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Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS)

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ABSTRACT

As a widely consumed beverage, coffee tends to be a target for intentional adulteration. This study describes the application of modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) coupled to liquid chromatography-high-resolution mass spectrometry (LC-HRMS) for simultaneous screening, identification, and quantification of undeclared phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes (ICPs). The mass spectrometer was operated in auto MS/MS acquisition for simultaneous MS and MS/MS experiments. Qualitative establishments from the suspected-target screening and targeted identification processes led to an unambiguous analyte assignment from the protonated molecule ($[M+H]^+$) precursor ion which is subsequently used for quantification of 23 targeted PDE5 inhibitors. The analytical method validation covered specificity, linearity, range, accuracy, limit of detection (LOD), limit of quantification (LOQ), precisions, matrix effect (ME), and extraction recovery (RE). The specificity was established using the optimised chromatographic separation as well as the distinguishable $[M+H]^+$ precursor ion. The linearity of each target analyte was demonstrated with a coefficient of determination (r^2) of > 0.9960 over the expected range of sample concentrations. The accuracy ranged from 88.1%–119.3% with LOD and LOQ of < 70 ng/mL and 80 ng/mL, respectively. Excellent precisions were established within 0.4%–9.1% of the relative standard deviation. An insignificant ME within −5.2% to +8.7% was achieved using three different strategies of chromatography, sample extraction, and sample dilution. The RE was good for all target analytes within 84.7%–123.5% except for N-desethylacetildenafil at low (53.8%) and medium (65.1%) quality control levels. The method was successfully applied to 25 samples of ICPs where 17 of them were found to be adulterated with PDE5 inhibitors and their analogues. Further quantification revealed the total amount of these adulterants ranged from 2.77 to 121.64 mg per sachet.

1. Introduction

Coffee is among the most favoured beverages throughout the world [1], leading to the advent of instant coffee premixes (ICPs) which typically packaged in a single serving sachet. These coffee products often comprise other ingredients such as creamer, sugar and ingredients to enrich flavour and texture [2,3]. Sometimes, they are fortified with vitamins and minerals [4]. Unfortunately, ICPs are also known to be adulterated with synthetic drugs which claim to enhance male sexual performance such as phosphodiesterase 5 (PDE5) inhibitors and their analogues.

An analogue of PDE5 inhibitors is synthesised by minor modifications to the parent structure of the approved drugs which will alter their physical and chemical properties [5]. Additionally, there are no clinical studies performed on these analogues to ensure their efficacy and safety [6]. To date, more than 90 unapproved analogues of PDE5 inhibitors have been discovered and described in the literature as adulterants. Since 2010 up to the end of 2018, the United States Food and Drug Administration has issued seven warnings regarding ICPs tainted with PDE5 inhibitors and their analogues [7], specifically those that were made in Malaysia [8].

Liquid chromatography (LC) coupled with mass spectrometry (MS) has been most popular in the detection and analysis of PDE5 inhibitors and their analogues. Although low-resolution MS was frequently used [9–11], high-resolution mass spectrometry (HRMS) proves to be superior [12–14] as it delivers full spectral information with excellent mass accuracy on top of isotopic reliability, aiding suspected-target screening [15] and targeted identification processes [16]. It also

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enables embedding non-targeted screening into a developed method for retrospective and prospective applications [17]. To date, analysis of PDE5 inhibitors has been primarily targeting health supplements, particularly in pharmaceutical dosage form [18]. Due to the relatively high concentration of analytes in these products and the relatively simple matrix involved, these published methods are not applicable in the analysis of PDE5 inhibitors in ICPs. The low analyte level and the complex matrix nature of ICPs in combination with the growing number of novel PDE5 analogues available for adulteration represent a real analytical challenge for forensic drug testing laboratories.

This study focused on developing an LC-HRMS based analytical method that is capable of accurately detecting and quantifying PDE5 inhibitors and their analogues down to trace levels in ICPs. Method development involved optimisation of chromatographic separation, MS conditions, and sample preparation, described in Ref. [19]. Method validation covered specificity, linearity, range, accuracy, limit of detection (LOD), limit of quantification (LOQ), precisions, matrix effect (ME), and extraction recovery (RE). The method was applied to real sample analysis incorporating suspected-target screening, targeted identification, quantification, and non-targeted screening approaches. To the best of our knowledge, this is the first report to comprehensively address the analytical challenge for a reliable determination of PDE5 inhibitors as adulterants in ICPs.

