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Rapid determination of sildenafil and its analogues in dietary supplements using gas chromatography–triple quadrupole mass spectrometry

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ABSTRACT

Application of gas chromatography–triple quadrupole mass spectrometry for identification, confirmation and quantification of 6 phosphodiesterase-5 (PDE-5) inhibitors (sildenafil, dimethylsildenafil, homosildenafil, thiosildenafil, thiodimethylsildenafil and thiohomosildenafil) in dietary supplements was investigated. The MS was operated in multiple reaction monitoring mode, for better sensitivity and selectivity. In this manner, the method is adequate to reduce background noise with less interference from co-eluting compounds in the samples. Two different ionisation techniques, electron ionisation (EI) and chemical ionisation (CI), were studied and compared. The chromatographic separation was performed on a short 10 m non-polar capillary column without any derivatisation step. This permitted fast analysis for all analogues with retention time less than 11 min, for both techniques. Use of backflushing can aid method retention time reduction and improves column maintenance. Evaluation of method validation included limit of detection (LOD), lower limit of quantitation (LLOQ), linearity, precision and recovery were performed for both EI and CI techniques. The LOD obtained varied from 0.03 to 1.50 μg/g and the LLOQ ranged from 0.10 to 5.00 μg/g. Good calibration linearity was obtained for all analogues for both techniques, with correlation coefficients (r^2) higher than 0.99. Mean recoveries of all analogues using CI show higher values (83.4–108.8%) than that of EI (61.9–91.1%). The intra- and inter-assay precisions were evaluated for all analogues at spiked concentration of 10 μg/g and the relative standard deviation was less than 15% for both methods. These methods were then successfully applied to dietary supplement samples without prior derivatisation, confirming that the samples were adulterated with sildenafil and/or its analogues.

1. Introduction

Sildenafil citrate is a synthetic phosphodiesterase type 5 enzyme inhibitor (PDE-5), used to treat male erectile dysfunction (ED) and pulmonary arterial hypertension (PAH). It is widely marketed under the name Viagra® (manufactured by Pfizer) [1]. The U.S Food and Drug Administration (FDA) has approved this drug, together with vardenafil (Levitra®, manufactured by Bayer) and tadalafil (Cialis®, developed and marketed by Lilly ICOS). However, several sildenafil analogues are increasingly found as adulterants in food and nutrient supplements outside the official health system. These analogues are structurally modified in the piperazine moiety or carbonyl in pyrazolopyrimidine moiety substituted with a thiocarbonyl group [2]. For instance, thioketone analogues may be synthesised by heating sildenafil with phosphorus pentasulfide (P_2S_5) and the C=O bond exchanged to C=S bond. Furthermore, such analogues are not generally registered as drug substances by the European Medicine Agency (EMA) and U.S Food and Drug Administration (FDA), but may be present as adulterants [3]. They can be considered as "designer drugs" and have no established safety profile. It is important to monitor these drugs because they can cause problems including adverse effects on cardiovascular function such as arterial systemic blood pressure reduction, headaches, facial flushing, dyspepsia, visual disturbances and back pain [4].

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Table 1
Significant peaks in EI and CI full scan MS for sildenafil and its analogues by GC–QQQMS with the relative ion abundances (%). Most abundant peak for each compound is shown in bold.

Analyte	Molecular ion, M^+[a]	Abundance peaks in full scan MS in order of relative abundance (%)	
		EI	CI
Sildenafil	474.2	**99.1** (100), 56.1 (30), 404.3 (10), 474.7 (0.1)	**475.3** (100), 313.2 (12.3)
Dimethylsildenafil	488.2	**113.2** (100), 70.1 (20), 488.4 (0.1)	**489.3** (100), 313.2 (18.1)
Homosildenafil	488.2	**113.1** (100), 70.1 (47.5), 404.3 (10), 488.4 (0.3)	**489.4** (100), 313.3 (14.9)
Thiosildenafil	490.2	**99.1** (100), 56.1 (32.5), 420.2 (7.5), 490.3 (0.8)	**491.3** (100), 223.1 (15.2), 299.2 (12.7)
Thiodimethylsildenafil	504.2	**113.2** (100), 70.1 (17.5), 504.3 (0.3)	**505.4** (100), 329.1 (8.4)
Thiohomosildenafil	504.2	**113.1** (100), 70.1 (32.5), 420.2 (5), 504.4 (1.2)	**505.3** (100), 313.3 (7.5), 223.2 (5.2)

[a] Molecular ion for each compound according to isotopic distribution (m/z).

Recently, numerous websites have been launched, offering various formulations for sale that potentially contain PDE-5. Interestingly, they often claim that the products (e.g., herbal products, dietary supplements or food products) are safe by labeling them as "all natural". Products may be sold at cheaper price, as compared to other health products, which have been approved by the FDA and other national regulatory bodies. They are adulterated by minor modification of the parent structure of approved PDE-5 inhibitors [5]. Development of a sensitive and accurate method to screen and confirm the illegal adulterants in dietary supplements, herbal and food products are of urgent priority.

