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

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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 desire 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. The presence of undeclared ingredients can cause risks to consumers. PDE-5 inhibitors can interact with nitrates found in some prescription drugs and may lower blood pressure to unsafe levels. Patients with health conditions including high cholesterol or heart disease often take nitrates and would be unaware of the risks of consuming adulterated products.[3-4]

A rapid initial screen of coffee samples was carried out using direct analysis in real time (DART) with a single quadrupole mass detector followed by confirmation of results using UPLC-HRMS and PDA after extraction.

METHODS

Initial rapid screen using DART-QDa

Two coffee samples were obtained from internet vendors. The coffee was sampled directly using a melting point capillary for analysis using DART coupled with ACQUITY QDa (Figure 1). The DART interface software controlled the ionization settings and MassLynx was used for data acquisition and data processing.

DART conditions: positive ionization mode, temp. 400°C and grid voltage 350V

ACQUITY QDa (Performance option) conditions: positive ion mode, mass range *m/z* 90 to 600, cone voltage 15V (low) or 55-80V (high) and sampling rate 5 Hz

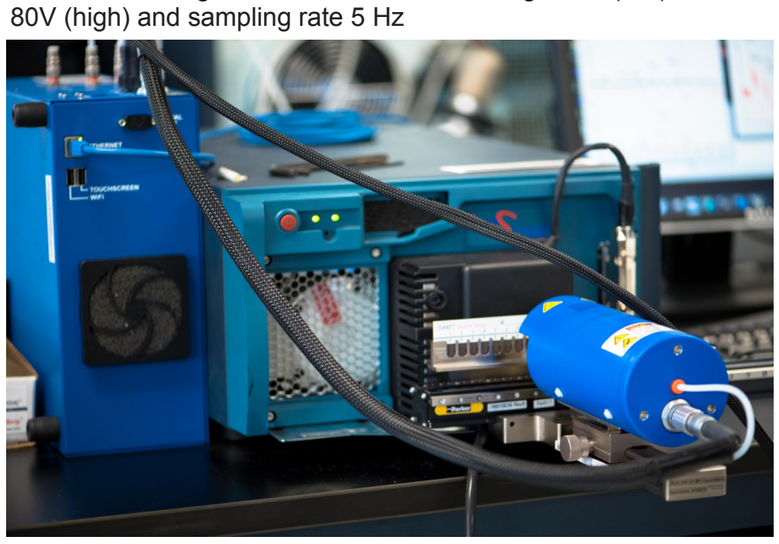

Figure 1. DART/QDa single quadrupole mass detector shown configured for automated multi-sample analysis.

Confirmation by UPLC-PDA/HRMS Analysis

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 and diluted 1:100 prior to analysis

ACQUITY H-Class UPLC: CORTECS C18, 2.1 x 100 mm, 1.6 µm, solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid in acetonitrile, gradient: 0 min 5% B, 10 min 95% B, 11 min 95% B, return to initial conditions. Flow rate: 0.50 mL/min, column temp.50 °C, inj volume: 1.0 µL and PDA detection 210 to 400 nm

Xevo G2-XS QToF conditions: electrospray in positive ion mode, capillary voltage 3.5 kV, cone voltage 40 V, source/desolvation temp. 150/400 °C, cone/desolvation gas flow 50/1000 L/Hr, mass range *m/z* 50 to 950, CE 20-45 eV ramp or 35 eV and MS^E mode.

UNIFI Scientific Information system software.

RESULTS AND DISCUSSION

Initial Rapid Screen using DART/QDa

Sample 1 was analyzed using the DART/QDa system. Two MS experiments were acquired simultaneously with low and high cone voltage settings. Ions consistent with protonated molecular ions of caffeine (*m/z* 195), tadalafil (*m/z* 390) and sildenafil (*m/z* 475) and associated fragment ions were observed in the mass spectra (Figure 3). The fragmentation patterns of the PDE-5 inhibitors have been widely reported in the literature.[5,6] In Sample 2, a prominent ion at *m/z* 505 was detected, which is characteristic of sildenafil analogue adulterants previously reported.[5-8]