2. Materials and methods

2.1. Chemicals and reagents

Certified reference materials were purchased from TLC Pharmaceutical Standards Ltd. (Aurora, Ontario, Canada). They are desmethylcarbodenafil (1), carbodenafil (2), N-desethylacetildenafil (3), acetildenafil (4), hydroxyvardenafil (5), dimethylacetildenafil (6), vardenafil (7), sildenafil (8), homosildenafil (9), dimethylsildenafil (10), propoxyphenyl-hydroxyhomosildenafil (11), udenafil (12), propoxyphenyl-sildenafil (13), hydroxythiovardenafil (14), tadalafil (15), mirodenafil (16), mutaprodenafil (17), thiosildenafil (18), thiohomosildenafil (19), dithiodesmethylcarbodenafil (20), thiodimethylsildenafil (21), propoxyphenyl-thiohydroxyhomosildenafil (22), and propoxyphenyl-thiodimethylsildenafil (23). Their chemical structures are presented in Fig. S1. LC-MS grade methanol and acetonitrile were purchased from Chem-Supply Pty Ltd. (Gillman, SA, Australia). LC-MS grade formic acid and analytical grade ammonium formate were purchased from Sigma Aldrich Pty Ltd. (Castle Hill, NSW, Australia). Ultrapure water (18.2 MΩ-cm) was obtained from a Sartorius arium® pro ultrapure water system (Goettingen, Germany). Restek Q-sep QuEChERS extraction salts (EN 15662) was purchased from LECO Australia Pty Ltd. (Castle Hill, NSW, Australia).

2.2. Standard solution preparation

All 23 individual stock solutions of PDE5 inhibitors were prepared separately in LC-MS grade methanol at 1 mg/mL and stored in the dark at 4 °C until analysis. A mixture of all standards (working solution) was prepared fresh for each analysis from the stock solutions by further dilution in methanol to make up to 25 μg/mL concentration.

2.3. Sample collection and storage

A total of 25 distinct brands of ICPs were acquired from Malaysia. These samples are highly suspected to be adulterated with PDE5 inhibitors based on the references to male sexual performance in their brand names, label claims, images, botanical ingredients, or advertising materials. Out of the total, 13 samples were kindly donated by the Pharmacy Enforcement Division, Ministry of Health Malaysia, obtained from surveillance activities (7 samples), and by confiscation at the international airport (2 samples) and international seaport (4 samples) during routine inspections by pharmacy enforcement officers. The other 12 samples were purchased through online shopping platforms in Malaysia. All distinct samples were coded and labelled as SPL001 to SPL025. These samples were deposited in a plastic zip lock bag separately and then stored in an airtight container in the dark. A blank ICP, free from any analyte of interests was sourced from a local supermarket and used for method development and validation.

2.4. Sample preparation

First, the whole content of a sachet of an ICP sample was weighed. Then, 100 mg of the sample was dissolved in 5 mL of acetonitrile and methanol (50:50, v/v). The resulting solution was then transferred into a tube prefilled with QuEChERS salts for the extraction procedure. Finally, the upper layer was filtered and diluted with methanol at 1:10 dilution level for analysis. The blank ICP was treated in the same manner as the steps described for the sample analysis. The full extraction procedures can be found in Section 2.1 of Ref. [19].

2.5. LC-HRMS conditions

The chromatographic separation was performed using an Agilent Technologies (Santa Clara, CA, USA) 1290 Infinity II LC system coupled to an Agilent Technologies 6510 quadrupole time of flight-mass spectrometer (QTOF-MS). The LC system was fitted with a reverse phase high-performance LC column from Merck KGaA (Darmstadt, Germany) Chromolith® High-Resolution RP-18 end-capped (100 × 4.6 mm, 2.0 μm) with solvent A (10 mM ammonium formate in ultrapure water) and solvent B (acetonitrile). Both solvents were acidified with 0.1% v/v formic acid as the binary mobile phase system. The QTOF-MS was operated in positive electrospray ionisation mode with auto MS/MS acquisition. Specific details on the LC-HRMS conditions are described in Section 2.2 of Ref. [19].

2.6. Method validation and data analysis

Method validation was performed in accordance with the guideline set by the International Conference on Harmonisation [20] covering specificity, linearity, range, accuracy, LOD, LOQ, and precisions. The ME and RE were also evaluated for each target analyte in the blank ICP matrix following the published procedures [21]. All analyses were done in triplicate.

The specificity was assessed for each target analyte based on their chromatographic resolution and their unique accurate mass of the protonated molecule ($[M+H]^+$) precursor ion from the MS experiment. The presence of two fragment ions corresponding to each targeted PDE5 inhibitors was established from the MS/MS experiment. To further confirm the identity of each target analyte, the average intensity ratio between the first and the second fragment ion was compared to those obtained from the linearity assessment with an acceptable value of ± 30%. The effects of interferences, especially from the blank ICP matrix, were ascertained by the evaluation of three levels of quality control (QC) analytes and analyte-free extracted blank matrix.

Six-point external calibration curves were constructed for each target analyte by diluting the working solution in methanol at concentrations ranged from 0.08 to 1.2 μg/mL. The individual analyte peak areas, from the $[M+H]^+$ precursor ion versus analyte concentrations, were utilised to construct an external calibration curve. A regression analysis was done to determine the linearity based on the coefficient of determination (r^2) and the regression equation was used to calculate the QC analytes and samples concentrations. The linear range was established based on the lower (trace level) and upper (lowest recommended dose) concentrations of analyte expected in adulterated ICPs.

