

Identification of Antineoplastic and Neurotrophic Lignans in Medicinal Prairie Plants by Liquid Chromatography Electron Impact Mass Spectrometry (LC/EI/MS)

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INTRODUCTION

Acorus calamus or sweet flag has been long known for its medicinal value and is cultivated in Asia for this reason. The rhizomes of *Acorus calamus* contain aromatic oil that has been used medicinally since ancient times. The rhizomes are considered to possess anti-spasmodic, carminative and anthelmintic properties and are also used for the treatment of epilepsy, mental ailments, chronic diarrhea, dysentery, bronchial catarrh, intermittent fevers and glandular and abdominal tumors. They are also employed for kidney and liver troubles, rheumatism, sinusitis, and eczema. **The discovery of two plant lignans, epiuedesmin and galgravin, in the leaves of the plant potentially explains several of the purported activities attributed to *Acorus calamus*.** Epiuedesmin has been shown to have antineoplastic activity against the murine P388 lymphocytic leukemia cell line and several human cancer cell lines (BXP-3, MCF-7, SF268, NCI-H460, KM20L2, & DU-145).¹ Galgravin has demonstrated activity in preventing neuronal death and stimulating neurite growth. Structurally similar lignans have also shown neuroprotective activity in *in vitro* models for Alzheimer's and Parkinson's disease.² Both epiuedesmin and galgravin were identified in the methanolic extracts of *Acorus calamus* leaves by liquid chromatography electron impact mass spectrometry. This method permits the rapid assessment of plant extracts for the presence of medicinally active non-volatile compounds with a minimum of pre-purification. Extracts are separated by RP-HPLC prior to volatilization of the LC effluent and introduction into the electron impact ionization chamber. The advantage to acquiring EI fragmentation data lies within the subsequent ability to use existing deconvolution and search programs to match results with well-established and extensive commercially available EI mass spectral databases.

SAMPLE PREPARATION FOR MASS SPECTROSCOPY

The *Acorus calamus* leaves were chopped (~1cm pieces) and the organic constituents extracted with methanol overnight with stirring. The leaves were removed by suction filtration and the filtrate shaken overnight with hexane/dichloromethane (60:40). The aqueous layer was removed in a separatory funnel, the organic solvent was washed with saturated sodium chloride, dried over sodium sulfate and the solvent was rotary evaporated to obtain a viscous dark oil. Samples of oil were dissolved in acetonitrile for LC/EI/MS or in acetone for the GC-MS.

INSTRUMENTATION – PARTICLE BEAM LC/EI/MS INTERFACE

For particle beam LCMS, the system included the following components. The liquid chromatograph used was an Agilent Model 1100 modular system with quaternary pump, vacuum degasser, 100 vial autosampler and variable wavelength detector. The HPLC column used was a Zorbax SB-C18 (Agilent pn 830990-902), narrow bore 2.1 x 150 mm 3.5 micron. The Genesis II particle beam interface (CSS Analytical Co Inc.) was attached to an Agilent 5973 MSD so that samples can be analyzed by LC/MS with electron impact and chemical ionization. The Genesis II is an improved particle beam interface, which delivers a higher amount of analyte to the ion source, when compared to previous commercial interfaces. The mass spectrometer used was an unmodified Agilent 5973 Mass Selective Detector (Agilent Technologies, Inc., Palo Alto California) with turbo molecular pump. The Agilent 5973 is a bench top quadrupole mass spectrometer with mass range of 1.6 to 800 mass units, 10,000 volt HED, and is available with EI or EI/CI capabilities.

INSTRUMENTATION – GCMS

The GC utilized for the analysis was a Agilent 5890 equipped with a Zebron ZB-1 column (15m x 0.25mm x 0.25um) (Phenomenex Torrance, CA). The mass spectrometer used was an unmodified bench top quadrupole Agilent 5971 Mass Selective Detector (Agilent Technologies, Inc., Palo Alto California). The 5971 was controlled by Eighty-X[®] data system (CSS Analytical Co Inc.) with Agilent G1701BA Chemstation running on Microsoft Windows 2000. The conditions for the GC were initial oven temperature of 40°C, injector 250°C, transfer line 280°C, a solvent delay of 2.00 min, the temperature was ramped at 10°C/min to a final temperature of 230°C and held for 1.00 min.

