1.0 INTRODUCTION

The aim of this work is to validate the analytical method of quantifying Sildenafil, Vardenafil, Tadalafil and their analogues (Hydroxyhomosildenafil, Norneosildenafil, Acetildenafil, Homosildenafil, Aminotadalafil, Thiosildenafil, N-Desmethylacetildenafil, Thiohomosildenafil, Gendenafil, Carbodenafil, Hydroxythiohomosildenafil, N-Desethylvardenafil, N-Desmethylsildenafil, Udenafil, Hydroxyacetildenafil, Piperiacetildenafil, Dimethylsildenafil, Chloropretadalafil, and Noracetildenafil) using the Liquid Chromatography Tandem Mass Spectrometer (LC-MS/MS) system. This method has been accredited and used daily in the forensic division of the Department of Chemistry (DOC), Malaysia.

Sildenafil Citrate (Viagra®), Vardenafil (Levitra®) and Tadalafil (Cialis®) are the only phosphodiesterase-5 (PDE5) enzyme inhibitors approved by the Food and Drugs Administration (FDA) in United State of America to treat male sex problem function or erectile dysfunction if and only if the consumption of those drugs need to be followed and supervised by physicians due to its harmful side-effects. A Phosphodiesterase type 5 inhibitor, often shortened to PDE5 inhibitor, are a drug used to block the degradative action phosphodiesterase-5 enzyme on cyclic GMP in the smooth muscle cells lining the blood vessels supplying one of the part of the penis named corpus cavernosum. Corpus cavernosum is one of a pair of sponge like regions of erectile tissue which contain most of the blood in penis during penile erection (Marshall).

Analogue is the term refers to structural derivative of a parent compound that often differs from it by a single element. Or in simple terms can describe as a substance that has major chemical structures in common with another chemical. Unfortunately, no toxicological data of these analogues published and the safety of its largely unknown

and unpredictable (James, 2007). US Federal Analog Act of 1986 (also known as Designer Drug Act) stated that:

- Chemical structure substantially similar to Schedule I or II substance AND EITHER
- Pharmacological effects similar to Schedule I or II substance
- Someone intentionally represents substance as having the effect of Schedule I or II substance

It means that any efforts in productions of these analogues are prohibited and can be charged under this Act.

The current method is using High Performance Liquid Chromatography with Ultra Violet detector (HPLC-UV) method but it has a limitation because it gives a similar UV spectrum for analogues and their parent. This HPLC-UV method only can determine to a minimum of 1µg/mL of these drugs in various type of samples. Due to its sensitivity properties, this LC-MS/MS method is developed to detect simultaneously these drugs and their analogues in single analysis. It has carried out to demonstrate this new instrument technique is suitable for its purpose to analyze these drugs in these matrices.

This liquid chromatography tandem mass spectrometry method was developed for the simultaneous identification of Sildenafil, Vardenafil and Tadalafil and their analogues. The aim of this research is to validate the established method to identify the presence of these drugs and their analogues in adulterated herbal medicinal preparations, herbal vitality products, supplements and foods and beverages. This method covers the qualitative analysis and specifies criteria for the identification of these synthetic drugs.

This work consists of two main parts. The first, theoretical part gives a short overview of Sildenafil, Vardenafil, Tadalafil, and their analogues. The second part focuses on the particular LC-MS/MS method and to the analyses used to achieve the validation. During the validation process several analyses has been done to evaluate the linearity of the calibration graph, to estimate the limit of detection, limit of quantification, reproducibility, repeatability, selectivity and finally the uncertainty.

2.0 LITERATURE REVIEW

2.1 Sildenafil, Vardenafil, Tadalafil and their analogues

Sildenafil is known as first generation (approved by FDA on 1998) and Vardenafil and Tadalafil are the second generation (approved by FDA on 2003) of PDE-5 inhibitors. And the variety of analogues Sildenafil, Vardenafil and Tadalafil are the third generation of these inhibitors. It is also reasonable to predict that the analogues of these drugs will have the same primary pharmacological effects and acts as PDE-5 inhibitors (Evan *et al*., 2004). A few side effects were observed when consumed Sildenafil such as headache, flushing, nasal congestion, dyspepsia and the potential for serious adverse effects to occur including impaired liver function, abnormal vision, hypotension and death (Anonymous, February 2009).

Recently, many company used the analogues of Sildenafil, Vardenafil and Tadalafil, due to its similar function as its original structural framework. They used it to avoid detection by regulatory or private laboratory testing. These compounds shared the same properties. Most consumers didn't aware of drug adulteration in their products until they feel the symptoms (James, 2007).

At present, only New Zealand allows the manufacturers include these analogues in dietary supplements. Scheduling of these analogues as prescription medicines would allow regulatory action to be taken against the manufacturers and/or suppliers of such products under the Medicines Act 1981.

With just a little modification to the sildenafil structure, a large number of different sildenafil analogues can be synthesised. These modifications are focused on three areas of the molecule:

- the piperazine moiety, comprising a number of piperidine-type derivatives and/or changes in substitution around the piperazine ring eg. from *N*-methyl to *N*-ethoxy or *N*-ethyl, or methylation.
- the sulfonyl group – substitution with a carbonyl group.
- the carbonyl group – substitution of the pyrimidone carbonyl with a sulfonyl group.

Vardenafil analogues are fewer in number but modifications focus on two areas of the molecule, namely the piperazine moiety and the sulfonyl group. And aminotadalafil is the only tadalafil analogue that can be identified may be due to its reactivity. The synthesis of both the sildenafil and vardenafil analogues is relatively straightforward. Both routes involve convergent syntheses, bringing together two halves of the molecule towards the end of the synthesis. Tadalafil analogues can also easily be produced, in a four step linear synthesis starting from an amino acid such as tryptophan. From a chemistry perspective, this means that the production of new analogues is relatively straightforward (Anonymous, February 2009). With just a little modification, it seems that the potential of structural modification to a new analogue is endless.

Figure 1 show the analogue of Tadalafil where methyl group already replaces by amine group.

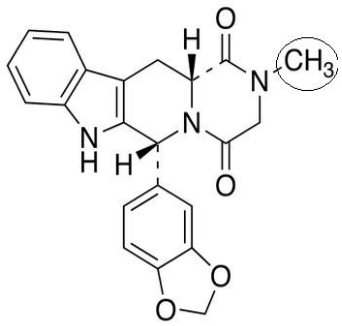
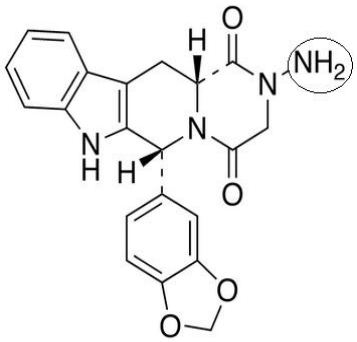
Tadalafil	Aminotadalafil
	
Molecular Mass : 389.40 g/mol	Molecular Mass : 390.39 g/mol

Figure 1: Molecule structure of Tadalafil and its analogue, Aminotadalafil.

