**Analysis of *Symphytum officinale* (Comfrey) root Extract Using LC-QToF-MS method**

Names of investigators: Bharathi Avula, Kumar Katragunta

Affiliation: National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS 38677, USA

Analysis date: December 13, 2021

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Common Botanical Name | CAS No. | Lot No. | Container ID | Net Weight |
| ***Symphytum offcinale* (Comfrey)** |  | RK-3-23-1-SO-C  RK-3-23-1-SO-D |  |  |

|  |
| --- |
| Sample storage condition until analysis |
| **2-8°C** |

**Quantitative/Targeted method:**

|  |  |
| --- | --- |
| **UHPLC-QToF-MS Method** | |
| UHPLC conditions | Mass spectrometry |
| System: Agilent 1290 series  Column: Poroshell 120 EC-C18 (2.1 X 150mm, 2.7µm) (Agilent technologies, Palo Alto, CA, USA)  Mobile phase A: Water+0.1% formic acid  Mobile phase B: Acetonitrile +0.1% formic acid  Flow rate: 0.23 mL/min  Column temperature: 35°C  Gradient   |  |  | | --- | --- | | Time (min) | Mobile phase B (%) | | 0.0 | 01 | | 3.0 | 01 | | 40.0 | 40 | | 45.0 | 100 | | System: QToF-MS 6530A series (Agilent technologies, Palo Alto, CA, USA)  Ionization: ESI  Polarity: Positive mode/Negative mode  Main Interface:  · Nebulizing gas flow: 11 L/min  . Gas temperature: 325°C  . Nebulizer: 30 psig  . Sheath gas temperature: 300°C  . Sheath gas flow: 11L/min  Capillary voltage: 3.5 kV  Fragmentor: 100V/150V   |  |  |  | | --- | --- | --- | | Compound | Precursor ion (*m/z*)  [M+H]+ | Retention time (min) | | Intermedine | 300.1805 | 12.3 | | Lycopsamine | 300.1805 | 12.6 | | Intermedine N-oxide | 316.1755 | 13.8 | | Lycopsamine N-oxide | 316.1755 | 14.2 | | 7-acetylintermedine | 342.1911 | 17.65 | | 7-acetyllycosamine | 342.1911 | 17.78 | | 7-acetylintermedine N-oxide | 358.1858 | 17.9 | | 7-acetyllycosamine N-oxide | 358.1858 | 18.1 | | Rosmarinic acid | DAD@330 nm | 26.6 | |

**Quantitative results**

|  |  |  |
| --- | --- | --- |
| **Compound** | **Concentration in extract (mg/g)**  **RK-3-21-1-SO-C** | **Concentration in extract (mg/g)**  **RK-3-21-1-SO-D** |
| Intermedine | 3 mg/g | 3 mg/g |
| Lycopsamine | 3 mg/g | 3 mg/g |
| Intermedine N-oxide | 3.7 mg/g | 3.7 mg/g |
| Lycopsamine N-oxide | 3.7 mg/g | 3.7 mg/g |
| 7-acetylintermedine | 0.2 mg/g | 0.2 mg/g |
| 7-acetyllycopsamine | 0.2 mg/g | 0.2 mg/g |
| 7-acetylintermedine N-oxide | 3 mg/g | 3 mg/g |
| 7-acetyllycopsamine N-oxide | 5 mg/g | 5 mg/g |
| Rosmarinic acid | 17 mg/g | 17 mg/g |