The accuracy was established at low, medium, and high QC levels. All target analytes were spiked into an extracted blank ICP, and the

resulting peak area of the $[M+H]^+$ precursor ion was fitted to the regression equation of the external calibration curve to determine its concentration. Comparison of the observed analyte concentration versus the expected concentration at the same QC level was expressed as a percentage of accuracy with an acceptable value of ± 25%.

The LOD and LOQ were determined experimentally. For LOD, solutions were prepared with an initial 100 ng/mL concentration of target analytes. The solutions were then decreased by 10 ng/mL each down to the final solution of 10 ng/mL. The LOD was set at the lowest concentration of target analyte that can be reliably detected based on the presence and the average intensity ratio of two fragment ions described in the specificity assessment. Meanwhile, the LOQ was defined as the lowest concentration of the calibration curve, where each target analyte can be quantified with an acceptable percentage of accuracy of ± 25% and precision based on the percentage of relative standard deviation (% RSD) of less than 20%.

Using the same QC analytes in an extracted blank matrix, precisions were determined based on repeatability and intermediate precision at low, medium, and high QC levels. Repeatability and intermediate precision were established at intra- and inter-day, respectively, and expressed as a %RSD of the peak areas of the $[M+H]^+$ precursor ion with an acceptable value of less than 20%.

The ME was evaluated based on the post-extraction addition method by comparing the slopes of the matrix-matched calibration curve versus those of the external calibration curve in a neat solution as expressed by Eq. (1). Both calibration curves were constructed using the same concentration as the QC analytes. The percentage of ME was then categorised in accordance with the set criteria of insignificant (0% to ± 10%), acceptable (± 10% to ± 20%), moderate (± 20% to ± 50%), and severe (−50% < > +50%), where positive value indicates ionisation enhancement while negative value indicates ionisation suppression.

$$ME\ (\%) = \left[\frac{S\ matrix\ matched}{S\ neat\ solution} - 1\right] \times 100 \quad (1)$$

where, S = the slope of the calibration curve.

The RE was determined by comparing the peak areas of the $[M+H]^+$ precursor ion of target analytes spiked into the blank ICP matrix before extraction versus those spiked into an extracted blank matrix at the same concentration. The RE was expressed in percentage at low, medium, and high QC levels with an acceptable value of ± 25%.

All qualitative and quantitative data were processed using Agilent Technologies Mass Hunter workstation software version B.07.00 and personal compound database and library (PCDL) manager software version B.04.00. All other calculations were done using Microsoft (Redmond, WA, USA) Excel 2016 MSO.

2.7. Workflow for determination of PDE5 inhibitors in ICPs

The targeted analysis workflow employed (1) the suspected-target screening, (2) the targeted identification, and (3) the quantification of identified PDE5 inhibitors. The non-targeted screening workflow covered both top-down and bottom-up approaches to identify novel PDE5 inhibitors. Fig. 1 summarises the LC-HRMS workflow for the targeted analysis and the non-targeted screening employed in this study.

The initial suspected-target screening workflow was based on a matching algorithm when an observed accurate mass of the $[M+H]^+$ precursor ion was compared to those theoretical ones in the database for a possible match and thus possible presence of a PDE5 inhibitor. Moreover, the isotope distribution pattern was also compared for a match based on its abundance and spacing. For this purpose, a personal MS compound database was created using the PCDL software based on the currently known PDE5 inhibitors found as adulterants in literature. The database contained a total of 95 PDE5 inhibitors with a comprehensive collection of the compound name, molecular formula and structure, and exact mass. The mass accuracy for the MS matching was set at 5 ppm windows with isotope abundance distribution and spacing score of more than 80%. A positive match of the suspected PDE5 inhibitors will be subjected to the targeted identification workflow while a negative match will be further investigated using the non-targeted screening workflow.

The targeted identification workflow relied on the matching of the observed retention time and two observed fragment ions with those of target analytes stored in the same database which included only the 23 PDE5 inhibitors. The same database comprises additional information on the retention time and MS/MS spectral library of target analytes at different collision energies (CEs). The mass accuracy for the MS/MS matching was set at 20 ppm windows with a retention time difference of up to ± 0.25 min.

