FOOD ANALYSIS

ANALYTICAL STRATEGY FOR THE DETECTION OF UNDECLARED STIMULANTS AND ANORECTIC COMPOUNDS IN DIETARY SUPPLEMENTS USING THE AGILENT 6550 IFUNNEL Q-TOF LC/MS

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Authors

Jennifer P. Pascali, PhD.
dtoLABS, Analytical Excellence Center.

Anna Calì,
Agilent Technologies.

ABSTRACT

The growing market of herbal remedies worldwide could pose severe problems to consumers' health due to the possible presence of potentially harmful, undeclared synthetic substances or analogues of prescription drugs. The present work shows a simple but effective approach to unequivocally identify synthetic anorectic compounds in allegedly 'natural' herbal extracts, by exploiting Q-TOF LC/MS technology, with the help of the Agilent's Forensic Accurate Mass Library (Forensic PCDL AM), Agilent's Auxiliary Softwares, MFG (Molecular Formula Generator) and MSC (Molecular Structure Correlator).

INTRODUCTION

Adulteration of botanical food supplements with undeclared synthetic drugs to improve their effectiveness is becoming a widespread and mostly uncontrolled problem in many countries.

Among them, slimming functional food, such as teas, coffee and 'natural' pills, are commercially readily available to a vast population, not always aware of the health risks. At the moment, there are no established analytical protocols for the systematic detection of synthetic adulterants in these products but a large body of literature is converging to the target screening approach, either by liquid chromatography or gas chromatography.

However, this approach may not be suitable due to the sheer number of chemicals. Furthermore, an accurate mass approach during acquisition allows for what is generally called "retrospective analysis" in post-acquisition data mining. For this reason, high-resolution high-accuracy mass spectrometry (HRMS), enabling accurate-mass determination of ionic species (and metabolites) with an accuracy of at least 4 decimal digits, offers the potential to overcome the limitations of multi-target screening.

The concept of HRMS is not novel at all and in recent years its use has become more widespread due to technological improvements. The present work shows a simple but effective approach to detect synthetic anorectic compounds in allegedly 'natural' herbal extracts by exploiting Q-TOF LC/MS technology in a simple procedure with the help of the Agilent's Forensic accurate mass Library [Forensic PCDL AM].

Briefly, the MS scan acquisition and the 'Find by formula' data mining algorithm were firstly used to identify possible adulterant agents in sample material. Then, acquisition in 'All Ion' mode approach, with the evaluation of coelution scores of detected fragments, was helpful in reducing the number of matched hits to a few candidates. At this point, the MassHunter MSC (Molecular Structure Correlator) program was used to correlate accurate mass MS/MS fragment ions for specific candidate compounds with one or more proposed molecular structures for that compound. Finally, the candidates were confirmed by the use of analytical standards and quantitative data was produced.

In this way, the number of possible candidates was substantially reduced, minimizing costs and time-consuming data mining. The overall procedure confirmed the presence of stimulants such as caffeine, as well as undeclared sibutramine, a synthetic anti-obesity drug, in two out of the three slimming products.

EXPERIMENTAL

Instrumentation

MS Parameters	
Instrument	*Agilent 6550 iFunnel Q-TOF LC/MS*
Ionization Mode	*ESI +*
Drying Gas Temperature	*125°C*
Drying Gas Flow Rate	*20 L/min*
Nebulizer Gas Pressure	*40 psi*
Sheath Gas Temperature	*325°C*
Sheath Gas Flow Rate	*12 L/min*
Vcap Voltage	*3500 V*
Nozzle Voltage	*300 V*
MS scan centroid + profile	*40-1000 m/z, rate 1 spectra/sec, 5993 transient/spectrum*
MS ALL ion	*40-1000 m/z, rate 1 spectra/sec, 5993 transient/spectrum* *Exp1 CE 0V* *Exp2 CE 10V* *Exp3 CE 20V* *Exp4 CE 40V*
Target MS/MS	*Sibutramine: m/z 280.1826, RT 10.4 ± 1 min* *CE 10V* *CE 20V* *CE 40V* *MS acquisition rate 1 spectrum/sec, 8154 transient/spectrum* *MS/MS acquisition rate 3 spectra/sec, 2663 transient/spectrum*
Reference masses	*121.050873 m/z, 922.009798 m/z*
Ion Funnel Voltages	*150/100 V*
TUNE Parameters	*Standard Tune m/z 3200, 4GHz, HiRes*