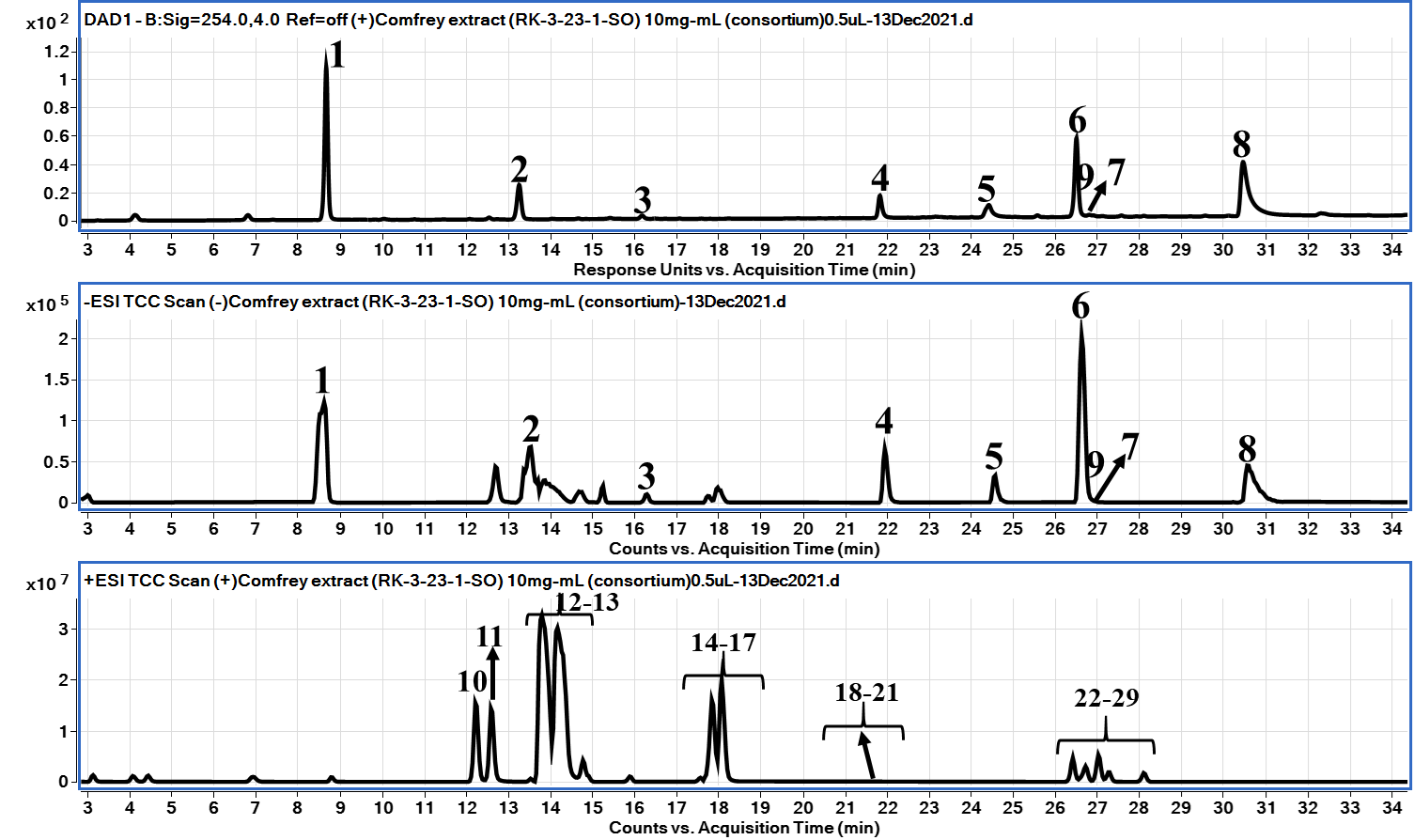
**Standards**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Name** | **Supplier** | **CAS Number** | **Catalog number** | **Purity %** |
| Intermedine | Millipore Sigma | 10285-06-0 | PHL82424 | 99% |
| Lycopsamine | Millipore Sigma | 10285-07-1 | PHL89726 | 99% |
| Intermedine N-oxide | Millipore Sigma | 95462-14-9 | PHL83446 | 99% |
| Lycopsamine N-oxide | Millipore Sigma | 95462-15-0 | PHL83447 | 99% |
| 7-acetylintermedine | Millipore Sigma | 74243-01-9 | PHL83759 | 99% |
| 7-acetylintermedine N-oxide | Millipore Sigma | 685132-59-6 | PHL83795 | 99% |
| 7-acetyllycopsamine N-oxide | Millipore Sigma | 685132-58-5 | PHL84275 | 99% |
| Rosamarinic acid | Millipore Sigma | 20283-92-5 | R4033 | 98% |

