METHODOLOGY FOR DETECTION AND STRUCTURAL CHARACTERIZATION OF PHOSPHODIESTERASE-5 (PDE-5) INHIBITOR ADULTERANTS IN AN HERBAL COFFEE PRODUCT

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INTRODUCTION

In recent years, there has been increased interest by consumers for foods that are considered "natural" and that can provide added health benefits. This convergence in consumer desires for improved health and wellness has resulted in the continued growth of the functional/fortified food business.[1]

Functional foods, are defined as foods that, provide the benefits of nutrients as well as beneficially affecting one or more target functions in the body and thus acting to promote health.[2]

Functional foods require a safe supply of ingredients with verified authenticity. Analysis of coffee products claiming to be a natural solution to erectile dysfunction and to be derived from herbs including Tonkat Ali, have been found to contain synthetic PDE-5 inhibitors (Figure 1). The presence of undeclared ingredients can cause risks to consumers (Figure 2). PDE-5 inhibitors can interact with nitrates found in some prescription drugs and may lower blood pressure to unsafe levels. Male patients with health conditions including high cholesterol, or heart disease often take nitrates and would be unaware of the risks of consuming this adulterated product.[3-4]

A rapid initial screen using direct analysis in real time (DART) with a single quadrupole mass detector was performed followed by confirmation of results for the suspected adulterated products using UPLC-HRMS and PDA. Ions corresponding to the protonated molecular ions of caffeine, sildenafil and tadalafil at *m/z* 195, 475 and 390 respectively, were observed in the DART-MS spectrum resulting from the analysis of one coffee sample. In a second coffee sample, ions consistent with other PDE-5 inhibitors previously reported in the literature were observed with the most prominent ion being *m/z* 505. Based on this information, coffee samples were extracted with solvent to perform further study.

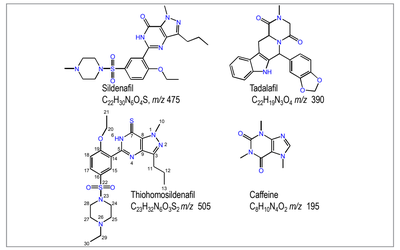

Figure 1. *Empirical formulas and structures for sildenafil, tadalafil, thiohomosildenafil and caffeine that were analyzed in the study.*

METHODS

Initial Rapid Screen

Instrumentation and software

Ambient ionization was performed using the DART source with subsequent mass detection using the ACQUITY QDa (Figure 6). MassLynx Software was used for data acquisition and interpretation. The DART interface software was used to control the ionization settings.

DART conditions

Ionization mode: Positive Temp.: 400 °C
Grid voltage: 350 V

MS conditions

MS system: ACQUITY QDa (Performance option)
Ionization mode: Positive Mass range: 90 to 600 Da
Cone voltage: 15V (low) or 55-80V (high) Sampling rate: 5 Hz

Sample Information

Two coffee samples (Figure 2) were obtained from internet vendors. The coffee was sampled directly using a melting point capillary for analysis with the DART-MS in positive ion mode, and helium gas heated to 400°C.

Confirmation by UPLC-PDA/HRMS Analysis

Instrumentation and software

ACQUITY H-class and Xevo G2-XS QTOF with the UNIFI Scientific Information System Software.

MS conditions

Ionization mode: Positive.
Capillary Voltage: 3.5 kV
Cone Voltage: 40 V
Source/Desolvation Temp. 150 °C/400 °C:
Cone/ Desolvation Gas Flow-rate: 50/1000 L/Hr
Mass range: 50 to 950 Da. CE: 20-45 eV ramp or 35 eV
MS Experiment: Simultaneous collection of high and low collision energy (CE) spectra (MS^E mode).

Sample Preparation

A portion of the coffee (12.5 g) was weighed into a centrifuge tube (50 mL) and extracted with 50:50 acetonitrile/water (40 mL). The sample was shaken (10 min), centrifuged at 3000 rpm (5 min) and an aliquot was filtered into a vial in preparation for analysis. The samples were diluted 1:100 prior to analysis.

UPLC Conditions

Column: CORTECS C_{18} 2.1 x 100 mm, 1.6 µm
Solvent A: 0.1% formic acid in water Solvent B: 0.1% formic acid in acetonitrile
Flow rate: 0.50 mL/min: Column temp.: 50 °C:
Injection volume: 1.0 µL
PDA detection: 210 to 400 nm
Gradient conditions: 0 min 5% B, 10 min 95% B, 11 min 95% B, return to initial conditions.