Most studies reporting identification of sildenafil and its analogues have used liquid chromatography coupled with mass spectrometry (LC–MS) [6,7], high performance liquid chromatography (HPLC) [8,9], LC–tandem MS (LC–MS/MS) [9–12] and ultra-performance LC-time of flight MS [13]. Recently, high resolution benchtop quadrupole-Orbitrap (Q-Orbitrap) mass spectrometry was applied for the detection of illegal adulterants in herbal medicines and dietary supplements [14]. However, the conventional HPLC method has a limitation because the method cannot supply characteristic mass spectral library data, which can assist to discriminate between a wide range of analogues or chemicals. Application of analytical gas chromatography (GC) is largely restricted to low molecular mass, higher volatility and thermally stable compounds [5]. Thermal stability and volatility may be improved through derivatisation, but it is difficult to derivatise sildenafil analogues with standard silylation reagents [15]. Nevertheless, GC may be a preferred option because of simplicity, affordability, low maintenance and well defined MS library databases compared to LC–MS. GC with MS (GC–MS) has been applied to analyse sildenafil, tadalafil and vardenafil in several samples such as biological samples [16–20] food supplements [13,21], and herbal products [22] and pharmaceutical product [23]. However, few studies report determination of either thio or non-thio sildenafil analogues by GC–MS. Thus, further investigation is required because the safety of these analogues has not been clinically tested, and there is a lack of MS library data for sildenafil identification. Prior study identified several thioketone analogues of sildenafil by using GC–MS, focusing on fast identification of the analogues with characteristic mass fragmentations, without further investigation [2].

To improve the identification power of GC–MS, GC–MS/MS with a triple quadrupole instrument can be used to provide greater sensitivity and selectivity of identification because it allows the classic possibility to monitor only the analyte of interest through selection of appropriate ions. Operation in multiple reaction monitoring (MRM) mode (also termed selected reaction monitoring; SRM) is beneficial to accurate identification, confirmation and quantification of some components in a sample [24]. Using the first quadrupole for precursor ion selection, the second as collision cell, and the third quadrupole for product ion selection, high discrimination against background signals should be achievable [25].

Further, chemical ionisation (CI) can support ionisation of labile drugs, in contrast to electron ionisation (EI). The CI soft ionisation technique produces minimal fragmentation of molecular ions, so the molecular species can be more readily identified [26]. The CI mode is particularly useful in target analysis when the molecular weight is known, such as in determination of volatile fatty acids [27], amphetamines [28], amino acids [29] and others. Furthermore, use of CI and MRM mass spectra can be used as a supplemental database simultaneously with an EI mass spectrum database [30].

The aim of this study was to demonstrate rapid identification and quantification for sildenafil and its analogues without use of derivatisation, by using GC–QQQMS with both EI and CI techniques. The method was then applied to analysis of PDE-5 in dietary supplements. To date, little quantitative analysis has been applied to sildenafil and its analogues using GC.

2. Experimental

2.1. Chemicals and reagents

Standards of six sildenafil and its analogues (sildenafil, dimethylsildenafil, homosildenafil, thiosildenafil, thiodimethylsildenafil, and thiohomosildenafil) were obtained from TLC PharmaChem Inc. (Vaughan, Ontario, Canada). Their chemical structures are shown in Supporting information Fig. S1. Ethyl acetate was purchased from Merck kGaA (Darmstadt, Germany) while octacosane (C28) alkane used as internal standard (IS), was obtained from Sigma–Aldrich (St. Louis, USA). Stock solutions of each analyte were prepared as 100 μg/mL stock solutions in ethyl acetate and stored at −4 °C. Mixtures of lower concentration standard solutions were prepared via dilution of the stock solutions in ethyl acetate and prepared fresh daily when analysis was conducted.

2.2. Instrumentation

Analyses were determined using a Bruker Scion 456 TQ GC–MS/MS system (Bruker, Preston, Australia) operated by MS Workstation version 8. Separations were carried out using a BPX5 capillary column (10 m × 0.25 mm I.D × 0.1 μm film thickness (d_f); SGE Analytical Science, Australia). Under fast GC–MS/MS, the initial column temperature was set at 150 °C (0 min), increased to 320 °C at 20 °C/min and held for 5 min. An injection volume of 1 μL was employed in the splitless mode. Helium was used as carrier gas at a constant flow rate of 1.5 mL/min. Temperatures of the injector and the transfer line were held at 280 °C and 300 °C, respectively. The MS was operated in either full scan or MRM/SIM mode using electron ionisation (EI) energy of 70 eV with ion source temperature at 280 °C. The mass scan range for full scan mode was m/z 50–550 and the abundance ions obtained are listed in Table 1. The total run time was 13.50 min. MS/MS mode used argon (99.9999%

purity) as collision gas at 0.1 mTorr for collision-induced dissociation (CID); filament emission current of 80 μA. The analysis was performed with a solvent delay of 4 min to prevent filament damage. The quantification ion and qualification ion transitions, and optimised collision energy for each analyte are listed in Table 2. The NIST MS (Version 2.0) library was used to compare mass spectra only for the sildenafil compound, since the other analogues were not available in the database.

A comparative study was performed using an Agilent 7890A GC system coupled to an Agilent 7000 GC–MS triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, USA). All parameters for GC operation were similar as for EI-MS above. The Agilent MS/MS system was equipped with a chemical ionisation (PCI) source and was operated in positive ion mode with both full scan (mass range m/z 150–550) and multiple reaction monitoring (MRM). Methanol was used as the reagent gas and maintained at 20% flow (approximately 1 mL/min) for the positive CI mode. Nitrogen (99.9999% purity) was used as the collision gas with a flow rate at 1.5 mL/min, and helium (99.9999% purity) was mixed with the collision gas in the collision cell at a flow rate of 2.25 mL/min. The transfer line, source and quadrupole temperatures were 300, 300 and 150 °C, respectively. Other MS operating conditions remained the same. Collision energy was adjusted as required for best sensitivity for the selected transitions.

2.3. Sample preparation

The dietary supplements were purchased online, and the samples were presented in the form of capsules. The shells of capsules were removed, and approximately 0.2 g powder was accurately weighed. The sample was transferred to a vial, and 5 mL of ethyl acetate was added. The sample was mixed thoroughly by vortexing for 1 min, followed by 15 min ultrasonic treatment and cooled to room temperature. Then, the samples were filtered through a 0.45 μm membrane filter and diluted to an appropriate concentration for analysis.

