Fast, Robust and Reliable Method for the Identification and Quantitation of Sildenafil Residue in Honey using LC-MS/MS

Praveen K. Sharma[1], Neha Bhasin[1], Prasanth Joseph[1], Manoj G Pillai[1], and André Schreiber[2]
[1]AB SCIEX Gurgaon, Haryana (India) and [2]AB SCIEX, Concord, Ontario (Canada)

Overview

In recent years, natural products and herbal medicines are increasing in popularity all over the world. However, adulteration of natural products with synthetic adulterants is a serious concern and can impose deleterious health issues in human. Many reports suggest the adulteration of Sildenafil in honey based products with the intention of boosting the effects of products. A fast, robust and reliable LC-MS/MS method has been developed to identify, quantify and confirm traces of Sildenafil in honey samples using the AB SCIEX 4000 QTRAP® system. The method presented here can be routinely employed to screen for sildenafil in raw honey and herbal drug preparations.

Introduction

Honey is one of the precious food commodities from ancient times and there is high market demand for natural honey. According to the European Union (EU); international food standards Codex Alimentarius and other international honey standards - honey stipulates a pure product that does not allow for the addition of any other substance. Sildenafil citrate marketed as Viagra, a medicine for treatment of erectile dysfunction is very often added to honey based products and marketed to public in order to increase the popularity of these products. Adverse effects of Sildenafil especially cardiovascular risk are still under controversy. This highlights the need for herbal medicine manufactures to utilize a fast, reliable and unambiguous method to detect the low levels of Sildenafil residues in honey samples.

We developed an LC-MS/MS method using AB SCIEX 4000 QTRAP® system operated in Multiple Reaction Monitoring (MRM) mode to identify and quantify Sildenafil in honey with high selectivity and sensitivity.

The developed method was validated in-house as per European Commission Decision 2002/657/EC. Specificity, limit of detection and quantitation (LOD and LOQ), linear dynamic range, accuracy, repeatability, and limit of decision and detection capability (CCα and CCβ) were evaluated.

Multiple MRM transitions were monitored to use the ratio of quantifier and qualifier transition for identification of Sildenafil in samples. In addition, MRM-triggered MS/MS using the Enhanced Product Ion (EPI) mode was utilized to gain additional confidence in identification of positive findings. MS/MS spectra were acquired fully automatic using the logic provided by Information Dependent Acquisition (IDA), Dynamic Background Subtraction (DBS), and Dynamic Fill Time (DFT).

Full scan MS/MS spectra were interpreted using PeakView® software version 2.0, searched against mass spectral libraries using MasterView™ software version 1.1. Quantitative data were evaluated in MultiQuant™ software version 3.0.

Experimental

Chemicals and Honey Samples

Sildenafil Citrate certified reference material (CRM) was purchased from Sigma Aldrich. MS grade methanol was procured from J.T. Baker and formic acid from Fluka.

Honey samples were procured from the local markets of Punjab and Delhi, and were kept under at room temperature until completion of analysis.

Sample Preparation

Approximately 1 g of homogenized honey was weighed and fortified with 50 µL of the Sildenafil working standard to obtain

dilutions of 0.1 to 1000 ng/mL. Spiked samples were extracted with 20 mL of methanol/water (80:20), vortexed to get homogenized mixture and sonicated for 5 minutes to achieve maximum extraction efficiency. Samples were centrifuged at 4000 rpm; the supernatant was collected and filtered through 0.45 µm filter. Filtered aliquots were transferred into the autosampler vials for LC-MS/MS analysis.

LC Separation

LC separation was achieved with a reverse phase C18 ACQUITY UPLC BEH column having particle size of 1.7 µm. Isocratic elution was employed over a short runtime of 4.5 min with an aqueous phase of 10 mM ammonium formate in water and an organic phase of methanol with addition 0.1% of formic acid at a ratio of 80:20. Optimized flow rate of 0.2 mL/min with column temperature maintained at 42°C was used for separation.

The injection volume was set to 20 µL.

