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No. AD-0101

Healthcare Product / LCMS-IT-TOF

Un-targeted Screening Method for Detection of Synthetic PDE-5 Inhibitors and Analogues Adulterated in Health Supplements on LCMS-IT-TOF

ASMS 2015 TP102

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Development of Un-targeted Screening Method for Detection of Synthetic PDE-5 Inhibitors and Analogues Adulterated in Health Supplements on LCMS-IT-TOF

Introduction

Sildenafl and other synthetic phosphodiesterase type 5 enzyme (PDE-5) inhibitors are used for treatment of erectile dysfunction (ED) in men. Sildenafl, tadalafl, vardenafl, avanafl and udenafl are the approved PDE-5 drugs in different countries [1]. However, adulteration of PDE-5 inhibitors and their analogues are found in dietary supplements and health products in recent years [1-3]. The adulteration is illegal and dangerous for consumers because none of the analogues is approved offcially for medical use. Analytical methods based on HPLC and LC/MS/MS [1-3] are used for detection of targeted PDE-5 inhibitors, but it is challenging to detect unknown adulterants in actual samples. We present here a novel un-targeted screening method using LCMS-IT-TOF and a MetID program with database function. A multi-event method was used to acquire HR-TICs with enhanced sensitivity. An un-targeted screening approach was established using a concerned compound database of thirty-two PDE-5 inhibitors and their analogues and applied to fve health supplements obtained from local market.

Experimental

Thirty-two synthetic PDE-5 inhibitors and analogues were obtained from TLC PharmaChem (Canada). These compounds were dissolved in MeOH at 100ppm or 10ppm as stocks and were used as standards. Five health supplements in capsules obtained from local markets were selected as matrixes to prepare spiked samples for method performance evaluation. These samples are named as M, C, PP, TA and RK for convenience. Sample pre-treatment includes extraction of the samples at a ratio of 1.0 gram of powders in 10 mL of pure MeOH. The mixture was sonicated for 20 minutes, followed by fltration with 0.2um PTFE flter before analysis. A high resolution LCMS-IT-TOF coupled with a UFLC (Shimadzu Corporation, Japan) was employed in this study to develop un-targeted screening method based on high resolution (HR) and accurate mass measurement. A Shim-pack XR-ODS-III UHPLC column (150x2mm, 2.2µm) was used and a gradient elution program was optimized to achieve separation of these thirty-two compounds. The mobile phases used were milli-Q water (A) and acetonitrile (B), both with 0.1% formic acid. The gradient program adopted was B (%): 10% (0.01min) → 75% (18-20min) → 10% (20.01-25min). A multievent TIC data acquisition method was used with each event covering 50Da only to improve the detection sensitivity. Standard ESI conditions in positive mode were applied. The injection volume used was 5uL unless indicated separately.

Results and Discussion

Comparison of multi-event method with single-event method

In an un-targeted screening analysis using LC-TOF, total ion chromatograms (TICs) of fullspectrum are acquired to cover all concerned masses. This requires the TOF-MS capable of acquiring data with high accuracy, high sensitivity and excellent selectivity to reduce the chance of false negative detection of concerned compounds that may be present in the samples studied. However, a TIC usually consists of a high background baseline and interference peaks, which may cause small peaks submerged under the baseline and become not detectable. In this work, a multi-event TIC method was adopted to acquire data with a narrow range in each event (50Da). The full range of m/z 200-600 was covered by eight events. To evaluate the sensitivity of this method, a comparison study of the multi-event method and the single event method was performed with mixture standards of 0.1ppm and 0.5ppm. The results (Figure 1) revealed that the peak areas of multi-event TIC increased twice on average than that acquired with a single event method (m/z 200-600) in detection of the 32 PDE-5 inhibitors and analogues (Table 1). The extract ion chromatogram (EIC) method using the multi-event was compared with that of the single event method (Figure 2).