2.2 Liquid Chromatography Tandem Mass Spectrometer (LC-MS/MS)

A High Performance Liquid Chromatography (HPLC) coupled with triple quadrupole system has been used in this study. This HPLC system was chosen due to the simple extraction method can be applied by using this technique. No derivatization step needed and the separation between peaks also resolved compared to gas chromatograph system. The limitation in HPLC-UV contributed to the development of this method. Triple quadrupole mass analyzer has been used in this study because the drugs are targeted and at the same time the capability of this instrument also better than HPLC –UV. It is also excellent for detecting adulteration of known compounds when the reference materials are available.

Most laboratories are used HPLC coupled with Ion Trap Mass Spectrometer (MS^n) detector. There is a method that combined Photo Diode Array Detector (PDA) and Ion MS^n detector which offer the best chance of detecting these drug adulterations. The PDA is a simple tool that can detect the adulteration in dietary supplements and

MS^n is an excellent tool for detecting adulteration of known and novel compounds without standards. The PDA signal compliments with MS^n data are hardly to dispute and it just can be interpreted by experienced analysts. But by combined both techniques, it's really help in rapid study for unknown compounds and extremely sensitive (James, 2007).

Most of the literature reviews are using Electrospray ionization (ESI) in positive ion mode because it produced prominent pseudomolecular ions in MS and characteristic fragmentation patterns in MS/MS (John *et al*., 2006).

Forensic Chemistry Centre, Ohio develop a method that combined of MS^n and Fourier Transform Ion Cyclotron Resonance (FT-ICR-MS) technologies to elucidate the analogue structure of Sildenafil. This hybrid system provides high resolution, mass accuracy and sensitivity. The analysis is done by using direct infusion or direct analysis in real time (DART). It is very useful by giving an accurate mass (up to five decimal points) and x-ray crystal structure of these analogues (Samuel, 2007).

3.0 METHODOLOGY

3.1 Instruments and Reagents

The entire certified reference materials (CRMs) were purchased from TLC PharmaChem, Canada- Sildenafil Citrate (99.8% pure), Tadalafil (100%pure), Vardenafil HCl (99.5% pure), Hydroxyhomosildenafil (99.2% pure), Norneosildenafil (100% pure), Homosildenafil (99.9% pure), Aminotadalafil (99.2% pure), Thiosildenafil (98.9% pure), Thiohomosildenafil (100% pure), Hydroxythiohomosildenafil (99.8% pure), Carbodenafil (99.3% pure), Gendenafil (98.9% pure), N-Desmethyl Sildenafil (99.8% pure), N-DesethylVardenafil (99.6% pure), Chloropretadalafil (99.7% pure), Udenafil (99.1% pure), Acetildenafil (99.2% pure), N-DesmethylAcetildenafil (98.2% pure), Hydroxyacetildenafil (99.5% pure), Piperiacetildenafil (99.1% pure), Noracetildenafil (99.6% pure) and Thiodimethylsildenafil (99.9% pure) in powder form. All these CRMs are provided with its Certificate of Analysis (COA) and Material Safety Data Sheet (MSDS) as references.

All other chemical and reagents must have purity level for analysis or higher: Methanol and Acetonitrile are LCMS grade (Fisher, USA), Ammonium Acetate (Merck Germany) and Acetic Acid (Fisher, USA) for preparation of mobile phase.

Argon gas has been used as a collision gas with purity 99.999%. Nitrogen gas with purity 99.999% was generated by nitrogen Generator (Dominic Hunter) together with compressed air by air compressor (Hitachi).

For reference standard solutions, the Class A volumetric flasks was used. Micropippettes (Eppendorf, USA) with different adjustable volumes range 10 to 1000 µL was used during standard solution preparation. And 2mL glass vial (Waters, USA) was used prior sample injection.

In sample preparation, mortar and pestle were used for sample homogenisation. Micropippettes (Eppdendorf, USA) with different adjustable volumes of 5000 µL, 0.45 µm Nylon syringe filter (VertiClean, Thailand) for pre-injection filtration and 2mL glass vial (Waters, USA) were used prior sample injection.

For weighing, a digital scale B3002-S (Mettler Toledo) was used to weigh 100mg of samples. A solvent dispenser (Dispensette Organic) was used to dispense 5mL of methanol into the sample. Ultrasonic Sonicator (Branson 5210) and Centrifuge (Beckman Coulter) were used for sample extraction and sedimentation.

3.2 Liquid Chromatography with Tandem Mass Spectrometry Detector

Samples were analysed using HPLC system Waters Alliance 2695 (Waters, UK) and mass spectrometry was performed using triple quadrupole Quattro Micro with Z-spray and standard electrospray probe (Waters, UK) (Appendix 1). Separation was achieved using a Chromolith Monolithic RP-18 Column (Merck) 3.00 x 100 mm eluted gradiently at a flow rate 0.25 mL/min. Data were recorded in the Multiple Reaction Monitoring (MRM) mode using Waters Masslynx 4.0.

One of the advantages when using MS/MS system is it can minimize the false positive result compared when using single MS analyzer. Single quadrupole is not really helpful if there are two compounds that having similar molecular weight and polarity characteristic because it will elute at the same retention time unless the use of chiral column to separate it.

The advantages of LC-MS/MS, apart from the high sensitivity and specificity of the technique, are the possibility of significantly shorter the analysis time. By using a shorter analytical column with a monolith technology, it can reduce the solvent consumption thereby reducing the column backpressure. Gradient mode has been used to separate these 23 analytes in based on their polarity.

3.3 The Methods

3.3.1 Parameters for Liquid Chromatography Tandem Mass Spectrometry

Auto sampler parameters: Injection volume 10 µL, methanol and water were use for seal wash and 100% methanol for needle wash.

Inlet parameters: This HPLC system has been equipped with online degasser. Quaternary solvent manager has been used to flow the mobile phase at 0.25ml/min. The mobile phase consisted of 0.1 % Acetic acid in 10 mM ammonium acetate (eluent A) and acetonitrile (eluent B) using a gradient program. 40 % eluent B increased to 70% B in the first 8 minutes followed by 70 % B to 40 % B in 0.01 min before equilibrated for 6.09 minutes at 40 % B. Total run time was 15 minutes. All the mobile phase

has been filtered with 0.45µm Nylon membrane filter to prevent the excess of any impurities into the inlet system.

