Applications of Mass Spectrometry to the Analysis of Chemical Components Found in Botanical Dietary Supplements

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Current Studies on Botanical Dietary Supplements

- Characterization of authenticated raw botanical materials (Example Below).
  - *Acacia rigidula*
  - Leaves used in Weight loss products
  - Phenethylamine, tyramine and tryptamine derivatives
  - Non natural compound found in 9 of 21 dietary supplements
    - β-methylphenethylamine
    - Can be misidentified as amphetamine using LC-MS
    - Independent GC-MS analysis able to confirm presence of β-methylphenethylamine and absence of amphetamine

Current Studies on Botanical Dietary Supplements (Cont.)

- Toxicity (hepatotoxicity) of specific components.
  - Example: pyrrolizidine alkaloids (from comfrey root).
- Adulteration and contamination of herbal dietary supplements (Discussion to follow)
- Screening for potential active pharmaceutical ingredients (API) adulterants and analogues (Discussion to follow)
Herbal dietary supplements

- Sales of herbal dietary supplements have increased steadily over the last decade.
- Dietary supplements represent valuable commodities with relatively high retail price.
- Consumers may assume that use of dietary supplements is a safer and more natural alternative to traditional medical treatments.

Source: New Hope Natural Media (http://newhope360.com)
Risks

- Adulteration of dietary supplements with active pharmaceutical ingredients (API’s)
- Potential interactions between natural constituents and prescription drugs
- Presence of chemical contaminants in dietary supplements
  - Plant toxins, mycotoxins
  - Pesticides
  - Heavy metals, etc.

Adulteration with API’s

- The aim is typically to intensify claimed biological effect
  - High concentration close or above the therapeutic doses
  - Addition of multiple API’s
  - Withdrawn drugs, designer drugs analogues

<table>
<thead>
<tr>
<th>Category</th>
<th>Status</th>
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<tbody>
<tr>
<td>WEIGHT LOSS SUPPLEMENTS</td>
<td>(major)</td>
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<tr>
<td>SPORTS SUPPLEMENTS</td>
<td>(major)</td>
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<tr>
<td>MALE ENHANCEMENT SUPPLEMENTS</td>
<td>(major)</td>
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<tr>
<td>BLOOD SUGAR SUPPORT SUPPLEMENTS</td>
<td>(growing)</td>
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<td>ANTI-INFLAMMATION SUPPLEMENTS</td>
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Courtesy of Dr. Lukas Vaclavik
Adulteration with API’s - Example

- Adulterants reported in weight loss supplements

<table>
<thead>
<tr>
<th>ANOREXICS</th>
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<th>ANTIDEPRESSANTS</th>
<th>DIURETICS</th>
<th>LAXATIVES</th>
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Contamination with plant toxins

- Usually a result of accidental (unintentional) substitution of plant material containing toxins
- May also be performed intentionally, especially in case authentic plants are either not available or too expensive

ARISTOLOCHIC ACID IN HERBAL SLIMMING PILLS

ANISATIN IN HERBAL TEA

EPHEDRA ALKALOIDS IN DIET AID SUPPLEMENTS

Courtesy of Lukas Vaclavik
Development of LC–MS methods

- Simple and rapid methods for simultaneous determination of multiple pharmaceuticals, plant toxins / alkaloids

### Anti-hypertension drugs
- Amlodipine besylate
- Benazepril
- Chlorthalidone
- Clonidine
- Felodipine
- Metoprolol tartrate
- Minoxidil
- Phentolamine mesylate
- Prazosin
- Valsartan

### Anti-diabetic drugs
- Acetohexamide
- Bumetanide
- Chloropropamide
- Gliclazide
- Glipizide
- Gliquidone
- Glibenclamide
- Metformin
- Nateglinide
- Phenformin
- Pioglitazone
- Repaglinide
- Rosiglitazone
- Tolazamide
- Tolbutamide

### Weight-loss drugs
- Bumetanide
- Fenfluramine
- Orlistat
- Phentermine
- Rimonabant
- Sertraline
- Sibutramine
- Topiramate

### Other drugs
- Diazepam
- Fluoxetine
- Ibufrofen
- Phenytoin
- Sertraline
- Warfarin

### NSAIDs
- Indomethacin
- Ketoprofen
- Mefenamic acid
- Naproxen
- Phenylbutazone

### Diuretic drugs
- Bendroflumethiazide
- Hydrochlorothiazide
- Hydroflumethiazide
- Indapamide
- Methyclothiazide
- Metolazone

### Other toxins and alkaloids
- Aristolochic acid I
- Aristolochic acid II
- Anisatin
- Colchicine
- Evodiamine
- Lycorine
- Picrotin
- Picrotoxinin
- Ricinine
- Strychnine
- Yohimbine

### Psychostimulants
- Amphetamine
- β-Methylphenethyamine
- Cathine
- 1,3-Dimethylamylamine
- Ephedrine
- Methamphetamine
- Norephedrine
- Phentermine