The quantification workflow was only applied to samples positive in the targeted identification process. The final dose of the adulterants in each ICP sachet was calculated based on Eq. (2).

$$Final\ dose = \frac{Average\ conc.\ from\ regression\ eq.}{(Analysis\ conc.\ \times Dilution\ level)} \times Weight\ per\ sachet \quad (2)$$

where,

Average conc. from regression eq. = concentration of target analyte calculated from the regression equation of the external calibration curve (n = 3)
Analysis conc. = concentration of an ICP used in sample preparation
Dilution level = level of dilution from the initial analysis concentration
Weight per sachet = total weight of ICP per sachet

The quantification levels were divided into subtherapeutic, therapeutic, and supratherapeutic based on the dose recommended by the approved PDE5 inhibitors. For the comparative purpose of this study, the quantification levels of unapproved PDE5 inhibitors analogues were linked to the therapeutic dosage of their corresponding approved drugs, i.e. 25–100 mg for sildenafil and 5–20 mg for vardenafil and tadalafil. The determination of trace concentrations was based on a definition set by the International Union of Pure and Applied Chemistry (IUPAC) [22].

The non-targeted screening workflow was employed for further investigation of negative samples from the suspected-target screening. The non-targeted screening approach used in this study was adapted and modified according to the critical review by Pasin et al. [17]. Based on the visual inspection of the chromatographic peak, the top-down and bottom-up approaches were both employed to detect any novel PDE5 inhibitors. A top-down approach was utilised for visible chromatographic peaks. All visible peaks within the base peak chromatogram (BPC) were integrated and extracted to reveal the mass spectra. Each mass spectrum was interrogated with the highest abundance peak selected as a possible $[M+H]^+$ precursor ion of novel PDE5 inhibitors. The relationship between the selected $[M+H]^+$ precursor ion was established with the fragment ions of target analytes via product ion scan at MS/MS level of the Mass Hunter workstation software to reveal any common fragmentation pattern. Conversely, a bottom-up approach was utilised for non-visible chromatographic peaks where the extracted ion chromatograms (EICs) were generated based on the fragment ions of target analytes at different CEs. Using this approach, no prior knowledge of the $[M+H]^+$ precursor ion is available. Therefore, all possible $[M+H]^+$ precursor ions generated from the MS experiment were considered as novel PDE5 inhibitors. The presence of class-specific EICs of the product ion scan at MS/MS level may reveal the presence of novel PDE5 inhibitors which can be further interrogated and linked with their distinct $[M+H]^+$ precursor ion. Both of these approaches aimed to reveal any common fragmentation pattern that could be linked to any known PDE5 inhibitors and thus, deduce the potential of identifying novel PDE5 inhibitors.

