})	Spiked concentration (μg mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
6	Benzylsildenafil	1.000	0.35	2	98.7	95.3	0.21	0.29
				10	100	104	0.15	0.62
				20	104	101	1.15	0.63
7	BMPEA	1.000	0.30	2	97.5	91.4	1.37	0.53
				10	97.7	99.7	0.47	0.35
				20	98.1	101	0.79	0.42
8	Carbodenafil	0.999	0.27	2	92.5	95.2	0.09	0.19
				10	103	106	0.22	1.26
				20	99.9	104	0.48	0.42
9	Cascarosides (A, B, C, D)	0.999	0.32	2	94.1	92.9	1.92	1.35
				10	102	97.5	0.31	0.41
				20	103	99.3	0.95	0.76
10	Chlorodenafil	0.999	0.17	2	106	104	0.11	0.12
				10	102	104	0.43	0.52
				20	102	101	0.72	0.30
11	Chloropretadalafil	1.000	0.30	2	99.9	94.9	0.30	0.44
				10	101	103	0.17	0.26
				20	104	101	1.07	0.52
12	Chlorosibutramine	0.999	0.34	2	110	105	0.73	1.07
				10	90.4	97.0	0.88	0.49
				20	100	101	0.21	0.28
13	Chlorosipentramine	0.994	0.30	2	114	108	1.05	0.48
				10	95.4	95.9	0.08	0.30
				20	102	102	0.38	0.28
14	Cinnamyldenafil	0.999	0.29	2	80.1	85.1	0.54	0.44
				10	99.8	97.5	0.37	1.12
				20	96.5	101	0.16	0.26
15	*cis*-Cyclopentyltadalafil	0.999	0.28	2	91.7	94.7	0.14	0.26
				10	103	105	0.26	0.98
				20	99.7	105	1.14	0.64
16	*trans*-Cyclopentyltadalafil	0.999	0.31	2	91.8	93.9	0.35	0.43
				10	103	105	0.31	0.86
				20	100	105	1.32	0.73
17	Cyclopentynafil	1.000	0.34	2	99.5	94.4	0.30	0.24
				10	100	102	0.05	0.70
				20	104	101	1.26	0.53
18	Demethylhongdenafil	0.999	0.37	2	85.5	85.3	0.14	0.17
				10	96.0	94.3	0.20	0.42
				20	98.4	94.0	0.59	0.28
19	Demethyltadalafil	0.999	0.40	2	94.7	93.8	0.28	0.40
				10	100	100	0.47	0.36
				20	102	99.0	0.81	0.36
20	Descarbonsildenafil	1.000	0.40	2	97.7	92.0	0.11	0.30
				10	100	102	0.12	0.26
				20	104	101	0.85	0.47

Table 2. Continued.

No	Compounds	Linearity (r^2)	LOQ (μg mL^{-1})	Spiked concentration (μg mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
21	Desmethylpiperazinylpropoxysildenafil	0.999	0.29	2	79.7	87.0	1.39	0.54
				10	95.0	96.4	0.42	0.36
				20	101	104	0.61	0.50
22	Desmethylsibutramine	0.999	0.35	2	97.8	98.6	1.46	0.59
				10	107	104	0.14	0.35
				20	109	105	0.30	0.23
23	Desulfonylchlorosildenafil	0.999	0.31	2	92.5	95.4	0.29	0.24
				10	103	105	0.21	1.26
				20	100	104	0.93	0.50
24	Desulfovardenafil	0.999	0.34	2	93.8	89.5	1.43	0.90
				10	100	97.2	0.55	0.42
				20	102	97.6	0.47	0.37
25	Dichlorodenafil	0.999	0.19	2	93.8	89.8	0.52	0.35
				10	100	98.4	0.29	0.40
				20	102	97.4	0.60	0.33
26	Didesmethylsibutramine	0.996	0.29	2	83.8	94.1	0.39	0.33
				10	104	103	0.31	0.26
				20	109	106	0.25	0.27
27	Dimethylsildenafil	1.000	0.26	2	97.5	92.3	0.37	0.45
				10	100	102	0.10	0.19
				20	104	101	1.05	0.50
28	Dimethylthiosildenafil	0.999	0.29	2	94.1	94.0	0.66	0.54
				10	98.0	98.9	0.22	0.94
				20	100	104	0.59	0.56
29	Dithiopropylcarbodenafil	0.999	0.24	2	97.9	93.5	1.08	0.46
				10	102	99.6	0.33	0.32
				20	104	99.1	0.74	0.41
30	Ephedrine	1.000	0.15	2	103	98.9	0.31	0.15
				10	105	103	0.49	0.40
				20	111	106	0.17	0.12
31	Fenfluramine	0.999	0.50	2	104	105	4.28	2.21
				10	113	97.8	1.40	0.93
				20	114	103	0.33	0.53
32	Fluoxetine	0.999	0.21	2	96.0	91.1	0.91	0.51
				10	97.3	98.6	0.41	0.53
				20	99.9	101	0.80	0.42
33	Gendenafil	0.999	0.22	2	93.1	94.0	0.13	0.23
				10	104	104	0.18	1.21
				20	100	105	0.59	0.36
34	Glibenclamide	0.999	0.36	2	98.6	94.7	0.17	0.25
				10	98.2	98.5	0.12	0.23
				20	97.6	99.1	0.60	0.43
35	Gliclazide	0.993	0.28	2	103	102	0.62	0.58
				10	91.5	95.2	0.36	0.56
				20	87.3	97.9	0.84	0.77

Table 2. Continued.