2.4. Method validation

The performance of the method was assessed by estimation of several parameters such as limit of detection (LOD), lower limit of quantitation (LLOQ), linearity, precision and recovery. Method validation for assaying sildenafil and its analogues in dietary supplements was conducted according to US Food and Drug Administration (USFDA) guidelines [31]. The LOD was determined as the minimum concentration at a signal-to-noise ratio of at least 3 (S/N > 3) while LLOQ was the lowest concentration of the calibration curve, giving a signal-to-noise ratio of at least 10 (S/N > 10) with acceptable accuracy and precision. The linearity of the method was assessed by the coefficient of correlation (r^2) of a 5-point calibration curve that was constructed by plotting relative response (analyte/IS, peak area) according to analyte concentration. Each calibration point was prepared in triplicate for each analogue. To assay the accuracy of the method, mean recoveries were studied. The recovery and precision experiments were performed by spiking a known amount of mixed standard into a blank sample at concentration of 10 μg/g. The experiment was conducted by injecting the sample into the system 6 times for intra-assay, and this procedure was repeated with a fresh spiked sample daily, over 6 days, for inter-assay validation. Then, the average percentage of recovery and relative standard deviation (RSD) were calculated for each analogue.

3. Results and discussion

3.1. Identification and confirmation of each analogue

Here, a fast approach was implemented with high initial column temperature (150 °C) and relatively high temperature ramp rate (20 °C/min) was applied. A non-polar short capillary column (10 m) was chosen to reduce upper oven T and prolong column life [22]. In addition, a thinner film phase column was used to minimise retention of the high boiling point analogues; they still eluted at T > 300 °C. In this study, acceptable separations were observed for all analogues in both EI and CI methods with retention time less than 11 min. In a method variation, backflushing by use of a Deans switch can protect the column by removing low volatility compounds that might only slowly travel through the column. As soon as the analytes of interest are eluted, backflushing for a few column volumes will clean the column, ready for the next injection.

Each standard was injected directly into the GC using splitless injection to provide MS identification of each compound, and establish their retention time in both techniques. However, most compounds apparently were not included in the NIST libraries; only sildenafil was observed in the NIST MS library software. Thus, identification of the analogues was performed by interpreting each fragmentation pattern obtained from both EI and CI techniques. To support identification of each analogue, reference to previous studies was made [2,15].

Table 2
Selected EI and CI MRM transitions for each compound with the best collision energy (CE).

Analyte	EI MRM transition			CI MRM transition		
	Precursor ion[a]	Product ion	CE[b] (eV)	Precursor ion[a]	Product ion	CE[b] (eV)
Sildenafil	404	311.9	30	475.3	99.9	40
	474	99.2	10	475.3	282.9	40
Dimethylsildenafil	488	311.9	40	489.4	98.9	40
	488	112.9	10	489.4	112.8	40
Homosildenafil	488	112.9	20	489.3	98.9	40
	488	403.7	40	489.3	112.9	40
Thiosildenafil	490	420.2	40	491.3	342.8	30
	490	98.2	10	491.3	326.9	30
Thiodimethylsildenafil	504	112.8	20	505.4	326.9	20
	504	328	40	505.4	113	40
Thiohomosildenafil	504	112.6	20	505.3	342.8	30
	504	421.3	40	505.3	326.8	30

[a] Precursor ion chosen ± 0.5 Da.
[b] Collision energy (eV); argon gas.