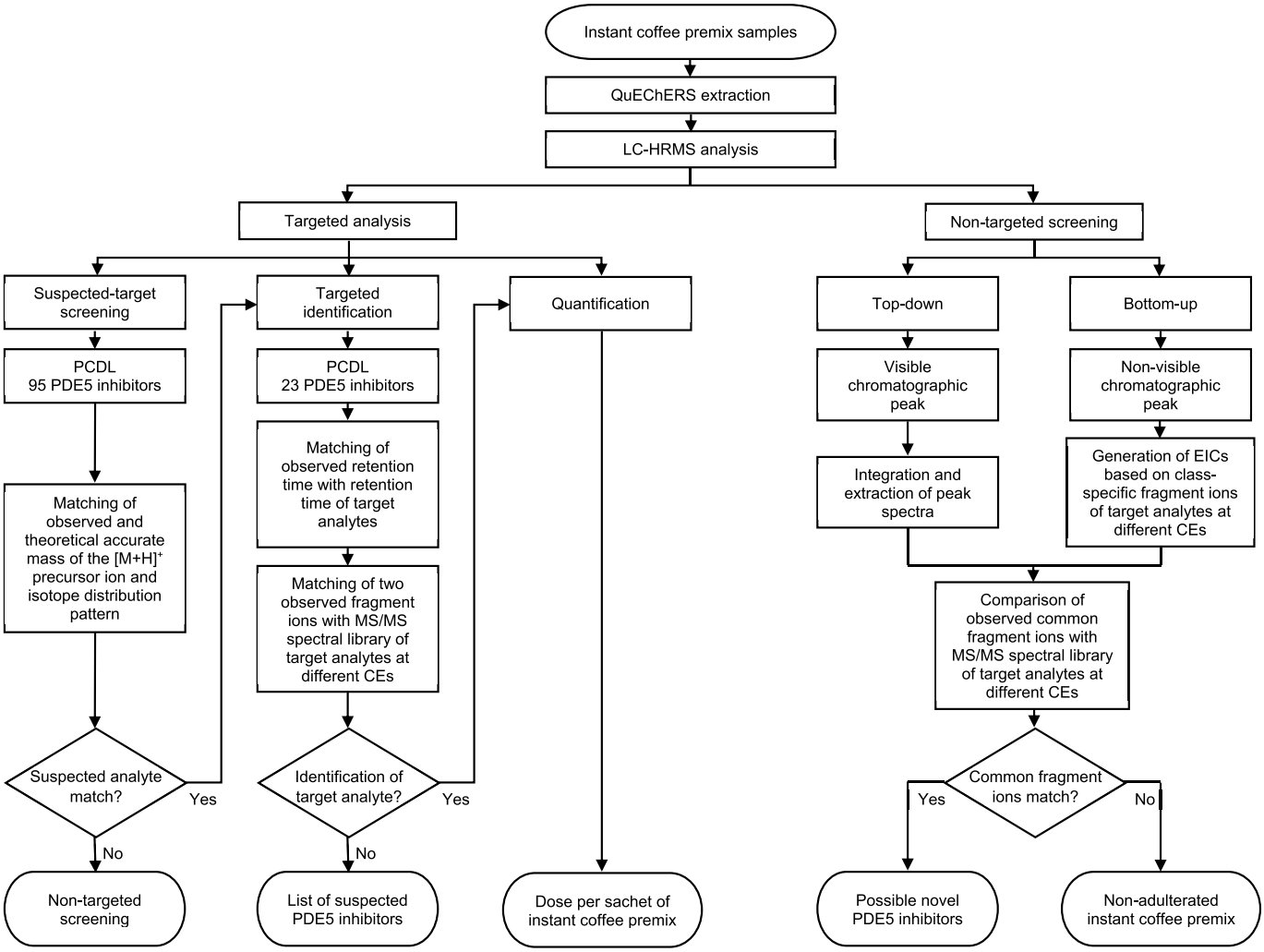

Fig. 1. LC-HRMS workflow for the determination of PDE5 inhibitors in ICPs.

3. Results and discussion

3.1. Analytical method optimisation and validation

The analytical method optimisation as a whole addressed the issue of MEs from complex matrices such as ICPs. Also, the presence of four different groups of structural isomers was tackled chromatographically, leading to a baseline chromatographic separation, enhancing the specificity of each isomeric analyte. Other chromatographic optimisation discussed in Section 2.3 of Ref. [19] resulted in improved peak shape and resolution, and reproducible retention time for each target analyte. The presence of sodium adducts was addressed during the MS optimisation and thus improved the selectivity and sensitivity of the MS and MS/MS experiments. The modified QuEChERS extraction procedure was successfully developed following poor MEs using the conventional dilute and shoot technique during the sample preparation optimisation. In conclusion, the success of the analytical method optimisations discussed in this study is significant for a definitive screening, identification, and quantification of PDE5 inhibitors and their analogues from ICPs.

The specificity was successfully demonstrated using the developed chromatographic separation as presented in Fig. 2. Target analytes in extracted blank ICP at all QC levels could be correctly identified using the distinguishable $[M+H]^+$ precursor ion without any interference from the matrix components. Conversely, the analyte-free extracted blank matrix returned insignificant signals corresponding to all target analytes at their retention times. The presence of two fragment ions correspondingly ensured the specificity of the method and the average intensity ratio confirmed the identity of the target analytes. These data are presented in Table 2 of Ref. [19].

The linearity of the method was confirmed for each target analyte with a coefficient of determination (r^2) larger than 0.9960. The selected range proved to suffice for quantification of target analytes ranging from trace level up to supratherapeutic concentrations from the ICP matrix. The percentage of accuracy ranged from 88.1%–119.3% at low; 94.8%–110.3% at medium; and 100.6%–109.3% at high QC level. The LOD and LOQ for all target analytes ranged from 10–70 ng/mL and 80 ng/mL, respectively. These results are presented in Table 3 of Ref. [19].

Table 4 of Ref. [19] shows the results of precisions, ME and RE. The method produced good repeatability at low, medium, and high QC levels with the %RSD ranging from 0.4%–7.3%; 1.0%–6.2%; and 0.6%–3.1%, respectively. In agreement with the repeatability results, the intermediate precision was calculated to be within 0.6%–7.2% at low; 0.6%–7.7% at medium; and 0.5%–9.1% at high QC level. Insignificant MEs were observed for all target analytes within −5.2% to +8.7% whereas the RE proved to be satisfactory at all QC levels within 84.7%–123.5% except for N-desethylacetildenafil at low (53.8%) and medium (65.1%) QC levels.