Column: Chromolith Monolithic RP-18 Column (Merck) 3.00 x 100 mm equipped with its guard column to prolong its lifetime and operated at a temperature of 25°C.

Mass spectrometer parameters: The HPLC system was coupled with Waters® Micromass® Quattro Micro™ fitted with an Electrospray ionization source. Argon as a collision gas was set at a cell pressure of 3.6 x 10-3 mbar. Nitrogen has been used as a desolvation gas. The source and desolvation temperature has been set at 120°C and 350°C with desolvation gas flow at 500L/hr. Capillary and cone voltage were set at 3.00 kV and 42 V. ESI collision-induced dissociation (CID) was conducted by direct infusion of 200 ng/mL sample solution at 15uL/min and the optimised parameter are summarizing in (**Table 1**). Two transitions are monitored for each analyte. Ions having the highest abundance (primary daughter ion) were used for quantitation and the secondary daughter ions were used as a confirmation in any analysis when using MS/MS mode (**Figure 2 and 3**). The ion ratios were used for confirmatory purpose. All the transitions were recorded in the Multiple Reaction Monitoring (MRM) mode using positive ESI mode. All aspects of data acquisition were controlled using MassLynx NT 4.0 software with automated data processing using QuanLynx program (Waters).

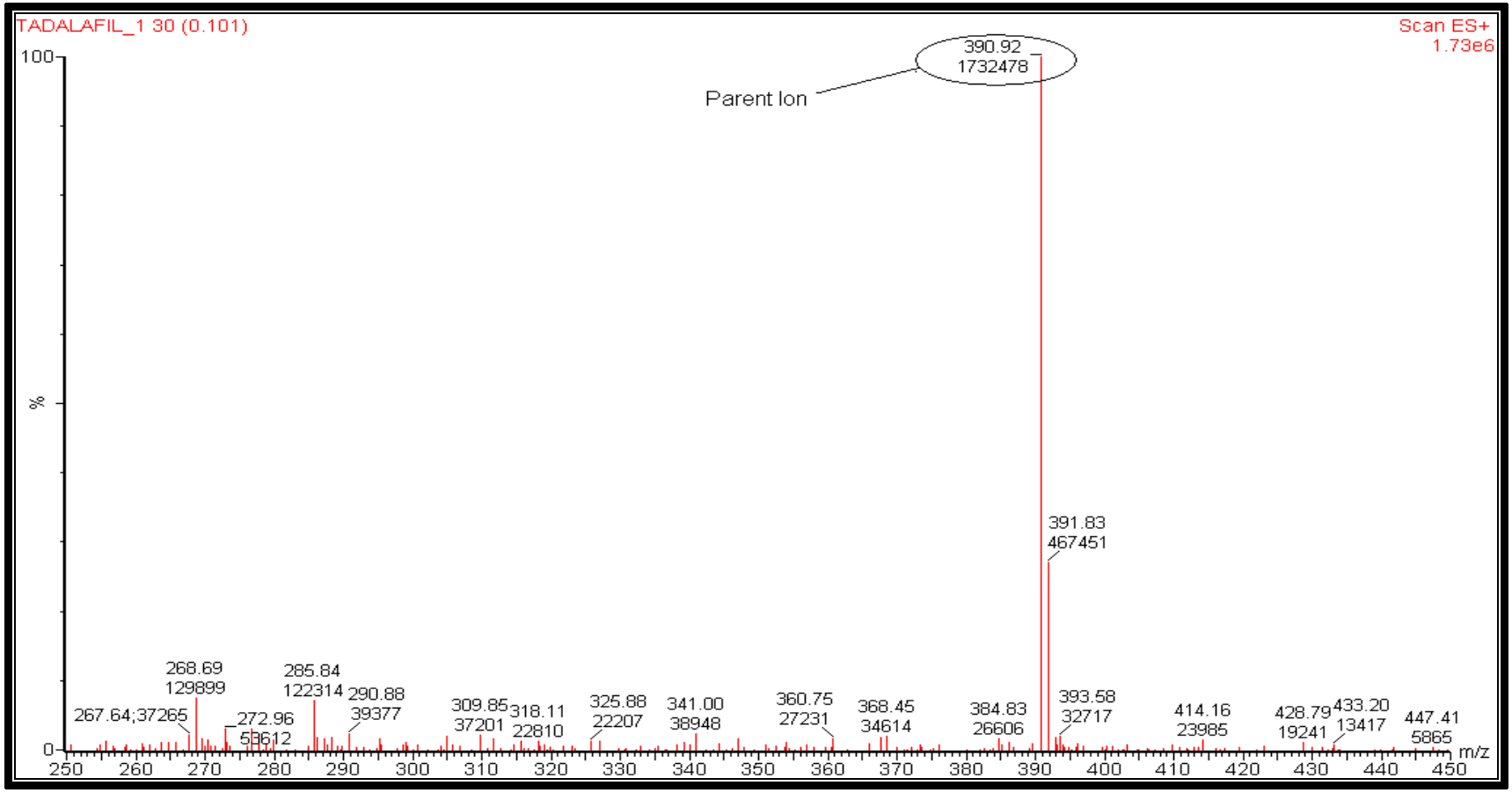


Figure 2: MS Spectrum for Tadalafil

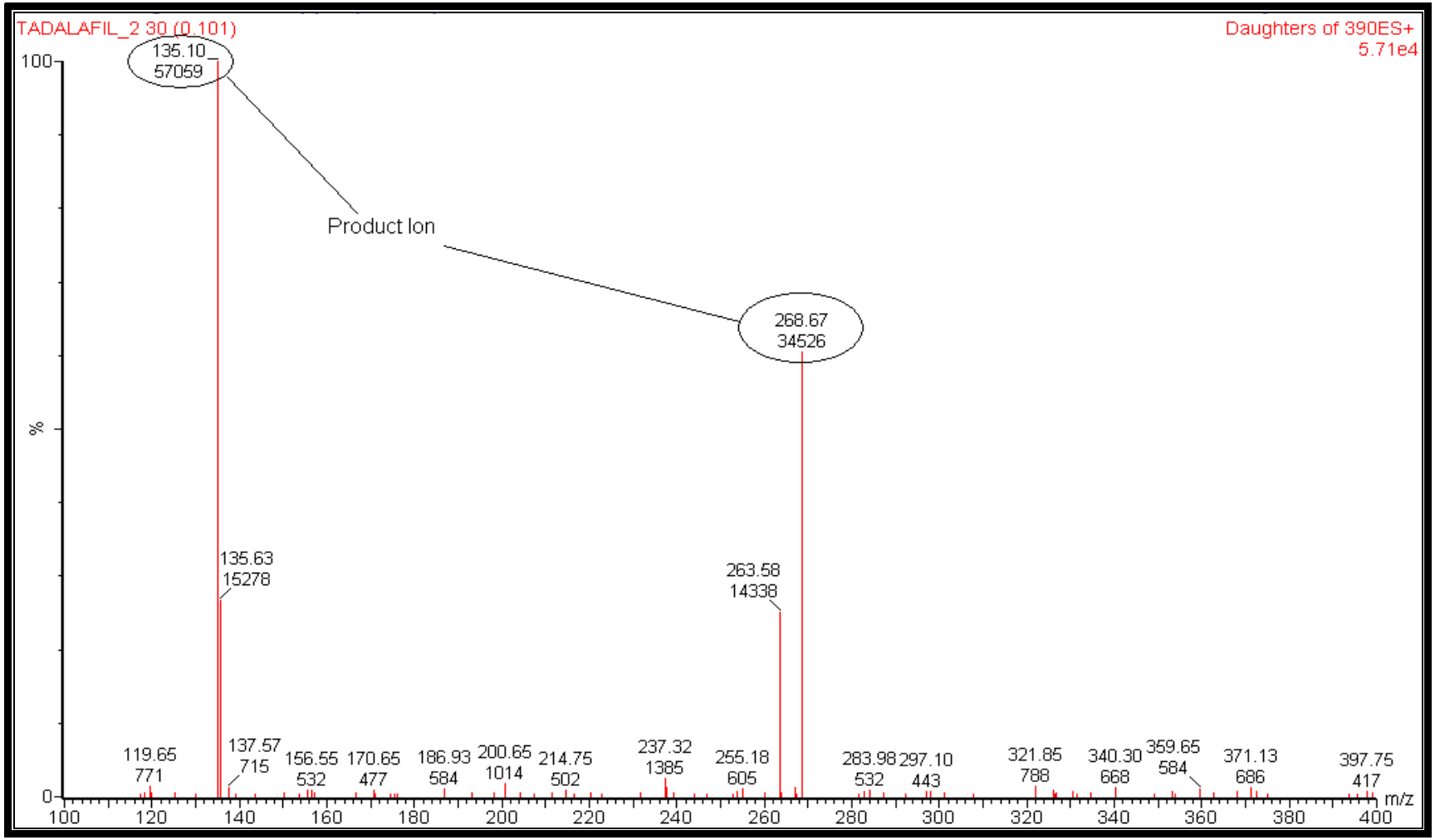


Figure 3: MS/MS Spectrum of Tadalafil

3.3.2 Sample Preparation

All the samples (sample: capsule, tablet or powder) must be first homogenized manually using mortar and pestle (**Figure 4**). About 100mg of homogenized sample or 50µL of liquid sample was weighed and transferred into 10mL glass vial. 5mL of methanol was dispensed into the samples and the solution was sonicated for about 5 minutes at room temperature. It was then centrifuged at 2000 rpm for 5 minutes. The sample was filtered through 0.20µm Nylon syringe filters into the 2mL vial prior for LC-MS/MS analysis (**Figure 5**) to prevent the blockage to the column thus, prolong its lifetime.

Figure 4: Samples were homogenized by mortar and pestle

Figure 5: Sample was filtered prior to LC-MS/MS injection

Table 1: MRM conditions for 23 Analytes

No.	Standards	Parent Ion	Daughter Ion	Cone Voltage (V)	Collision Energy (eV)
1	Sildenafil	475.30	100.05	42	30
			283.13	42	41
