**Analysis of Usnea Extract Using UPLC-MS/MS**

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|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Common Botanical Name | CAS No. | Lot No. | Container ID | Net Weight |
| Usnea lichen |  |  |  |  |

|  |
| --- |
| Sample storage condition until analysis |
| -80 ℃ |

**Quantitative/Targeted method:**

|  |
| --- |
| **UPLC-MS Method** |
| UHPLC conditions | Mass spectrometry |
| System: **Waters Acquity**Column: **Waters Acquity Ultra C18 (2.1 x 100 mm, 1.7 um)**Mobile phase A: **H2O + 0.1% FA**Mobile phase B: **CH3CN + 0.1% FA**Flow rate: **0.3 mL/min**Column temperature: **50 ℃**Gradient

|  |  |
| --- | --- |
| Time (min) | Mobile phase B (%) |
| **0** | **3** |
| **1.0** | **3** |
| **5.0** | **75** |
| **5.5** | **75** |
| **6.0** | **3** |
|  |  |
|  |  |
|  |  |
|  |  |

 | System: **Waters TQS**Ionization: **Electrospray (ESI)**Polarity: **Positive**Main Interface: · Nebulizing gas flow: **150 L/hr** · Heating gas flow: **500 L/hr** · Interface temperature: **150 ℃** · Capillary voltage: **2.5 kV** · Cone voltage: **50 V**Ion guide: · Drying gas flow: **0.15 mL/min** · Desolvation line temperature: **500 ℃**  · Heat block temperature: SRM dwell time:

|  |
| --- |
| Selected Reaction Monitoring (SRM) events |
| Compound | Precursor ion (*m/z*) | Product ion (*m/z*) | Collision energy (V) | Retention time (min) |
| **Atranorin** | 373.09 | - | 45 | 3.35 |
| **p-coumaric acid** | 165.05 | - | 45 | 3.72 |
| **Ellagic acid** | 303.01 | - | 45 | 3.39 |
| **Gallic acid** | 171.03 | - | 45 | 0.50 |
| **Usnic acid** | 345.10 | - | 45 | 5.55 |
| \*Quantifier, †Qualifier |

 |

**Quantitative results**

|  |  |
| --- | --- |
| **Compound** | **Concentration in extract (mg/g)** |
| Atranorin | **2.419 ± 0.070** |
| p-coumaric acid | **0.560 ± 0.038** |
| Ellagic acid | **227.2 ± 2.8** |
| Gallic acid | **26.72 ± 0.32** |
| Usnic acid | **141.6 ± 1.6** |

**Standards**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Name** | **Supplier** | **CAS Number** | **Catalog number** | **Purity %** | **Batch/Lot number** |
| Atranorin | Phytolab | 479-20-9 | 84203-10mg | >98% | 118154304 |
| p-coumaric acid | Sigma | 501-98-4 | C9008-1g | >98% | BCCF4731 |
| Ellagic acid | Sigma | 476-66-4 | E2250-1g | >98% | BCCF5486 |
| Gallic acid | Sigma | 149-91-7 | G7384-100g | >97.5% | SLCJ6385 |
| Usnic acid | Sigma | 7562-61-0 | 329967-5g | >98% | MKCN5004 |

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



**Chromatograms**

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**Calibration**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Compound | Lower limit of quantitation (LLOQ) **ug/mL** | Calibration range (**ug/mL**) | Number of Calibration Points | R2 | Concentration mg/g **in extract** (n = 3) |
| **Atranorin** | 0.001 | 0.001 – 2.187 | 8 | 0.9991 | 2.419 ± 0.070 |
| **Usnic acid** | 0.001 | 0.001 – 0.729 | 7 | 0.9997 | 141.6 ± 1.6 |
| **Ellagic acid** | 0.003 | 0.003 – 19.683 | 9 | 0.9979 | 227.2 ± 2.8 |
| **Gallic acid** | 0.081 | 0.081 – 19.683 | 7 | 0.9989 | 26.72 ± 0.32 |
| **p-coumaric acid** | 0.001 | 0.001 – 0.729 | 7 | 0.9972 | 0.560 ± 0.038 |