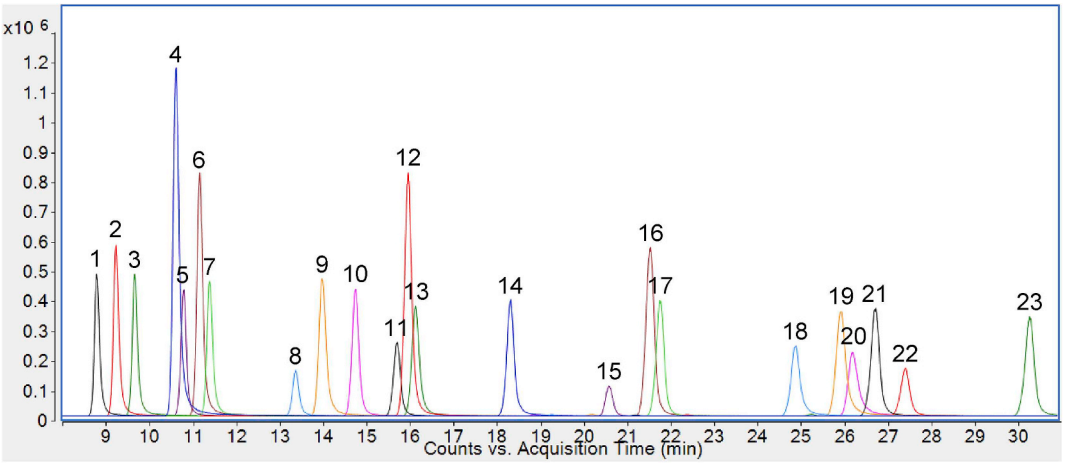

Fig. 2. Representative EIC of the $[M+H]^+$ precursor ion of 23 targeted PDE5 inhibitors mixture in neat solution at a concentration level of 1.2 µg/mL.

3.2. Analysis of PDE5 inhibitors in ICPs

A total of 25 ICP samples were submitted to the LC-HRMS analysis for the determination of PDE5 inhibitors. The initial suspected-target screening resulted in 17 positive samples, of which 15 were further confirmed using the targeted identification process and quantified. The non-targeted screening workflow detected no suspicious compounds, so there were no analytes of novel PDE5 inhibitors flagged from the ICP samples. In summary, 9 samples were adulterated with one PDE5 inhibitor, 2 samples with two inhibitors, and the rest 6 samples with three and four inhibitors for each 3 samples, respectively, as shown in Fig. S2 (A, B).

Collectively, eight distinct PDE5 inhibitors were determined using the targeted identification workflow while another two highly suspected adulterants were detected through the suspected-target screening workflow. The most prominent adulterant was sildenafil which was identified in 4 samples as a single adulterant and 5 samples in combinations with other PDE5 inhibitors. Other adulterants of PDE5 inhibitors discovered in this study included dimethylsildenadil, thiodimethylsildenafil, and thiosildenafil (5 samples each), tadalafil (3 samples), desmethylcarbodenafil (2 samples), and propoxyphenylthiodimethylsildenafil and propoxyphenyl-sildenafil (1 sample each) either in combination with each other or as a single adulterant.

Only 15 samples were quantified with these adulterants found at subtherapeutic levels up to supratherapeutic concentrations ranged from 2.77 to 121.64 mg per sachet of the ICP sample. Although distinct PDE5 inhibitors may be quantified at trace, subtherapeutic, and therapeutic levels, a combination of these adulterants in one sachet of ICP may subsequently result in supratherapeutic concentrations as presented in SPL004, SPL015, SPL019, SPL020, and SPL024. The sample dilution approach employed in this study proved to be excellent for the determination of PDE5 inhibitors at trace and subtherapeutic levels. For quantification of adulterants at therapeutic and supratherapeutic concentrations, the dilution level of up to 1:100 was deemed to be sufficient. However, the fact that multiple adulterants may be present in a sample and often at different concentration levels, required at least another further sample dilution for accurate and precise quantification of each target analyte. A detailed content of each sachet of ICP samples is presented in Table 1 and Fig. S2 (C) summarises the results.

Qualitative establishments from the suspected-target screening and targeted identification processes had revealed two highly suspected PDE5 inhibitors in three different samples. SPL002 and SPL006 exhibited a prominent peak at 23.63 and 23.65 min, respectively, for each of their BPC. Each of these samples was initially matched with two possible structural isomers, i.e. hydroxythiohomosildenafil and hydroxythiovardenafil based on its $[M+H]^+$ precursor ion at *m/z* 521.1999, with mass errors of 0.00 ppm for SPL002 and 0.19 ppm for SPL006. Moreover, the isotope abundance distribution and spacing score of more than 90% correspondingly approved the matched compounds. The suspected compound has a similar fragmentation pattern with thiohomosildenafil at three different CEs and hence, construed its identity as a possible analogue of thiohomosildenafil. Due to the additional 16 Da mass unit of hydroxythiohomosildenafil, which corresponds to an oxygen atom, their fragmentation patterns are expected to be the same [23].

In contrast, the BPC of SPL005 revealed a prominent peak at 27.85 min which was initially assigned as an unknown compound X with *m/z* 499.2310 for its $[M+H]^+$ precursor ion as shown in Fig. 3 (A). The suspected-target screening revealed matching for two possible structural isomers, namely 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil [24] and dithiopropylcarbodenafil [25] with a mass error of 0.40 ppm for their $[M+H]^+$ precursor ion. Further investigation of the collision-induced-dissociation (CID) process of compound X revealed two unique fragment ions at *m/z* 371.0995 and *m/z* 343.0682 which were also present in the CID spectrum of dithiodesmethylcarbodenafil (20) run at 10, 20, and 40 eV CEs, shown in Fig. 3 (B) as a representative at 20 eV CE. The data suggest strongly that compound X is a structural analogue of dithiodesmethylcarbodenafil with an extra 28 Da mass unit (C_2H_4). Only 2 isomers are shown in Fig. 3 (C) with varying R groups, although many other possible R group variations may exist for the structure.