No	Compounds	Linearity (r^2)	LOQ (μg mL^{-1})	Spiked concentration (μg mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
36	Glimepiride	0.999	0.34	2	91.3	91.6	0.20	0.27
				10	96.6	96.1	0.50	0.43
				20	97.6	99.1	0.09	0.28
37	Glipizide	0.999	0.22	2	96.1	91.3	0.13	0.29
				10	98.1	100	0.13	0.13
				20	99.1	101	0.14	0.13
38	Homosildenafil	0.999	0.38	2	93.1	88.2	0.24	0.47
				10	100	97.6	0.44	0.41
				20	102	97.5	0.53	0.34
39	Homotadalafil	1.000	0.28	2	98.0	90.7	0.43	0.48
				10	100	99.1	0.11	0.46
				20	104	101	0.98	0.57
40	Hongdenafil	1.000	0.19	2	92.7	89.0	0.27	0.22
				10	98.7	102	0.20	0.80
				20	98.7	98.9	1.68	0.66
41	Hydroxychlorodenafil	0.999	0.22	2	93.8	89.2	0.16	0.36
				10	100	98.3	0.27	0.39
				20	102	97.5	0.71	0.39
42	Hydroxyhomosildenafil	1.000	0.27	2	97.0	93.4	0.59	0.38
				10	99.7	102	0.22	0.55
				20	103	101	2.41	0.95
43	Hydroxyhongdenafil	1.000	0.24	2	92.5	88.0	0.28	0.18
				10	98.6	103	0.21	0.68
				20	99.7	99.6	1.34	0.64
44	Hydroxythiohomosildenafil	1.000	0.42	2	98.7	94.1	0.45	0.54
				10	101	102	0.13	0.42
				20	105	101	1.30	0.76
45	Hydroxyvardenafil	0.999	0.34	2	90.3	88.6	0.91	0.85
				10	99.2	98.1	0.67	0.79
				20	102	97.9	0.38	0.25
46	Icariin	0.999	0.35	2	80.6	93.9	0.12	0.26
				10	95.0	105	0.33	0.55
				20	101	105	0.59	0.46
47	Icaritin	0.999	0.38	2	95.0	93.2	0.66	0.64
				10	103	105	0.40	1.46
				20	101	104	1.03	0.59
48	Imidazosagatriazinone	0.999	0.20	2	92.8	88.9	0.23	0.25
				10	100	98.4	0.35	0.48
				20	103	97.5	0.58	0.36
49	Isopropylnortadalafil	1.000	0.40	2	97.1	92.0	0.20	0.46
				10	99.7	102	0.15	0.29
				20	104	101	1.14	0.54
50	Levothyroxine (T4)	0.999	0.23	2	89.4	90.8	2.76	1.25
				10	96.0	96.1	0.15	0.17
				20	99.3	99.4	1.50	0.73

Table 2. Continued.

No	Compounds	Linearity (r^2)	LOQ (μg mL^{-1})	Spiked concentration (μg mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
51	Liothyronine (T3)	0.999	0.25	2	94.1	93.4	1.59	0.73
				10	97.2	97.4	0.64	0.35
				20	100	99.9	1.36	0.54
52	Methylhydroxyhomosildenafil	0.999	0.37	2	93.1	96.3	0.11	0.36
				10	103	106	0.15	1.18
				20	100	104	0.63	0.49
53	Mirodenafil	1.000	0.33	2	97.8	92.0	0.95	0.51
				10	100	102	0.08	0.78
				20	104	101	1.17	0.62
54	Nitrodenafil	1.000	0.20	2	98.3	93.6	0.23	0.27
				10	100	103	0.15	0.24
				20	104	101	1.08	0.48
55	N-Nitrosofenfluramine	0.999	0.41	2	102	102	0.93	0.50
				10	98.0	100	0.45	0.52
				20	108	105	0.36	0.18
56	Norneosildenafil	1.000	0.36	2	98.7	93.7	0.28	0.27
				10	100	102	0.15	0.22
				20	104	101	1.19	0.48
57	Norneovardenafil	0.999	0.35	2	95.8	92.0	0.12	0.28
				10	101	98.7	0.41	0.42
				20	103	98.8	0.51	0.34
58	N-Octylnortadalafil	0.999	0.28	2	92.3	88.8	0.88	0.42
				10	100	98.2	0.15	0.39
				20	102	97.3	0.73	0.39
59	Orlistat	0.999	0.29	2	78.9	89.9	1.01	1.07
				10	85.5	94.3	1.51	1.18
				20	98.5	99.3	0.90	0.74
60	Oxohongdenafil	0.999	0.32	2	90.2	86.2	0.50	0.45
				10	99.1	96.5	0.36	0.60
				20	102	95.8	0.28	0.26
61	β-PEA	1.000	0.26	2	94.3	89.6	1.39	0.59
				10	97.6	99.2	0.83	0.61
				20	99.2	101	0.91	0.49
62	Phenolphthalein	0.999	0.23	2	89.9	88.8	1.35	0.65
				10	98.2	99.5	0.56	0.24
				20	102	102	1.80	0.75
63	Piperidinohongdenafil	1.000	0.22	2	96.9	98.6	0.49	0.42
				10	101	107	0.33	0.84
				20	101	100	1.05	0.63
64	Propoxyphenylthioaildenafil	0.999	0.35	2	93.4	95.3	0.44	0.37
				10	103	105	0.07	1.23
				20	99.7	104	0.47	0.43
65	Propoxyphenylthiohomosildenafil	1.000	0.35	2	99.3	95.4	1.66	0.75
				10	100	102	0.61	0.52
				20	101	99.7	1.13	0.49