Untargeted Analysis

|  |
| --- |
|  |
| UHPLC conditions | Mass spectrometer conditions |
| System: Thermo VanquishColumn: Waters Acquity Ultra C18 (1.7 um, 1.0 x 150 mm)Mobile phase A: H2O w/ 0.1% FAMobile phase B: ACN w/ 0.1% FAFlow rate: 0.100 mL/minColumn temperature: 55℃Gradient:

|  |  |
| --- | --- |
| Time (min) | Mobile phase B (%) |
| 0 | 5 |
| 1 | 5 |
| 12.5 | 98 |
| 13.5 | 98 |
| 12.6 | 5 |
| 15 | 5 |

 | System: Thermo Orbitrap Fusion LumosMode: ESIPolarity: positive/negativeMain-Interface: · Interface voltage: 3500/2500 · Nebulizing gas flow: 25 · Heating gas flow: 5 · Interface temperature: 325 ℃ · Desolvation temperature: 350 ℃Ion guide: · Drying gas flow: 1 · DL temperature: 350 ℃ · Heat block temperature |

|  |
| --- |
| **Table 1: Proposed identification of constituents of Usnea** |
| Peak No. | RT (min) | Exp. *m/z* | Mass accuracy (ppm) | Proposed ID’s (Confirmed with Std in **green**)(Structure with correlated MS2 spectra in **orange**)(Most probable ID is **blue**) | Molecular Formula |
| 1 | 10.79 | 149.0598 | -0.67 | Cinnamic acid | C9H8O2 |
| 2 | 10.52 | 373.0930 | -1.88 | **Atranorin** | C19H18O8 |
| 3 | 1.12 | 153.0759 | 2.61 | Arabitol | C5H12O5 |
| 4 | 12.40 | 345.0969 | 1.45 | **Usnic Acid** | C18H16O7 |
| 5 | 7.05 | 387.0357 | -1.29 | Salazinic Acid | C18H12O10 |
| 6 | 12.04 | 359.1136 | -1.67 | Barbatic Acid | C19H20O7 |
| 7 | 12.39 | 709.1541 | -1.13 | Isousnic Acid | C18H16O7 |
| 8 | 10.47 | 639.1708 | 0.78 | Secalonic Acid | C32H30O14 |
| 9 | 8.14 | 373.0566 | -1.88 | Menegazziaic Acid/Protocetaric Acid | C18H14O9 |
| 10 | 10.06 | 467.0988 | -2.14 | Gyrophoric Acid | C24H20O10 |
| 11 | 10.4 | 487.1958 | 2.05 | Oxyphysodic Acid | C26H30O9 |
| 12 | 10.94 | 375.3257 | -95.38 | 3-Ketocholanic Acid | C24H38O3 |
| 13 | 9.53 | 274.2741 | 1.82 | N-Lauryldiethanolamine | C16H35NO2 |
| 14 | 13.07 | 339.2530 | 1.47 | 5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid | C20H34O4 |
| 15 | 11.33 | 315.1954 | 1.90 | 7-Oxodehydroabietic acid | C20H26O3 |
| 16 | 13.58 | 439.3571 | 1.14 | Oleanolic Acid | C30H48O3 |
| 17 | 13.9 | 637.3046 | -3.14 | (132S, 17S, 18S)-132-hydroxy-20-chloro-ethylpheophorbide a | C37H40N4O6 |
| 18 | 10.41 | 165.0548 | -1.21 | **p-coumaric acid** | C9H8O3 |
| 19 | 1.76 | 171.0345 | -33.33 | **Gallic acid** | C7H6O5 |
| 20 | 9.2 | 181.0495 | 0.00 | Caffeic acid | C9H8O4 |
| 21 | 10.08 | 333.0969 | 0.00 | Evernic acid | C17H16O7 |
| 22 | 1.38 | 353.0878 | 0.00 | Chlorogenic acid | C16H18O9 |
| 23 | 9 | 371.041 | -0.54 | Norstictic acid | C18H12O9 |
| 24 | 9.32 | 373.0916 | 0.54 | cryptostictinolide | C19H16O8 |
| 25 | 11.46 | 373.1292 | 0.00 | Diffractic acid | C20H22O7 |
| 26 | 8.31 | 387.071 | 0.00 | Stictic acid | C19H14O9 |
| 27 | 8.08 | 387.0722 | -0.26 | Cryptostictic acid | C19H16O9 |
| 28 | 9.35 | 391.102 | 1.02 | Squamatic acid | C19H18O9 |
| 29 | 6.65 | 401.0512 | 0.25 | Constictic acid/Peristictic acid | C19H14O10 |
| 30 | 12.32 | 417.1556 | -0.48 | Sekikaic acid | C22H26O8 |
| 31 | 12.35 | 429.3361 | 0.47 | Ergosterol peroxide | C28H44O3 |
| 32 | 8.75 | 429.0466 | -0.70 | Galbinic acid | C20H14O11 |