2	Vardenafil	489.30	151.11	50	45
			169.16	50	38
3	Tadalafil	390.17	268.10	18	15
			135.02	18	20
4	Hydroxyhomosildenafil	505.32	99.01	52	38
			487.22	52	23
5	Norneosildenafil	460.25	283.12	55	33
			299.13	55	38
6	Acetildenafil	467.32	111.08	55	32
			297.13	55	43

No.	Standards	Parent Ion	Daughter Ion	Cone Voltage (V)	Collision Energy (eV)
7	Homosildenafil	489.33	113.10 283.12	53 53	30 40
8	Aminotadalafil	391.11	268.96 169.03	18 18	12 33
9	Thiosildenafil	491.15	341.08 299.02	45 45	28 38
10	Thiohomosildenafil	505.15	355.07 327.04	43 43	30 30
11	Hydroxythiohomosildenafil	521.15	503.10 327.05	58 58	25 33
12	Hydroxyacetildenafil	483.28	127.09 143.13	49 49	30 31
13	Carbodenafil	453.27	339.08 311.05	48 48	24 32

No.	Standards	Parent Ion	Daughter Ion	Cone Voltage (V)	Collision Energy (eV)
14	Piperiacetildenafil	438.22	297.13 341.13	50 50	37 29
15	Gendenafil	355.17	285.11 327.08	46 46	29 24
16	Dimethylsildenafil	489.11	113.05 311.04	48 48	30 30
17	Udenafil	517.23	112.09 283.05	49 49	35 39
18	Chloropretadalafil	427.07	135.03 274.04	30 30	20 33
19	N-Desmethyl Sildenafil	461.20	283.06 311.10	46 46	35 29
20	N-Desmethylacetildenafil	439.27	99.03 353.12	41 41	26 27

No.	Standards	Parent Ion	Daughter Ion	Cone Voltage (V)	Collision Energy (eV)
21	N-Desethylvardenafil	461.06	151.06 169.14	45 45	48 37
22	Noracetildenafil	453.31	296.99 353.04	52 52	38 26
23	Thiodimethylsildenafil	505.11	113.03 298.93	45 45	28 39

4.0 RESULTS AND DISCUSSION

4.1 Chromatographic Separation

The previous HPLC with UV detector method has many weaknesses, which are mainly related to validation process: the calibration graph was developed using single calibration point and the quality control system was deficient. The UV spectrum achieved weren't acceptable since it gives a similar UV spectrum for analogues and their drug, thus the analysts faced a lot of problem regarding to report these drugs. By HPLC-UV method, they need to separate these 23 drugs to three groups because their UV spectrums overlap. But by using this LC-MS/MS method, hundreds of analytes can be analysed simultaneously.

The Chromolith column used in this method has been succeeding by separating some drugs and their analogue. For example, aminotadalafil is eluted at 4.0 minutes and tadalafil is at 5.5 minutes (**Figure 6**). Aminotadafil is eluted faster than tadalafil since it is more polar with the presence of amine group in its structure.