Table 2. Continued.

No	Compounds	Linearity (r^2)	LOQ (μg mL^{-1})	Spiked concentration (μg mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
66	Propoxyphenylthiohydroxyhomosildenafil	0.999	0.37	2	101	97.9	0.36	0.58
				10	102	101	0.40	0.43
				20	102	99.7	0.44	0.21
67	Propoxyphenylthiosildenafil	0.999	0.27	2	92.6	99.2	0.45	0.51
				10	103	108	0.05	1.41
				20	100	105	0.47	0.41
68	Pseudovardenafil	0.999	0.34	2	94.1	95.2	1.06	0.78
				10	103	105	1.30	1.33
				20	99.8	105	0.67	0.51
69	Sennosides (A, B)	0.999	0.29	2	104	103	0.45	0.29
				10	107	103	0.11	0.23
				20	109	105	0.12	0.18
70	Sibutramine	0.999	0.33	2	112	109	0.44	0.26
				10	110	104	0.22	0.40
				20	104	103	0.27	0.33
71	Sildenafil	0.999	0.37	2	88.8	88.8	0.59	0.43
				10	99.5	98.1	0.33	0.52
				20	102	97.8	0.49	0.31
72	Tadalafil	0.999	0.33	2	81.0	88.0	1.10	0.43
				10	95.4	98.2	0.34	0.19
				20	102	104	0.85	0.52
73	Thiohomosildenafil	0.999	0.32	2	105	104	0.32	0.35
				10	101	103	0.94	0.59
				20	102	101	0.55	0.31
74	Thioquinapiperifil	1.000	0.14	2	97.7	92.5	0.12	0.87
				10	100	103	0.32	0.40
				20	104	102	1.10	0.74
75	Thiosildenafil	1.000	0.29	2	98.2	95.4	0.24	0.45
				10	100	101	0.17	0.39
				20	102	100	1.01	0.50
76	Udenafil	0.999	0.35	2	92.5	94.9	0.23	0.28
				10	103	105	0.46	1.29
				20	99.9	104	0.48	0.44
77	Vardenafil	0.999	0.42	2	90.6	88.2	0.32	0.58
				10	99.1	97.8	0.56	0.88
				20	102	97.5	0.36	0.48
78	Xanthoanthrafil	0.999	0.37	2	94.2	83.9	3.36	1.43
				10	102	97.8	0.54	0.55
				20	103	103	0.59	0.49
79	Yohimbine	1.000	0.33	2	82.1	87.9	2.35	1.07
				10	94.8	97.9	0.18	0.69
				20	101	104	0.79	0.41
80	Rauwolscine (α-Yohimbine)	0.999	0.44	2	91.3	93.5	0.15	0.33
				10	104	105	0.16	1.08
				20	100	104	0.64	0.59

1) RSD represents relative standard deviation.

Table 3. Linearity, limit of quantification (LOQ), accuracy, and precision of 80 unapproved compounds using LC-MS/MS.