Development of Un-targeted Screening Method for Detection of Synthetic PDE-5 Inhibitors and Analogues Adulterated in Health Supplements on LCMS-IT-TOF

Table 1: Comparison of multi-event method and single-event method in detection of 32 PDE-5 inhibitor and analogues (0.1ppm)

name	Formula	CAS	$[M+H]^+$	RT (min)	A (Multi-event) /A (Single-event)
YOHIMBINE	$C_{21}H_{26}N_2O_3$	146-48-5	355.2016	4.58	1.2
ACETYLVARDENAFIL	$C_{25}H_{34}N_6O_3$	1261351-28-3	467.2765	5.01	1.9
CARBODENAFIL	$C_{24}H_{32}N_6O_3$	944241-52-5	453.2609	5.80	1.9
N-DESMETHYL ACETILDENAFIL	$C_{23}H_{30}N_6O_3$	n.a.	439.2452	5.84	2.8
HYDROXYHOMOSILDENAFIL	$C_{23}H_{32}N_6O_5S$	139755-85-4	505.2228	5.86	5.7
VARDENAFIL	$C_{23}H_{32}N_6O_4S$	224785-90-4	489.2279	5.95	3.3
HYDROXYACETILDENAFIL	$C_{25}H_{34}N_6O_4$	147676-56-0	483.2714	6.01	2.9
NORACETILDENAFIL	$C_{24}H_{32}N_6O_3$	949091-38-7	453.2665	6.13	2
ACETILDENAFIL	$C_{25}H_{34}N_6O_3$	831217-01-7	467.2765	6.29	1.6
PIPERIACETILDENAFIL	$C_{24}H_{31}N_5O_3$	147676-50-4	438.2456	6.69	1.6
AVANAFIL	$C_{23}H_{26}ClN_7O_3$	330784-47-9	484.1858	6.94	1.2
HYDROXYVARDENAFIL	$C_{23}H_{32}N_6O_5S$	224785-98-2	505.2227	7.02	2.6
SILDENAFIL	$C_{22}H_{30}N_6O_4S$	171599-83-0	475.2127	7.07	2.8
HOMOSILDENAFIL	$C_{23}H_{32}N_6O_4S$	642928-07-2	489.2278	7.19	2.2
DIMETHYLSILDENAFIL	$C_{23}H_{32}N_6O_4S$	1416130-63-6	489.2277	7.42	2.3
UDENAFIL	$C_{25}H_{36}N_6O_4S$	268203-93-6	517.2592	7.71	2.7
N-DESETHYL VARDENAFIL	$C_{21}H_{28}N_6O_4S$	448184-46-1	461.2041	9.12	1.4
BENZYLSILDENAFIL	$C_{28}H_{34}N_6O_4S$	n.a.	551.2435	9.12	2.8
HYDROXYTHIOHOMOSILDENAFIL	$C_{23}H_{32}N_6O_4S_2$	479073-82-0	521.1999	9.32	3.3
THIOSILDENAFIL	$C_{22}H_{30}N_6O_3S_2$	479073-79-5	491.1894	9.41	2.7
THIOHOMOSILDENAFIL	$C_{23}H_{32}N_6O_3S_2$	479073-80-8	505.205	9.56	3.1
THIODIMETHYLSILDENAFIL	$C_{23}H_{32}N_6O_3S_2$	856190-47-1	505.2009	9.75	2.9
AMINOTADALAFIL	$C_{21}H_{18}N_4O_4$	385769-84-6	391.1401	9.76	0.9
NORTADALAFIL	$C_{21}H_{17}N_3O_4$	171596-36-4	376.1292	9.96	0.5
VARDENAFIL intermediate	$C_{17}H_{20}N_4O_2$	n.a.	313.1659	9.97	1.2
TADALAFIL	$C_{22}H_{19}N_3O_4$	171596-29-5	390.1448	10.81	0.8
PSEUDOVARDENAFIL	$C_{22}H_{29}N_5O_4S$	224788-34-5	460.2013	12.51	2
GENDENAFIL	$C_{19}H_{22}N_4O_3$	147676-66-2	355.1765	12.99	1.8
N-BUTYL TADALAFIL	$C_{25}H_{25}N_3O_4$	171596-31-9	432.2034	13.99	0.4
CHLOROPRETADALAFIL	$C_{22}H_{19}ClN_2O_5$	171489-59-1	427.1055	14.38	0.8
NORNEOSILDENAFIL	$C_{22}H_{29}N_5O_4S$	371959-09-0	460.2014	15.98	2.4
N-OCTYL NORTADALAFIL	$C_{29}H_{33}N_3O_4$	1173706-35-8	488.2518	18.52	0.5