LIQUID CHROMATOGRAPHIC SEPARATION

The constituents were separated using a water (A) and acetonitrile (B) gradient. Initial conditions were 5% acetonitrile (0-3min) increasing to 95% acetonitrile at 50 minutes holding to 65 minutes and returning to starting conditions at 70 min. The detection wavelength was 254nm.

MASS SPECTRAL ANALYSIS

The data collected from the GC-MS and the Particle Beam LC/EI/MS was analyzed with AMDIS (Automated Mass Spectral Deconvolution and Identification System), version 2.1, DTRA/NIST, 2002. The Total Ion Chromatograph (FIGURE 5) is delayed by approximately 0.2 minutes relative to the HPLC trace as determined by comparison of the two respective data sets. Since no library was available for searching for the ESI, spectral matches were made by comparing the parent ion molecular weight with those obtained for the Particle Beam EI ionization.

FIGURE 1. EPIEUDESMIN

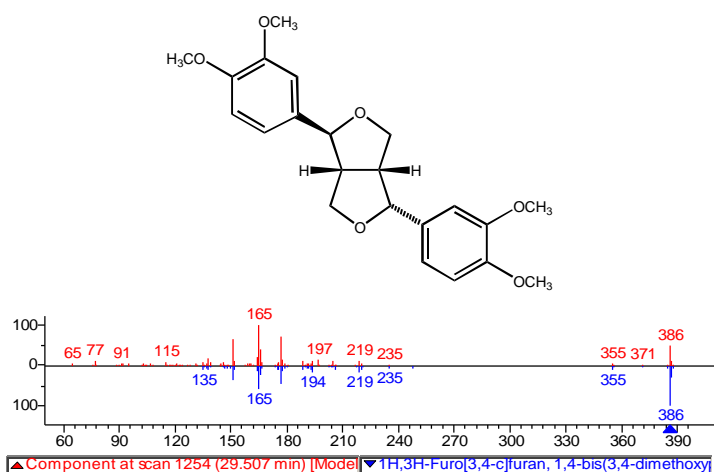


FIGURE 2. LIBRARY MATCH FOR EPIEUDESMIN

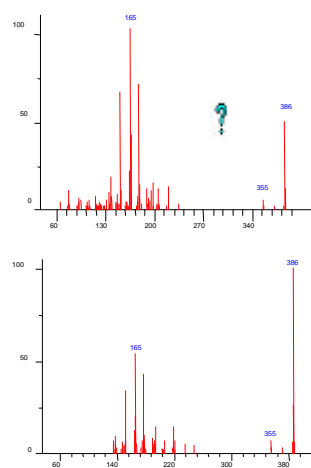


FIGURE 3. GALGRAVIN

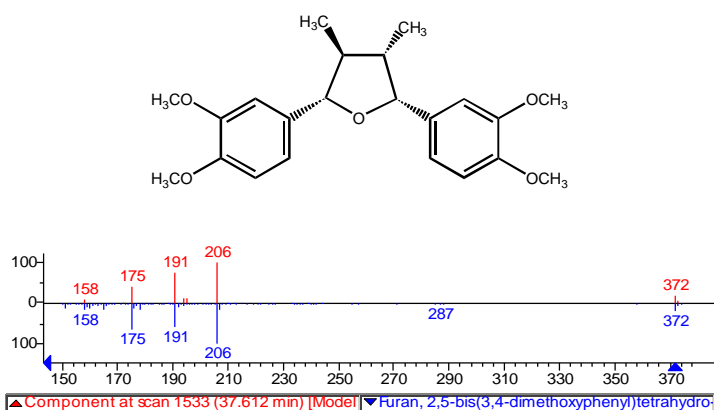


FIGURE 4. LIBRARY MATCH FOR GALGRAVIN

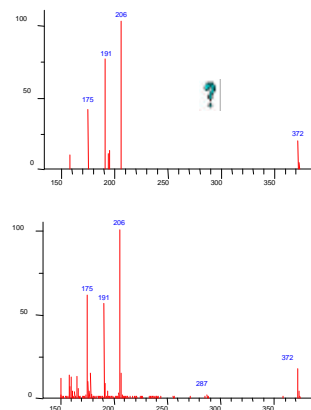
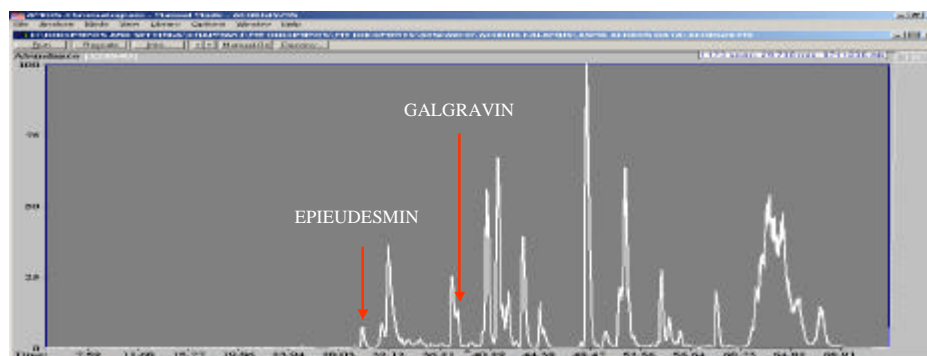


FIGURE 5. TOTAL ION CHROMATOGRAPH FROM PB-LC/EI/MS



RESULTS

Interesting results were obtained from both the GCMS and the LCMS analysis of the *Acorus calamus* leaf extract. The GCMS was able to separate ~47 constituents from the extract. By HPLC, a chromatographic method was successfully developed using a reverse-phase column with UV detection at 254 nm that could resolve ~35 peaks from the extract. When this separation was coupled with the Particle Beam LC/EI/MS mass spectrometry, the Total Ion Chromatogram shown in FIGURE 5 was obtained. The collected data for the GCMS and Particle Beam LC/EI/MS was submitted for deconvolution and extracted ion analysis using the AMDIS program. The deconvolution program found 189 ion signatures in the Particle Beam TIC for the organic extract of the leaves.

The deconvolution program isolates and identifies every component ion signature that it believes is unique and frequently will match the same component several times to one peak in the TIC. This exhaustive analysis does produce some redundancies, but is critical to the discovery and investigation of co-eluting component peaks. Our evaluation of the EI data yielded about 50-60 completely unique ion signatures in the analysis of the *Acorus calamus* leaf constituents from the Particle Beam LC/EI/MS. Using the combined information from the GCMS and the Particle Beam LC/EI/MS, we were able to identify 29 constituents by mass spectral matching and several of these assignments were further supported by comparison to previously published accounts of the constituents. While the matching does postulate the presence of the constituent, it alone is not proof of the absolute identity. We were able to confirm the identity of epieudesmin by comparing the retention time and fragmentation under the same ionization conditions to an authentic synthesized standard provided by Dr. Brown's laboratory.

To this point we have identified only a fraction of the chemical constituents of the *Acorus calamus* leaves. While we were able to find several previously identified constituents, we did identify several constituents that have not previously been reported in the literature. With one exception, all of the compounds that were identified as novel components were discovered with the aid of the PB-LC/EI/MS. The PB-LC/EI/MS provided an analytical tool for exploring constituents of plant extracts not amenable to analysis by standard GCMS or LC/ESI/MS techniques.