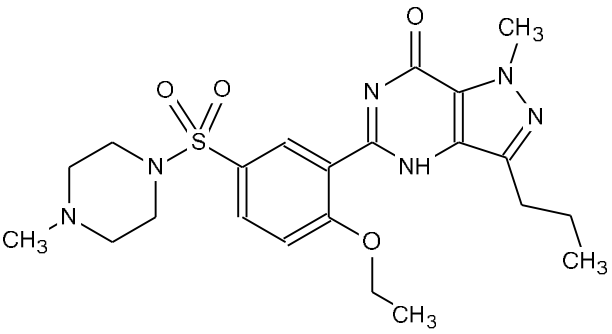

Figure 1. Chemical structure of sildenafil

MS/MS Detection

An AB SCIEX 4000 QTRAP® system equipped with Turbo V™ source was used, in positive Electrospray Ionization (ESI) mode. The presented method uses three MRM transitions: 475/100 as quantifier transition and 475/283 475/311 as qualifier transition.

Table 1. MRM transitions MS/MS Parameters for Sildenafil

Analyte	MRM transition	DP	CE
Sildenafil 1	475 / 100	40	54
Sildenafil 2	475 / 283	40	44
Sildenafil 3	475 / 311	40	44

Table 1 summarizes optimized compound dependent parameters such as Declustering Potential (DP) and Collision Energy (CE).

IDA was used to acquire MRM-triggered MS/MS spectra which aids in compound identification. CE was set to 35 V with a Collision Energy Spread (CES) of 15 V

Results and Discussion

A representative chromatogram of quantifier and qualifier MRM transitions is shown in Figure 2. MRM ratios were calculated automatically in MultiQuant™ software. The average MRM ratio of all standard injections with tolerance matching 2002/657/EC is displayed in the peak review window.

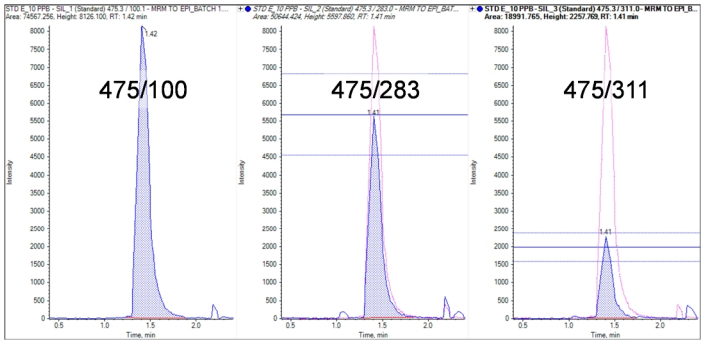

Figure 2. MRM transitions of Sildenafil at a concentration of 10 ng/mL, the MRM ratio was automatically calculated in MultiQuant™ software (Sildenafil 2: 0.686 ± 20%, Sildenafil 3: 0.210 ± 25%)

Lowest injected concentration of 0.1 ng/ml showed a signal-to-noise ratio (S/N) of 18.9 and was considered as the LOD whereas the LOQ was established at 0.5 ng/mL (Figure 3).

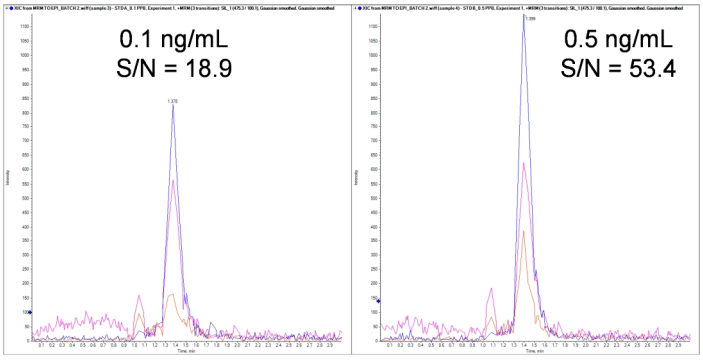

Figure 3. S/N for Sildenafil at the LOD (0.1 ng/mL) and LOQ (0.5 ng/mL)

Calibration lines were generated by using matrix matched calibration standards spiked within the linearity range of the 0.5 to 1000 ng/mL. Matrix matched calibration lines were found to be linear with correlation coefficient (r) of 0.995 or higher as shown in Figure 4.

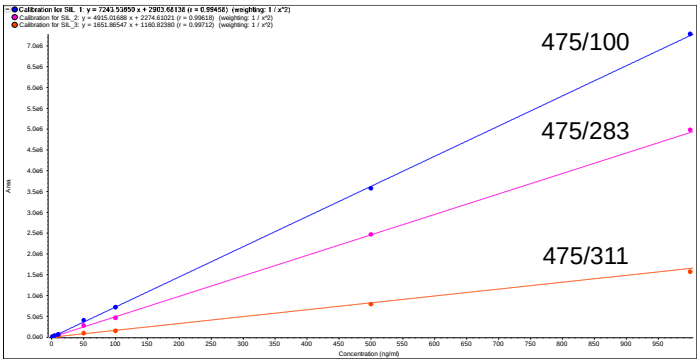

Figure 4. Calibration curve of Sildenafil from 0.5 to 1000 ng/mL with a regression > 0.995 for all three MRM transitions

Accuracy (% recovery) and precision (repeatability) were evaluated at four concentration levels of the LOQ (0.5 ng/mL), 2xLOQ, 5xLOQ and 10xLOQ with repeat injections (n=6). The mean recovery at the four levels was obtained above 85% as shown in Table 2.