No	Compounds	Linearity (r^2)	LOQ (ng mL^{-1})	Spiked concentration (ng mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
1	Acetaminotadalafil	0.999	0.20	1	80.9	88.1	2.93	5.94
				5	104	97.0	7.28	3.43
				10	104	97.8	2.86	3.19
2	Acetil acid	0.999	0.10	1	75.3	87.7	2.70	3.86
				5	104	101	2.89	2.84
				10	108	104	3.14	2.92
3	Acetylvardenafil	0.993	0.50	1	118	108	3.59	3.58
				5	109	104	1.85	4.30
				10	108	105	2.94	3.85
4	Aminotadalafil	0.997	0.10	1	101	93.5	3.90	7.01
				5	110	103	3.69	3.65
				10	109	103	3.89	3.02
5	Avanafil	0.998	0.10	1	75.7	78.3	3.85	7.32
				5	108	106	1.00	1.90
				10	110	109	1.44	1.87
6	Benzylsildenafil	0.998	0.20	1	101	89.6	3.80	3.17
				5	93.7	101	3.95	3.02
				10	104	105	3.37	3.14
7	BMPEA	0.998	0.50	1	79.7	83.0	3.16	3.60
				5	107.	99.0	3.97	4.80
				10	96.5	97.9	2.43	3.14
8	Carbodenafil	0.999	0.10	1	86.0	85.6	2.52	6.40
				5	106	105	1.85	2.64
				10	108	107	4.10	3.01
9	Cascarosides (A, B, C, D)	0.999	2.00	10	94.8	85.5	1.43	1.18
				50	104	97.1	1.99	2.28
				100	106	99.0	1.77	2.86
10	Chlorodenafil	0.997	0.02	1	71.7	89.7	8.45	6.53
				5	109	101	4.82	3.19
				10	109	101	4.87	3.34
11	Chloropretadalafil	0.999	0.10	1	90.0	89.4	9.12	7.74
				5	100	97.7	5.86	4.54
				10	99.8	101	7.64	3.71
12	Chlorosibutramine	0.996	0.20	1	84.2	82.8	1.68	3.04
				5	101	101	2.88	2.65
				10	105	103	7.11	3.97
13	Chlorosipentramine	0.996	0.20	1	98.8	85.1	1.51	4.05
				5	112	101	2.58	2.92
				10	111	105	5.26	4.05
14	Cinnamyldenafil	0.987	0.50	1	117	105	2.67	4.75
				5	102	105	2.91	4.31
				10	99.2	104	4.24	5.62
15	*cis*-Cyclopentyltadalafil	0.999	0.50	1	110	94.6	0.95	3.92
				5	110	105	4.97	3.85
				10	110	110	5.57	4.37

Table 3. Continued.

No	Compounds	Linearity (r^2)	LOQ (ng mL^{-1})	Spiked concentration (ng mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
16	*trans*-Cyclopentyltadalafil	0.999	0.50	1	108	99.9	1.53	3.49
				5	110	99.1	3.77	2.60
				10	110	98.3	4.32	3.59
17	Cyclopentynafil	0.997	0.20	1	85.7	91.3	3.01	5.03
				5	108	107	2.54	2.63
				10	111	105	3.14	2.44
18	Demethylhongdenafil	0.995	0.20	1	111	105	3.79	5.70
				5	105	102	4.18	4.85
				10	109	104	7.45	4.09
19	Demethyltadalafil	0.998	0.10	1	94.9	91.8	3.43	3.89
				5	104	102	3.64	3.76
				10	103	100	4.54	3.39
20	Descarbonsildenafil	0.996	0.50	1	98.7	98.7	4.75	5.46
				5	99.8	99.2	4.13	3.12
				10	103	100	2.18	2.31
21	Desmethylpiperazinylpropoxysildenafil	0.999	0.50	1	98.6	89.7	3.70	3.90
				5	109	102	2.66	3.11
				10	107	102	3.81	3.36
22	Desmethylsibutramine	0.998	0.30	1	81.9	95.2	3.56	3.29
				5	106	99.0	2.41	3.30
				10	103	99.1	7.05	5.20
23	Desulfonylchlorosildenafil	0.998	0.05	1	103	91.7	1.25	1.71
				5	112	110	7.48	4.65
				10	111	96.5	11.7	7.19
24	Desulfovardenafil	0.998	0.05	1	86.1	95.7	5.90	5.02
				5	107	104	3.73	2.67
				10	104	102	6.76	5.19
25	Dichlorodenafil	0.998	0.01	1	84.1	96.7	7.40	4.66
				5	108	102	3.65	5.28
				10	103	102	4.87	3.80
26	Didesmethylsibutramine	0.998	0.10	1	94.4	88.0	3.95	4.36
				5	108	98.9	3.21	4.08
				10	98.6	98.6	4.54	3.11
27	Dimethylsildenafil	0.998	0.20	1	102	95.4	5.93	7.63
				5	102	99.9	2.48	2.14
				10	105	103	2.23	2.22
28	Dimethylthiosildenafil	0.993	0.20	1	99.6	93.9	4.18	5.56
				5	101	105	4.31	2.34
				10	104	105	5.06	4.83
29	Dithiopropylcarbodenafil	0.993	0.10	1	102	106	3.56	2.72
				5	111	102	1.97	2.06
				10	118	105	4.46	3.26
30	Ephedrine	0.997	0.10	1	92.2	92.6	9.97	5.39
				5	98.4	96.9	6.48	4.86
				10	101	98.2	4.98	3.48

Table 3. Continued.