It was observed that drugs with high molecular weight were relatively stable and higher cone voltage and collision energy were needed for their fragmentation. For example, hydroxythiohomosildenafil gained the highest cone voltage at 58V. It is reasonable because of the stability of its main structure. Aminotadalafil showed lowest cone energy at 18V. The reason for its easy fragmentation is the stability of the fragment ion of aminotadalafil at m/z 268.

It seems that the development of these drugs using other chromatography technique such as gas chromatography (GC) are not really work since most of the compounds have high molecular weight. It is because of the limitation in GC column that are not capable to detect high molecular weight compounds.

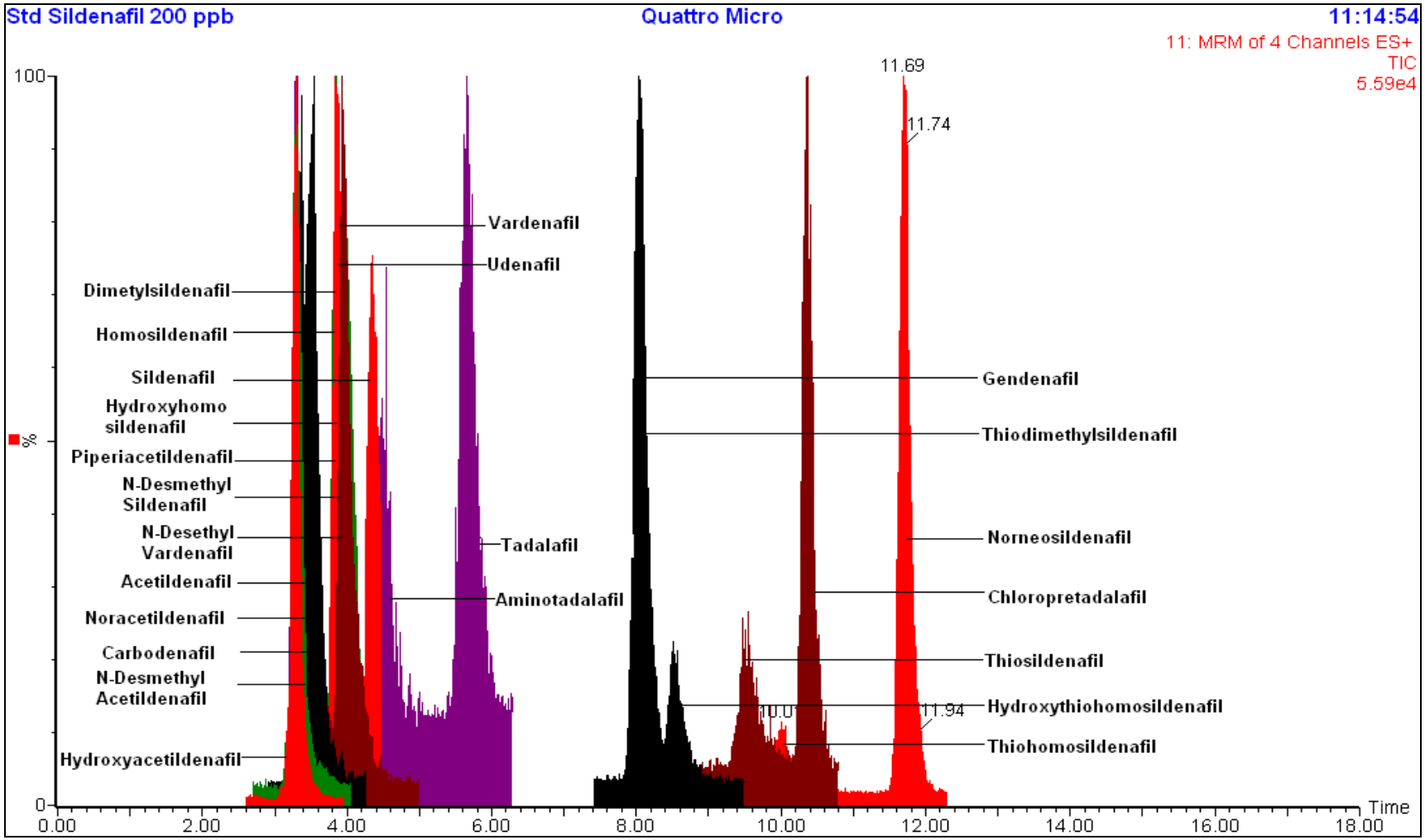


Figure 6: Total Ion Chromatogram (TIC) for 23 analytes

4.2 Sample Preparation

Previous HPLC method used 1gram of sample and 5mL methanol as an extraction solvent. And the solution was directly transferred to HPLC vial for injection. No centrifugation and filtration step that may lead to reduce the lifetime of the HPLC column.

New sample preparation procedure didn't consume time and the centrifugation and filtration steps make the sample solution more clear and indirectly may prolong the lifetime of column and reduce the time to do maintenance on the ionisation source.

A few extraction solvent have been studied in this method. Some of them were 100% water, 100% acetonitrile, 100% methanol, the mixture of methanol and water and the mixture of acetonitrile and water. But methanol has been chosen as an extraction solvent because of its ability to extract all these 23 analytes and gives the best chromatographic separation.

Water has been the worst extraction solvent especially for coffee matrix. It will need a further clean up if water is used as an extraction solvent. It is because it also extracted the impurities from coffee matrix and it is really hard to filter the solution prior to LC-MS/MS injection.

4.3 Validation

4.3.1 Linear Range and Working Range

Calibration solutions were made at concentration: 10.0, 20.0, 50.0, 100.0 and 200.0ng/mL. The calibration graph is linear with correlation coefficient is more than 0.99 for all analytes. The calibration graph of N-Desmethylsildenafil is shown in the **Figure 7**. Two transitions as well as the ion ratios were used for confirmatory purpose (**Table 1**). The ion ratio of standards will be calculated using formula below. A calibration graph of individual analytes will be constructed by plotting response values against the concentration (µg/mL) of the standards:

$$\text{Response} = \frac{Area\ \text{Primary}\ Daughter}{Area\ Secondary\ Daughter}$$