**Chemical structures of standards used for quantitative analysis**



**Chromatograms**



**Calibration**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Compound | Limit of detection (LOD) ng/mL | Limit of quantitation (LOQ) ng/mL | Calibration range (mg/g) | Number of Calibration Points | *r*2 | Concentration  mg/g (n = 3)  RK-3-21-1-SO-C | Concentration  mg/g (n = 3)  RK-3-21-1-SO-D |
| Intermedine | 0.5 ng/mL | 1.0 ng/mL | 0.1 – 2.5 μg/mL | 5 | 0.9993 | 3.4 ± 0.28 | 3.4 ± 0.71 |
| Lycopsamine | 0.5 ng/mL | 1.0 ng/mL | 0.1 – 2.5 μg/mL | 5 | 0.9995 | 3.1 ± 0.32 | 3.2 ± 1.66 |
| Intermedine N-oxide | 1.0 ng/mL | 2.5 ng/mL | 0.1 – 2.5 μg/mL | 5 | 0.9995 | 3.7 ± 2.30 | 3.7 ± 0.51 |
| Lycopsamine N-oxide | 0.5 ng/mL | 1.0 ng/mL | 0.1 – 2.5 μg/mL | 5 | 0.9995 | 3.7 ± 0.33 | 3.7 ± 0.53 |
| 7-acetylintermedine | 0.5 ng/mL | 1.0 ng/mL | 0.1 – 2.5 μg/mL | 5 | 0.9994 | 0.2 ± 1.38 | 0.2 ± 2.22 |
| 7-acetyllycopsamine | 1.0 ng/mL | 2.5 ng/mL | 0.1 – 2.5 μg/mL | 5 | 0.9994 | 0.2 ± 1.61 | 0.2 ± 2.12 |
| 7-acetylintermedine N-oxide | 0.5 ng/mL | 1.0 ng/mL | 0.1 – 2.5 μg/mL | 5 | 0.9995 | 3.5 ± 0.62 | 3.5 ± 0.61 |
| 7-acetyllycopsamine N-oxide | 1.0 ng/mL | 2.5 ng/mL | 0.1 – 2.5 μg/mL | 5 | 0.9995 | 4.8 ± 0.35 | 4.7 ± 1.25 |
| Rosamarinic acid | 10 ng/mL | 50 ng/mL | 0.5 – 25 μg/mL | 5 | 0.9996 | 16.8 ± 0.10 | 16.9 ± 0.24 |