Figure 1: Chromatograms

































































Structures of compounds in Table 1





|  |
| --- |
| **Table 3: HRMS product ions for the peaks of the botanical extract** |
| Peak No. | RT (min) | *m/z* | Proposed ID’s (Confirmed with Std in **green**)(Structure with correlated MS2 spectra in **orange**)(Most probable ID is **blue**)CAS ID | Product Ions*m/z* Rel. Int.. |
| 1 | 10.79 | 149.0598 | Cinnamic acid |

|  |  |
| --- | --- |
| 67.0285 | 16 |
| 79.0532 | 7 |
| 91.0543 | 7 |
| 95.049 | 64 |
| 105.0333 | 100 |

 |
| 2 | 10.52 | 373.0930 | **Atranorin** |

|  |  |
| --- | --- |
| 80.1094 | 23 |
| 119.0493 | 50 |
| 133.0292 | 51 |
| 163.0396 | 100 |
| 177.0211 | 42 |
| 200.4178 | 28 |

 |
| 3 | 1.12 | 153.0759 | Arabitol |

|  |  |
| --- | --- |
| 53.0385 | 5 |
| 55.0176 | 5 |
| 57.0333 | 79 |
| 59.0489 | 3 |
| 61.0283 | 5 |
| 69.0333 | 100 |
| 70.9416 | 1 |
| 70.9811 | 1 |
| 71.0126 | 17 |
| 71.0489 | 20 |
| 73.0283 | 13 |
| 75.0442 | 1 |
| 76.775 | 1 |
| 80.2019 | 1 |
| 81.0334 | 5 |
| 87.0441 | 3 |
| 99.0441 | 83 |
| 111.9685 | 6 |
| 111.9839 | 1 |
| 112.0058 | 1 |
| 116.2628 | 1 |
| 117.0548 | 33 |
| 135.0656 | 14 |
| 153.0763 | 1 |

 |
| 4 | 12.40 | 345.0969 | **Usnic Acid** |

|  |  |
| --- | --- |
| 71.0126 | 3 |
| 85.0274 | 3 |
| 118.068 | 2 |
| 121.0287 | 2 |
| 131.329 | 2 |
| 133.6191 | 2 |
| 177.0542 | 5 |
| 187.0761 | 4 |
| 191.0703 | 8 |
| 201.0534 | 6 |
| 205.0477 | 3 |
| 205.087 | 4 |
| 215.07 | 37 |
| 219.0653 | 22 |
| 233.0808 | 100 |
| 235.0905 | 2 |
| 239.0711 | 3 |
| 243.0652 | 20 |
| 257.0802 | 14 |
| 261.0758 | 42 |
| 263.0725 | 4 |
| 267.0646 | 7 |
| 275.0928 | 4 |
| 281.0813 | 10 |
| 284.0702 | 2 |
| 285.0763 | 17 |
| 291.0688 | 3 |
| 294.0504 | 3 |
| 297.0386 | 5 |
| 299.0906 | 5 |
| 303.089 | 7 |
| 309.0764 | 17 |
| 312.0625 | 11 |
| 315.0515 | 13 |
| 327.0868 | 21 |
| 330.0748 | 10 |