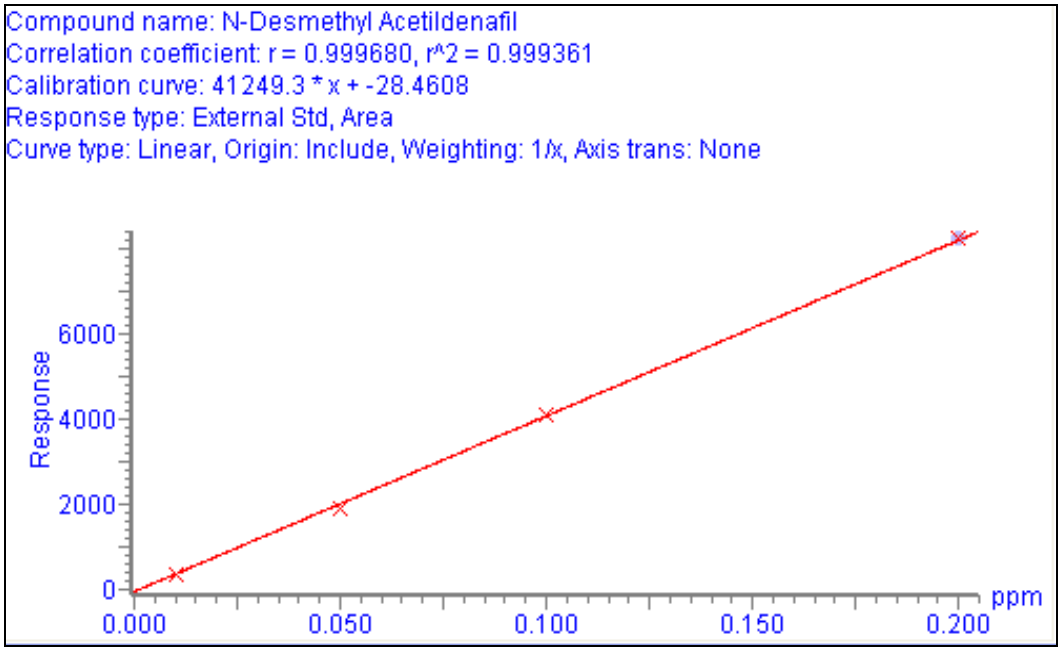


Figure 7: The calibration graph of N-Desmethylsildenafil

This method also linear ranged from 1 to 500ng/mL as shown in **Figure 8**.

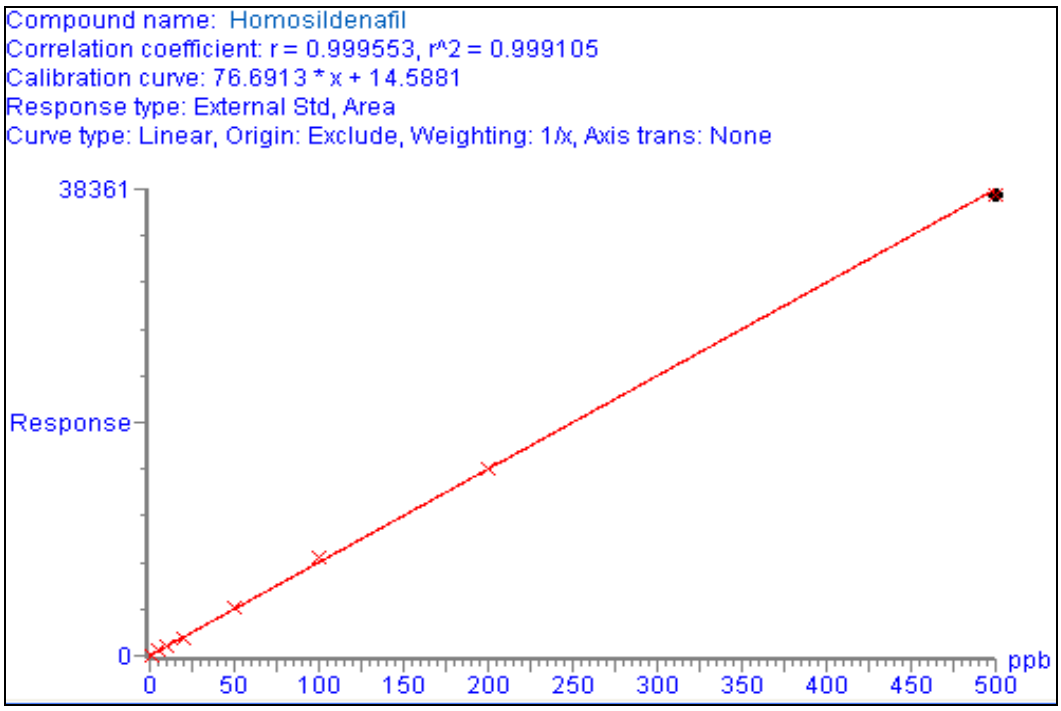


Figure 8: Linearity study for Homosildenafil

4.3.2 Limit of Detection and Limit of Quantification

The precision (standard deviation, SD) of the method was determined from 7 replicates. Limit of detection obtained from 3 times SD and the limit of quantification from 10 times SD. The LOD ranged from 20 to 146 ng/ml in coffee matrix, 1 to 10ng/ml in herbal matrix and 1 to 9ng/ml in candy matrix (See **Appendix B**).

The average LoD, LoQ and recovery study in these three matrixes also can be seen in **Table 2**. The average recovery for coffee matrix ranged from 51.21% to 118.57% and from 74.29% to 131.67% in herbal matrix and ranged from 82.86% to 104.29% in candy matrix. Any result detected below than their LoD will report as a not detected and when it fall below than LoQ will report as below than their LoQ. And it is not necessary to detect the analytes than below than their LoQ.

From the results obtained, it can be observed that there is an unstable result for piperiacetildenafil analysis in coffee matrix. The recovery of this compound is only 51.21%. It is believed that this is due to the matrix effect of caffeine in coffee matrix to this compound. This procedure had been repeated for a few times by spiking single standard of piperiacetildenafil in blank coffee, but still the same results obtained. These spiking samples must be analysed in 30 minutes after extraction. If not, the recovery of these samples will be reducing to 0 %. Some efforts has been put by changed the extraction solvent, used hot water but the result remained the same.

It has been noted that the LoD of dimethylsildenafil and hydroxyacetildenafil in coffee matrix are quite high compared to other analytes. It is believed that the matrix effect of caffeine in coffee matrix has contributed to these results. But this value is still acceptable because normally the adulteration of these drugs in real samples is quite high.

There are also problems for analyst if the analysed sample showed positive result for homosildenafil or dimethylsildenafil because the peaks for these analytes will elute at the same retention time. They also shared same molecular mass (*m/z*=489) and the polarity of these compounds also similar. The other mass analyzer such as Time of Flight (ToF) should be used since this mass analyzer has a capability to differentiate and give an accurate mass for these analytes up to four points.