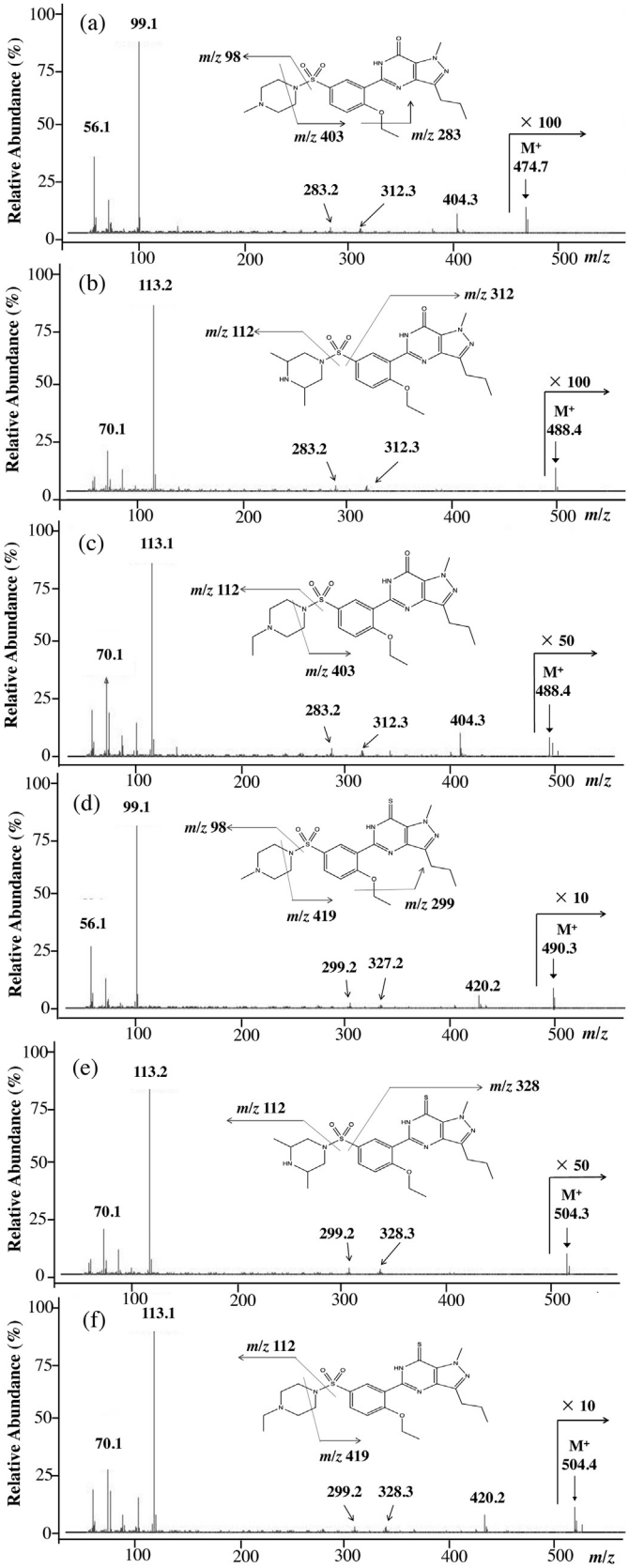

Fig. 1. EI full scan mass spectra with interpreted fragmentation pattern for each analogue; (a) sildenafil, (b) dimethylsildenafil, (c) homosildenafil, (d) thiosildenafil, (e) thiodimethylsildenafil and (f) thiohomosildenafil.

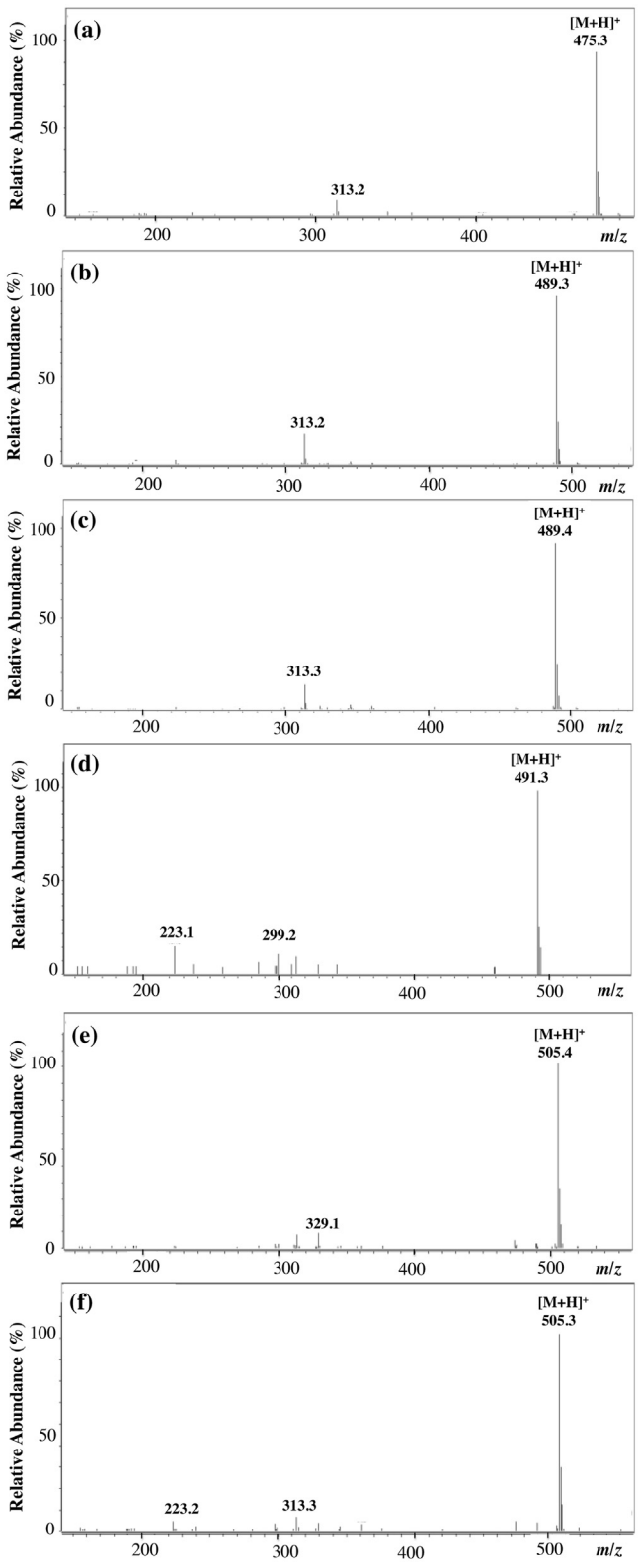

Fig. 2. CI full scan mass spectra for each compound with relative ion abundances (%); (a) sildenafil, (b) dimethylsildenafil, (c) homosildenafil, (d) thiosildenafil, (e) thiodimethylsildenafil and (f) thiohomosildenafil.