Although the ultimate identity of compound X cannot be concluded with the obtained data, the presence of either 3,5-dimethylpiperazinyl-dithiodesmethylcarbodenafil, dithiopropylcarbodenafil, or any other possible structural isomers as an adulterant in an ICP has not been reported in the literature before. To unambiguously confirm the structure of compound X, the use of complementary techniques, such as nuclear magnetic resonance (NMR), would be highly valuable following analyte isolation and purification. Alternatively, identification might be achieved if the certified reference materials of various structural isomers are available. Future investigation for full structural elucidation is warranted as compound X might be a novel PDE5 inhibitor.

4. Conclusions

A modified QuEChERS extraction procedure coupled to LC-HRMS analysis was fully optimised and validated to determine PDE5 inhibitors and their analogues found as adulterants in ICPs. The process of screening, identification, and quantification were done simultaneously with detailed procedures and examples discussed in this study. These adulterants were comprehensively screened via the suspected-target and non-targeted approaches, utilising the full spectral information of

Table 1
The contents of PDE5 inhibitors in each sachet of ICP samples.

Label	Weight per sachet (g)	Identified analytes (average dose per sachet in mg - quantification level)				
		Analyte 1	Analyte 2	Analyte 3	Analyte 4	Total analyte
SPL001	20.21	Desmethylcarbodenafil (106.02 - SPR)	ND	ND	ND	106.02 - SPR
SPL002	24.81	Thiosildenafil (2.77 - SUB)	Hydroxythiohomosildenafil*	ND	ND	2.77 - SUB
SPL003	23.37	Dimethylsildenafil (0.85 - TRC)	Propoxyphenyl-thiodimethylsildenafil (4.12 - SUB)	Thiodimethylsildenafil (20.39 - SUB)	ND	25.36 - THE
SPL004	19.75	Tadalafil (27.03 - SPR)	Sildenafil (41.86 - THE)	ND	ND	68.89 - SPR
SPL005	25.50	Compound X*	ND	ND	ND	NA
SPL006	24.40	Hydroxythiohomosildenafil*	ND	ND	ND	NA
SPL007	20.88	Sildenafil (84.93 - THE)	ND	ND	ND	84.93 - THE
SPL008	20.31	ND	ND	ND	ND	ND
SPL009	25.50	ND	ND	ND	ND	ND
SPL010	30.06	ND	ND	ND	ND	ND
SPL011	8.26	ND	ND	ND	ND	ND
SPL012	21.61	Sildenafil (83.69 - THE)	ND	ND	ND	83.69 - THE
SPL013	25.03	Sildenafil (86.56 - THE)	ND	ND	ND	83.56 - THE
SPL014	29.67	ND	ND	ND	ND	ND
SPL015	19.23	Dimethylsildenafil (0.60 - TRC)	Sildenafil (0.85 - TRC)	Thiodimethylsildenafil (29.15 - THE)	Thiosildenafil (91.04 - THE)	121.64 - SPR
SPL016	17.59	ND	ND	ND	ND	ND
SPL017	19.66	Desmethylcarbodenafil (9.47 - SUB)	ND	ND	ND	9.47 - SUB
SPL018	24.13	ND	ND	ND	ND	ND
SPL019	19.18	Dimethylsildenafil (1.32 - TRC)	Thiosildenafil (22.18 - SUB)	Thiodimethylsildenafil (91.55 - THE)	ND	115.05 - SPR
SPL020	20.12	Propoxyphenyl-sildenafil (Detected)	Tadalafil (2.33 - SUB)	Sildenafil (97.82 - THE)	ND	100.15 - SPR
SPL021	18.08	Tadalafil (36.02 - SPR)	ND	ND	ND	36.02 - SPR
SPL022	19.81	Sildenafil (68.90 - THE)	ND	ND	ND	68.90 - THE
SPL023	24.39	ND	ND	ND	ND	ND
SPL024	20.06	Dimethylsildenafil (Detected)	Sildenafil (1.11 - TRC)	Thiodimethylsildenafil (31.40 - THE)	Thiosildenafil (84.16 - THE)	117.32 - SPR
SPL025	23.47	Dimethylsildenafil (3.08 - SUB)	Sildenafil (4.43 - SUB)	Thiodimethylsildenafil (8.59 - SUB)	Thiosildenafil (40.50 - THE)	56.60 - THE

Notes: ND: not detected, TRC: trace, SUB: subtherapeutic, THE: therapeutic, SPR: supratherapeutic, NA: not applicable, *suspected-target screening.