LC Parameters		
Instrument	*Agilent 1290 LC System*	
Column	*Agilent ZORBAX Eclipse Plus C18, RRHT 2.1 mm x 150 mm, 1.8 µm*	
Mobile Phases	*A: water +0.01% formic acid* *B: methanol +0.01% formic acid*	
Flow rate	*0.2 mL/min*	
Temperature	*40°C*	
Injection Volume	*5 µL*	
Gradient:	*Time (min)*	*% B*
	0-1	*5*
	1-12	*95*
	12-15	*95*
	15.1	*5*
Post Run Time	*3 minutes*	

SAMPLE PREPARATION

Samples were acquired by regular on-line shopping. For general screening, powders (samples 1 and 3) and a liquid sample (sample 2, soft-gel capsules) were weighed to 400 mg and suspended in 16 mL of water under agitation. After centrifugation, 50 µL of sample was diluted in 150 µL of water and directly injected. A second aliquot of 1 mL of each sample was liquid/liquid extracted using ToxiTube A devices. The supernatant was dried and then reconstituted in 200 µL of water. The extracted samples were analyzed for confirmation purposes. For quantitation, samples were diluted 1/10, 1/100 and 1/1000 in water. External standard calibration curves were used to quantitate the confirmed analytes.

STANDARDS AND SOLVENTS

Ultrapure water was from Purelab Ultra (Elga Labwater, High Wycombe, UK). Methanol was of LC/MS grade from Sigma Aldrich, St. Louis, MO, USA. Sibutramine and caffeine were from Cerilliant Corporation (Round Rock, Texas, USA).

SOFTWARE

- MassHunter acquisition
- Qualitative
- Quantitative.

RESULTS AND DISCUSSION

A specific Personal Compound Database and Library (PCDL), from Agilent's Forensic Accurate Mass Library (Forensic PCDL AM), comprising 80 amphetamines and amphetamines-like compounds with anorexigenic effects, was built. All compounds were reported with acquired fragment spectra at collision energies of 10, 20 and 40V.

Dissolved and diluted samples were firstly acquired in SCAN mode, using the described analytical parameters. The data mining algorithm 'Find by formula' and database search (with fixed Δppm of 10 and [M-H+] adduct) were used to screen samples for the compounds present in the PCDL. Blank injections were used to eliminate false positive results from the system. Results with scores > 80% were considered consistent and further investigated. The score of a compound or spectrum is based upon the score for each of the identification techniques applied to it. The contribution to overall score is calculated from the Mass Match, isotope abundance score, spacing match and retention time score.

Using this technique, sibutramine was found as a preliminary identification result in two of the samples. For sample 2 (soft gel capsule), the Mass and Isotope scores for sibutramine were greater than 91%, Δppm was 1.08 and the overall score was greater than 96% (Fig. 1). Caffeine and other compounds were also preliminary identified; all preliminary data results are reported in Table 1.