### Other drugs
- Diazepam
- Fluoxetine
- Ibuprofen
- Phenytoin
- Sertraline
- Warfarin

### Cardiac glycosides
- Cymarin
- Digoxigenin
- Digitoxin
- Digoxin
- Ouabain
Sample preparation

- Analytes with different physico-chemical properties
- Expected concentrations ranging from µg/kg to g/kg
- Complex matrices (tablets, softgels, liquids)

Courtesy of Dr. Lukas Vaclavik
Sample preparation

- QuEChERS procedure
- Acetonitrile-water extraction followed by partition step
- Polar matrix components remain in aqueous layer
Sample preparation

TABLETS / CAPSULES
- Homogenized sample (1g) soaked with 1% FA in H_2O (10mL) for 30 min
- Addition of MeCN (10mL) and extraction by shaking (30 min)
- Addition of salts (4g MgSO_4 & NaCl)
- Hand shaking (1 min)
- Centrifugation (4,500 rpm, 5 min)
- dSPE of MeCN extract (2mL) with C_18 sorbent (100mg) and MgSO_4 (300mg)
- Filtration

SOFTGELS
- Shaking of homogenized sample (1g) with n-hexane (4 mL)
- Addition of 1% FA in H_2O (10mL)
- Addition of MeCN (10mL) and extraction by shaking (30 min)
- Addition of salts (4g MgSO_4 & NaCl)
- Hand shaking (1 min)
- Centrifugation (4,500 rpm, 5 min)
- dSPE of MeCN extract (2mL) with C_18 sorbent (100mg) and MgSO_4 (300mg)
- Filtration

LIQUIDS
- Addition of MeCN containing 1% FA (10mL) to liquid sample (10mL)
- Extraction by shaking (30 min)
- Addition of salts (4g MgSO_4 & NaCl)
- Hand shaking (1 min)
- Centrifugation (4,500 rpm, 5 min)
- dSPE of MeCN extract (2mL) with C_18 sorbent (100mg) and MgSO_4 (300mg)
- Filtration

SALTS
WATER
MATRIX
ACETONITRILE
(analytes)
Liquid chromatography

- UPHLC separation
  - **Column**: Acquity UPLC HSS T3 reversed phase column
    (100 mm x 2.1 mm i.d., 1.7 µm particle size)
  - **Mobile phase**: A – 5 mM ammonium formate + 0.1% formic acid in water
    B – 5 mM ammonium formate + 0.1% formic acid in methanol

Gradient Elution

- Analytes eluted in 0.7 - 10.5 min
- Typical peak widths: 5 - 12 s
- RT stability: 0.15–3.5% (RSD, n = 50)
Instrumentation - HR MS

- Hybrid HR mass spectrometer
- Quadrupole-orbital ion trap / ESI source / UHPLC system
- RP 17,500 – 140,000 FWHM
- Enables to obtain HR product ion spectra of selected precursor ion

Source: Thermo Scientific Inc.
MS detection - QExactive (1)

- ESI+, full MS – data dependent MS/MS acquisition mode
- Inclusion list ($m/z$, retention time window, collision energy)

Source: Figure provided by Dr. J. Wong
Identification and confirmation - HRMS

- Interference-free extracted ion chromatograms and product ion ion spectra typically obtained
- Interfering ions co-isolated by the quadrupole and their fragments were resolved from diagnostic ions of analytes

EIC: Chloropropamide (m/z 277.0408)

Product ion spectrum: Chloropropamide (NCE = 20%)

SPIKE 500 µg/kg into DS intended for BLOOD PRESSURE MGMT

EIC: Chloropropamide (m/z 277.0408)

Product ion spectrum: Chloropropamide (NCE = 20%)

SPIKE 500 µg/kg into DS intended for ANTI-INFLAMMATION

Courtesy of Dr. Lukas Vaclavik
IDENTIFICATION/CONFIRMATION REQUIREMENTS - HRMS

- ≥ 2 diagnostic ions (preferably including the quasi molecular ion). Mass accuracy below 5 ppm, at least one fragment ion
- Requirements can be met for all analytes in complex matrices
- Mass accuracy in full MS and dd-MS/MS mode was below 3 and 5 ppm, respectively

![Graph showing number of analytes vs number of fragment ions](image)

Product ion spectrum:
Ephedrine (NCE = 10%)

Product ion spectrum:
Metoprolol tartrate (NCE = 40%)

Source: Method Validation and Quality Control Procedures for Pesticides Residues Analysis in Food and Feed, Document No. SANCO/12495/2011
**OPTIMIZATION – EXTRACTION / CLEAN-UP**

- Extraction at lower pH improves recoveries of pyrrolizidine alkaloids, their N-oxides and some other analytes
- Defatting slightly decreases recoveries of some non-polar analytes
- C\textsubscript{18} sorbent does not bind target analytes