**Linearity profiles**

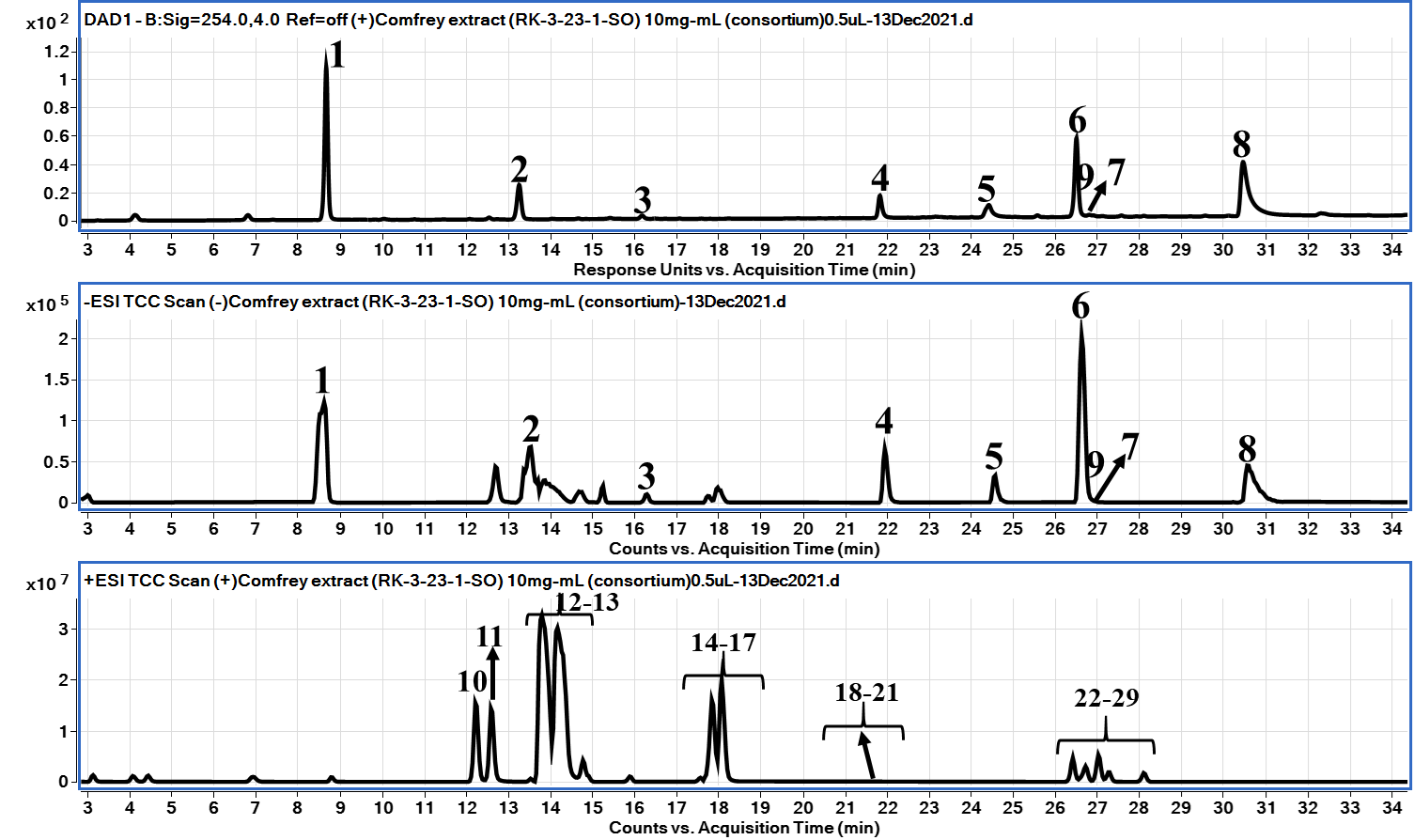
****

**Untargeted Analysis**

|  |  |
| --- | --- |
|  | |
| UHPLC conditions | Mass spectrometer conditions |
| System: Agilent 1290 series  Column: Poroshell 120 EC-C18 (2.1 X 150mm, 2.7µm) (Agilent technologies, Palo Alto, CA, USA)  Mobile phase A: Water+0.1% formic acid  Mobile phase B: Acetonitrile +0.1% formic acid  Flow rate: 0.23 mL/min  Column temperature: 35°C  Gradient:   |  |  | | --- | --- | | Time (min) | Mobile phase B (%) | | 0.0 | 01 | | 3.0 | 01 | | 40.0 | 40 | | 45.0 | 100 | | System: QToF-MS 6530A series (Agilent technologies, Palo Alto, CA, USA)  Ionization: ESI  Polarity: Positive mode  Main Interface:  · Nebulizing gas flow: 11 L/min  . Gas temperature: 325°C  . Nebulizer: 30 psig  . Sheath gas temperature: 300°C  . Sheath gas flow: 11L/min  Capillary voltage: 3.5 kV  Fragmentor: 100V/150V |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Table 1: Proposed identification of constituents of *Symphytum officinale* root extract showing RT, *m/z*, ppm, tentative compound name and molecular formula** | | | | | |
| Peak No. | RT (min) | Exp. *m/z* | Mass accuracy  (ppm) | Proposed ID’s  (Confirmed with Std in **green**)  (Most probable ID is **yellow**) | Molecular Formula |
| **Phenolic compounds [M-H]-** | | | | | |
| 1 | 8.5 | 599.1615 | -0.5 | **Hydroxybenzoic acid derivative** | C26H32O16 |
| 2 | 13.5 | 161.0818 | -0.6 | **Viridifloric acid** | C7H14O4 |
| 3 | 16.3 | 179.0347 | -1.7 | **Caffeic acid** | C9H8O4 |
| 4 | 21.9 | 537.1036 | -0.4 | **Globoidnan B** | C27H22O12 |