No	Compounds	Linearity (r^2)	LOQ (ng mL^{-1})	Spiked concentration (ng mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
				1	75.6	83.8	1.78	4.01
31	Fenfluramine	0.999	0.05	5	110	99.4	3.32	2.66
				10	108	100	5.07	4.68
				1	90.0	93.9	2.16	5.06
32	Fluoxetine	0.995	0.10	5	107	104	1.52	4.46
				10	105	108	7.18	4.27
				1	101	95.2	0.99	2.36
33	Gendenafil	0.997	0.10	5	108	99.8	4.95	3.66
				10	108	99.9	1.85	2.10
				1	97.6	92.4	6.13	7.62
34	Glibenclamide	0.999	0.50	5	99.5	99.9	1.70	5.34
				10	103	101	3.13	3.83
				1	114	102	2.27	6.02
35	Gliclazide	0.999	0.50	5	101	93.9	2.80	3.80
				10	101	92.6	1.68	2.67
				1	93.3	92.9	2.16	5.27
36	Glimepiride	0.998	0.20	5	99.3	104	4.30	5.20
				10	99.6	103	3.88	4.29
				1	113	102	4.02	8.60
37	Glipizide	0.996	0.20	5	103	102	2.95	5.26
				10	101	109	1.69	4.78
				1	83.3	91.7	3.90	5.81
38	Homosildenafil	0.992	0.20	5	78.0	94.8	8.55	4.98
				10	114	106	3.95	2.92
				1	109	95.9	7.99	5.46
39	Homotadalafil	0.998	0.20	5	109	102	7.08	4.24
				10	106	102	9.70	6.42
				1	111	105	1.96	4.99
40	Hongdenafil	0.999	0.20	5	104	105	2.62	2.30
				10	101	104	2.24	2.89
				1	98.7	91.4	2.01	2.55
41	Hydroxychlorodenafil	0.999	0.10	5	109	102	5.02	3.32
				10	102	100	1.80	2.07
				1	76.5	79.9	5.13	6.16
42	Hydroxyhomosildenafil	0.998	0.20	5	103	103	2.98	2.32
				10	106	105	2.40	2.55
				1	83.4	90.9	6.58	7.31
43	Hydroxyhongdenafil	0.996	0.20	5	103	98.2	6.58	7.62
				10	105	101	2.76	5.80
				1	77.4	92.6	6.51	5.15
44	Hydroxythiohomosildenafil	0.996	0.20	5	101	102	3.28	3.18
				10	107	104	4.03	3.03
				1	106	93.8	4.69	6.95
45	Hydroxyvardenafil	0.995	0.50	5	109	100	5.06	5.28
				10	110	105	1.73	5.43

Table 3. Continued.

No	Compounds	Linearity (r^2)	LOQ (ng mL^{-1})	Spiked concentration (ng mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
				1	98.6	91.2	1.93	3.19
46	Icariin	0.999	0.10	5	108	100	4.34	4.30
				10	106	100	5.96	4.93
				1	98.0	95.4	12.5	8.13
47	Icaritin	0.997	0.50	5	85.9	89.8	1.72	5.24
				10	77.0	84.9	4.83	7.50
				1	86.8	92.9	0.49	2.48
48	Imidazosagatriazinone	0.999	0.05	5	106	99.2	3.59	2.30
				10	107	99.8	3.18	2.44
				1	99.7	91.5	2.99	4.83
49	Isopropylnortadalafil	0.998	0.20	5	110	103	1.94	2.64
				10	109	105	2.37	2.40
				1	89.7	95.3	11.3	11.1
50	Levothyroxine (T4)	0.985	0.50	5	111	95.0	11.8	10.5
				10	106	96.9	7.62	7.67
				1	92.8	89.9	10.7	11.3
51	Liothyronine (T3)	0.999	0.50	5	106	99.0	3.43	4.76
				10	106	104	2.37	2.88
				1	81.7	90.6	4.71	5.05
52	Methylhydroxyhomosildenafil	0.999	0.50	5	105	103	3.24	2.90
				10	104	101	3.21	2.36
				1	94.3	87.0	7.46	8.06
53	Mirodenafil	0.995	0.50	5	102	101	4.01	4.98
				10	106	102	4.53	5.34
				1	83.0	101	1.71	1.47
54	Nitrodenafil	0.999	0.01	5	105	107	4.68	3.42
				10	108	108	4.52	2.92
				1	82.2	89.7	1.34	1.18
55	N-Nitrosofenfluramine	0.998	0.08	5	107	101	3.13	2.43
				10	103	101	5.95	3.30
				1	84.1	94.4	1.81	1.93
56	Norneosildenafil	0.999	0.10	5	93.7	98.0	6.10	4.15
				10	107	102	1.62	2.93
				1	88.2	86.1	3.17	3.18
57	Norneovardenafil	0.999	0.05	5	102	92.5	5.05	4.50
				10	108	111	2.86	5.16
				1	98.2	99.1	4.59	3.10
58	N-Octylnortadalafil	0.994	0.10	5	111	94.0	9.14	8.93
				10	114	93.8	9.78	6.58
				1	91.3	89.2	5.90	6.05
59	Orlistat	0.996	0.50	5	82.8	84.3	6.77	5.17
				10	88.1	91.0	7.07	5.44
				1	109	101	3.08	9.19
60	Oxohongdenafil	0.998	0.20	5	102	103	2.32	9.15
				10	105	101	4.02	4.00

Table 3. Continued.