Fig. 1 shows full scan EI spectra with postulated fragmentation pathways produced by each analogue. For sildenafil, an abundant *m/z* 99 ion in the mass spectrum results from cleavage of the S—N bond between the piperazine ring and the sulfonyl group. Ion *m/z* 404 results from the cleavage of two C—C bonds of the piperazine moiety [32], as shown in Fig. 1. Dimethylsildenafil varies from parent sildenafil by an additional methyl group attached to the piperazine moiety. It alters the fragment ion mass by producing a *m/z* 113 ion (2-methyl bonded) for dimethylsildenafil compared to that of the analogous fragment ion *m/z* 99 for sildenafil. Although both dimethylsildenafil and homosildenafil have the same nominal molar mass (488 g/mol), they can be differentiated by the fragment ion *m/z* 404, which only arises in the MS result of homosildenafil. From their structures, they differ by the alkyl group(s) bonded to piperazine moiety; an ethyl group for homosildenafil compared to two methyl groups for dimethylsildenafil. As reported previously [2], *m/z* 312 and 283 result from fragmentation of the pyrazolopyrimidine moiety from sulfoxide and subsequent loss of the ethyl group on the ethoxy substituent of the phenyl ring, respectively. This fragmentation not only occurred in sildenafil, but a similar pattern also can be observed in dimethylsildenafil and homosildenafil. Due to the presence of sulfur in thioketone analogues, the mass fragmentation pattern theoretically generates *m/z* 328 and 299, corresponding to 16 mass units greater than non-thioketone analogues.

The difference between non-thioketone analogues and thioketone analogues is the substitution of oxygen by the sulfur atom at the pyrazolopyrimidine moiety. The fragmentation pattern for thiosildenafil was similar to that of sildenafil, but the only difference is addition of 16 mass units arising from the presence of sulfur. Thiodimethylsildenafil and thiohomosildenafil share the same molar mass of 504 g/mol but again differ by the alkyl substitution on the piperazine moiety. Both thio-analogues can be differentiated by the presence of peak *m/z* 420, which only appear in the mass spectra of thiohomosildenafil. This ion resulted from the cleavage of two C—C bonds of the piperazine moiety of thiohomosildenafil. All the fragment ions and molecular ions for each compound are listed in Table 1.

Based on Fig. 1, the molecular ion obtained from the EI technique for each compound showed very low abundance compared to the base peak. Thus, a CI source was applied to provide molecular ion information for each compound, with minimal fragmentation. Table 1 demonstrates that the most abundant ion is contributed by the protonated molecular ions, $[M+H]^+$. The presence of the protonated molecular ion can assist identification of each analogue, and provides highly specific detection of analytes of interest when combined with retention information. As shown in Fig. 2, all mass spectra are simple, with considerably less fragmentation than when using EI, with a dominant protonated molecular ion as base peak. Other fragment ions appeared, but in very low relative abundance (<10%). For non-thioketone analogues, the *m/z* 313.2 fragment ion was obtained as shown in the mass spectra of sildenafil, dimethylsildenafil and homosildenafil. This ion results from cleavage of the pyrazolopyrimidine moiety from sulfoxide. Furthermore, CI mass spectra can be used as supplementary library database information together with EI mass spectra. As compared with the EI technique, the identification of each analogue by CI provides high selectivity and high sensitivity due to lower interference from the matrix.

3.2. GC–QQQMS optimisation

Analysis of sildenafil and it analogues by using MS/MS provides greater sensitivity and selectivity. Furthermore, the identification of detected analytes is more reliable at low concentration, with greater confidence for confirmation compared to conventional full scan GC–MS. Several parameters were evaluated such as precursor ion (Q1) and product ion (Q3) selection, and collision energy (CE) to achieve the above. Time windows were set corresponding to retention time of each analogue, with the selected ion masses. Multiple windows allow more data points per peak because fewer compounds are present in the window, and the number of transitions will be fewer, therefore allowing faster cycle times. The MRM method was developed with both EI and CI techniques, based on respective full scan mass spectra data. At first, the most abundant ion in the mass spectrum was chosen as precursor ion. However, if the result did not provide acceptable sensitivity or selectivity, a few factors can be taken into account such as specificity of the transition, and relative abundances of selected ion masses. In this study, the molecular ion for each analyte was chosen as the precursor ion since this provides better fragmentation. Also, most of the compounds have a common base peak, i.e., either *m/z* 99.1 or 113. Then, a product ion scan was performed at various collision energies (10, 20, 30 and 40 eV) to select the highest abundance product ion. In addition, the choice of product ions was usually made on the basis of the two most abundant fragments at the respective CE value, where nearly complete dissociation of the chosen precursor ion occurred (Supporting information Fig. S2–S7). However, in some cases, the second most intense ion transition is too weak, so it is necessary to use a different precursor ion, to generate a more intense product ion for qualitative identification purposes. This arises for sildenafil where two precursor ions (*m/z* 404 and 474) were selected to attain better subsequent fragmentation. Thus, several factors need to be considered to select the best ion transition which is unique only to certain compounds with freedom from interferences [33].

Two selected fragmentation reactions were chosen for method implementation, such that the first SRM transition was selected for quantification, and the second SRM transition was chosen for qualification to support confirmation of each compound. Therefore, MRM transitions of one or two precursor ions and two product ions were assigned for each analyte. From Table 2, it can be seen that thiodimethylsildenafil and thiohomosildenafil shared similar first transitions (quantification) but can be differentiated by the second transition (qualification). Having different retention times ensures there is no overlap of the quantification ion. While thiosildenafil and thiodimethylsildenafil have very close elution times, they have quite different MRM, and so can be readily differentiated. The optimised MRM transition conditions are listed in Table 2. Fig. 3 displays the EI MRM chromatogram of each compound including respective retention times at specific time windows. Each peak was obtained from the first ion transition at appropriate collision energies (Table 2).