 |
| 5 | 7.05 | 387.0357 | Salazinic Acid |

|  |  |
| --- | --- |
| 74.3278 | 28 |
| 84.5254 | 30 |
| 118.6729 | 31 |
| 121.0291 | 58 |
| 151.0403 | 46 |
| 178.3161 | 59 |
| 181.9935 | 35 |
| 213.0567 | 36 |
| 215.8074 | 38 |
| 225.0554 | 50 |
| 227.0329 | 81 |
| 241.0505 | 88 |
| 243.0276 | 100 |
| 269.0423 | 80 |

 |
| 6 | 12.04 | 359.1136 | Barbatic Acid |

|  |  |
| --- | --- |
| 63.4371 | 11 |
| 119.0497 | 22 |
| 120.0538 | 16 |
| 137.0601 | 100 |
| 138.064 | 58 |
| 150.4137 | 13 |
| 163.0398 | 78 |
| 164.0428 | 56 |
| 181.0497 | 72 |
| 182.0533 | 60 |
| 340.0687 | 13 |
| 241.0505 | 33 |
| 243.0276 | 37 |
| 269.0423 | 30 |

 |
| 7 | 12.39 | 709.1541 | Isousnic Acid |

|  |  |
| --- | --- |
| 83.0159 | 26 |
| 91.8227 | 3 |
| 113.785 | 3 |
| 122.5103 | 3 |
| 231.0713 | 23 |
| 232.0725 | 9 |
| 259.0667 | 81 |
| 260.0698 | 43 |
| 299.0997 | 10 |
| 300.0998 | 12 |
| 301.0722 | 8 |
| 302.0768 | 7 |
| 313.037 | 5 |
| 328.0652 | 100 |
| 329.0683 | 98 |
| 343.0894 | 31 |
| 344.0912 | 24 |

 |
| 8 | 10.47 | 639.1708 | Secalonic Acid |

|  |  |
| --- | --- |
| 95.0492 | 16 |
| 123.0442 | 46 |
| 141.0559 | 34 |
| 151.0392 | 29 |
| 169.0498 | 97 |
| 172.9659 | 15 |
| 183.0655 | 38 |
| 193.05 | 42 |
| 285.6843 | 16 |
| 346.91 | 16 |
| 377.0702 | 100 |
| 455.1151 | 44 |
| 469.099 | 32 |
| 483.1107 | 45 |
| 501.1237 | 58 |
| 502.1313 | 40 |
| 511.1093 | 18 |
| 529.0942 | 17 |
| 543.1223 | 17 |

 |
| 9 | 8.14 | 373.0566 | Menegazziaic Acid/Protocetaric Acid |

|  |  |
| --- | --- |
| 62.5342 | 59 |
| 142.6553 | 61 |
| 256.0666 | 79 |
| 268.0692 | 100 |

 |
| 10 | 10.06 | 467.0988 | Gyrophoric Acid |

|  |  |
| --- | --- |
| 73.4358 | 3 |
| 97.0183 | 4 |
| 167.0341 | 35 |
| 178.3149 | 10 |
| 317.0648 | 100 |

 |
| 11 | 10.4 | 487.1958 | Oxyphysodic Acid |

|  |  |
| --- | --- |
| 71.0848 | 8 |
| 84.1846 | 3 |
| 95.0849 | 4 |
| 177.0896 | 3 |
| 219.0645 | 10 |
| 273.0418 | 4 |
| 275.0604 | 3 |
| 301.0328 | 4 |
| 317.1368 | 14 |
| 328.0949 | 12 |
| 329.1014 | 9 |
| 373.2081 | 6 |
| 399.1049 | 10 |
| 427.1762 | 100 |
| 427.3089 | 6 |

 |
| 12 | 10.94 | 375.3257 | 3-Ketocholanic Acid |

|  |  |
| --- | --- |
| 67.0539 | 73 |
| 69.0696 | 40 |
| 79.0541 | 44 |
| 81.0698 | 61 |
| 91.0543 | 23 |
| 93.0697 | 100 |
| 94.0735 | 29 |
| 95.0856 | 61 |
| 96.0888 | 25 |
| 101.8679 | 17 |
| 107.0857 | 43 |
| 119.0856 | 20 |
| 121.1011 | 39 |
| 122.1051 | 22 |
| 311.9981 | 22 |
| 376.3295 | 29 |