No	Compounds	Linearity (r^2)	LOQ (ng mL^{-1})	Spiked concentration (ng mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
				10	81.6	80.0	3.48	3.76
61	β-PEA	0.996	2.00	50	107	102	3.27	3.95
				100	100	100	6.26	5.39
				1	107	94.3	3.52	4.72
62	Phenolphthalein	0.997	0.10	5	109	102	3.89	4.12
				10	105	99.9	4.86	2.98
				1	83.4	93.4	4.31	3.77
63	Piperidinohongdenafil	0.999	0.10	5	106	103	4.15	4.56
				10	109	107	3.06	5.37
				1	108	102	5.14	7.58
64	Propoxyphenylthioaildenafil	0.992	0.50	5	110	106	1.64	1.49
				10	114	108	2.32	2.90
				1	78.0	84.1	3.96	6.63
65	Propoxyphenylthiohomosildenafil	0.996	0.50	5	97.0	101	2.79	4.06
				10	103	104	3.18	4.46
				1	98.3	97.2	3.96	6.25
66	Propoxyphenylthiohydroxyhomosildenafil	0.997	0.50	5	98.4	104	3.01	3.35
				10	104	107	1.67	3.02
				1	111	97.3	4.98	5.57
67	Propoxyphenylthiosildenafil	0.993	0.20	5	103	102	2.84	4.26
				10	102	104	7.10	4.13
				1	83.9	96.2	1.67	1.83
68	Pseudovardenafil	0.999	0.01	5	98.1	99.9	1.06	2.02
				10	102	99.9	11.4	5.92
				10	96.9	91.0	5.67	3.53
69	Sennosides (A, B)	0.999	2.00	50	111	102	2.09	4.47
				100	111	102	1.78	3.79
				1	79.3	86.5	4.05	3.82
70	Sibutramine	0.994	0.05	5	108	102	2.03	2.73
				10	108	104	7.11	4.11
				1	80.3	96.4	9.50	6.78
71	Sildenafil	0.996	0.50	5	106	103	6.86	5.65
				10	107	102	2.50	5.08
				1	101	90.6	5.19	6.76
72	Tadalafil	0.999	0.50	5	108	103	3.60	4.37
				10	107	104	5.80	3.58
				1	103	98.4	4.07	4.12
73	Thiohomosildenafil	0.990	0.50	5	101	106	2.44	4.86
				10	114	114	2.63	2.17
				1	119	105	4.67	4.24
74	Thioquinapiperifil	0.995	0.10	5	105	103	7.00	6.66
				10	106	94.7	8.06	5.63
				1	116	101	6.28	12.0
75	Thiosildenafil	0.995	0.20	5	94.8	99.9	7.33	4.75
				10	103	103	12.2	7.39

Table 3. Continued.

No	Compounds	Linearity (r^2)	LOQ (ng mL^{-1})	Spiked concentration (ng mL^{-1})	Accuracy (Recovery, %)		Precision (RSD[1], %)	
					Intra-day	Inter-day	Intra-day	Inter-day
76	Udenafil	0.998	0.10	1	103	90.7	4.98	5.40
				5	106	102	3.20	3.52
				10	106	104	4.41	3.94
77	Vardenafil	0.989	0.50	1	104	90.6	5.37	5.44
				5	101	103	1.71	3.62
				10	101	103	3.14	4.33
78	Xanthoanthrafil	0.999	0.05	1	100	93.6	2.14	6.47
				5	110	102	1.59	3.11
				10	106	105	3.91	3.03
79	Yohimbine	0.999	0.05	1	111	87.9	0.85	2.24
				5	101	100	3.94	4.04
				10	96.6	101	4.30	4.17
80	Rauwolscine (α-Yohimbine)	0.999	0.05	1	77.5	82.1	3.28	3.47
				5	105	100	1.61	1.80
				10	103	97.7	3.60	4.51

1) RSD represents relative standard deviation.

Analysis of samples using HPLC-PDA and LC-MS/MS methods

The developed methods were applied to 51 dietary supplements collected in 2019–2021 through MFDS's alert system. The product category of samples was classified into sexual enhancement product (39.2%), weight-loss product (49%), and muscular strengthening product (11.8%). Table 4 provides the number and concentration (range, mg g^{-1}) of illicit compounds detected in dietary supplements by HPLC-PDA and LC-MS/MS. Of all monitored products, the most detected compound in dietary supplements was icariin (9 products), sibutramine (9 products), and yohimbine (9 products), followed by sildenafil (8 products), tadalafil (7 products), sennosides (A, B) (6 products), cascarosides (A, B, C, D) (4 products), and phenolphthalein (4 products).

Icariin is an active flavonoid extracted from horn goat weed (*Epimedium koreanum*) and used in traditional Chinese medicine for the treatment of erectile dysfunction (ED). Icariin as an adulterant in dietary supplements has been used for the purpose of enhancing sexual and sporting

Table 4. Analytical results for illicit compounds in 51 sample of dietary supplements collected between 2019 and 2021 through MFDS alert system.