For CI, since protonated molecular ions constituted the most abundant peak in the mass spectra, thus they were obvious choices as precursor ion for each compound. As in the EI MRM method, the precursor ion was studied with product ion scans attained at different collision energies (10, 20, 30 and 40 eV). The product ion spectra for each analogue are shown in Supporting information Fig. S8. Then, the two most abundant product ions were selected with the best collision energy. The optimised parameters for each compound using CI are listed in Table 2. Most of the product ions obtained from CI MS/MS are similar to those in the EI MS/MS method. Although dimethylsildenafil and homosildenafil generated the same transitions, good separation can be obtained by assigning them to specific time windows (see Fig. 4). Since the most common ionisation product in CI is the $[M+H]^+$ ion, lower LOD can be achieved by the MS/MS set up configuration. This method holds great potential when analysing samples with trace levels of analytes, to provide a high sensitivity and selectivity screening method. Man et al. reported the only study of GC–MS for thioketone analogue identification. However, GC–MS with an EI source suffers from lack of spectrum information due to structural similarity and co-eluting compounds, and no reproducibility was discussed [2]. In this study,

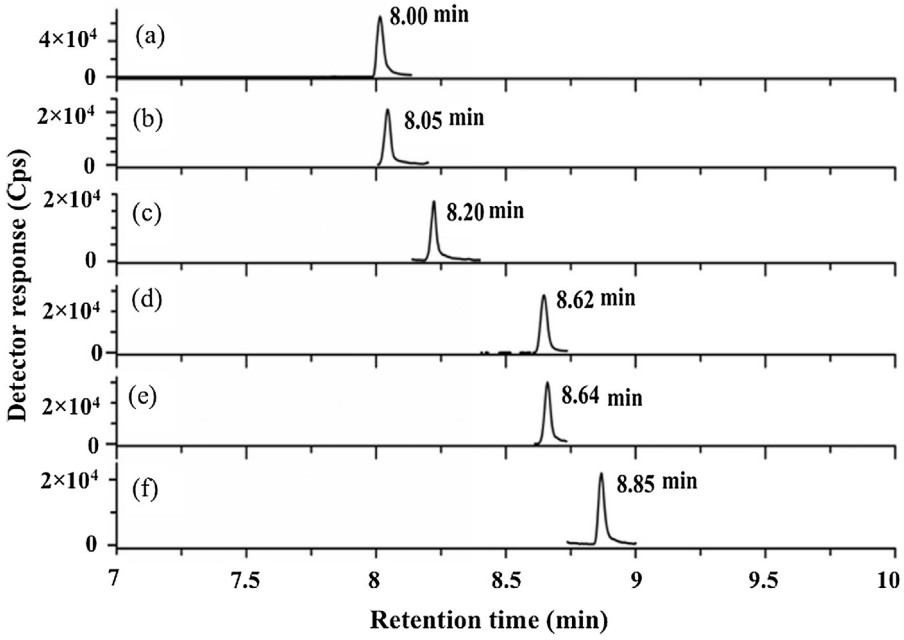

Fig. 3. EI MRM chromatograms for all standard analogues obtained from GC–EI–QQQMS with retention times; (a) sildenafil, (b) dimethylsildenafil, (c) homosildenafil, (d) thiosildenafil, (e) thiodimethylsildenafil and (f) thiohomosildenafil using first ion transitions according to Table 2.

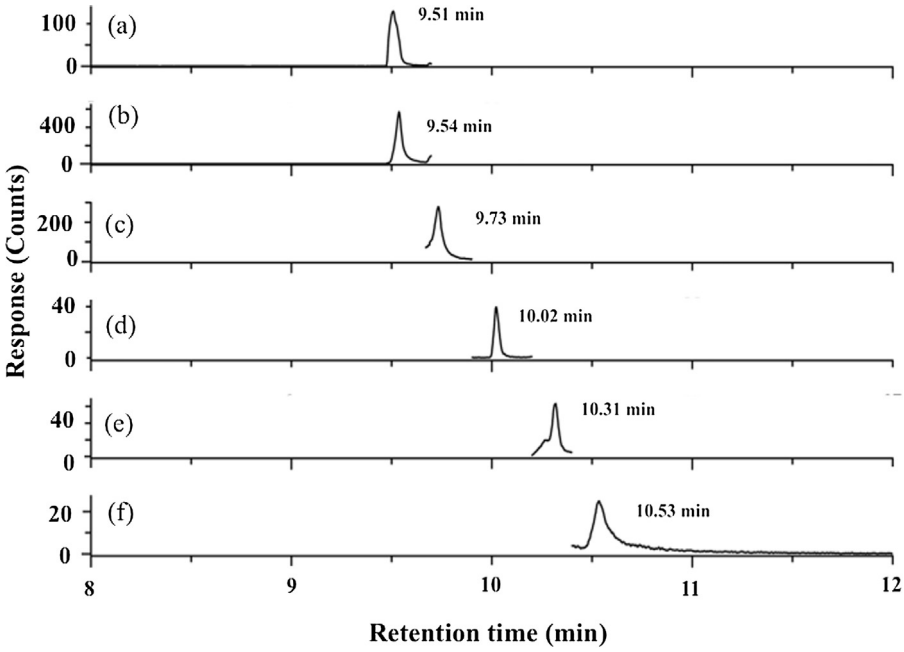

Fig. 4. CI MRM chromatograms for all standard analogues obtained from GC–CI–QQQMS with retention times; (a) sildenafil, (b) dimethylsildenafil, (c) homosildenafil, (d) thiosildenafil, (e) thiodimethylsildenafil and (f) thiohomosildenafil using first ion transitions according to Table 2.

GC–MS/MS proved to be a novel method compared to previous GC methods because it provides rapid analysis of sildenafil, non-thioketone and thioketone analogues with mass fragmentation information by using both EI and CI modes, in conjunction with the reproducibility study. Whilst no derivatisation step was conducted here, most previous GC methods employed derivatisation to analyse the compounds, according to the low volatility of sildenafil as reported by Saisho et al. [16] and Pyo et al. [15] with EI–MS.