Table 2. Accuracy data based on mean % recovery of 6 replicate injections at four different concentration levels

Analyte	Concentration (ng/mL)	Accuracy (%)
Sildenafil 1	0.5 (LOQ)	113.4
	1.0	89.6
	2.5	95.7
	5.0	94.2
Sildenafil 2	0.5 (LOQ)	117.6
	1.0	85.5
	2.5	94.5
	5.0	96.1
Sildenafil 3	0.5 (LOQ)	105.3
	1.0	109.9
	2.5	92.6
	5.0	105.8

Repeatability (precision) is defined in terms of the coefficient of variation (%CV). Repeatability of the method was determined using an independently spiked honey matrix at four different levels. In one day the set of four levels with six repetitions was measured to determine intra-day %CV. Two additional sets at same concentration levels with six repetitions were measured over the next two days for the determination of inter-day repeatability. Precision results were found satisfactory at all four levels of concentrations with %CV well below 15%. The results of intra-day and inter-day precision are summarized in Table 3.

Table 3. Repeatability data obtained by injecting 6 replicates over period of 3 days at four different concentration levels

Concentration (ng/mL)	Intra-day %CV	Inter-day %CV
0.5 (LOQ)	10.3	13.0
1.0	8.6	8.8
2.5	5.4	3.2
5.0	6.2	4.7

Both CCα (decision limit) and CCβ (detection capability) were determined following commission decision 2002/657/EC.

CCα was established by analyzing blank honey (n=60) at a level of 0.36 µg/kg. CCβ was established by analyzing blank honey spiked at 0.36 µg/kg (n=60) at a level of 0.44 µg/kg.

The developed method was subjected for screening and quantification of locally procured honey samples. Out of 15 samples analyzed, only one sample showed the traces of sildenafil and was quantified ~ 1.0 µg/kg.

Identification using QTRAP® Full Scan MS/MS Spectra and Mass Spectral Library Searching

Full scan MS/MS spectra were acquired for additional confidence in compound identification. During the method development step MS/MS spectra of Sildenafil were processed using the fragment prediction tool in PeakView® software (Figure 5). A tentative interpretation of the fragmentation pathway is shown in Figure 6.

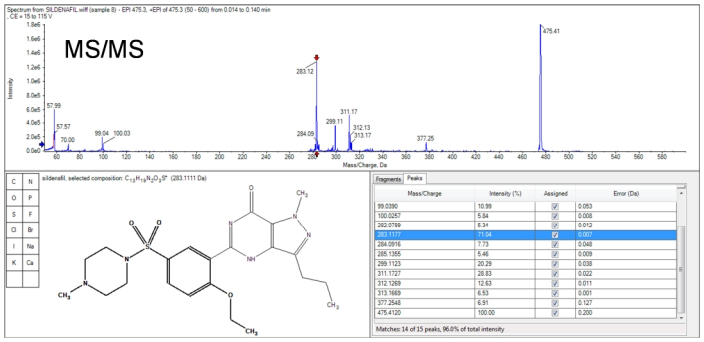

Figure 5. Automatic interpretation of the fragmentation pathway of Sildenafil in PeakView® software (CE ramp from 10 to110 V)