Product type	Compounds	Detected number	Detected sample No.	Concentration range (mg g^{-1})	
				HPLC-PDA	LC-MS/MS
Sexual enhancement product (*n = 20*)	Icariin	6	S-24, S-25, S-26, S-27, S-28, S-29	0.12–8.80	0.10–8.68
	Sildenafil	8	S-1, S-2, S-3, S-4, S-5, S-6, S-7, S-14[1]	3.97–217	3.41–235
	Tadalafil	7	S-8, S-9, S-10, S-11, S-12, S-13, S-14[1]	0.57–27.0	0.70–40.5
	Cascarosides	4	S-48, S-49, S-50, S-51	14.2–41.4	12.8–40.5
Weight-loss product (*n = 25*)	Icariin	2	S-30, S-31	3.15–3.56	3.21–3.30
	Phenolphthalein	4	S-20[1], S-21[1], S-22[1], S-23[1]	6.66–27.2	7.17–28.2
	Sennosides	6	S-42, S-43, S-44, S-45, S-46, S-47	6.79–54.6	7.56–55.4
	Sibutramine	9	S-15, S-16, S-17, S-18, S-19, S-20[1], S-21[1], S-22[1], S-23[1]	0.89–31.6	0.72–46.8
	Yohimbine	4	S-33, S-34, S-35, S-36	0.28–6.88	0.14–4.74
Muscular strengthening product (*n = 6*)	Icariin	1	S-32	1.45	0.21
	Yohimbine	5	S-37, S-38, S-39, S-40, S-41	0.43–4.11	0.49–2.19
Total (*n = 51*)	8	56	S-51	0.12–217	0.10–235