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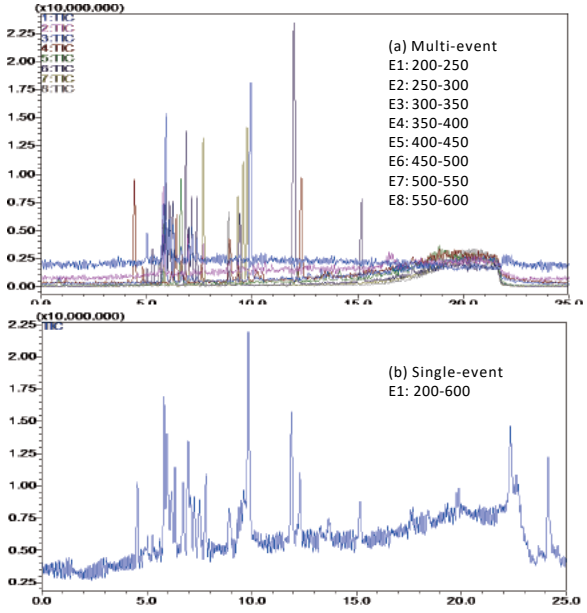

Fig 1: Comparison of TICs of 32 mixed standard of PDE-5 inhibitor drugs and analogues of 0.1ppm acquired by multievent TIC method (a) and single event TIC method (b).

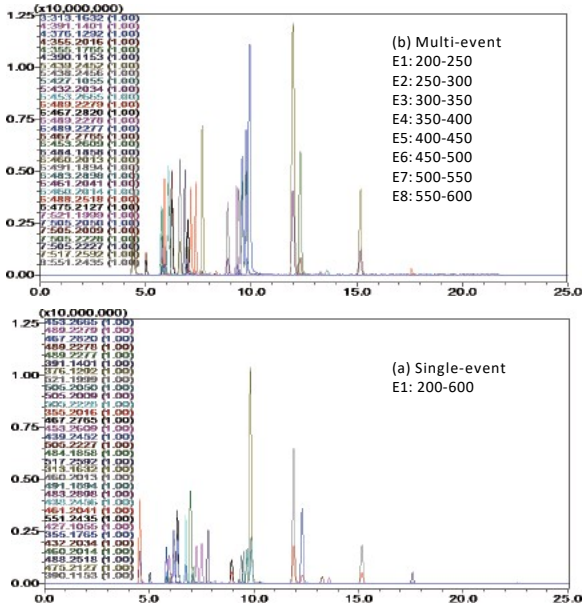

Fig 2: HR-EICs of mixed standard of thirty-two PDE-5 inhibitor drugs and analogues of 0.1ppm acquired by multi-event method (a) and the single event method (b).

Un-targeted screening method on LCMS-IT-TOF

Un-targeted screening worklow: The workflow based on LCMS-IT-TOF and MetID Solution program is shown in Figure 3. First, a concerned compound database with their accurate masses was created in the MetID Solution program. The thirty-two PED-5 inhibitors and analogues studied are registered into the database, which includes the information of names, formula and accurate masse, but without retention times. A method for data analysis and database search is set up, which includes appropriate peak detection and peak picking parameters, and database search criteria like mass error allowed (e.g., +/- 5ppm). After a raw data fle acquired with Multi-event method is submittted to the MetID solution program, data analysis and database search is performed without restriction of RTs and other specifc parameters related to particular compounds. The above work-flow was applied to the MeOH extracts of the fve health products & their spiked samples with the 32 PDE-5 inhibitors & analogues studied. The TICs of the extract samples are very complicated because most components are detectable by mass spectrometer. Figure 4a shows the complex chromatogram (by single-event method) of MeOH extract of sample M, which was confrmed to be free of the thirty-two compounds. Figure 4c is the chromatograms of 0.5ppm mixed standards spiked in the same matrix (M). This spiked sample was also analysed by multi-event method (Figure 4d) and the raw data was subjected to un-targeted screening analysis. Data analysis of un-targeted screening is a process of peak detection, peak picking and database search to generate a found lihealth supplement samples as st of candidates. Table 3 shows the found list in sample M spiked with 0.1ppm of thirty-two PDE-5 inhibitors and analogues. It is worth to note that this data analysis process may result in a long list of candidates if the peak detection (integration parameters), peak picking and database settings (mass error margin etc) are in a more sensitive range. In contrast, a shorter found list will be generated if these settings are less sensitive. But this may result in false negative detection result.