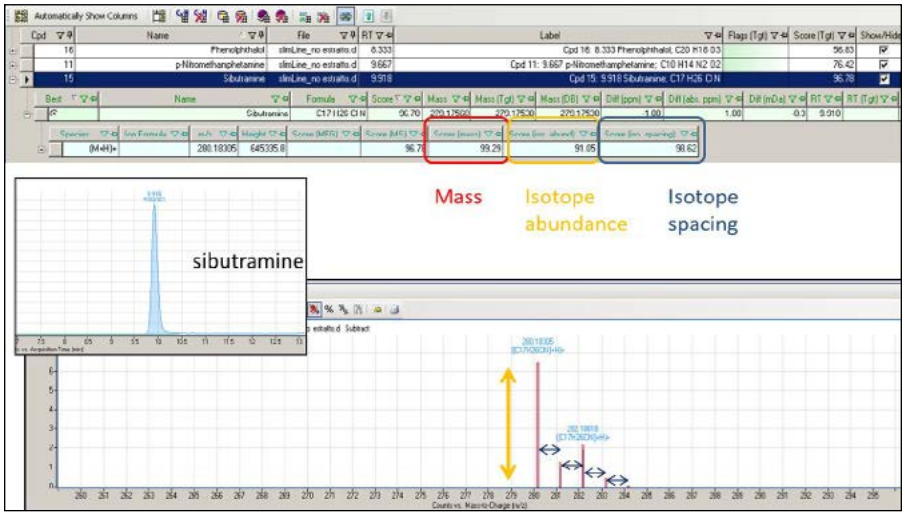

Figure 1. Preliminary identification of Sibutramine in Sample 1 using 'Find by formula' data mining and Database Search, with a Score > 96%.

Table 1 Preliminary Results.

Compound	Formula	RT	Sample #	SCORE %
Caffeine	*C8H10N4O2*	*6.5*	*1*	*87*
Caffeine	*C8H10N4O2*	*6.5*	*3*	*89*
Cathine/4-hydroxyamphetamine	*C9H13NO*	*6.3*	*2*	*98*
Ephedrine	*C10H15NO*	*5.5*	*3*	*95*
MDA	*C10H13NO2*	*4.3*	*2*	*88*
Sibutramine	*C17H26ClN*	*10.3*	*1*	*96*
Sibutramine	*C17H26ClN*	*10.3*	*2*	*97*
Theophylline	*C7H8N4O2*	*5.7*	*1*	*93*
Trimethoxyamphetamine	*C13H21NO3*	*3.4*	*2*	*82*

The samples were then analyzed in ALL ION acquisition mode, in order to assess the accurate mass fragments and coelution score values contribution to the preliminary analytes identification. Sibutramine and caffeine were still evaluated as possible candidates by the presence of significant fragments in the ALL ION acquisition mode (Fig. 2A and Fig. 2B). Seven sibutramine fragments were confirmed by good coelution scores values (> 70%) and a Δppm of 2.5 between target and measured mass was achieved. The use of fragment coelution scores helped to reduce the number of total presumptive candidates from 7 to 3.

Target MS/MS runs were then performed, in order to acquire spectral information. The 'find by target MS/MS' data mining algorithm was used, with averaged background subtraction on the acquired data (Fig. 3). The MFG (Molecular Formula Generator) was then applied to the extracted compound 1 (Fig. 4), in order to generate a panel of possible formulae from the acquired spectra, resulting in the formula C17H26ClN, with an overall score of 97.17% and Δppm of 0.39.