**DEFATTING (tested on softgels)**

<table>
<thead>
<tr>
<th>Recovery (%)</th>
<th>0 - 40</th>
<th>40-70</th>
<th>70-120</th>
<th>&gt;120</th>
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</thead>
<tbody>
<tr>
<td>No defatting</td>
<td>2</td>
<td>5</td>
<td>89</td>
<td>0</td>
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<tr>
<td>Defatting</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**DS intended for BLOOD PRESSURE MGMT (tablet)**

- 1,3-DMAA
- Monocrotaline
- Monocrotaline N-oxide

**dSPE (all matrices)**

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</thead>
<tbody>
<tr>
<td>No defatting</td>
<td>0</td>
<td>0</td>
<td>67</td>
<td>0</td>
</tr>
<tr>
<td>Defatting</td>
<td>0</td>
<td>22</td>
<td>94</td>
<td>0</td>
</tr>
</tbody>
</table>

**Average recovery - all matrices**

- dSPE PSA/MgSO\textsubscript{4}
- dSPE C\textsubscript{18}/MgSO\textsubscript{4}

_Courtesy of Dr. Lukas Vaclavik_
Detectability of analytes is strongly matrix-dependent

Criteria for estimation of LOQ:

- At least 8 full MS scans at RT of analyte
- Signal-to-noise ratio ≥ 9 (when noise was present in the extracted ion chromatogram)
- Analyte intensity > threshold for triggering of product ion scan
- Criteria for analyte identification passed

Courtesy of Dr. Lukas Vaclavik
• Recoveries for the majority of analytes at concentrations ≥ LOQ were 70-120%

• Repeatability (RSD, n = 5) was below 25% for all experiments

• Highly polar analytes (metformin, tropine, ouabain) are not transferred into acetonitrile layer during partition step

• Recoveries of non-polar analytes are slightly decreased during defatting step

Courtesy of Dr. Lukas Vaclavik
QUANTIFICATION

- The extent of matrix effects (mainly signal suppression) differs significantly between respective herbal dietary supplements

COMPENSATION OF MATRIX EFFECTS

- Matrix-matched external calibration ✗
- Method of standard additions ✓
- Method of internal standardization ✗

MINIMIZATION/REMOVAL OF MATRIX EFFECTS

- Selective extraction ✗
- Effective sample clean-up ✗
- Optimization of chromatographic separation ✗
- Dilution of sample extract ✓

• Approach provides results and performance comparable to those obtained by matrix-matched calibration

• Post-extraction addition at three concentrations (25, 100, and 500 µg/kg)

• Time-demanding and laborious
• Successfully applied to quantification of analytes at higher concentrations
• Dilution followed by quantification using solvent standards (dilution factors ≥ 25 should be used)
• Straightforward approach

GLIBENCLAMIDE (0.25 mg/g)
DS intended for BLOOD SUGAR MANAGEMENT

Matrix effect (%)

Dilution factor (DF)

Quantification based on solvent standards

Product ion spectrum: SAMPLE

m/z

Relative abundance (%)
Instrumentation - LR MS

- Hybrid mass spectrometer
- QqQ-LIT / ESI source / UHPLC system
- Combines capabilities of QqQ and IT

Source: AB Sciex
Performance of the method - DETECTABILITY

BLOOD SUGAR MANAGEMENT DS: SPIKE 100 µg/kg

**ACETOHEXAMIDE**

- **MS/MS**
  - 324.9 > 243.0
  - 324.9 > 91.0

- **HR MS(/MS)**
  - 325.1216 ± 2.5 ppm

**DIGITOXIGENIN**

- **MS/MS**
  - 392.0 > 339.1
  - 392.0 > 91.0

- **HR MS(/MS)**
  - 375.2529 ± 2.5 ppm
Identification and confirmation - LRMS

Aristolochic acid II
329.2 > 268.2
329.2 > 163.2

Aristolochic acid II
329.2 > 268.2 > 238.2

Source: Vaclavik et. al Food Additives & Contaminants 31 (2014) 784-791
Post-run screening

EXAMPLE: SIBUTRAMINE & ANALOGUES IN WEIGHT LOSS SUPPLEMENT

Sibutramine

Benzylsibutramine

N-Desmethylsibutramine

" EXAMPLE: SIBUTRAMINE & ANALOGUES IN WEIGHT LOSS SUPPLEMENT

Sibutramine

Benzylsibutramine

N-Desmethylsibutramine
Conclusions

- LR and HR MS techniques provide complementary information that allow identification and confirmation of target analytes.

- The developed method allowed for achieving acceptable performance characteristics in terms of both detectability and recoveries.

- The post-run screening without analytical standards based on accurate mass measurements can reveal presence of new API’s and analogues.
Acknowledgments

- Lukas Vaclavik, FDA-CFSAN
- Rahul Pawar, FDA-CFSAN
- Hemlata Tamta, FDA-CFSAN
- Erich Grundel, FDA-CFSAN
- Jun Ma, FDA-CFSAN
- Jon W. Wong, FDA-CFSAN
- James B. Wittenberg, FDA-CFSAN
Thank you for your attention!

Courtesy of R. Chris Clark (Coastal Point)