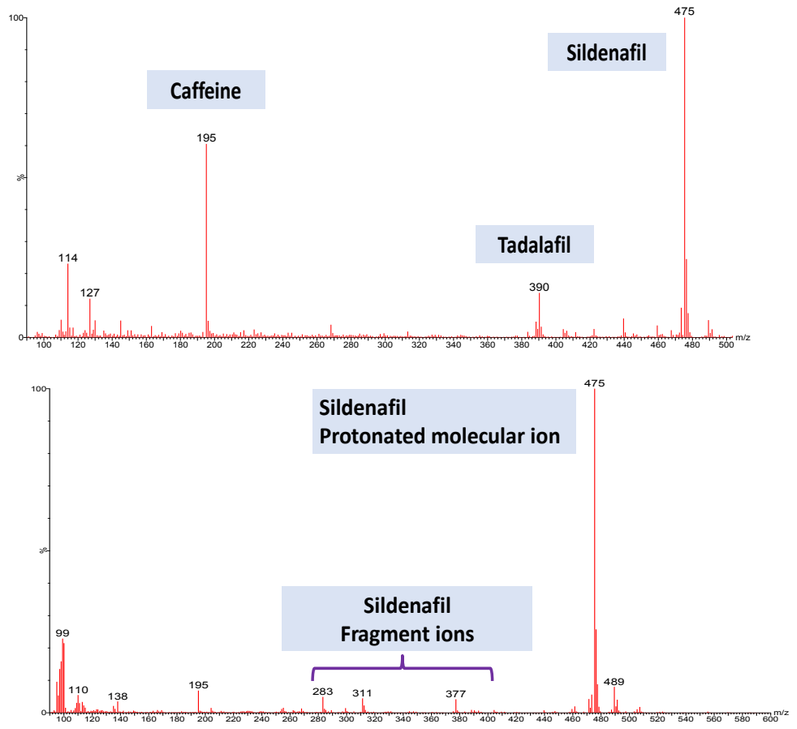

Figure 3. Mass spectra from DART/QDa analysis of Sample 1 showing protonated molecular ions and fragments ions for caffeine, tadalafil and sildenafil.

Confirmation of the PDE-5 adulterants detected by DART-QDa 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 extracts were 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 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 that comprehensively catalogs component precursor and fragment ions within a single injection (*e.g.* Figure 4).

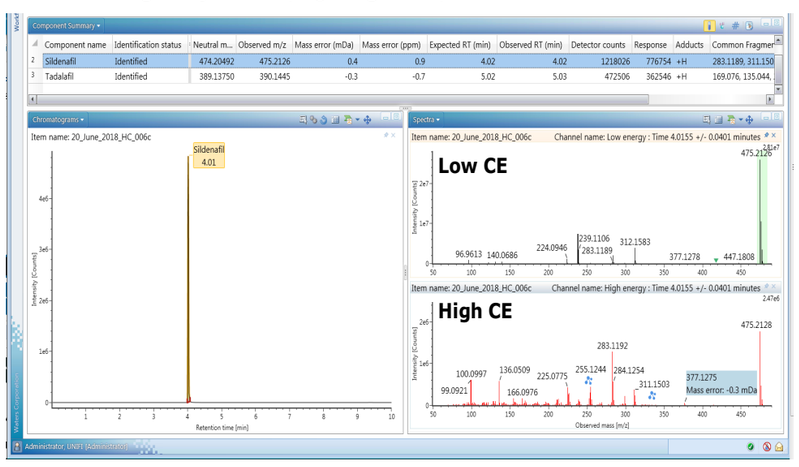

Figure 4. Confirmation by LC-HRMS of the earlier assignment of component as sildenafil from DART-QDa screening experiments

Identification of unknown PDE-5 analogue using LC-PDA-HRMS and NMR spectroscopy

Further data analysis was performed using the structural elucidation tools in the UNIFI software, which links directly to the ChemSpider structural database.[9] A database search was performed and the most likely structure proposed was the PDE-5 analogue, thiohomosildenafil. The Fragment Match algorithm was used to perform a comparison between fragments observed in the high collision energy spectrum and the theoretical fragments possible with the proposed structure. In this case, 34 fragment matches (highlighted in blue in Figure 5) were observed indicating a high probability that the unknown and thiohomosildenafil share common structural features.