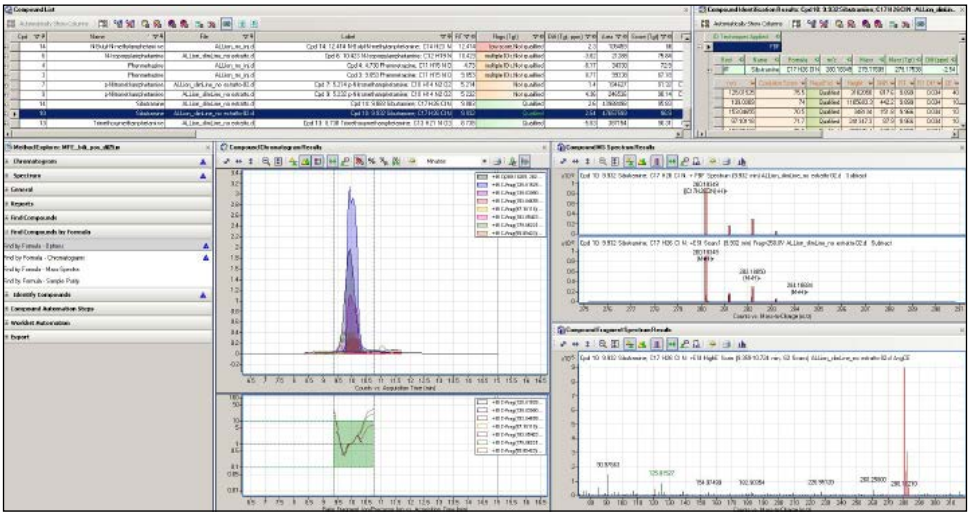

Figure 2A. Sibutramine peak in a real sample. The 'ALL ION' acquisition mode was used to obtain accurate mass fragments and coelution score (CE) values.

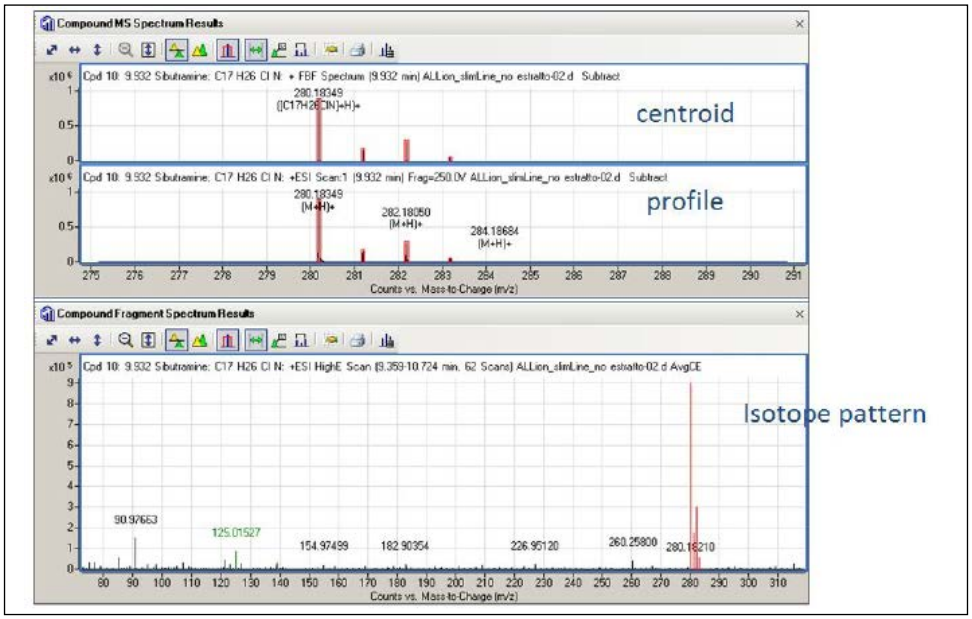

Figure 2B. Sibutramine spectra and fragments in a real sample. Data was acquired in both profile and centroid. The Isotope pattern is highlighted in red, fragments are in green.