 |
| 13 | 9.53 | 274.2741 | N-Lauryldiethanolamine |

|  |  |
| --- | --- |
| 57.0697 | 61 |
| 58.0653 | 5 |
| 59.3362 | 4 |
| 70.065 | 85 |
| 71.0853 | 17 |
| 78.7121 | 4 |
| 82.8639 | 5 |
| 88.0756 | 100 |
| 102.0913 | 24 |
| 106.0864 | 34 |
| 256.2649 | 12 |
| 274.2759 | 17 |

 |
| 14 | 13.07 | 339.2530 | 5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid |

|  |  |
| --- | --- |
| 67.054 | 54 |
| 69.0695 | 81 |
| 71.0854 | 35 |
| 81.0696 | 100 |
| 83.0848 | 43 |
| 93.0696 | 56 |
| 95.0852 | 51 |
| 109.1012 | 27 |
| 116.9715 | 36 |
| 121.1019 | 37 |
| 135.1167 | 30 |

 |
| 15 | 11.33 | 315.1954 | 7-Oxodehydroabietic acid |

|  |  |
| --- | --- |
| 66.499 | 2 |
| 69.0698 | 2 |
| 87.1659 | 2 |
| 98.9858 | 2 |
| 99.7033 | 2 |
| 107.0855 | 3 |
| 117.0706 | 5 |
| 129.0686 | 4 |
| 131.0854 | 11 |
| 143.0854 | 3 |
| 145.0645 | 9 |
| 147.0807 | 9 |
| 157.0642 | 9 |
| 171.0803 | 14 |
| 171.1102 | 2 |
| 173.0953 | 4 |
| 187.1118 | 100 |
| 190.6516 | 2 |
| 199.1118 | 14 |
| 209.1322 | 6 |
| 213.1277 | 14 |
| 227.1426 | 4 |
| 318.0153 | 2 |

 |
| 16 | 13.58 | 439.3571 | Oleanolic Acid |

|  |  |
| --- | --- |
| 81.0703 | 30 |
| 95.0856 | 51 |
| 107.0865 | 31 |
| 109.1018 | 45 |
| 119.0852 | 48 |
| 121.1002 | 54 |
| 133.1004 | 38 |
| 147.1173 | 41 |
| 163.1487 | 28 |
| 189.1635 | 56 |
| 189.3654 | 31 |
| 203.1808 | 100 |
| 239.3945 | 26 |

 |
| 17 | 13.9 | 637.3046 | (132S, 17S, 18S)-132-hydroxy-20-chloro-ethylpheophorbide a |

|  |  |
| --- | --- |
| 83.0484 | 18 |
| 91.0528 | 3 |
| 91.4687 | 3 |
| 109.2185 | 2 |
| 116.5271 | 2 |
| 141.0693 | 4 |
| 147.116 | 26 |
| 163.0137 | 3 |
| 175.0142 | 4 |
| 227.0445 | 6 |
| 239.0454 | 13 |
| 260.9916 | 5 |
| 283.1035 | 3 |
| 337.024 | 100 |
| 337.1167 | 5 |
| 393.0859 | 36 |
| 413.0583 | 6 |
| 469.1185 | 20 |
| 525.1804 | 25 |
| 581.2383 | 6 |

 |
| 18 | 10.41 | 165.0548 | **p-coumaric acid** |

|  |  |
| --- | --- |
| 59.4256 | 21 |
| 65.94 | 50 |
| 74.1178 | 20 |
| 83.9297 | 100 |
| 84.0117 | 24 |
| 101.9409 | 39 |
| 110.0691 | 22 |

 |
| 19 | 1.76 | 171.0345 | **Gallic acid** |

|  |  |
| --- | --- |
| 53.9389 | 12 |
| 55.0541 | 4 |
| 55.9344 | 4 |
| 60.9863 | 44 |
| 60.9934 | 5 |
| 70.942 | 2 |
| 72.9368 | 11 |
| 78.9969 | 100 |
| 88.9522 | 4 |
| 89.9636 | 6 |
| 97.0075 | 49 |
| 100.1328 | 2 |
| 102.0132 | 6 |
| 106.9921 | 4 |
| 120.0243 | 6 |

 |
| 20 | 9.2 | 181.0495 | Caffeic acid |

|  |  |
| --- | --- |
| 54.9471 | 5 |
| 56.7179 | 5 |
| 56.9647 | 42 |
| 57.9346 | 100 |
| 67.0176 | 12 |
| 79.0543 | 7 |
| 91.47 | 5 |
| 98.9607 | 15 |
| 107.049 | 5 |
| 116.9719 | 33 |
| 126.9686 | 8 |
| 135.0445 | 47 |

 |
| 21 | 10.08 | 333.0969 | Evernic acid |

|  |  |
| --- | --- |
| 72.0929 | 72 |
| 113.6758 | 66 |
| 226.062 | 81 |
| 254.0544 | 100 |
| 322.8821 | 74 |

 |
| 22 | 1.38 | 353.0878 | Chlorogenic acid | n/a |
| 23 | 9 | 371.041 | Norstictic acid |

|  |  |
| --- | --- |
| 81.1114 | 84 |
| 110.0152 | 100 |

 |
| 24 | 9.32 | 373.0916 | cryptostictinolide | n/a |
| 25 | 11.46 | 373.1292 | Diffractic acid |

|  |  |
| --- | --- |
| 68.6623 | 23 |
| 82.3379 | 25 |
| 103.3434 | 29 |
| 135.0813 | 100 |
| 145.391 | 27 |
| 178.2756 | 47 |
| 281.6184 | 26 |

 |
| 26 | 8.31 | 387.071 | Stictic acid |

|  |  |
| --- | --- |
| 149.5851 | 37 |
| 164.1086 | 42 |
| 178.3198 | 85 |
| 209.0617 | 47 |
| 254.0553 | 96 |
| 267.0312 | 100 |
| 284.3924 | 43 |

 |
| 27 | 8.08 | 387.0722 | Cryptostictic acid |

|  |  |
| --- | --- |
| 119.9679 | 17 |
| 121.0294 | 70 |
| 201.0572 | 20 |
| 213.0543 | 44 |
| 220.3165 | 18 |
| 225.0555 | 69 |
| 227.0337 | 100 |
| 228.0608 | 21 |
| 239.0333 | 51 |
| 241.0482 | 52 |
| 243.0294 | 60 |
| 251.033 | 46 |
| 253.0489 | 30 |
| 256.0677 | 31 |
| 269.0432 | 56 |
| 289.928 | 21 |
| 314.1177 | 21 |

 |
| 28 | 9.35 | 391.102 | Squamatic acid |

|  |  |
| --- | --- |
| 119.0497 | 15 |
| 127.1115 | 21 |
| 137.0606 | 50 |
| 143.1074 | 12 |
| 155.1095 | 14 |
| 157.1226 | 13 |
| 163.0399 | 74 |
| 171.1018 | 17 |
| 181.051 | 38 |
| 187.0977 | 13 |
| 195.1349 | 11 |
| 201.1129 | 72 |
| 211.1314 | 15 |
| 213.1493 | 21 |
| 240.6411 | 10 |
| 371.2781 | 46 |
| 387.2723 | 59 |
| 389.2882 | 100 |

 |
| 29 | 6.65 | 401.0512 | Constictic acid/Peristictic acid | n/a |
| 30 | 12.32 | 417.1556 | Sekikaic acid |

|  |  |
| --- | --- |
| 79.0967 | 7 |
| 108.1354 | 9 |
| 110.0875 | 7 |
| 149.0604 | 19 |
| 165.0544 | 17 |
| 193.0499 | 100 |
| 400.9641 | 9 |

 |
| 31 | 12.35 | 429.3361 | Ergosterol peroxide | n/a |
| 32 | 8.75 | 429.0466 | Galbinic acid | n/a |
|  |  |  |  |  |
|  |  |  |  |  |
|  |  |  |  |  |
|  |  |  |  |  |
|  |  |  |  |  |