Table 2: LoD, LoQ and recovery study in matrix coffee, herbal products and candy in ng/mL level.

No.	Compounds	LOD (ng/mL)			LOQ (ng/mL)			Recovery (100 %)		
		Coffee	Herbal	Candy	Coffee	Herbal	Candy	Coffee	Herbal	Candy
1	Dimethylsildenafil	6.7	4.2	1.7	20.0	13.0	5.0	111.67	108.57	95.71
2	Piperiacetildenafil	34.5	1.7	3.6	3.0	5.0	11.0	51.21	84.29	100.00
3	N-Desethylvardenafil	3.0	1.5	2.4	9.0	5.0	7.0	98.57	85.71	82.86
4	Hydroxyacetildenafil	146.2	1.5	1.5	439.0	5.0	5.0	78.49	97.14	92.86
5	N-Desmethylacetildenafil	89.8	2.5	1.5	269.0	7.0	5.0	86.14	85.71	97.14
6	Carbodenafil	5.1	1.0	1.2	15.0	3.0	4.0	94.29	99.14	102.86
7	Udenafil	2.8	2.4	3.8	8.0	7.0	11.0	118.57	102.86	98.57
8	Chloropretadalafil	2.6	3.7	3.8	8.0	11.0	11.0	90.00	131.67	88.57
9	Hydroxythiohomosildenafil	4.7	1.2	2.2	14.0	4.0	7.0	101.43	98.57	98.57
10	Gendenafil	1.7	5.1	1.7	5.0	15.0	5.0	91.43	111.43	91.43
11	N-Desmethyl Sildenafil	5.6	4.1	3.6	17.0	12.0	11.0	94.29	100.00	104.29

No.	Compounds	LOD (ng/mL)			LOQ (ng/mL)			Recovery (100 %)		
		Coffee	Herbal	Candy	Coffee	Herbal	Candy	Coffee	Herbal	Candy
12	Hydroxyhomosildenafil	3.9	2.4	1.8	12.0	7.0	5.0	98.57	102.86	100.00
13	Thiodimethylsildenafil	6.6	3.5	6.8	20.0	10.0	20.0	115.71	108.57	98.00
14	Sildenafil	6.5	4.4	2.6	19.0	13.0	8.0	96.43	124.29	100.00
15	Acetildenafil	38.0	1.2	3.0	114.0	4.0	9.0	86.86	102.86	97.14
16	Homosildenafil	18.2	2.5	4.2	55.0	7.0	13.0	87.14	95.71	98.57
17	Tadalafil	5.7	3.0	4.0	17.0	9.0	12.0	108.57	107.14	95.71
18	Vardenafil	1.7	2.4	2.2	5.0	7.0	7.0	114.29	74.29	101.43
19	Aminotadalafil	19.7	10.1	9.3	59.0	30.0	28.0	112.57	100.86	95.71
20	Norneosildenafil	1.5	1.2	0.8	5.0	4.0	2.0	105.71	97.14	100.29
21	Thiosildenafil	27.9	5.0	5.6	84.0	15.0	17.0	79.43	94.57	98.00
22	Thiohomosildenafil	5.7	6.2	3.1	17.0	19.0	9.0	115.00	93.33	95.71
23	Dimethylsildenafil	290.0	2.8	4.6	870.0	8.0	14.0	78.90	88.57	101.43

4.3.3 Repeatability and Reproducibility

For repeatability evaluation, 7 replicates of analytes from the same reference material source have been injected and their Relative Standard Deviation (RSD) was as shown in Table 3. The average RSD for candy matrix ranged from 0.04 to 0.14% and 0.04 to 0.20% for herbal matrix. And for coffee matrix, it ranged from 0.05 to 0.33%.

Table 3: RSD of repetability test in matrix coffee, herbal products and candy in ng/mL level.

No.	Standard/Compound	RSD (%)		
		Candy	Herbal	Coffee
1	Dimethylsildenafil	0.06	0.12	0.19
2	Piperiacetildenafil	0.12	0.06	0.11
3	N-Desethylvardenafil	0.09	0.11	0.05
4	Hydroxyacetildenafil	0.05	0.05	0.12
5	N-Desmethylacetildenafil	0.05	0.09	0.07
6	Carbodenafil	0.07	0.07	0.17
7	Udenafil	0.12	0.07	0.08
8	Chloropretadalafil	0.14	0.09	0.09
9	Hydroxythiohomosildenafil	0.07	0.04	0.07

No.	Standard/Compound	RSD (%)		
		Candy	Herbal	Coffee
10	Gendenafil	0.12	0.29	0.12
11	N-Desmethylsildenafil	0.11	0.13	0.09
12	Hydroxyhomosildenafil	0.06	0.07	0.06
13	Thiodimethylsildenafil	0.04	0.05	0.09
14	Sildenafil	0.08	0.11	0.11
15	Acetildenafil	0.10	0.07	0.14
16	Homosildenafil	0.14	0.08	0.33
17	Tadalafil	0.13	0.09	0.08
18	Vardenafil	0.07	0.20	0.05
19	Aminotadalafil	0.06	0.06	0.11
20	Norneosildenafil	0.05	0.08	0.09
21	Thiosildenafil	0.04	0.03	0.22
22	Thiohomosildenafil	0.10	0.21	0.08
23	Noracetildenafil	0.14	0.10	0.15

For reproducibility test, 7 replicates sample were prepared analysed by different analysts and it gave not very much difference in RSD which it ranged from 0.04 to 0.40 % for all matrices.

4.3.4 Selectivity

By using Multiple Reaction Monitoring (MRM) mode, this method is very selective since it can differentiate 23 analytes simultaneously in single run time. **Figure 6** show total ion chromatogram (TIC) for these 23 standards and their analogues. The first peak eluted at 3.36 minutes and the last peak at 11.69 minutes.

It means that this method can easily detect the presence of these drugs in 15 minutes and no cross talk from previous run observed during this study.

4.3.5 Measurement Uncertainty

Uncertainty for this study in 3 matrices was measured by used the data from validation procedure (reproducibility estimation) and from the certificate of CRM (all analytes).

Combined relative uncertainty (uR) was calculated using the following formula:

$$uR = \sqrt{(uRm)^2 + (uRs)^2}$$ where

uRm is the correction uncertainty of the experimental value and uRs is the uncertainty of sample effect (in this study: candy, herbal and coffee) calculated by pooled standard deviation of these matrices.

Expanded uncertainty was calculated by multiplying combined uncertainty with the coverage factor, k=2.