Figure 2. *Photograph of coffee sample. The package did not disclose the presence of the PDE-5 adulterants however there was a consumer responsibility statement listing where this food product would not be recommended.*

RESULTS AND DISCUSSION

Initial Rapid Screen using DART/QDa Mass Detector

Sample 1 was rapidly analysed without sample preparation using the DART/QDa system. Ions consistent with protonated molecular ions of caffeine (*m/z* 195), tadalafil (*m/z* 390) and sildenafil (*m/z* 475) were observed in the mass spectrum from the coffee sample (Figure 3). Two MS experiments were acquired simultaneously with low and high cone voltage settings. The fragmentation patterns of the PDE-5 inhibitors have been widely reported in the literature.[5,6] The diagnostic fragments with *m/z* 283, 311 and 377 were clearly visible in the high cone voltage spectrum of sample 1 providing increased confirmation of the presence of a PDE-5 inhibitor such as sildenafil in this sample, Figure 4. In Sample 2, a prominent ion with *m/z* 505 was detected, Figure 5. This ion is characteristic of sildenafil analogue adulterants that were previously reported.[5-8]

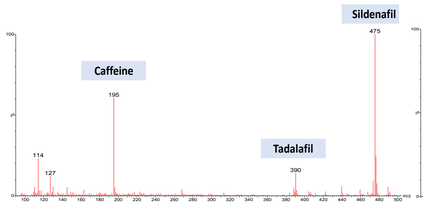

Figure 3. *DART/QDa MS analysis of Sample 1 at low cone voltage showing characteristic ions for caffeine, tadalafil and sildenafil.*

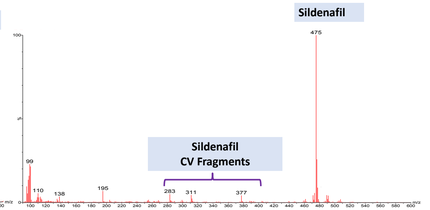

Figure 4. *DART/QDa MS analysis of Sample 1 at high cone voltage showing characteristic fragment ions m/z* ***m/z*** *283, 311 and 377 for sildenafil.*

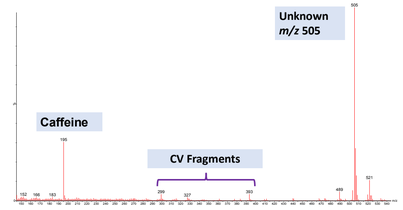

Figure 5. *DART/QDA MS analysis of Sample 2 showing prominent m/z 505 ion.*

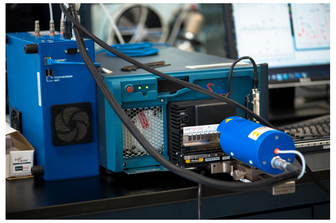

Figure 6. *DART/QDa single quadrupole mass detector shown configured for automated multi-sample analysis.*

Confirmation using LC-PDA-HRMS

UNIFI software employs the use of a library to help identify detected compounds. The Scientific Library has the ability to store structures and other information associated with the compounds such as retention time, masses of molecular species, fragment ions and isotope patterns. Data from the analysis of coffee was componentized and a search was made for a target list of PDE-5 adulterants created from the Scientific Library. Using all of this information together greatly helps improve the confidence of the identifications and reduces the number of false positives. The identity of the PDE-5 adulterants that were tentatively identified in the DART/QDa analysis, was confirmed from review of the UPLC-HRMS data. Authentic standards were used to provide further retention time, accurate mass and fragment matching for caffeine, sildenafil and tadalafil (data not shown). The Xevo G2-XS QTof was operated in a manner where all precursor and product ion data is acquired in a data independent acquisition mode called MS^E which comprehensively catalogs component precursor and fragment ions within a single injection. High and low CE spectra for sildenafil are shown in Figure 7. PDA spectral matching with authentic standards increased the confidence in the identifications (data not shown).

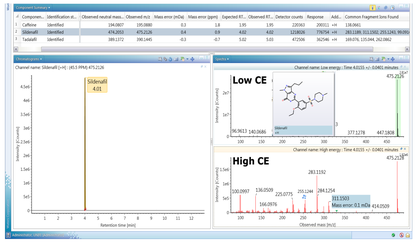

Figure 7. *Confirmation of the sildenafil and tadalafil tentatively identified in the DART/QDa screening experiments of Sample 1. Sildenafil chromatogram and high/low CE spectra shown.*