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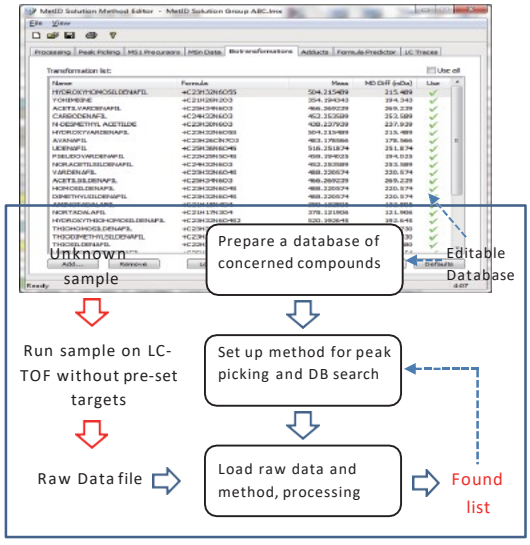

Fig. 3: Un-targeted screening workflow on LCMS-IT-TOF with MetID Solution program for screening analysis of PDE-5 inhibitor drugs and analogues (32 compounds)

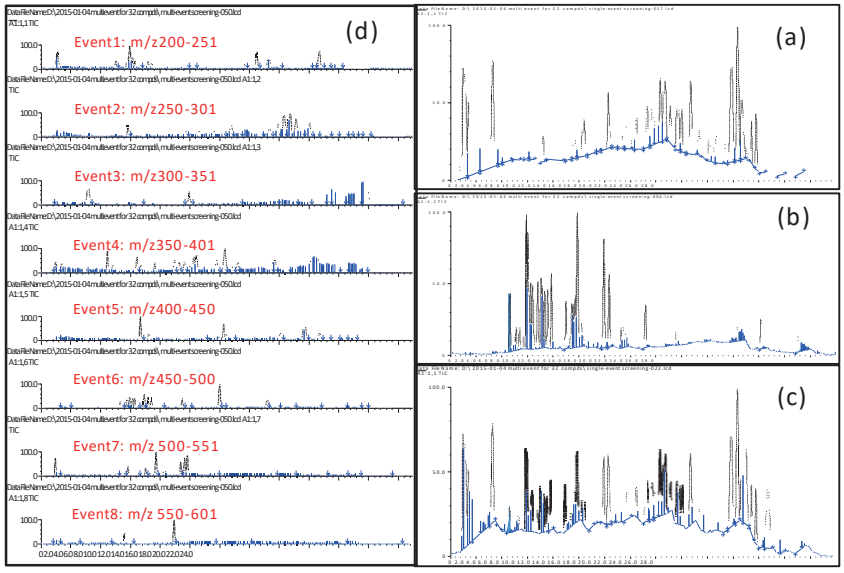

Fig. 4: TICs of spiked 0.5ppm Sample M analysed by multi-event method (d, left) and (a) single event method of blank extract of Sample M, (b) 0.5ppm mixed standards and (c) 0.5ppm spiked Sample M.

Un-targeted screening for family analogue: There are only seven synthetic PDE-5 inhibitors being offcially approved in different countries. The supply of approved PDE-5 inhibitors is restricted due to limited manufacture. This may explain the adulterations found often by use of synthetic analogues of the PDE-5 inhibitors which are synthesized by minor modifcation of the parent structures [1]. The MetID program enables to search for any possible analogues of a "parent compound", for example, tadalafl analogues having a common frame structure same as Tadalafl with different molecular moieties. Therefore, a tadalafl family database can be established and used for un-targeted screening. As shown in Table 2, the six members of the tadalafl family was found successfully in spiked sample M. This method is potentially useful in fnding suspected family members which is not registered in compound data-base.