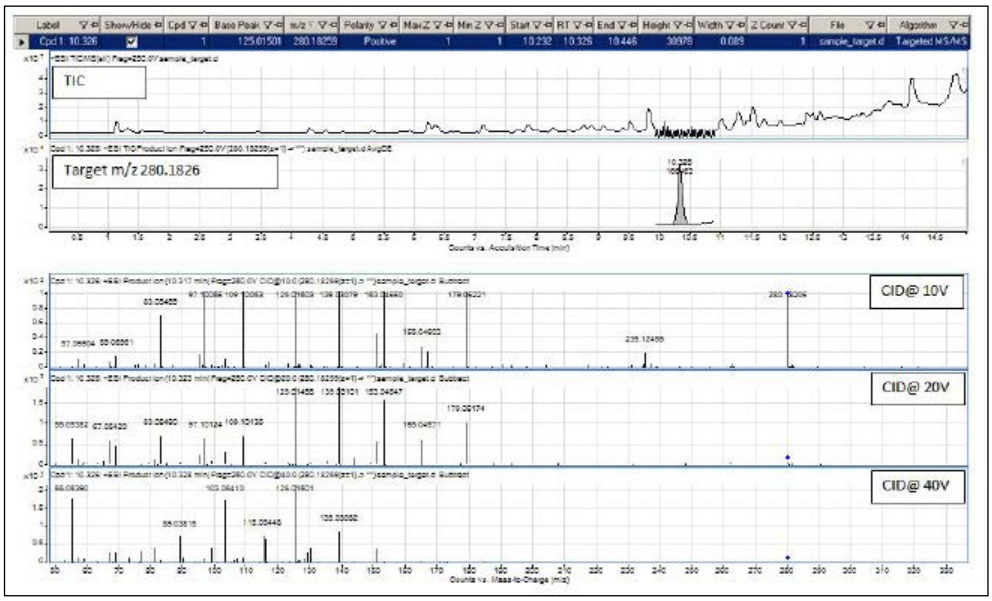

Figure 3. Target MS/MS runs at collision energies of 10, 20 and 40V. The data mining algorithm 'find by target MS/MS' was used. Compound found at RT 10.326 minutes.

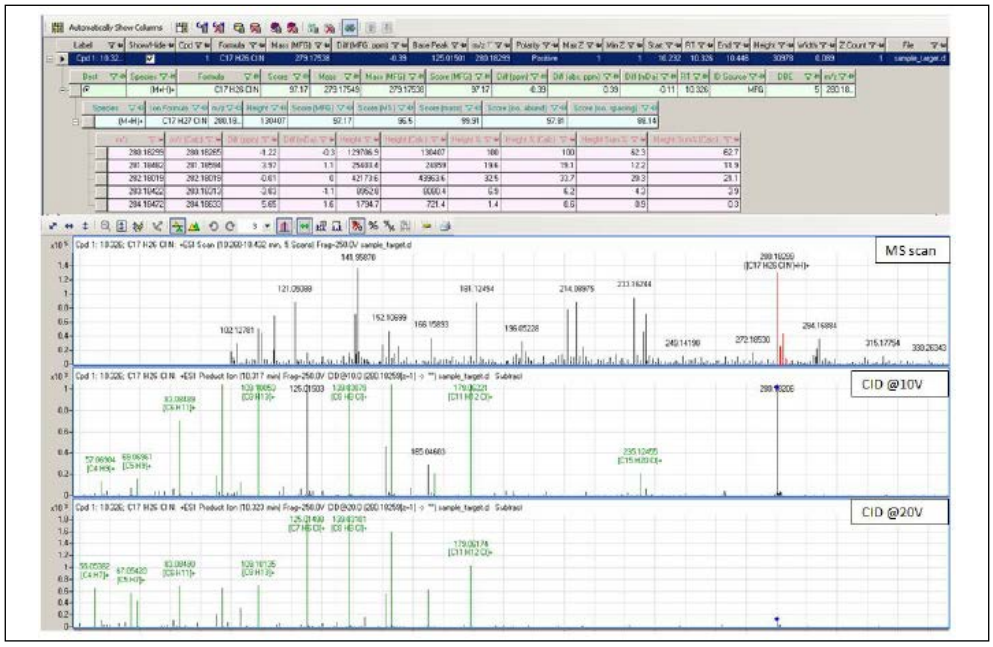

Figure 4. MFG (molecular formula generator) software was applied to the extracted Compound 1. The resulting formula C17H26ClN was obtained with an overall score of 97.17%.

For the MFG score, the scoring of the generated formula is based on three factors:

Mass: how well the measured mass (or m/z) compares with the value predicted from the proposed formula; Abundance: how well the abundance pattern of the measured isotope cluster compares with the values predicted from the proposed formula; Spacing: how the m/z spacing between the lowest m/z ion and the A+1 and A+2 ions compare with the values predicted from the proposed formula. These individual factors are computed as match probabilities between 0 (no probability) and 1 (certainty). The overall score is combined as a weighted average and reported on a scale of zero to 100. In Fig. 4, the MGF score and spectra at CID 10 and 20V are reported. Recognized fragments are highlighted in green in the acquired spectra.