3.3. Method validation

The methodology was verified through assessment of linearity, LOD, LLOQ, precision and recovery. Table 3 shows the linear range of the calibration curve for each analyte in the concentration range of interest to this analysis, along with correlation coefficient, LODs and LLOQs. Three replicate injections were conducted for each analysis. For EI, the linearity of a five-point calibration was examined over the concentration range of 0.1–20 μg/g while for CI the concentration range was 2–20 μg/g. As can be seen in Table 3, the

Table 3
Linear ranges, coefficient of correlation, LLOQ and LOD for each analogue using EI and CI methods.

Analyte	EI				CI			
	Linear range (μg/g)	Coefficient of correlation (r^2)	LLOQ (μg/g)	LOD (μg/g)	Linear range (μg/g)	Coefficient of correlation (r^2)	LLOQ (μg/g)	LOD (μg/g)
Sildenafil	0.1–10	0.9987	0.1	0.03	2–20	0.9929	2	0.6
Dimethylsildenafil	0.1–10	0.9974	0.1	0.03	2–20	0.9964	2	0.6
Homosildenafil	0.1–10	0.9974	0.1	0.03	2–20	0.9948	2	0.6
Thiosildenafil	1–20	0.9958	1	0.3	5–20	0.9971	5	1.5
Thiodimethylsildenafil	1–20	0.9963	1	0.3	5–20	0.9905	5	1.5
Thiohomosildenafil	1–20	0.9921	1	0.3	5–20	0.9911	5	1.5

Table 4
Intra-assay precision and recovery ($n = 6$) and 6 inter-assay precision data for each analogue in spiked samples at concentration of 10 μg/g for GC–EI–MS/MS and GC–CI–MS/MS.

Compounds	Recovery (%)		Precision, RSD (%)			
			Intraday ($n = 6$)		Interday ($n = 6$ days, 6 replicates/day)	
	EI	CI	EI	CI	EI	CI
Sildenafil	76.9	83.4	12.5	2.1	14.7	1.5
Dimethylsildenafil	67.8	100.3	9.8	1.2	13.3	1.9
Homosildenafil	67.4	100.8	7.7	1.1	13.1	2.4
Thiosildenafil	91.1	101.9	5.1	4.2	6.8	3.3
Thiodimethylsildenafil	61.9	108.8	8.2	1.9	8.3	3.1
Thiohomosildenafil	76.6	103.9	9.2	2.5	9.1	2.9

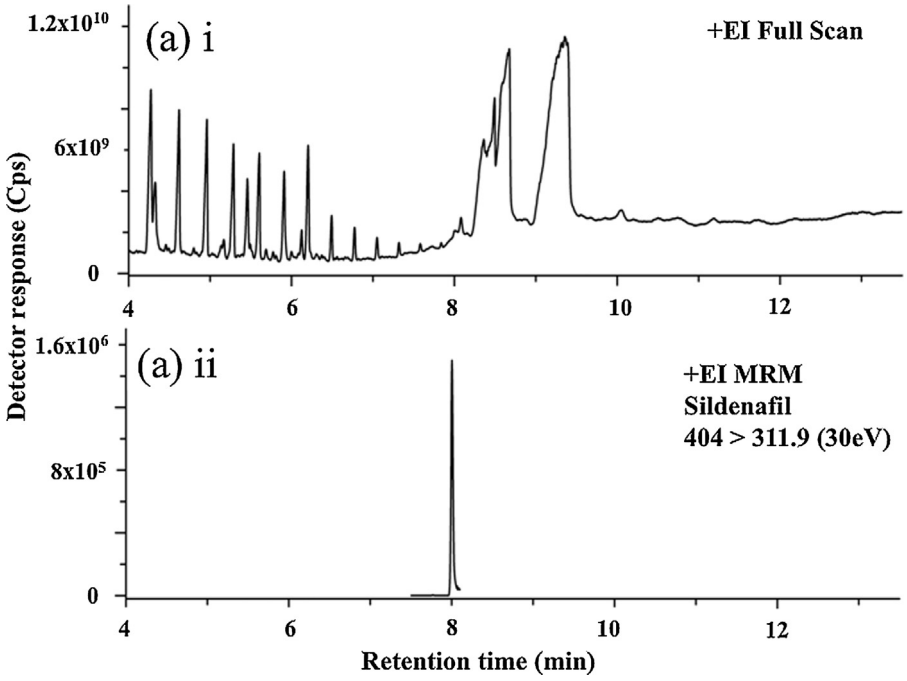

Fig. 5. Chromatograms of positive sample B with (a) i Full scan mode and (a) ii MRM mode by GC–EI–QQQMS, in which sildenafil was detected in the sample.

Table 5
Results for sildenafil and analogues detected in 5 samples (μg/g), $n = 6$.

Sample	Sildenafil		Thiosildenafil		Thiodimethylsildenafil	
	EI	CI	EI	CI	EI	CI
A	0.8 ± 0.1	2.6 ± 0.1	ND	ND	ND	ND
B	4.7 ± 1.0	4.5 ± 0.3	ND	ND	ND	ND
C	7.3 ± 2.3	20.3 ± 2.9	ND	ND	ND	ND
D	ND[a]	ND	3.9 ± 0.4	6.5 ± 0.5	ND	ND
E	ND	ND	ND	ND	2.4 ± 0.1	3.2 ± 0.1

[a] Not detected.