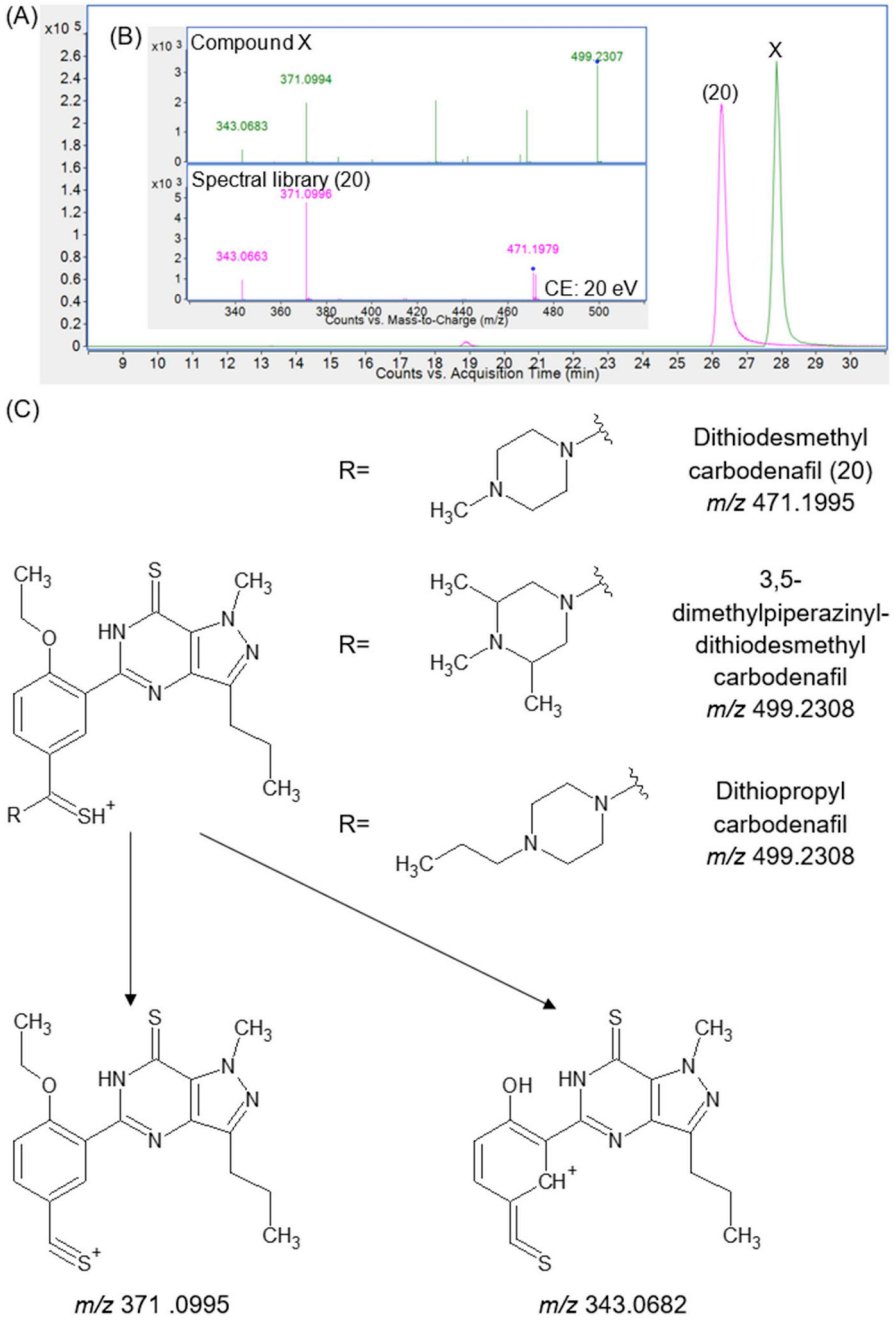

Fig. 3. Comparison between dithiodesmethylcarbodenafil (20) from the PCDL spectral library with the unknown compound X of sample SPL005 using the suspected-target screening approach with (A) overlaid EIC of the $[M+H]^+$ precursor ion, (B) comparison of fragment ions based on common fragments at 20 eV CE, and (C) proposed common fragmentation pattern shared by dithiodesmethylcarbodenafil (20) and compound X (only showing two possible isomers, 3,5-dimethylpiperazinyl dithiodesmethylcarbodenafil and dithiopropylcarbodenafil, among other isomeric variations).

the simultaneous MS and MS/MS experiments. The optimisation of chromatography, sample extraction, and sample dilution led to the minimisation of ME for all 23 targeted PDE5 inhibitors [19]. The applicability of the developed method was then demonstrated using 25 ICP samples. Typically, consumers tend to take extra precaution when taking health supplements, especially in pharmaceutical dosage form compared to consumable products, such as ICPs. Therefore, this kind of adulterated products will put the public at the absolute risk owing to its easy accessibility, either through conventional or online markets. The strategies proposed in this study would be beneficial to tackle the problems of adulterated ICPs, especially with PDE5 inhibitors and their analogues to safeguard the public health.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.05.078.

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