Theoretical and experimental fragment correlation was obtained with a supplemental tool, MSC (Molecular Structure Correlator). The MassHunter MSC program correlates accurate mass MS/MS fragment ions for a compound of interest with one or more proposed molecular structures for that compound. MSC accomplishes this by trying to explain each observed fragment ion into the proposed structure using a 'systematic bond-breaking' approach, as described by Hill and Mortishire-Smith[8]. The input for MSC is an accurate mass MS/MS fragment spectrum, a molecular formula for the compound of interest, and one or more candidate molecular structures. The MSC then uses the selected formula, retrieves one or more possible structures from a .mol file, an .sdf file, a MassHunter compound database (PCD, PCDL) or ChemSpider (web) and scores on how well each candidate structure correlates with the MS/MS spectrum.

The overall correlation score gets calculated from individual scores for each fragment ion signal. For each fragment ion one or more substructure candidates may be suggested and a 'penalty' assigned, based on type and number of bonds that need to be broken in order to generate that substructure. Breaking two bonds, a double bond or even an aromatic ring carries a higher penalty (requires more energy and therefore is less likely) than just breaking one single bond. Two other factors impacting the overall correlation score are the mass accuracy of the observed fragment ions and the overall percentage of fragment ion intensity that can be plausibly explained with the substructures. MSC results obtained for sibutramine are depicted in Fig. 5. Theoretical structures retrieved for fragments comparison were from .mol file (for sibutramine), toxicological PCDL and ChemSpider. In all cases, sibutramine was recognized as the candidate.

Caffeine and sibutramine were finally confirmed using reference standards and subsequently quantitated by an external standard quantitation method (Fig. Fig. 6). Data results are summarized in Table 2. Sibutramine was found in two out of the three analyzed samples. Caffeine, a stimulant substance, probably present as a naturally occurring compound, was confirmed in two out of the three samples. However, external fortification of caffeine, in order to improve the products 'slimming' effect, could not be excluded.

The total caffeine content of the 2 samples (mg per 5 g pack) was also evaluated in relation to a 'generic' cup of coffee (typically 80-150 mg for a French press or plunger cup of coffee). The caffeine content varied from 5 to 100 mg per pack for the two products. Although one pack contained what could be considered a negligible amount, the second pack contained 100 mg of caffeine, which could consistently increase the consumer's total daily caffeine intake, leading to caffeine adverse effects

Table 2 Summary of results

Compound	Formula	RT	Sample #	ALL ION Confirmation	Standard Confirmation	Results
Caffeine	*C8H10N4O2*	*6.5*	*1*	*yes*	*yes*	*0.1 %*
Caffeine	*C8H10N4O2*	*6.5*	*3*	*yes*	*yes*	*2.0 %*
Cathine or 4-hydroxyamphetamine	*C9H13NO*	*6.3*	*2*	*no*	*no*	
Ephedrine	*C10H15NO*	*5.5*	*3*	*no*	*no*	
MDA	*C10H13NO2*	*4.3*	*2*	*no*	*no*	
Sibutramine	*C17H26ClN*	*10.5*	*1*	*yes*	*yes*	*15 µg/mg*
Sibutramine	*C17H26ClN*	*10.5*	*2*	*yes*	*yes*	*26 µg/mg*
Theophylline	*C7H8N4O2*	*5.7*	*1*	*yes*	*Not tested*	
Trimethoxyamphetamine	*C13H21NO3*	*3.4*	*2*	*no*	*no*	