Table 2: Results of un-targeted screening for Tadalafl family in sample M spiked with 0.5ppm standards of thirty-two PDE-5 inhibitors and analogues

Name	RT (min)	Area	Meas. m/z	Tadalafil & moiety	Formula (M)	Ion	Diff (mDa)
Tadalafl	10.30	1932463	390.1425	Tadalafl (T)	$C_{22}H_{19}N_3O_4$	$[M+H]^+$	-2.3
Aminotadalafl	9.32	1069945	391.1421	T-CH_3+NH_2	$C_{21}H_{18}N_4O_4$	$[M+H]^+$	2.0
Nortadalafl	9.50	230080	376.1256	T-CH_2	$C_{21}H_{17}N_3O_4$	$[M+H]^+$	-3.6
N-butyl tadalaf	13.27	1306904	432.1912	T+C_3H_6	$C_{25}H_{25}N_3O_4$	$[M+H]^+$	-0.6
Chloropretadalafl	13.61	658308	427.1048	T-N+ClO	$C_{22}H_{19}N_2O_5Cl$	$[M+H]^+$	-0.7
N-octyl nortadalafl	17.59	2072446	488.2548	T+C_7H_{14}	$C_{29}H_{33}N_3O_4$	$[M+H]^+$	0.4

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Detection reliability of un-targeted screening: Evaluation of the method performance was focused on the detection reliability of the compounds in different sample matrixes due to matrix effect and peak interference. The compositions of different health supplements are very different because of different recipes and different materials used. This will result in very different matrix effects and peak interferences. The matrix effect and interference were evaluated with 0.1ppm and 0.5ppm spiked samples in MeOH extracts of fve health supplement samples M, C, PP, TA and RK. None of the thirty-two compounds were detected in the blank matrixes. The matrix effect (%) was determined by comparison of the peak areas of spiked samples and the peak areas of neat mixed standards of same concentrations. The results of 0.5ppm spiked samples are shown in Table 4. The matrix effect values of almost all samples are below 100%, which may be indicate that peak interferences are extremely less. This is believed due to the high resolution of MS detection, which provides excellent mass selectivity in data acquisition [mass window of (+/-) 50ppm]. However, matrix effects of the samples are signifcant and varied with compounds. A few compounds like aminotadalafl (m/z391.1401), piperiacetildenafl (m/z438.2456) and Tadalafl (m/z390.1153) in sample C exhibited very strong matrix effect to less than 10%. However, the same compounds exhibited much less matrix effect in other samples. For 0.5ppm spiked samples (M, C, PP, TA and KR), all of the thirty-two compounds were detected by un-targeted screening method. However, a few compounds could not be found from the 0.1ppm spiked samples due to matrix effect. Therefore, a general detection limit of the un-targeted screening method for the thirty-two PDE-5 inhibitors and analogues on LCMS-IT-TOF is suggested tentatively to be 0.5ppm.

Table 3: The found list of un-targeted screening of sample M spiked with 0.5ppm standards of thirty-two PDE-5 inhibitors and analogues (repeated detected peaks are removed manually)