The combined uncertainty for each analytes was differing to each other because it depends to several factors such as the recovery obtained during reproducibility test and the sample effect to difference matrices. For example: The relative combined uncertainty of the method for hydroxythiohomosildenafil was estimated to be uR = 2.18% and relative expanded uncertainty, U = 5.1%; (k=2) (See **Appendix C**).

4.3.6 Screening Sample

Some samples have been submitted for screening purposes. The chromatogram obtained was shown in **Figure 9**. Sample preparation procedure has been followed and the sample has been analysed using this LC-MS/MS method.

It was observed that the presence of tadalafil, homosildenafil and dimethylsildenafil as shown in this chromatogram. As mentioned before, there are some challenges to analyst while reporting this sample because there is not much difference between homosildenafil and dimethylsildenafil since these peaks are coeluted together. Further research should be made to solve this issue. Some positive samples that have been analysed have been showed in **Appendix D**.

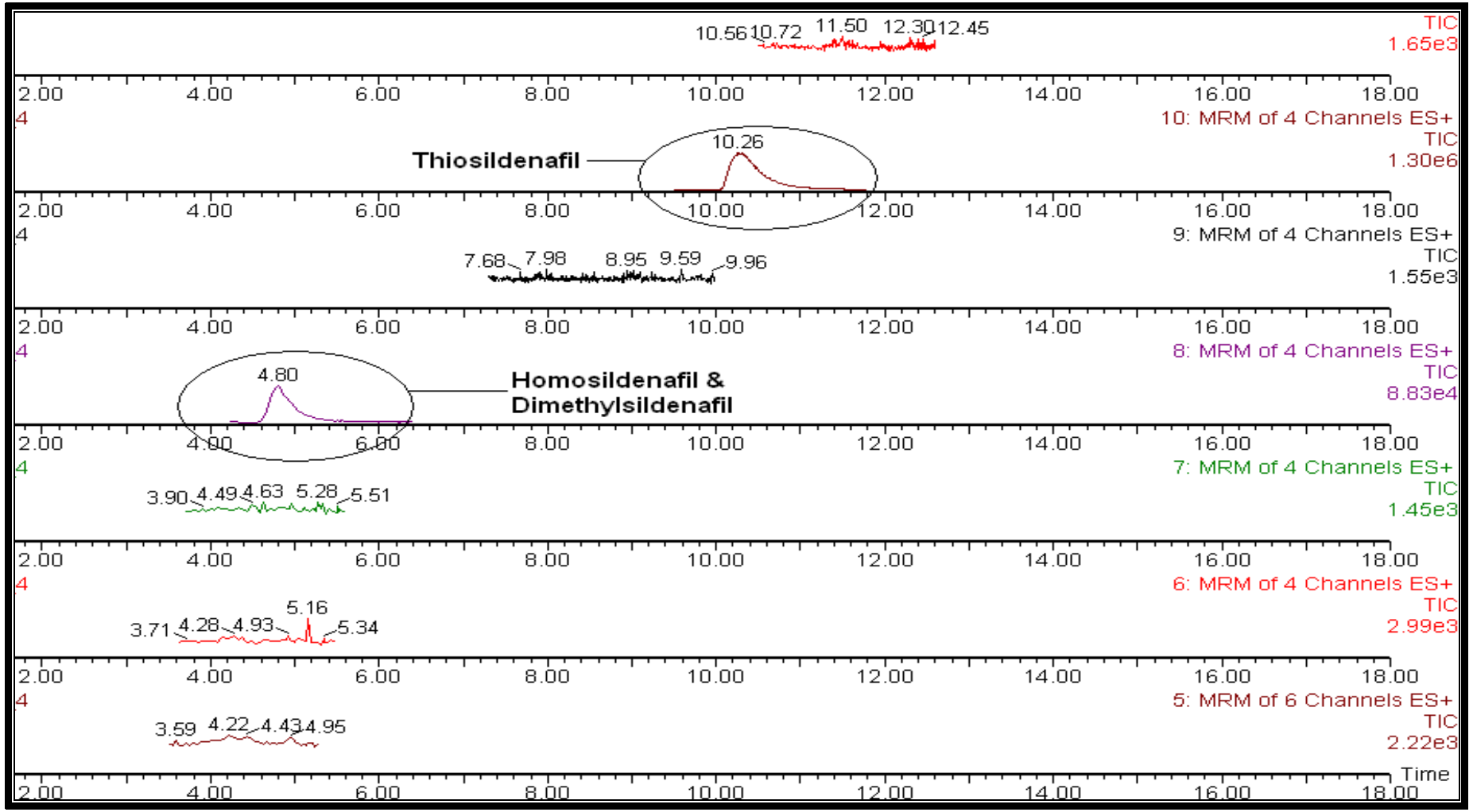


Figure 9: Chromatogram of Positive sample

5.0 CONCLUSION

The purpose of this master's theses is to validate a method to quantificate Sildenafil, Vardenafil, Tadalafil and their analogues (Hydroxyhomosildenafil, Norneosildenafil, Acetildenafil, Homosildenafil, Aminotadalafil, Thiosildenafil, N-Desmethylacetildenafil, Thiohomosildenafil, Gendenafil, Carbodenafil, Hydroxythiohomosildenafil, N-Desethylvardenafil, N-Desmethylsildenafil, Udenafil, Hydroxyacetildenafil, Piperiacetildenafil, Dimethylsildenafil, Chloropretadalafil, and Noracetildenafil) in adulterated herbal medicinal preparations, herbal vitality products, supplements and foods and beverages using LC-MS/MS system.

For validation purposes, the following parameters were evaluated: the properties of calibration graph including linear and working range, LoD and LoQ, repeatability and reproducibility, selectivity and measurement uncertainty of the method.

There are some challenges for detection of these analogues because most of the CRMs are not available. It's also difficult to detect without the target and related to the legal issues where the classification of substance as an analogues (Ying *et al*., March 2008). Nowadays, more than 100 analogues are spread over worldwide in our products because of the limitation in detection. The actual analogues used are likely to change over time as regulators develop test methods to detect and rapidly screen for known analogues. So, the combination of the analytical properties and technology are needed to fully characterise these analogues.

As a conclusion, it may be stated that the validated method is appropriate for quantification of Sildenafil, Vardenafil, Tadalafil and their analogues by LC/MS-MS. There are some interesting aspects in this work that can be used for further. This LC/MS-MS method is very sensitive and can be adapted to body fluid analysis such as blood and urine but with a little change in sample preparation procedure. This method can easily detect, confirm and quantify these analogues sildenafil concentrations in a single analysis. The chromatographic separation was achieved in less than 13 minutes, with the total run time of 15 minutes.

6.0 REFERENCES

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