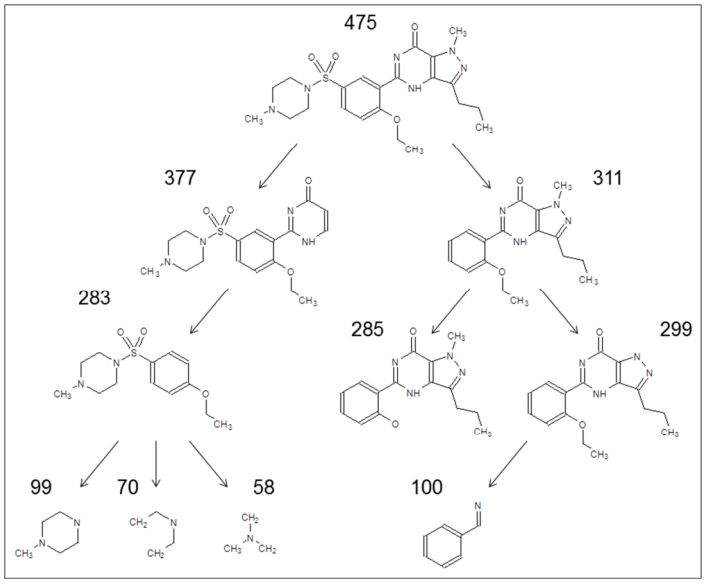

Figure 6. Tentative interpretation of the fragmentation pathway of Sildenafil based on QTRAP® full scan MS/MS data, proposed structures do not include the location of the charge and positions for double bond formation after H removal

An MS/MS spectrum of Sildenafil using the standardized settings for CE and CES was added to a mass spectral library.

Matrix spikes from 0.5 to 1000 µg/kg were analyzed using the MRM-triggered MS/MS approach. Confident identification of Sildenafil was achieved based on retention time matching and MS/MS library searching in MasterView™ software (Figure 8).

The retention error was well below 2.5% and the library search Purity above 95% with the exception of the 0.5 and 1000 µg/kg matrix spike. The MS/MS intensity at the lowest concentration was too low for library searching and some space charge was observed at the highest spiking level resulting in a change of the ion ratios and a lower Purity score of 74%.

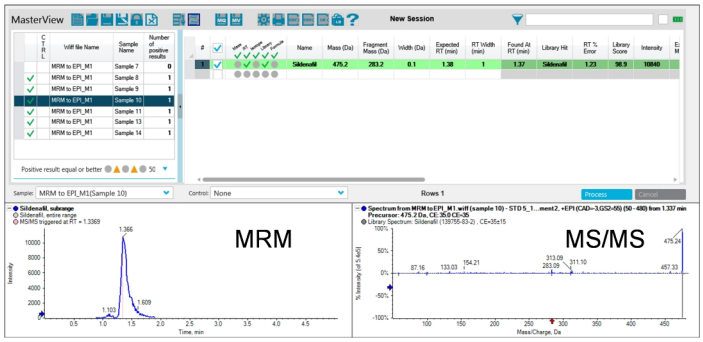

Figure 8. Automatic interpretation of the fragmentation pathway of Sildenafil in PeakView® software (CE ramp from 10 to110 V)

Summary

The method and data presented here showcase the fast, simple, and accurate solutions for the analysis of sildenafil in honey using the AB SCIEX 4000 QTRAP® system. The sensitivity and selectivity of LC-MS/MS allows minimal sample preparation and high throughput.

The method was validated as per European Commission Decision 2002/675/EC for a quantitative method. The decision limit (CCα) and detection capability (CCβ) was established at 0.36 µg/kg and 0.44 µg/kg, respectively. The linear dynamic range for quantitation was over 3 orders of magnitude for all 3 MRM transitions monitored. Confident compound identification was achieved by retention time matching, MRM ratio calculation and QTRAP® MS/MS library search.

References

1 FDA News Release 'Tainted products marketed as dietary supplements potentially dangerous' (2010) http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm236967.htm

2 Commission Decision 2002/657/EC 'concerning the performance of analytical methods and the interpretation of results' (2002)

3 M.J. Bogusz et al.: 'Application of LC–ESI–MS–MS for detection of synthetic adulterants in herbal remedies' Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 554-564

4 R.A. Kloner and J.P. Jarow: 'Erectile disfunction and sildenafil citrate and cardiologists' Am J Cardiol 83 (1999) 576-582

5 C.E. Dennehy et al.: 'Evaluation of herbal dietary supplements marketed on the internet for recreational use' Ann Pharmacother. 39 (2005) 1634-1639

6 J. Maria and M.I. Noordin: 'Fast detection of sildenafil in adulterated commercial products using differential scanning calorimetry' J Therm Anal Calorim 15 (2014) 1907-1914

7 N. Kannappan et al.: 'Method Development and Validation of stability indicating methods for assay of Tadalafil and Sildenafil Citrate by HPLC' Int.J. ChemTech Res. 2 (2010) 329-333

Publication number: 10880214-01

Headquarters
500 Old Connecticut Path, Framingham, MA 01701 USA
Phone 508-383-7700
www.absciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
www.absciex.com/offices