No	RT (min)	Meas. m/z	Compound	Formula (M)	Ion	Diff (mDa)
1	4.43	355.2010	YOHIMBINE	$C_{21}H_{26}N_2O_3$	$[M+H]^+$	-0.6
2	4.99	467.2761	ACETYLVARDENAFIL	$C_{25}H_{34}N_6O_3$	$[M+H]^+$	-0.4
3	5.74	453.2598	CARBODENAFIL	$C_{24}H_{32}N_6O_3$	$[M+H]^+$	-1.1
4	5.81	439.2466	N-DESMETHYL ACETILDENAFIL	$C_{23}H_{30}N_6O_3$	$[M+H]^+$	1.4
5	5.81	505.2199	HYDROXYHOMOSILDENAFIL	$C_{23}H_{32}N_6O_5S$	$[M+H]^+$	-2.9
6	5.93	489.2272	VARDENAFIL	$C_{23}H_{32}N_6O_4S$	$[M+H]^+$	-0.7
7	5.99	483.2692	HYDROXYACETILDENAFIL	$C_{25}H_{34}N_6O_4$	$[M+H]^+$	-2.2
8	6.11	453.2598	NORACETILDENAFIL	$C_{24}H_{32}N_6O_3$	$[M+H]^+$	-1.1
9	6.28	467.2757	ACETILDENAFIL	$C_{25}H_{34}N_6O_3$	$[M+H]^+$	-0.8
10	6.65	438.2504	PIPERIACETILDENAFIL	$C_{24}H_{31}N_5O_3$	$[M+H]^+$	0.4
11	6.90	484.1853	AVANAFIL	$C_{23}H_{26}N_7O_3Cl$	$[M+H]^+$	-0.5
12	6.99	505.2219	HYDROXYVARDENAFIL	$C_{23}H_{32}N_6O_5S$	$[M+H]^+$	-0.9
13	7.04	475.2113	SILDENAFIL	$C_{22}H_{30}N_6O_4S$	$[M+H]^+$	-0.9
14	7.17	489.2282	HOMOSILDENAFIL	$C_{23}H_{32}N_6O_4S$	$[M+H]^+$	0.3
15	7.40	489.2269	DIMETHYLSILDENAFIL	$C_{23}H_{32}N_6O_4S$	$[M+H]^+$	-1.0
16	7.70	517.2580	UDENAFIL	$C_{25}H_{36}N_6O_4S$	$[M+H]^+$	-1.2
17	8.93	551.2409	BENZYLSILDENAFIL	$C_{28}H_{34}N_6O_4S$	$[M+H]^+$	-2.6
18	8.93	461.1957	N-DESETHYL VARDENAFIL	$C_{21}H_{28}N_6O_4S$	$[M+H]^+$	-0.9
19	9.31	521.1994	HYDROXYTHIOHOMOSILDENAFIL	$C_{23}H_{32}N_6O_4S_2$	$[M+H]^+$	-0.5
20	9.33	391.1421	AMINOTADALAFIL	$C_{21}H_{18}N_4O_4$	$[M+H]^+$	2.0
21	9.46	491.1887	THIOSILDENAFIL	$C_{22}H_{30}N_6O_3S_2$	$[M+H]^+$	-0.7
22	9.50	376.1262	NORTADALAFIL	$C_{21}H_{17}N_3O_4$	$[M+H]^+$	-3.0
23	9.61	505.2033	THIOHOMOSILDENAFIL	$C_{23}H_{32}N_6O_3S_2$	$[M+H]^+$	-1.7
24	9.81	505.2038	THIODIMETHYLSILDENAFIL	$C_{23}H_{32}N_6O_3S_2$	$[M+H]^+$	-1.2
25	9.93	313.1655	VARDENAFIL INTERMEDIATE	$C_{17}H_{20}N_4O_2$	$[M+H]^+$	-0.4

No	RT (min)	Meas. m/z	Compound	Formula (M)	Ion	Diff (mDa)
26	10.31	390.1434	TADALAFIL	$C_{22}H_{19}N_3O_4$	$[M+H]^+$	-1.4
27	11.98	460.2007	PSEUDOVARDENAFIL	$C_{22}H_{29}N_5O_4S$	$[M+H]^+$	-0.6
28	12.33	355.1762	GENDENAFIL	$C_{19}H_{22}N_4O_3$	$[M+H]^+$	-0.3
29	13.27	432.1911	N-BUTYL TADALAFIL	$C_{25}H_{25}N_3O_4$	$[M+H]^+$	-0.7
30	13.61	427.1048	CHLOROPRETADALAFIL	$C_{22}H_{19}N_2O_5Cl$	$[M+H]^+$	-0.7
31	15.18	460.1994	NORNEOSILDENAFIL	$C_{22}H_{29}N_5O_4S$	$[M+H]^+$	-1.9
32	17.60	488.2560	N-OCTYL NORTADALAFIL	$C_{29}H_{33}N_3O_4$	$[M+H]^+$	1.6