| 5 | 24.58 | 717.1459 | -0.3 | **Rabdosin** | C36H30O16 |
| 6 | 26.6 | 359.0770 | -0.6 | **Rosmarinic acid** | C18H16O8 |
| 7 | 26.7 | 719.1619 | 0.1 | **Dihydrorabdosin** | C36H32O16 |
| 8 | 26.9 | 493.1143 | 0.6 | **Dihydrogloboidnan A** | C26H22O10 |
| 9 | 30.6 | 491.0989 | 1.0 | **Globoidnan A/Eritrichin** | C26H20O10 |
| **Pyrrolizidine alkaloids (PAs) [M+H]+** | | | | | |
| 10 | 12.3 | 300.1807 | 0.7 | **Intermedine** | C15H25NO5 |
| 11 | 12.6 | 300.1807 | 0.7 | **Lycopasamine** | C15H25NO5 |
| 12 | 13.8 | 316.1757 | 0.6 | **Intermedine-N-oxide** | C15H25NO6 |
| 13 | 14.2 | 316.1757 | 0.6 | **Lycopasamine N-oxide** | C15H25NO6 |
| 14 | 17.65 | 342.1912 | 0.3 | **7-Acetylintermedine** | C17H27NO6 |
| 15 | 17.78 | 342.1912 | 0.3 | **7-Acetyllycopsamine** | C17H27NO6 |
| 16 | 17.9 | 358.1858 | -0.6 | **7-Acetylintermedine *N*-oxide** | C17H27NO7 |
| 17 | 18.1 | 358.1858 | -0.6 | **7-Acetyllycopsamine *N*-oxide** | C17H27NO7 |
| 18 | 19.2 | 414.2123 | 0.2 | **7-Sarracinyl-9- trachelantyl-retronecine-*N*-oxide/ 7-Sarracinyl-9- viridiflorylretronecine-*N*-oxide/Echimidine-*N*-Oxide/**  **Heliosupine-*N*-oxide** | C20H31NO8 |
| 19 | 19.9 |
| 20 | 20.5 |
| 21 | 22.0 |
| 22 | 26.4 | 382.2223 | -0.3 | **Symphytine/ Symviridine/**  **Anadoline/Symlandine** | C₂₀H₃₁NO₆ |
| 23 | 26.7 |
| 24 | 26.9 |
| 25 | 27.2 |
| 26 | 26.5 | 398.2175 | 0.5 | **Echimidine/ Heliosupine/Symphytine *N*-oxide/ Symviridine *N*-oxide/ Anadoline-*N*-Oxide/ Symlandine *N*-oxide** | C20H31NO7 |
| 27 | 26.9 |
| 28 | 27.2 |
| 29 | 27.5 |