One of the newly identified constituents is epieudesmin, which belongs to the class of plant molecules known as lignans. Several of epieudesmin's confirmed pharmacological actions correlate to the purported herbal medicinal actions or uses of *Acorus calamus* leaves or rhizomes and its presence in the leaves would implicate it as the medicinally active agent. Another lignan, galgravin, was one of several lignans used for the treatment of malaria and rheumatism in addition to its neurotrophic effects.² Another constituent, sakuranin, has been shown to have antihyperlipidemic activity and plant extracts containing sakuranin have been used as a herbal treatment for diabetes. The flavanoid retusin was identified and has previously been shown to be an anti-tumor agent and psychoactive agent. Teas containing this compound are used as anti-inflammatory and analgesics and also used as a purgative, laxative, and cathartic.

NOVEL CHEMICAL CONSTITUENTS DISCOVERED BY PB-LC/EI/MS: (9E,12E,15E)-9,12,15-Octadecatrien-1-ol, Apigenin 4',7-dimethyl ether, Epieudesmin, Galgravin, 2-Allyl-5-ethoxy-4-methoxyphenol, Dehydroabietic acid, Retusin, and Sakuranin.

CONCLUSIONS

Almost all new commercial LC/MS systems today are atmospheric pressure ionization (API) instruments and use ionization techniques like Atmospheric Pressure Electrospray Ionization (AP-ESI) and Atmospheric Pressure Chemical Ionization (AP-CI). Despite this, it is important to remember that the analysis of a true unknown is an extremely difficult task and may require the utilization of more than one technique. At this time, matching obtained data to a commercially available (Wiley, NIST, EPA ...), well accepted and standardized database of mass spectra (produced by Electron Impact Ionization) is the surest way to identify an unknown. Commercial and standardized libraries are not yet prevalent for AP-CI and AP-ESI. While individual users of AP-CI and AP-ESI instruments are able to create "in-house" libraries, they (and instrument vendors) have not been able to create cross platform (instrument type and instrument manufacturer) libraries for use by the entire community of API instrument users like exists for the EI instrument users. Other techniques include using GCMS to investigate the essential oils obtained by steam distillation or the organic oils obtained by solvent extraction. This method of analysis works well for the volatile constituents obtained by either extraction route, but does not work for the non-volatiles without tedious derivatization steps. While derivatization is possible for a single molecule, it is extremely difficult for entire plant extracts and the failure to modify a constituent could mean that a possible lead is omitted. The combination of LC and EI mass spectral analysis exploits the best of each method. While the non-volatility of a component might result in thermal decomposition on-column or a failure to escape the column matrix, the same component would not be exposed to high temperatures in LC and the mobile phase could be adjusted to insure elution from the column. In principle, a gradient elution would separate and elute all of the components in a complex plant extract. When this non-destructive LC separation is coupled with the Particle Beam effluent volatilization it permits the exploration of plant fractions unavailable to GCMS. The EI fragmentation pattern for a molecule remains constant and does not depend on specific introduction routes (GC, LC, or direct probe). EI mass spectrometers operate within a relatively narrow set of analytical conditions with regards to ionizing voltage. Therefore, mass spectral searching is not limited to a specific instrument type nor to a single introduction route to the EI chamber. When the collected EI fragmentation data is combined with a deconvolution and search program PB-LC/EI/MS can provide vital information for the identification of medicinal and chemical constituents unavailable to GC or AP-ESI mass spectrometry.

In summary, an evaluation of our experiments showed the following:

1. The Particle Beam LC/EI/MS works well for the organic extract obtained from the leaves and eliminates the need for any preparatory derivatization steps that are typically required for the introduction of polar compounds into a GC.
2. The ability to search EI spectral libraries makes the identification of the constituents in the mixture much less tedious than the analysis of each component's fragmentation pattern by hand.
3. LC/EI/MS with Particle Beam sample introduction is capable of providing fragmentation information on molecules inaccessible to other techniques.
4. When coupled with a deconvolution and search program PB-LC/EI/MS can provide vital information for the identification of medicinal and chemical constituents displaying the utility and efficacy of the Particle Beam EI Interface as a vital tool of drug discovery.

FUTURE WORK

Our intentions are to utilize the Particle Beam LC/EI/MS to identify volatile and non-volatile polar and non-polar constituents in the rhizomes of the *Acorus calamus* plant by the same chromatographic separation.

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