Table 4: Matrix effect in screening analysis of 32 PDE-5 inhibitors and analogues spiked in health supplements (0.5ppm) by LC-TOF method

Compound Name	$[M+H]^+$ (m/z)	Matrix effect (%)				
		M	C	PP	TA	RK
YOHIMBINE	355.2016	65.3	27.5	77.0	78.7	33.4
ACETYLVARDENAFIL	467.2765	56.2	15.3	104.5	65.6	26.0
CARBODENAFIL	453.2609	78.2	22.8	81.9	84.5	31.0
N-DESMETHYL ACETILDENAFIL	439.2452	62.9	21.2	54.9	62.9	25.1
HYDROXYHOMOSILDENAFIL	505.2228	74.7	42.1	92.8	85.7	30.8
VARDENAFIL	489.2279	83.6	58.3	102.5	94.4	45.5
HYDROXYACETILDENAFIL	483.2898	61.6	10.1	70.7	63.0	13.8
NORACETILDENAFIL	453.2665	58.1	15.1	81.0	74.7	20.8
ACETILDENAFIL	467.2820	92.3	18.8	92.8	98.5	32.1
PIPERIACETILDENAFIL	438.2456	84.8	3.8	67.2	88.0	12.7
AVANAFIL	484.1858	88.3	57.6	101.2	96.3	65.4
HYDROXYVARDENAFIL	505.2227	69.1	38.1	92.7	85.8	32.3
SILDENAFIL	475.2127	73.6	32.3	86.3	85.8	29.1
HOMOSILDENAFIL	489.2278	84.7	54.2	100.8	99.4	55.1
DIMETHYLSILDENAFIL	489.2277	76.5	46.1	90.7	94.8	44.7
UDENAFIL	517.2592	76.8	50.1	99.9	98.6	43.5
BENZYLSILDENAFIL	551.2435	74.1	44.2	83.9	95.9	31.4
N-DESETHYL VARDENAFIL	461.2041	57.9	13.6	61.2	69.5	13.1
HYDROXYTHIOHOMOSILDENAFIL	521.1999	62.8	24.3	68.3	83.6	22.2
AMINOTADALAFIL	391.1401	54.4	9.3	51.7	73.8	10.0
THIOSILDENAFIL	491.1894	79.6	38.3	76.2	98.1	32.6
NORTADALAFIL	376.1292	42.8	21.7	8.6	76.2	6.8
THIOHOMOSILDENAFIL	505.2050	66.7	35.0	84.1	83.2	30.2
THIODIMETHYLSILDENAFIL	505.2009	66.2	32.1	59.3	84.5	25.9
VARDENAFIL INTERMEDIATE+ACo-	313.1632	78.1	62.7	72.4	86.2	46.3
TADALAFIL	390.1153	55.2	9.3	41.5	59.4	14.2
PSEUDOVARDENAFIL	460.2013	78.8	59.6	31.6	92.0	29.7
GENDENAFIL	355.1765	42.6	14.1	5.1	61.0	10.6
N-BUTYL TADALAFIL	432.2034	35.9	13.0	19.8	53.5	8.3
CHLOROPRETADALAFIL	427.1055	23.0	12.1	8.3	41.4	15.7
NORNEOSILDENAFIL	460.2014	56.0	39.4	20.5	80.7	24.5
N-OCTYL NORTADALAFIL	488.2518	40.0	28.4	30.3	45.4	32.6

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Conclusions

An un-targeted screening method for detection of adulterated PDE-5 inhibitor drugs and analogues in health supplements was developed on LCMS-IT-TOF with MetID Solution program. The method was proven to be effective for thirty-two compounds spiked in five different health supplement products randomly selected from local market. The detection reliability was evaluated with focusing on the matrix effect and peak interference. The results indicate that matrix effect is a key factor for the large variation in compositions of different samples due to their different recipes and materials used. A general detection limit of the screening method is proposed to be 0.5ppm based the evaluation performed in this study.

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First Edition: May, 2015

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