A PDE-5 Analogue Adulterant Confirmed in Coffee Sample

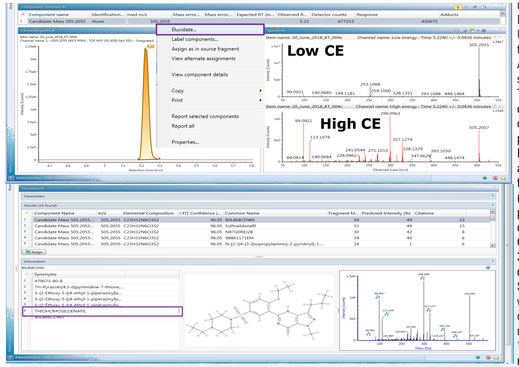

Figure 8. *Mass spectra and elucidation of m/z 505 (top) and ChemSpider proposals for this unknown compound (beneath). Thiohomosildenafil with its elemental composition $C_{23}H_{32}N_6O_3S_2$ is the most likely proposal.*

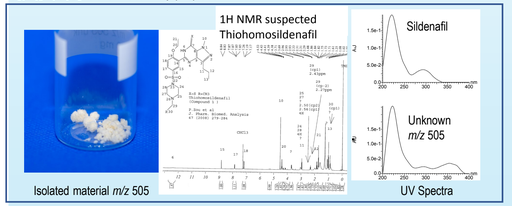

Figure 9. *Image of isolated material (pale yellow solid), ^{1}H NMR spectrum and UV spectra for sildenafil and unknown with m/z 505.*

The initial DART/QDa screening data indicated that an unknown component with *m/z* 505 was present in Sample 2. Further analysis using the structural elucidation tools in the UNIFI software was performed. The software links directly to the ChemSpider database which is a free structural database.[9] A search of the database was performed and the most likely structure proposal was the PDE-5 analogue thiohomosildenafil. The Fragment Match algorithm was used to perform a comparison between the fragments observed in the high collision energy spectrum and the theoretical fragments possible with the proposed structure.

Fragment matches observed in the spectrum are ranked within a specified mass tolerance and other scoring parameters. In the case of thiohomosildenafil, 34 fragment matches (highlighted in blue in Figure 8) were observed indicating a high probability that the unknown and thiohomosildenafil share common structural features.

Cone voltage fragments detected in the DART/QDa (*m/z* 393, 327 and 299, Figure 7) were also observed in the high CE data of the HRMS results.

Solid/liquid extraction was performed on Sample 2 and *m/z* 505 was isolated using mass directed preparative supercritical fluid chromatography (SFC) (results not shown). Sufficiently pure material was collected to enable structural assignments using ^{1}H, ^{13}C NMR spectroscopic studies, Figure 9. The H and C nuclei before S22 have very similar chemical shift values to those peaks published by Zou et al.[8] However the proton and carbon chemical shift values after S22 cannot be matched to this journal reference. It is hypothesized that the piperazine peaks are missing due to the existence of salts at the basic nitrogen atoms which can lead to peak broadening. Future experiments with the free base may allow for the peaks to reemerge for this group. However the signal for the remainder of the proposed structure matched exactly with the acquired NMR spectra.

The prominent HRMS fragments detected, could be correlated exactly with those observed by Zou et al. In addition, the UV spectrum (Figure 9) had the same absorption band and UV maxima reported previously.[8]

Quantitative analysis of the component with *m/z* 505 in the coffee was performed using the UV trace at 220 nm and sildenafil as a calibration reference compound. Each sachet was found to contain 60 mg of the analogue, which would be a therapeutic dose of sildenafil.

CONCLUSION

- PDE-5 inhibitors are effective drugs used in the treatment of erectile dysfunction; however their use requires a prescription from a licensed physician and their hidden presence in functional foods could lead to serious health issues.
- In the current study the DART coupled with the ACQUITY QDa was used for the rapid screening of coffee samples suspected to contain adulterants.
- Direct analysis of herbal coffee samples using DART-QDa, without any sample preparation or chromatography, allows samples to be analyzed very quickly and characteristic ions detected.
- PDE-5 inhibitors were detected in the coffee samples analyzed, despite none being declared on the label or the enclosed product information. Inaccurate or insufficient labeling increases the likelihood of adverse side effects.
- Accurate mass measurements provided by ACQUITY UPLC coupled with Xevo G2-XS QTof enables efficient identification through reference entries in a database.
- Unknown components can be efficiently isolated in the amounts needed for structural elucidation using preparative SFC.
- The isolated unknowns can be obtained in their pristine forms, thus enabling the simple application of HRMS and ^{1}H, ^{13}C NMR spectroscopic studies, allowing for structural elucidation.

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