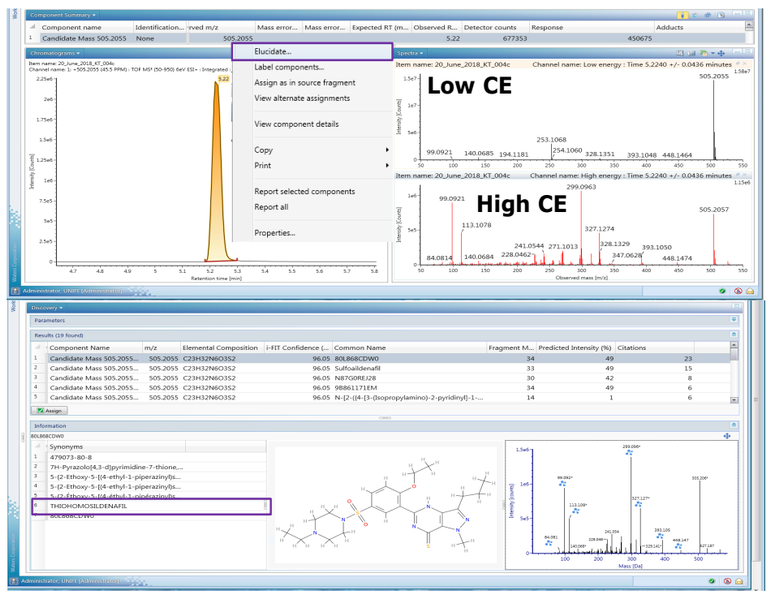

Figure 5. Chromatogram and mass spectra for the unknown peak, proposals from ChemSpider for the identification and interrogation of candidate by Fragment Match.

The unknown compound was isolated from the extract using mass directed preparative supercritical fluid chromatography. Sufficiently pure material was collected to enable structural assignment using ^1H and ^{13}C NMR spectroscopy (*e.g.* Figure 6). The H and C nuclei before S22 have very similar chemical shift values to those peaks published for thiohomosildenafil but the proton and carbon chemical shift values after S22 cannot be matched to this reference.[8] 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. 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*.[8] In addition, the UV spectrum (Figure 6) had the same absorption band and UV maxima reported previously.[8]

Quantitative analysis of the analogue in 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.

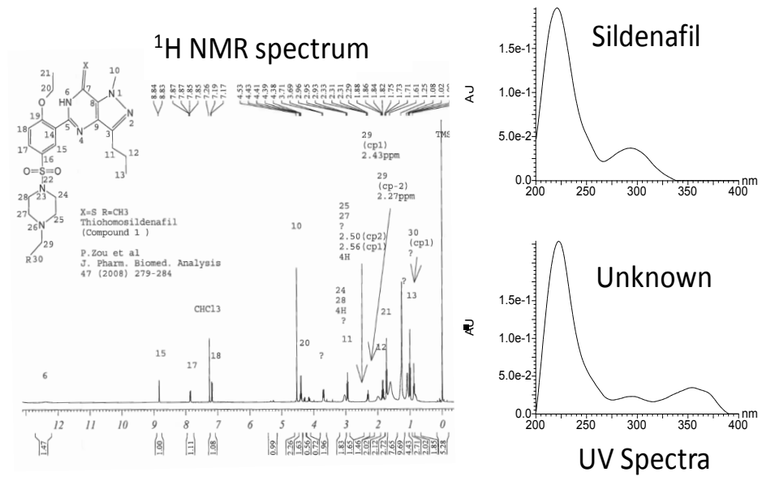

Figure 6 ^1H NMR and UV spectra for the unknown, isolated from Sample 2, suspected to be thiohomosildenafil

CONCLUSIONS

- **Direct analysis of herbal coffee samples using DART-QDa, without any sample preparation or chromatography, allowed samples to be analyzed very quickly.**
- **PDE-5 inhibitors were detected in the coffee samples, despite none being declared on the label or product information.**
- **Accurate mass measurements provided by LC-MS on Xevo G2-XS QTof enabled efficient identification through comparison with reference entries in a database. Identities were confirmed by comparison with reference standards.**
- **Other unknown components were efficiently isolated using preparative SFC for structural elucidation.**
- **Structural elucidation was carried out analysis of the isolated fractions using HRMS and ^1H and ^{13}C NMR.**

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