Confirmatory Analysis of Honey for Sildenafil and Tadalafil by LC-MS/MS

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ABSTRACT

This methodology outlines a rapid and robust confirmatory method for sildenafil also known as Viagra® and tadalafil also known as Cialis® in honey matrices. The method uses a simple dilution and filtration approach to accommodate a high sample throughput. The method developed in this LIB employs an LC-MS/MS instrument with electrospray ionization (ESI) in positive ion mode. The LC-MS/MS acquisition method can be completed within 4 minutes and is followed by a short column equilibration time. Method validation was performed by multiple analysts and was carried out over three non-consecutive days.

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INTRODUCTION

Impotence or erectile dysfunction (ED) is a fairly prevalent medical condition in men over the age of 40. Studies tend to show that as age increases, the likelihood of developing ED increases rapidly. The Massachusetts Male Aging Study conducted a survey of men 40 to 70 years old, and reported from its study that the combined prevalence of minimal, moderate and complete impotence was 52%[1-2]. In 2017, the John Hopkins institute stated that more than 18 million men in the United States are affected by ED. They also noted that the prevalence of erectile dysfunction was directly correlated with age, cardiovascular disease, diabetes, and lack of physical exercise[3].

It is not surprising that individuals with a history of heart disease have a higher risk for ED, as any disorder that impairs the arterial supply of blood adversely affects erectile function. In an evaluation of impotent men with organic causes for ED, 53% had evidence of an arterial lesion [4]. Therefore, it can be concluded that underlying health issues often are the cause of ED. These underlying symptoms are important factors that require careful considerations when these types of drugs are prescribed by physicians, which is why the United States requires a prescription when these drugs are administered to patients.

Tadalafil, sold under the brand name Cialis® and sildenafil, sold under the brand name Viagra® are both classified as phosphodiesterase 5 (PDE5) inhibitors. The PDE5 inhibitors cause relaxation of the blood vessels and increase blood flow. People may take PDE5 inhibitors to treat ED or pulmonary hypertension[5-8]. PDE5 inhibitors can often have adverse health risks and become potentially fatal if they are taken in combination with other drugs, or alcohol.

Tadalafil and sildenafil can be legally obtained in the United States only through a prescription by a licensed physician and are not allowed to be sold in the form of dietary supplements or foodstuff. The United States Food and Drug Administration (FDA) is aware of the importation of products identified as miracle honeys or similar names that are adulterated with these ED drugs. Sildenafil and tadalafil are not allowed in these products at any detectable concentration; therefore, any product which confirms the presence of these drugs is subject to regulatory action.

The FDA had developed two methods over 15 years ago. One method was for the detection of sildenafil, and the other for tadalafil. These methods are identified as LIB 4331 and LIB 4353[9-10]. Both of these older methods make use of different instrument platform, different columns, mobile phases composition and extraction procedures than this method. The previously developed methods did not have the ability to take advantage of technological advances which greatly enhance sample throughput. Whereas this method reduces instrument run time from as much as 21 minutes to 6 (accounting for instrument re-equilibration) minutes in certain cases. This allows for approximately 10 samples to be analyzed over an hour. Furthermore, extraction times are reduced and improved due to the use of modern equipment.

This study illustrates the method development and validation of a confirmatory method for the analysis of sildenafil and tadalafil in honey. The method employs a simple extraction procedure and is analyzed by a LC-tandem mass spectrometry (LC-MS/MS) technology with electrospray ionization.

EXPERIMENTAL

(Equipment and reagents have been provided for guidance. Equivalent products may be substituted as appropriate)

Equipment:

a) Mass Spectrometer: Sciex QTRAP 6500 and 6500+ Mass Spectrometers with a Turbo V source and electrospray ionization (Framingham, MA)
b) Liquid Chromatograph: Agilent 1260 Liquid Chromatograph (Santa Clara, CA)
c) Chromatographic Column: Agilent Zorbax Eclipse XDB-C18 column (1.8 µm, 4.6 X 50 mm)
d) Centrifuge: Must be capable of holding 50 mL centrifuge tubes, and approximately 4000 *g* (Fisher Scientific, Houston, TX)
e) 50 mL disposable polypropylene centrifuge tubes with screw cap lids (Sarstedt, Newton, NC)
f) Geno/Grinder capable of holding 50 mL centrifuge tubes (SPEX Sample Prep, Metuchen, NJ)
g) Auto-Sampler vials (Agilent Technologies)
h) Auto-Sampler screw caps (Agilent Technologies)
i) Polytetrafluoroethylene (PTFE) syringe filters: 0.45 µm X 13 mm (Fisher Scientific)
j) Disposable 1 mL syringes (Fisher Scientific)

Reagents and Standards:

a) Acetonitrile: LC/MS grade – (Fisher Scientific)
b) Acetonitrile: HPLC grade – (Fisher Scientific)
c) Methanol: HPLC grade – (Fisher Scientific
d) Water: LC/MS grade – (Fisher Scientific)
e) Water: 18 MΩ-cm or equivalent for extraction use only
f) Formic Acid: LC/MS grade – (Fisher Scientific)
g) Dimethyl sulfoxide (DMSO) – HPLC Grade (Fisher Scientific)
h) Sildenafil citrate – (Sigma Aldrich, St. Louis, MO)
i) Tadalafil – (Sigma Aldrich)
j) Sildenafil d_3 – (Cayman Chemical, Ann Arbor, MI)

METHOD

Suggested Reagent and Standard Preparation:

a) Extraction Solvent: 80/20 (v/v) acetonitrile (HPLC grade) and 18.2 MΩ-cm water or equivalent
b) Stock Internal Standard Solution (ISTD): 500 µg/mL for Sildenafil d_3 in DMSO. *(this solution should be stored in a refrigerator at ≤ 6 ˚C and have a shelf life of 1 year).*
c) Stock Analytical Standard Solutions: *(all stock standard solutions were prepared in DMSO and stored in a refrigerator at ≤ 6 ˚C and have a shelf life of at least 1 year)*
   i. 500 µg/mL for sildenafil
   ii. 500 µg/mL for tadalafil

d) Intermediate Internal Standard Solution as described in Table 1: *(this solution should be stored in a freezer at ≤ 0˚C and has a shelf life of at least 6 months)*

Table 1: Preparation of an Intermediate ISTD solution in Methanol

Internal Standard	Conc. Of Stock Solution	Volume Used	Final Volume	Final Conc.
Sildenafil d_3	500 µg/mL	500 µL	100 mL	2.50 µg/mL

e) Intermediate Analytical Standard Solution as described in Table 2: (*this solution should be stored in a freezer at ≤ 0˚C and has a shelf life of at least 6 months)*

Table 2: Intermediate Analytical Standard Solution in Methanol

Analytical Standard	Conc. Of Stock Solution	Volume Used	Final Volume	Final Conc.
Sildenafil	500 µg/mL	500 µL	100 mL	2.50 µg/mL
Tadalafil	500 µg/mL	500 µL	100 mL	2.50 µg/mL

Sample Preparation and Extraction:

1. Measure 1.00 gram (±0.03 grams) of homogenized honey into a 50 mL centrifuge tube.
2. Fortify all quality controls and unknown samples with 200 µL of the intermediate ISTD.
3. Fortify all matrix spikes and the matrix matched standard with 200 µL of the intermediate analytical standard.
4. Add 20 mL of the extraction solution and shake on the Geno/Grinder (2 minutes at 1400 strokes per minute).
5. Centrifuge the samples at 4000 *g* at ambient temperature for 5 minutes.
6. Filter approximately 1 mL of the supernatant through a PTFE syringe filter into an autosampler vial for analysis.

Chromatography:

Table 3: HPLC Gradient

Time (min)	Flow (µl/min)	%Mobile Phase A	% Mobile Phase B
0.0	750	90	10
2.00	750	20	80
4.00	750	20	80
4.10	750	90	10

**A 2-minute post run was used to re-equilibrate the column.*

Mobile Phase A: 0.1% formic acid in LC-MS grade water
Mobile Phase B: 0.1% formic acid in LC-MS grade acetonitrile
Column temperature: 40˚C
Injection volume: 5 µL
Autosampler temperature: 5˚C

Mass Spectrometry using Electrospray Ionization (ESI):

The mass spectrometer was tuned and calibrated in positive ion detection mode according to the manufacturer's instructions. The instrument was optimized by infusing the standards with mobile phase (50:50) 0.1% formic acid in water and 0.1% formic acid in acetonitrile, at an HPLC flow rate of 750 µL/min to optimize electronic voltages and gas flows. The triple quadrupole mass spectrometer was equipped with an electrospray ionization (ESI) source.

The source heater was set to 650 ˚C. The curtain gas, ion source gas 1, and ion source gas 2 were set to 20 psi, 40 psi, and 50 psi respectively. Electrical voltages were optimized for the capillary voltage at +5000 volts, the entrance potential at 5 eVolts, collision cell exit potential at 12 eVolts, and the declustering potential at 40 volts. The collision energy and SRM transition information are listed in Table 4[9-11].

Table 4: Collision energy and SRM transition information

Analyte	Q1	Q3	Time (minutes)	Collision Energy (V)
Sildenafil 1	**475**	**283**	**2.4**	**50**
Sildenafil 2	475	100	2.4	35
Sildenafil 3	475	311	2.4	42
Tadalafil 1	**390**	**268**	**2.9**	**42**
Tadalafil 2	390	135	2.9	24
Tadalafil 3	390	262	2.9	37
Sildenafil d_3	478	283	2.4	50

** Ions in bold are used to generate semi-quantitative results.*

Data Interpretation:

Although the method described herein is for qualitative purposes only, an internal standard was utilized to generate semi-quantitative results. The isotopically labeled sildenafil internal standard was used for both sildenafil and tadalafil. The semi-quantitative value should only be used for an estimated value to determine if the amount detected is above the established method detection limit.

For positive confirmation all product ions must be detected, the associated chromatographic peak must exhibit a retention time within ± 5% of the retention time of the calibration standard, and the product ion ratios must be within 20% of the product ion ratio obtained from the calibration standard[12]. All product ions must exhibit as signal to noise ratio of ≥ 3, and the semi-quantitative value should also be greater than or equal to the method detection limit.

Analysis of Reference Materials and Commercial Products:

Reference materials used were consumable honey products that were locally commercially available and previously analyzed honey products. Samples were analyzed using a matrix-matched extracted standard that was previously screened and determined to be free of the targeted residues. It should also be noted that a royal jelly honey sample

which contained an incurred residue was analyzed to verify that the methodology could confirm residues in honey or honey type matrices.

Limits of Detection Studies:

The estimated method detection limits (MDL) for each analyte were determined on the basis of replicate analyses (n=7). The MDL of each analyte was calculated by the multiplication of the standard deviation by the student's t-value at the 99% confidence level [13].

RESULTS AND DISCUSSION

Method Optimization:

Method optimization consisted of a 2-fold process. The first step was to develop an LC-MS/MS instrument acquisition method that could achieve and maintain proper sensitivity throughout multiple injections and not inhibit sample throughput with extended analysis times.

Sildenafil and tadalafil were optimized for response on the mass spectrometer by infusing them with the mobile phase to find the optimal voltages, temperatures, and gas flows. After looking at the chemical structure of the drugs, it was believed the analytes would be retained well by employing reverse phase chromatography. The majority of chemical assays performed at the Arkansas laboratory use C18 analytical columns. The Agilent C18 column that was used in this method was initially evaluated based upon its availability at our laboratory. This column performed well during the initial method development with specific regards to its reproducibility. The reproducibility was evaluated by examining the %RSD values for retention times and %RSD values for peak area. The %RSD values for retention time were consistently <2% and %RSD for peak area < 8% when analyzing ≥6 consecutive injections at the same concentration level. The column also demonstrated acceptable peak symmetry as shown in Figure 1. Since the initial column evaluated provided acceptable peak symmetry, and reproducibility it was chosen for the analysis and no other columns were evaluated.

A four-minute chromatographic run was developed that includes an organic flush at the end of the analysis to help remove residual buildup on the analytical column left by the honey matrix. This has been shown to help with reproducibility and ruggedness with the method. The mobile phase gradient is followed by a 2-minute chromatographic equilibration for the next sample in the sequence.

Once the instrument acquisition method was developed, our attention shifted to the extraction process. Because the concentrations of the drugs typically present in intentionally adulterated samples analyzed are at the parts per million or parts per thousand levels, it was believed that sample dilution and filtration could be used instead of employing more costly and complex cleanup procedures. This would also allow less labor-intensive measures of sample cleanup, which in turn increases sample throughput.

It was also discovered that the use of a Geno/Grinder agitated the honey samples more effectively than a multi-vortexer or shaking by hand. This not only reduces sample preparation time, but also did a much more efficient job of dissolving the honey samples

into solution. Even honey matrices that had proven in the past to be difficult to dissolve into water using multi-vortexers were easily dissolved into solution.

During the analysis it became apparent that the different chemical properties associated with certain types of honey matrices would either enhance or suppress recoveries. As a result, an isotopically labeled internal standard was used. Although the method that was developed is for qualitative purposes, the authors wanted the method to be capable of semi-quantitative data. This would help in establishing a method detection limit and give an estimated value for samples. The isotopically labeled sildenafil corrected the large fluctuation of response from matrix to matrix for sildenafil, and to a smaller extent tadalafil as well. However, it was noticed in the validation that tadalafil still saw some fluctuation in recoveries. Unfortunately, at the time of the validation an isotopically labeled tadalafil was not available.

Method Validation:

Three different honey matrices were evaluated when performing the method validation. A floral honey, an Acadia honey, and a wildflower honey were used in validation. Validation was performed utilizing the U.S. Food and Drug Administration guidance for industry for the mass spectrometry confirmation and identification of animal drug residues, and the FDA guidelines for chemical validation. The validation procedure consisted of a total of 39 matrix spikes and 36 matrix blanks. The semi-targeted calculated values to establish the limit of detection are illustrated in Table 5.

All 36 matrix spikes analyzed met the required confirmation criteria for both residues of interest. Nine of these spikes were at 250 ng/g, fifteen spikes were at 500 ng/g and nine other spikes were at 1000 ng/g. Furthermore, no false positives were observed in the 36 matrix blanks that were analyzed. A royal jelly incurred residue was analyzed using this method. The sample was analyzed and confirmed the presence of tadalafil.

CONCLUSION:

This method was written in response to an upcoming assignment to potentially analyze > 400 samples for regulatory purposes. As a result, a quick and robust confirmatory method was needed that met the needs of the U.S. Food and Drug Administration. The method described herein uses a simple sample dilution and filtration process to prepare the samples for analysis, which is followed by a quick LC-MS/MS acquisition.

During the validation study semi-quantitative results were generated for statistical purposes. However, the calculated results were extremely accurate in all matrices for sildenafil, and in certain matrices for tadalafil. If an isotopically labeled tadalafil internal standard had been utilized, it is the authors belief that the calculated concentrations would have mirrored those for sildenafil. Therefore, if quantitative results were ever desired in the future, the method could be potentially used for quantitation. Since the presence of these drugs are not allowed in food products or dietary supplements at any detectable concentration, only a qualitative method is needed at this time.

The adulterated products which will be tested are designed to have a concentration of one or more of the drugs that would illicit an increase of blood flow and combat current ED issues of the subject ingesting the product. These concentrations far exceed the method detection limits as seen in Table 5; therefore, there was no need to test how low the method could detect these drugs. With the simplicity, high-throughput, and robustness of this

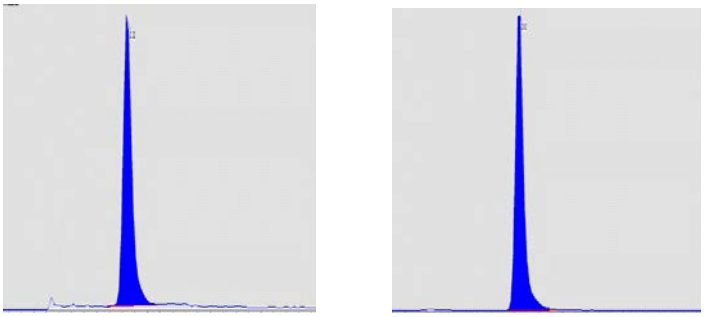
method, it makes it a viable option for laboratories performing analysis of honey for sildenafil and tadalafil.

Table 5

Analyte	MDL ng/g	Average recovery ng/g (Target concentration)	Average Percent Recovery	%RSD
Sildenafil	23.5	253 (250)	101	3.28
Tadalafil	237	301 (250)	121	27.9

Figure 1: Chromatograms of Fortified Blank at 250 ng/g

Sildenafil Tadalafil



References:

1. Feldman HA, G. I., Hatzichristou DG, Krane RJ, McKinlay JB, Impotence and its medical and psychosocial correlates: Results of the Massachusetts Male Aging Study. *J. Urology* **1994,** *151*, 54-61.
2. Johannes, C. B. A., A.B.; Feldman, H.A.; Derby, C.A.; Kleinman, K.P.; McKinlay, J.B., Incidence of erectile dysfunction in men 40 to 69 years old: longitudinal results from the Massachusetts male aging study. *Journal of Urology* **2000,** *163*, 460-463.
3. Selvin, E. B., A.; Platz, E.; 18 Million Men in the United States Affected by Erectile Dysfunction. https://www.jhsph.edu/news/news-releases/2007/selvin-erectile-dysfunction.html (accessed 12/29/2020).
4. Kloner, R. A. Z., R.M, Cardiovascular effects of sildenafil citrate and recommendations for its use. *The American Journal of Cardiology* **1999,** *84*, 11-17.
5. Sissons, B. What are PDE5 inhibitors? https://www.medicalnewstoday.com/articles/list-of-pde5-inhibitors (accessed 12/29).
6. Codevilla, C. F. C., T; Bergold, A.M, A review of analytical methods for the determinatin of four new phosphodiesterase type 5 inhibitors in biological samples and pharmaceutical preparations. *Brazillian Journal of Pharmaceutical Sciences* **2013,** *49*.
7. Inae Jang, J.-u. L., Jung-min Lee, Beom Hee Kim, Bongjin Moon, Jongki Hong, and Han Bin Oh, LC-MS/MS Software for Screening Unknown Erectile Dysfunction Drugs and Analogues: Artificial Neural Network Classification. Peak-Count Scoring, Simple Similarity Search, and Hybrid Similarity Search Algorithms. *Anal. Chem.* **2019,** *91*, 9119-9128.
8. Noordin, J. M. a. M. I., Fast detection of sidenafil in adulterated commercial products using differentail scanning calorimetry. *Thermal Analysis and Calorimetry* **2013,** *115*, 1907-1914.
9. Karbiwynk, C., Determination of Sildenafil in Tablets by LC/MS/MS. *United States Food and Drug Administration Laboratory Information Bulletin 4331*.
10. Holcomb, J., LC/MS^3 Screen for Tadalafil in Dietary Supplement Tablets. *United States Food and Drug Administration Laboratory Information Bulletin 4353*.
11. Praveen K. Sharma, N. B., Prasanth Joseph, Manoj G Pillai, and Andre Schreiber, Fast, Robust and Reliable Method for the Identification and Quantitation of Sildenafil Residue in Honey using LC-MS/MS.
12. U.S. Food and Drug Administration. Guideline for industry: Mass spectrometry for confirmation of the identity of animal drug residues.
. Fed. Regist., 2003; Vol. 68, pp 25617- 25618.
13. *Guidelines for the Validation of Chemical Methods in Food, Feed, Costmetics, and Veterinary Products 3rd Edition*. 3rd edition ed.; US FDA: Washington DC, 2019.