Figure 1: Chromatograms



Structures of compounds in Tables 1

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Table 2: HRMS product ions for the peaks of the botanical extract (*Symphytum officinale* root) showing RT, *m/z*, tentative compound name and fragment ions** | | | | |
| Peak No. | RT (min) | [M-H]-/[M+H]+  *m/z* | Proposed Compounds  (Confirmed with Std in **green**)  (Probable ID is **yellow**)  CAS Number | Product Ions  *m/z* |
| **Phenolic compounds [M-H]-** | | | | |
| 1 | 8.5 | 599.1615 | **Hydroxybenzoic acid derivative** | 137.0250  93.0354 |
| 2 | 13.5 | 161.0818 | Viridifloric acid | 135.0571  117.0556 |
| 3 | 16.3 | 179.0347 | Caffeic acid | 135.0448 |
| 4 | 21.9 | 537.1036 | Globoidnan B | 493.1141  339.0505  197.0451  135.0452 |
| 5 | 24.58 | 717.1459 | Rabdosin | 537.1006  519.0916  475.1019  339.0502  197.0456 |
| 6 | 26.6 | 359.0770 | Rosmarinic acid | 197.0461  161.0231 |
| 7 | 26.9 | 493.1143 | Dihydrogloboidnan A | 359.0746  295.0597  185.0234  161.0239 |
| 8 | 30.6 | 491.0989 | Globoidnan A/Eritrichin | 311.0563  179.0348  135.0450 |
| 9 | 26.7 | 719.1619 | Dihydrorabdosin | 359.0765  197.0456  161.0246 |
| **Pyrrolizidine alkaloids (PAs) [M+H]+** | | | | |
| 10 | 12.3 | 300.1807 | Intermedine | 156.1006 |
| 11 | 12.6 | 300.1807 | Lycopsamine | 156.1006 |
| 12 | 13.8 | 316.1757 | Intermedine-*N*-oxide | 172.0841 |
| 13 | 14.2 | 316.1757 | Lycopasamine N-oxide | 172.0841 |
| 14 | 17.65 | 342.1912 | 7-Acetylintermedine | 120.0629 |
| 15 | 17.78 | 342.1912 | 7-Acetyllycopsamine | 120.0629 |
| 16 | 17.9 | 358.1858 | 7-Acetylintermedine *N*-oxide | 214.1067  180.1007  120.0802 |
| 17 | 18.1 | 358.1858 | 7-Acetyllycopsamine *N*-oxide | 214.1067  180.1007  120.0802 |
| 18 | 19.2 | 414.2123 | 7-Sarracinyl-9- trachelantyl-retronecine-*N*-oxide/ 7-Sarracinyl-9- viridiflorylretronecine-*N*-oxide/Echimidine-*N*-Oxide/  Heliosupine-*N*-oxide | 298.1575  270.1307  236.1311  214.1048  154.0832  137.0810  120.0800 |
| 19 | 19.9 |
| 20 | 20.5 |
| 21 | 22.0 |
| 22 | 26.4 | 382.2223 | Symphytine/ Symviridine/  Anadoline/Symlandine | 138.0893  120.0809 |
| 23 | 26.7 |
| 24 | 26.9 |
| 25 | 27.2 |
| 26 | 26.5 | 398.2175 | Echimidine/ Heliosupine/Symphytine *N*-oxide/  Symviridine *N*-oxide/ Anadoline-*N*-Oxide/ Symlandine *N*-oxide | 254.1282  138.0893  120.0809 |
| 27 | 26.9 |
| 28 | 27.2 |
| 29 | 27.5 |

**References:**

1. Wuilloud JC, Gratze SR, Gamble BM, Wolnik KA. Simultaneous analysis of hepatotoxic pyrrolizidine alkaloids and N-oxides in comfrey root by LC-ion trap mass spectrometry. Analyst. 2004 Feb;129(2):150-6. doi: 10.1039/b311030c. Epub 2004 Jan 8. PMID: 14752559.
2. Trifan A, Opitz SEW, Josuran R, Grubelnik A, Esslinger N, Peter S, Bräm S, Meier N, Wolfram E. Is comfrey root more than toxic pyrrolizidine alkaloids? Salvianolic acids among antioxidant polyphenols in comfrey (*Symphytum officinale* L.) roots. Food Chem Toxicol. 2018 Feb;112:178-187.
3. Trifan A, Wolfram E, Esslinger N, Grubelnik A, Skalicka-Woźniak K, Minceva M, Luca SV. Globoidnan A, rabdosiin and globoidnan B as new phenolic markers in European-sourced comfrey (*Symphytum officinale* L.) root samples. Phytochem Anal. 2021 Jul;32(4):482-494. doi: 10.1002/pca.2996. Epub 2020 Oct 5. PMID: 33015885.
4. Avula B, Sagi S, Wang YH, Zweigenbaum J, Wang M, Khan IA. Characterization and screening of pyrrolizidine alkaloids and *N*-oxides from botanicals and dietary supplements using UHPLC-high resolution mass spectrometry. Food Chem. 2015 Jul 1;178:136-48. doi: