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Identification of Unknown Compound in *Apocynum cannabinum* by High Resolution Mass Spectrometry (HRMS) and 600 MHz NMR

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Abstract

High resolution mass spectrometry (HRMS) was able to determine that the *m/z* of the $[M+H]^+$ ion formed by an unknown compound that was purified by preparative scale liquid chromatography (LC) was 137.05966. This was done rapidly and easily by direct infusion. Neither a column nor chromatography was needed. This is within 0.4 ppm of the mass calculated by ThermoFisher's exact mass calculator for the protonated version of $C_8H_8O_2$. Based on this, the compound could have been 2'-, 3'- or 4'-hydroxyacetophenone or 2'-, 3'- or 4'-methoxybenzaldehyde. However, ^{1}H- and ^{13}C-NMR spectra of the isolated compound showed that it was 4'-hydroxyacetophenone, without needing a standard. Neither mass spectrometry (MS) nor NMR could have identified this compound by itself. Finally, HRMS was also able to verify that another peak isolated by preparative scale LC was apocynin, or 4'-hydroxy-3'-methoxyacetophenone.

Keywords: HRMS, nmr, 4'-hydroxyacetophenone, apocynin, non-targeted analysis

1. Introduction

Apocynum cannabinum L. (also known as dogbane and Indian hemp) is a native North American plant that can be 0.61−1.83 m tall, with elliptical leaves, small flowers and fruits that are 2.4−3.5 cm long, with a slender, cylindrical shape [1]. The rhizomes of *A. cannabinum* contain the bioactive compound apocynin, or 4'-hydroxy-3'-methoxyacetophenone that impart many important health effects [2, 3]. Apocynin and its dehydro dimer, diapocynin, inhibit the enzyme NADPH oxidase, which is bound to cell membranes and protects against the formation of reactive oxygen substances, also known as ROS [4]. Still, there might be other bioactive compounds in the rhizomes of *A. cannabinum* that have not yet been identified. At least one of them can be separated from apocynin by preparative scale LC. So, the objective of this study was to identify the unknown compound that was isolated from the rhizomes of *A. cannabinum*.

HRMS can measure the *m/z* of ions with an accuracy that is good enough to unequivocally determine the molecular formula of the parent compound. That is, some compounds may have the same masses to within ± 0.5 Dalton, such as cyclohexane (C_6H_{12}), cyclopentanone C_5H_8O and cyanopropylamine ($C_4H_8N_2$). However, their exact masses are 84.0939, 84.0575 and 84.0688, respectively. So, HRMS with a resolution of 140,000 at *m/z* of 200 and <1 ppm mass accuracy can distinguish between them. At the same time, HRMS can unequivocally determine the molecular formula from the accurate mass.

2. MATERIALS AND METHODS

2.1. Materials

Rhizomes of *A. cannabinum* were harvested in April, 2012 from a large naturally-occurring population growing in a bottomland area at the University of Missouri's Southwest Research Center (lat. 37.083110, long. -93.868260), then washed, air dried (2 d at ≈ 50 °C), and ground to prepare for analysis. They were extracted by sonication with methanol for several hours and then let set overnight at room temp.The mixture was centrifuged. Then, 1 mL of the supernatant was combined with 1 mL of water and analyzed by HPLC using Hitachi Model L-7100 pump with Hitachi Model L-7400 UV detector (set at 270 nm), Hitachi Model L-7200 autosampler with a Hitachi D-7000

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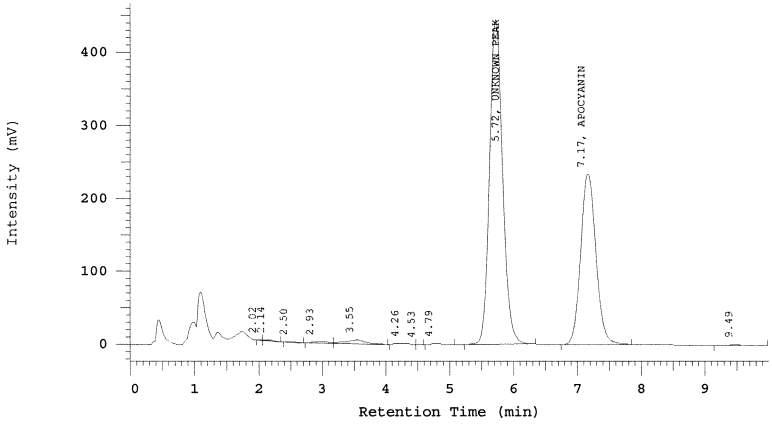

Figure 1: HPLC chromatogram of methanolic extract of *Apocynum cannabinum* showing the unknown peak eluting at 5.72 min and the peak due to apocynin at 7.17 min

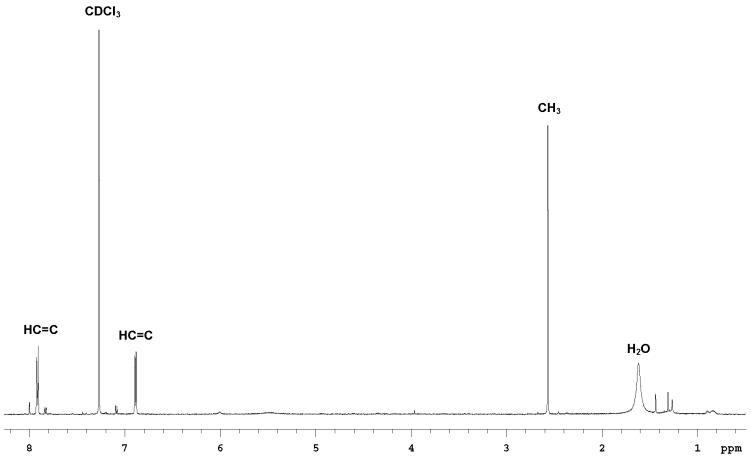

Figure 2: ^{1}H-NMR of the unknown compound that produced the peak eluting at 5.72 min

data acquisition interface and ConcertChrom software on a microcomputer. A Phenomenex Hyperclone 3μm BDS C18 column, 100 $\times$ 4.6mm, fitted with a C_{18} ODS SecurityGuard 4.0 $\times$ 3.0 mm guard column (Phenomenex) was used with a mobile phase of methanol:acetonitrile:water (25:25:50). Fractions were collected after about 5.2 to 6.4 min and 6.6 to 7.7 min, corresponding to responses from the UV detector. The solvent was evaporated off from both fractions. The dry residues were sent to the Kansas District Lab of the FDA for HRMS analysis and then to the University of Missouri−St. Louis for NMR analysis. For HRMS analysis, the purified sample was redissolved in methanol (CH_3OH). For NMR analysis, the purified sample was redissolved in deuterated chloroform ($CDCl_3$).

2.2. Method

The HRMS analysis was done using direct infusion into a Q-Exactive HPLC-MS from ThermoFisher (Sunnyvale, CA) in the positive ion mode. The molecular formula of the unknown compound was calculated from its *m/z* using Thermo Fisher's exact mass calculator, Qual Browser−Isotope Simulation, as part of their Xcalibur 3.0.63 software. ^{1}H and ^{13}C{^{1}H}-NMR spectra were obtained using an Agilent DD2 600 MHz NMR (Santa Clara, CA). A 30° pulse width and 1 sec pulse delay were used for the ^{1}H NMR, while a 30° pulse width and 2 sec pulse delay were used for the ^{13}C-NMR spectra. Chemical shifts were referenced to the $CDCl_3$ peaks at 7.27 and 77.23 ppm for ^{1}H and ^{13}C, respectively.

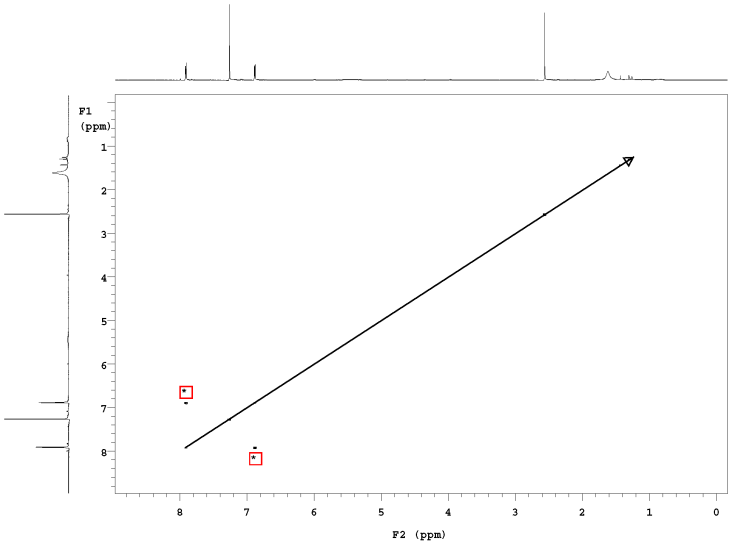

Figure 3: COSY NMR spectrum of the unknown compound that produced the peak eluting at 5.72 min. The line connects all the signals in the original ^{1}H-NMR spectrum. The two signals marked by asterisks (*) in boxes that do not lie on this line show the signals due to HC=C aromatic hydrogens that are on carbons that are adjacent to each other

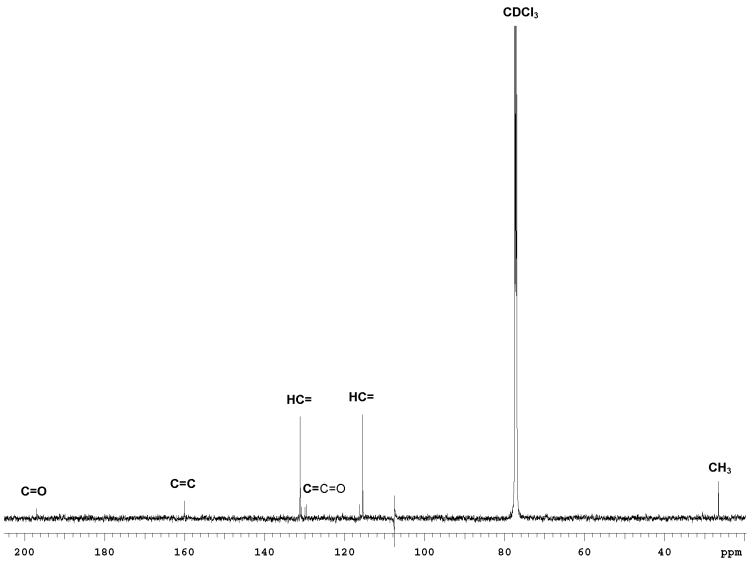

Figure 4: ^{13}C{^{1}H}-NMR spectrum of the unknown compound that produced the peak eluting at 5.72 min

3. RESULTS AND DISCUSSION

The HPLC chromatogram of the methanolic extract of *A. cannabinum* rhizomes is shown in Figure 1. Apocynin eluted at 7.17 min, as confirmed by injecting an apocynin standard. *m/z* of the purified compound was 137.05966. This is within 0.4 ppm of the mass calculated by ThermoFisher's exact mass calculator for the protonated version of $C_8H_8O_2$. Based on this, the compound could have been 2'-, 3'- or 4'-hydroxyacetophenone or 2'-, 3'- or 4'-methoxybenzaldehyde. In addition, a fragment with *m/z* of 95.04916 was seen. This was probably due to C_6H_7O (predicted m/z = 95.04914) but there are other possible candidates. Still, this does support the idea that the compound is a phenol instead of an aldehyde.

To distinguish between 2'-, 3'- and 4'-hydroxyacetophenone from 2'-, 3'- and 4'-methoxybenzaldehyde, NMR was required. The ^{1}H-NMR spectrum (Figure 2) had signals (or peaks) with chemical shifts of 2.564 ppm (singlet, due to methyl), 6.886

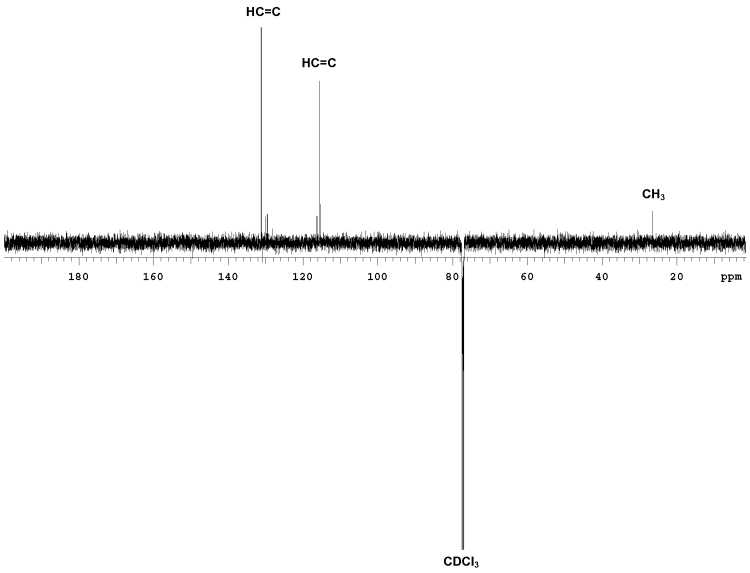

Figure 5: Attached proton test (APT) of the unknown compound that produced the peak eluting at 5.72 min. Carbons with one or three attached protons produce positive peaks

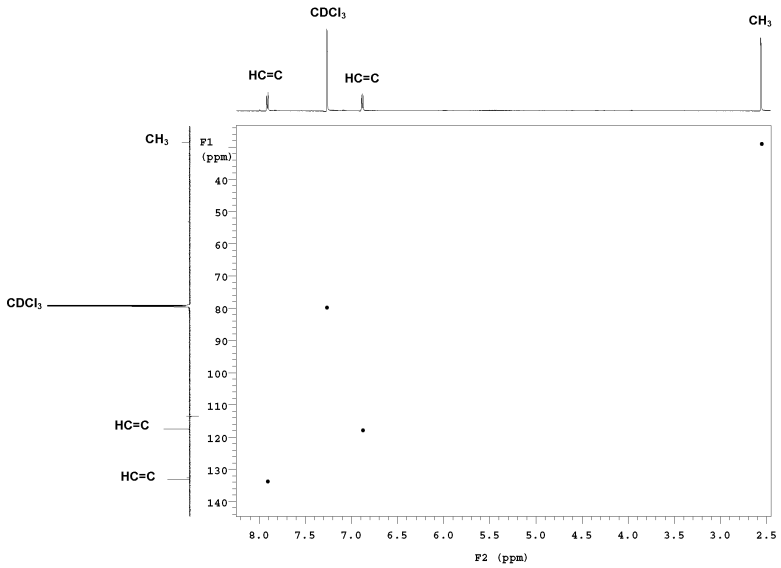

Figure 6: Heteronuclear Multiple-Quantum Correlation (HMQC) spectrum of the unknown compound that produced the peak eluting at 5.72 min. The top part shows the ^{1}H spectrum and the left shows the ^{13}C{^{1}H} spectrum. The four small dots in the middle show which signals in the ^{1}H and ^{13}C{^{1}H} spectra are correlated with each other

and 7.908 ppm (aromatic C-H). The correlation spectroscopy (COSY) spectrum (Figure 3) shows that the carbons with attached aromatic C-H are adjacent to each other. The ^{13}C{^{1}H}-NMR spectra (Figure 4) had signals with chemical shifts of 26.573 (methyl), 115.478, 130.536, 131.152, 160.283 (four aromatic carbons) and 197.021 (C=O) ppm. The signal at 130.536 ppm is due to carbon C-1'. That is, it is due to the C= carbon that is attached to the C=O. All of the chemical shifts are in good agreement with those that were published on a website [5]. The nomenclature ^{13}C{^{1}H}-NMR indicates that a proton decoupled ^{13}C-NMR spectrum was acquired. That is, the magnetizations due to protons are saturated with a continuous radio frequency (rf) transmission at 600 MHz. This decouples the magnetization of the protons from the ^{13}C nuclei. As a result, the relative sizes of the signals (or peaks) in the ^{13}C{^{1}H}-NMR that have attached hydrogens increase due

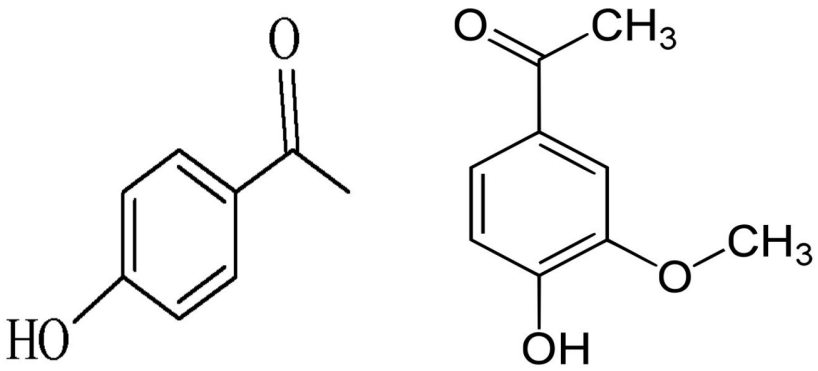

Figure 7: Structures of 4'-hydroxyacetophenone (left) and apocynin (4'-hydroxy-3'-methoxyacetophenone)

to the nuclear Overhauser effect (NOE). So, the signals due to the methyl and HC= carbons are larger than the signals due to C=C and C=O carbons that have no attached hydrogens. To further support the hypothesis that the unknown compound is 4'-hydroxyacetophenone, an attached proton test (APT) was performed. The APT spectrum (Figure 5) shows how many protons (hydrogens) are attached to each carbon. Carbons with one or three attached protons produce negative signals. So, this confirms that the signals at 26.573, 115.478 and 131.152 are due to carbons with one or three hydrogens attached. The chemical shifts indicate that the signal at 26.573 ppm was due to a carbon with three attached protons, while the signals at 115.478 and 131.152 were due to aromatic carbons with one attached proton. To add additional support, a heteronuclear multiple-quantum correlation (HMQC) spectrum was acquired (Figure 6). It shows that the signals in the ^{1}H-NMR spectrum at 2.564, 6.886 and 7.908 ppm were produced by hydrogens that were attached to the carbons that produced signals at 26.573, 115.478 and 131.152 ppm, respectively. These NMR results are consistent with the compound being 4'-hydroxyacetophenone and not any of its isomers. Due to molecular symmetry, the ^{1}H-NMR spectrum of 4'-hydroxyacetophenone has two sets of doublets in the phenyl region. Also, there are only four signals due to aromatic carbons in the ^{13}C{^{1}H}-NMR spectrum. The other isomers, being asymmetric would have produced four signals in the aromatic region of the ^{1}H spectrum and six in the aromatic region of the ^{13}C spectrum. Also, the presence of signals at 2.564 and 26.478 ppm in the ^{1}H- and ^{13}C{^{1}H}-NMR spectra shows that the single methyl is attached to a carbonyl carbon (as an acetophenone), as opposed to being attached to an oxygen, as in 2'-, 3'- and 4'-methoxybenzaldehyde [6].

So, the unknown compound is unequivocally identified as 4'-hydroxyacetophenone. It is like the bioactive compound, apocynin, or 4'-hydroxy-3'-methoxyacetophenone [2]. It eluted after 4'-hydroxyacetophenone and produced a $[M+H]^{+}$ ion with m/z = 167.07022. The expected m/z for apocynin is 167.07027, based on ThermoFisher's exact mass calculator. It can be metabolized to its dehydro dimer, diapocynin [6], which inhibits NADPH oxidase, like apocynin [7]. It is possible that 4'-hydroxyacetophenone can also be metabolized to its dehydro dimer, since it has the same reactive aromatic C-H that apocynin has [8]. Like apocynin, 4'-hydroxyacetophenone can inhibit NADPH oxidase [9]. Unlike apocynin, it has been found in soil bacteria (*Pseudomonas* species), a plant used in traditional medicine for treating liver diseases (*Artemisia capillaris*) and the rhizomes of the Asian traditional herbal medicine *Cynanchum wilfordii* [10–12]. An extract of *A. capillaris* prepared in boiling water was able to protect against fibrosis and liver damage in an animal model [11]. In another study, an extract of *C. wilfordii* and 4'-hydroxyacetophenone inhibited the expression of pro-inflammatory intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in human aortic muscle cells that were stimulated with the pro-inflammatory tumor necrosis factor-α (TNF-α) to mimic atherosclerosis [12]. So, 4'-hydroxyacetophenone and botanicals that contain it may be good additions to the diet for helping to prevent liver fibrosis, atherosclerosis and cardiovascular diseases [11, 12]. So, it is possible that some of the health effects that *A. cannabinum* exhibits may be due to not just apocynin and diapocynin, but also 4'-hydroxyacetophenone and its dimer. The structures of the monomers are shown in Figure 7.

It should be added that there is a book in which it was written that 4'-hydroxyacetophenone occurs in *Apocynum cannabinum*, without citing any peer reviewed research articles [13]. Moreover, to the best of our knowledge, no peer reviewed article has ever been published that showed that 4'-hydroxyacetophenone occurs in *Apocynum cannabinum*.

In conclusion, this work shows that we can do untargeted analyses by HRMS and NMR and that we have an active and productive collaboration with the University of Missouri.

This work should not be taken as reflecting FDA policy or regulations.

4. ACKNOWLEDGEMENT

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5. Article Information

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