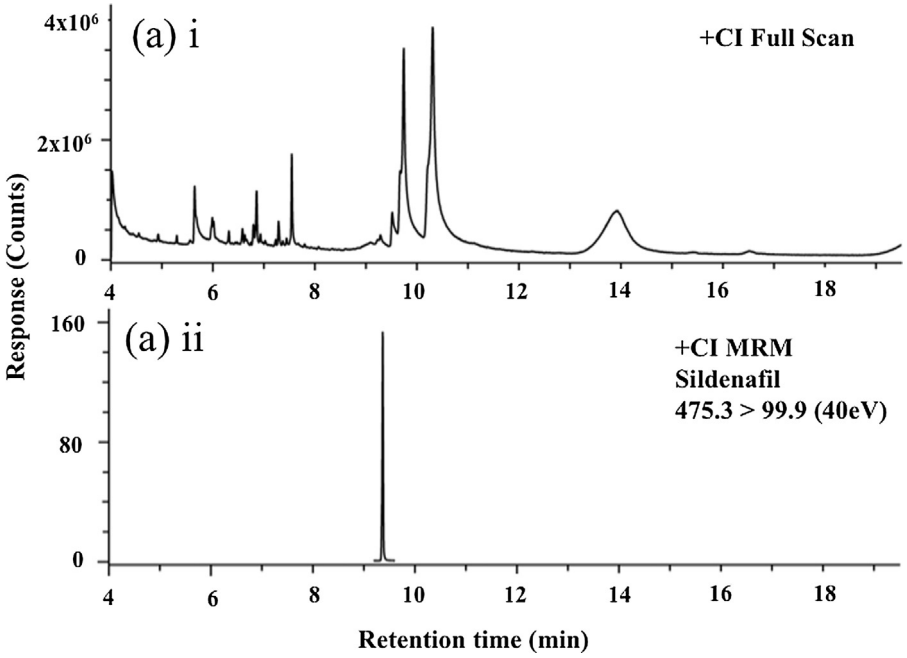

Fig. 6. Chromatograms of positive sample B with (a) i CI Full scan mode and (a) ii CI MRM mode by GC–CI–QQQMS, in which sildenafil was detected in the sample.

calibration curves vs IS response showed good response vs concentration linearity, with correlation coefficients (r^2) > 0.99 for all analytes. The LLOQ values are from 0.1–5 μg/g for both techniques while the LODs of all analogues were estimated to be in the range 0.03–0.3 μg/g and 0.6–1.5 μg/g for EI and CI, respectively. Through an examination of both techniques, EI–MS/MS was demonstrated to have a lower detection limit, and produces a more sensitive method than CI–MS/MS.

The precision and recovery of the methods were evaluated at a concentration of 10 μg/g for both EI and CI. Each sample was injected six times for intra-assay precision evaluation and repeated for six consecutive days (inter-assay). The recoveries and intra/interday precisions obtained by the two approaches are summarised in Table 4. Comparable precision and recovery was observed for both EI and CI methods. The percentage recovery of each analyte was calculated by comparing the peak area of the sample with the spiked analyte, to the peak area of the reference standard. Results indicated mean recoveries for all analogues were within the range of 61.9–91.1% for EI and 83.4–108.8% for CI. From Table 4, the mean recoveries of all compounds by using EI were lower, compared with CI, indicating the closeness of the response of the spiked sample to the response of the each standard analogue for CI. Intraday and interday precision were calculated based on RSD values obtained for each spiked sample. Evidently CI had lower RSD values (<5%) for all analogues, ranging from 1.1–4.2% to 1.5–3.3% for intra-assay and inter-assay validation, respectively compared to EI (<13%). Apparently, adequate method precision and robustness of the method is attained.

3.4. Analysis of dietary supplement samples

The proposed method was applied to check the presence of sildenafil and its analogues in five dietary supplements, which were purchased online in Singapore. For positive samples with concentrations higher than the working range, the sample solution was diluted to produce responses within the working range. The identification of target compounds was based on the MRM mode with specific time windows. The sample was considered negative if no signal was detected, or the signal was below the LOD levels within respective time windows. Results indicated that all samples were confirmed to be positive samples, adulterated with sildenafil and its analogues by both EI and CI techniques (see Figs. 5 and 6). The amount of each analogue detected was calculated from the calibration curve. Based on Table 5, it is noted that the sildenafil was detected in sample A–C using both ionisation mode at specific concentrations. Sample D and E were illegally adulterated with a thioketone analogue; thiosildenafil and thiodimethylsildenafil, respectively. Agreement between EI and CI was reasonable for the amount of sildenafil detected in Sample B. However, other samples demonstrated higher concentrations of target compounds were obtained by using CI than by EI (see Table 5), possibly due to higher mean recoveries for all analogues in CI as discussed in Section 3.3. The identification of the sildenafil analogues in adulterated dietary supplements is crucial since many cases have been reported recently on adulteration of food supplements, even though the FDA has banned the use of class I drugs in dietary supplements. Results proved that the proposed method was adequate for simultaneous identification, confirmation and quantitation of sildenafil and its analogues in dietary supplements.

4. Conclusion

This paper reports the development of a GC–QQQMS method for identification sildenafil and various analogues in dietary supplements with two different ionisation techniques. By using the triple quadrupole method with MRM mode, the determination of each analogue was shown to provide adequate sensitivity, selectivity and acceptable separation without a derivatisation step. It also highlighted that this method provides rapid analysis because all the target compounds were eluted within 11 min, for both EI and CI techniques. Experiments were carried out to achieve the best condition of MRM transition for each analogue. Validation parameters such as linearity, LLOQ, LOD, recovery and precision of this method were acceptable. EI–MS/MS method demonstrated more sensitive compared to CI–MS/MS. Nevertheless, the CI method plays an important role for the identification of each analogue by providing reduced fragmentation of molecular ions. The developed methods

were successfully applied to detect and confirm that five dietary supplements were adulterated with sildenafil and its analogues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2016.01.034.

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