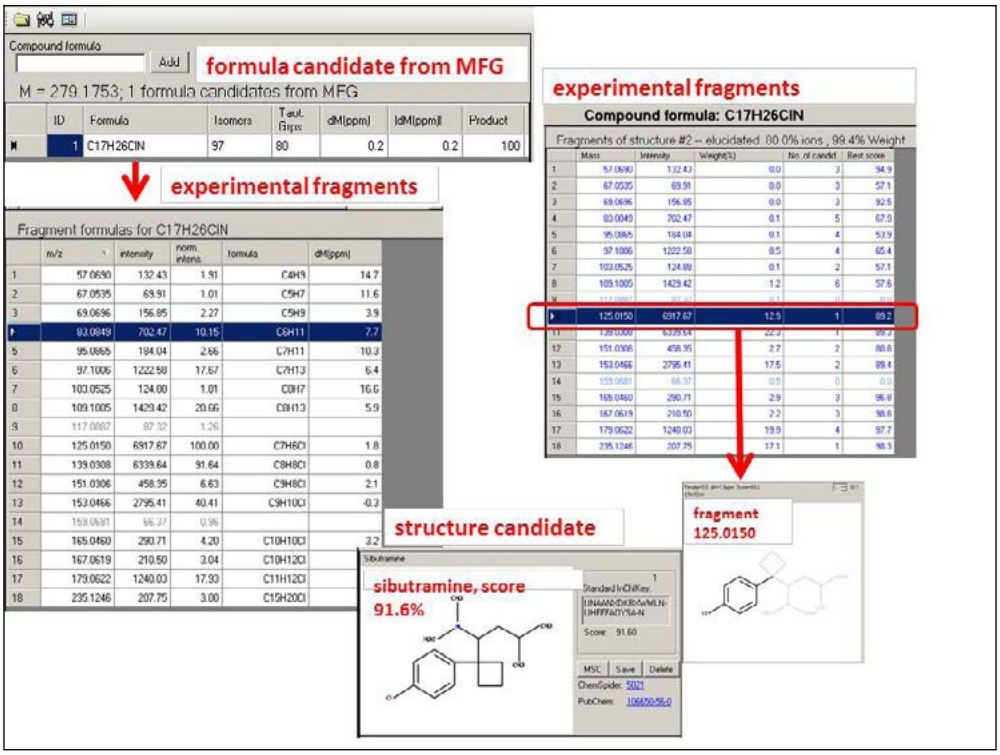

Figure 5.
Using the MSC (molecular structure correlator) program, the Sibutramine structure has been correlated with the 'unknown' formula, with a match score >91%

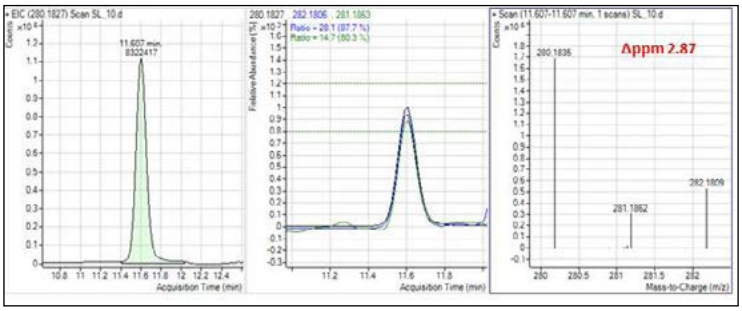

Figure 6.
Sibutramine peak in Sample 1 acquired in MS scan mode and quantitated through the MassHunter quantitation platform (left), Isotope ratio bars (middle) and sibutramine spectrum and total Δppm (right).

CONCLUSION

Adulterated herbal weight loss products containing undeclared synthetic drugs are common and responsible for many serious health issues. Synthetic adulterants are not mentioned on labels and therefore consumers are kept unaware of their presence and side effects. The most commonly undeclared ingredients, which are illegally added, include sibutramine, phenolphthalein, bumetanide, and phenytoin. Caffeine can also be found in these supplements. Sibutramine is known to be associated with psychosis and mood changes, cardiovascular problems and heart failure. For this reason, sibutramine has been withdrawn from the market in the USA, European Union, Australia, Canada and some Asian countries. In those countries where sibutramine is still available as a pharmaceutical drug, patients are regularly monitored. In this application, the applicability of accurate mass measurements (Q-TOF LC/MS) in the investigation of undeclared active compounds in commercial foodstuffs was demonstrated in an easy and rapid method set-up.

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