Adulterated product containing two illicit compounds

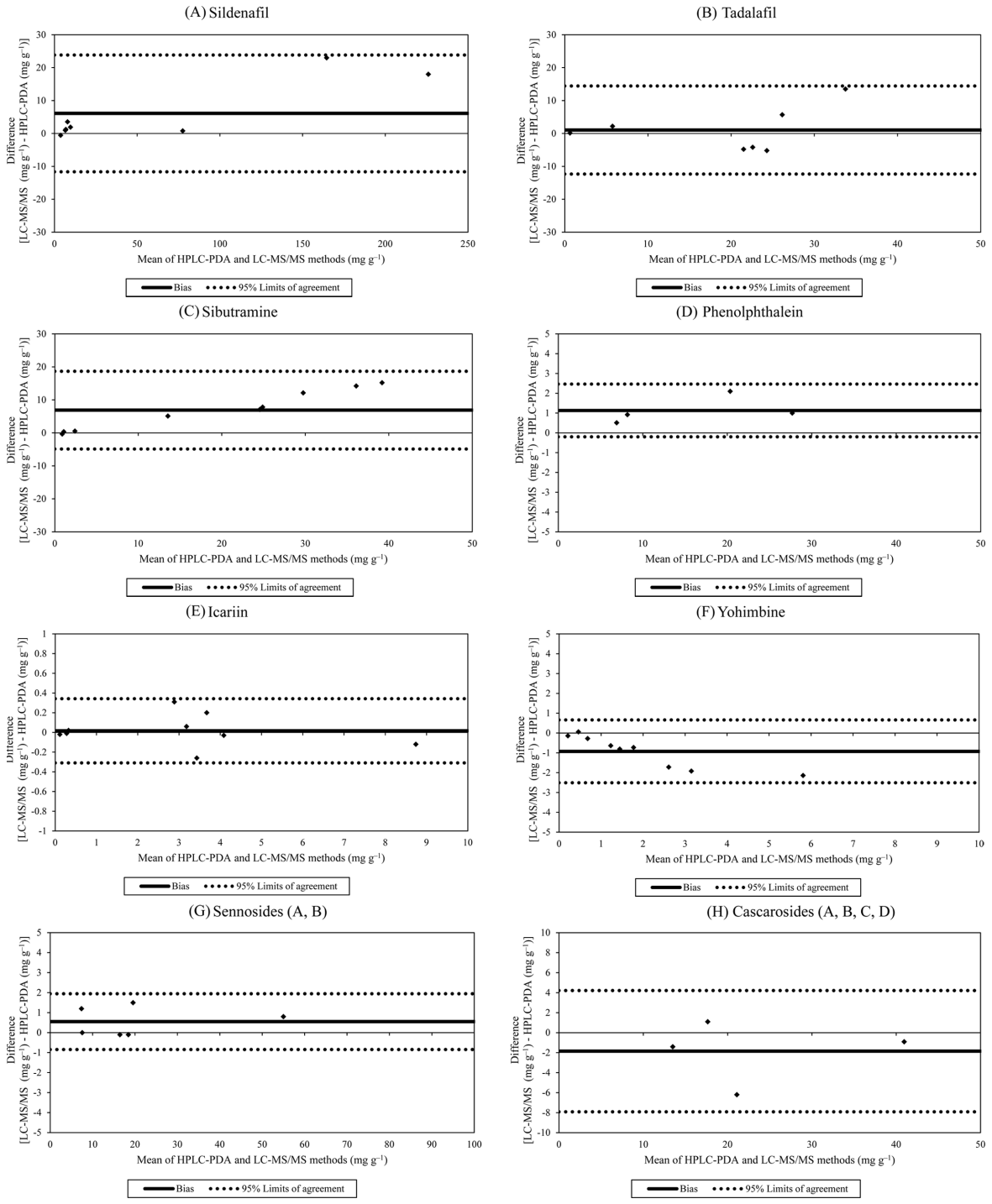

Figure 3. Bland-Altman plots showing agreement between the HPLC and the LC-MS/MS methods. (A) Agreement for sildenafil determination showed a mean bias of 6.1 mg g^{-1} (LC-MS/MS–HPLC-PDA), 95% limits of agreement: -12 to 24. (B) Agreement for tadalafil determination showed a mean bias of 1.0 mg g^{-1} (LC-MS/MS–HPLC-PDA), 95% limits of agreement: -12 to 14. (C) Agreement for sibutramine determination showed a mean bias of 6.9 mg g^{-1} (LC-MS/MS–HPLC-PDA), 95% limits of agreement: -5 to 19. (D) Agreement for phenolphthalein determination showed a mean bias of 1.1 mg g^{-1} (LC-MS/MS–HPLC-PDA), 95% limits of agreement: 0 to 2. (E) Agreement for icariin determination showed a mean bias of 0 mg g^{-1} (LC-MS/MS–HPLC-PDA), 95% limits of agreement: -0.3 to 0.3. (F) Agreement for yohimbine determination showed a mean bias of -0.9 mg g^{-1} (LC-MS/MS–HPLC-PDA), 95% limits of agreement: -3 to 1. (G) Agreement for sennosides determination showed a mean bias of 0.5 mg g^{-1} (LC-MS/MS–HPLC-PDA), 95% limits of agreement: -1 to 2. (H) Agreement for cascarosides determination showed a mean bias of -1.9 mg g^{-1} (LC-MS/MS–HPLC-PDA), 95% limits of agreement: -8 to 4.

performance. However, the toxicology of icariin has not been investigated and established for safe intake of icariin in food supplements.[1,2] Icariin was detected in sexual enhancement, weight-loss, and muscular strengthening products in this study. Yohimbine is a natural tryptamine alkaloid extracted from yohimbe bark (*Rausinystalia yohimbe*). Yohimbe bark is traditionally used in Africa as an aphrodisiac or yohimbine is a drug in veterinary medicine in livestock products in Korea. Due to a significant risk to consumers, yohimbine is banned in dietary supplement in several countries. However, so far, yohimbine is consumed to improve sexual satisfaction, athletic performance and weight loss as an adulterant in dietary supplements.[1,26] In this study, yohimbine was detected in weight-loss and muscular strengthening products. Sibutramine is an approved drug for long-term use in obesity treatment. But no longer available in dietary supplements because this drug may pose cardiovascular risks, such as heart attack, stroke and cardiac arrest.[1,2] In this study, sibutramine was detected in weight-loss products.

Comparison of HPLC-PDA and LC-MS/MS method

In this study, illicit compounds detected in 51 dietary supplements were determined by two different analytical approaches such as HPLC-PDA and LC-MS/MS. Similar concentrations were found by the two methods, showing icariin values in sexual enhancement products of 0.12–8.80 mg g^{-1} and 0.10–8.68 mg g^{-1} by HPLC-PDA and LC-MS/MS, respectively. Also, other compounds, including sibutramine, yohimbine, sildenafil, tadalafil, sennosides, cascarosides, and phenolphthalein, showed similar result values (Table 4).

The Passing and Bablok regression analysis indicated a high degree of linear correlation between two methodologies giving correlation coefficient (r^2>0.9). These results were also confirmed by the Bland-Altman analysis (Figure 3). The statistical results showed good agreement between two methods with a mean bias of 6.1 mg g^{-1} for sildenafil (95% limits of agreement: -12~24), 1.0 mg g^{-1} for tadalafil (95% limits of agreement: -12~14), 6.9 mg g^{-1} for sibutramine (95% limits of agreement: -5~19), 1.1 mg g^{-1} for phenolphthalein (95% limits of agreement: 0~2), 0 mg g^{-1} for icariin (95% limits of agreement: -0.3~0.3), -0.9 mg g^{-1} for yohimbine (95% limits of agreement: -3~1), 0.5 mg g^{-1} for sennosides (95% limits of agreement: -1~2), -1.9 mg g^{-1} for cascarosides (95% limits of agreement: -8~4). Both methods can be effectively used to monitor the unapproved ingredients in dietary supplements.

Conclusion

The objective of this study was to develop as reliable, easy and fast method for the simultaneous determination of 80 illicit compounds in dietary supplements using HPLC-PDA and LC-MS/MS. The unapproved compounds in 51 dietary supplements collected between 2019 and 2021 were successfully determined using two analytical methods. The validation results of developed method were satisfactory with the AOAC guidelines, and showed acceptable performances for accuracy and precision. The concentrations of detected compounds were re-analyzed using two different analytical approaches such as HPLC-PDA and LC-MS/MS. In addition, these values were compared using the Passing and Bablok analysis and Bland-Altman analysis, and we confirmed a good agreement between two methods. Therefore, it is suggested that developed methods can be complementarily used to monitor simultaneously the unapproved 80 ingredients in dietary supplements.

Acknowledgement

This work was supported by the Ministry of Food and Drug Safety (grant no. 20161MFDS052, 21161MFDS052) in 2020–2021.

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