

Chemical Constituents of *Acorus calamus* from a Kansas Prairie

James M. Chapman¹, Ryan Meyer¹, Kelly Kindscher², Scott Niemann³.

¹Rockhurst University, Kansas City, MO, ²University of Kansas, Lawrence, KS,

³CSS Analytical Company, Shawnee, KS

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 and has been harvested commercially. The rhizomes are considered to possess anti-spasmodic, carminative and anthelmintic properties and 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. Other virtues of this plant include its mature leaves, which act as an insect repellent when cut up and stored in dry foods.

SAMPLE PREPARATION FOR MASS SPECTROSCOPY

The *Acorus calamus* leaves were treated by two separate methods. The leaves were chopped (~1cm pieces) and the organic constituents extracted overnight by stirring with pentane/dichloromethane (50:50). The leaves were removed by suction filtration, a small amount of water was removed in a separatory funnel, the organic solvent was washed with saturated sodium chloride, and the solvent was rotary evaporated to obtain a viscous dark oil. Prior to GCMS this extract was applied to a preparative silica TLC plate with fluorescent indicator, developed in 50:50 ethyl acetate:hexane, the bands were visualized with a UV lamp, scraped from the plate, and extracted from the silica with ethyl acetate. Each band contained several constituents as evidenced by the data shown here for fraction AC01. The extract was submitted for ESI-LCMS and Particle Beam-LCMS without any prior separation procedures. Additionally, the leaves were steam distilled to obtain the volatile constituents. The distillate was extracted with ether, the ether washed with saturated sodium chloride, dried over magnesium sulfate, and rotary evaporated to obtain a light-colored oil. Samples of each oil were dissolved in acetonitrile for ESI-MS and PB-MS or in acetone for the GC-MS.

INSTRUMENTATION – PARTICLE BEAM LCMS 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 benchtop quadrupole mass spectrometer with mass range of 1.6 to 800 mass units, 10,000 volt HED, and is available with EI or EICI capabilities.

INSTRUMENTATION – HPLC/DAD/ESI-MS/MS

LC/ESIMS/MS experiments were performed on an Agilent MSD XCT ion trap mass spectrometer (Palo Alto, CA) equipped with an electrospray ionization (ESI) interface, 1100 HPLC, a DAD detector, and Chemstation software. The column used was a 150 x .5 mm i.d., Zorbax XDB-C18 3.5 μ m (Agilent, Palo Alto, CA). Flow rate was 5.000 μ L/min, injection volume was 0.5 μ L, and column temperature was 25 $^{\circ}$ C. The ESI parameters were as follows: nebulizer, 15 psi; dry gas (N_2), 5.00 L/min; dry temperature, 325 $^{\circ}$ C; trap drive, 34.7; skim 1, 40 V; lens 1, -5.00 V; octopole RF amplitude, 142.5 Vpp; capillary exit, 109.8 V. The ion trap mass spectrometer was operated in positive and negative (alternating) ion mode scanning from m/z 100 to m/z 2200 at a scan resolution of 13000 amu/s. Trap ICC was 200000 units and maximal accumulation time was 200000 μ s. MS-MS was operated at a fragmentation amplitude of 1.0 V, and threshold ABS was 20,000 units.

INSTRUMENTATION – GCMS

The GC utilized for the analysis was a Agilent 5890 equipped with a Zebron ZB-1 column (15m x 0.25mm x 0.25 μ m) (Phenomenex Torrance, CA). The mass spectrometer used was a benchtop quadrupole Agilent 5971 Mass Selective Detector (Agilent Technologies, Inc., Palo Alto California). The 5971 was controlled by the 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 $^{\circ}$ C, injector 250 $^{\circ}$ C, transfer line 280 $^{\circ}$ C, a solvent delay of 2.00 min, the temperature was ramped at 10 $^{\circ}$ C/min to a final temperature of 140 $^{\circ}$ 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. This separation method was utilized on both the ESI and PB instruments.

MASS SPECTRAL ANALYSIS

The data collected from the GC-MS and the Particle Beam EI Ionization of the chromatographic separation was analyzed with AMDIS (Automated Mass Spectral Deconvolution and Identification System), version 2.1, DTRA/NIST, 2002. The Total Ion Chromatograph (FIGURE 1) 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. TOTAL ION CHROMATOGRAPH FROM GCMS

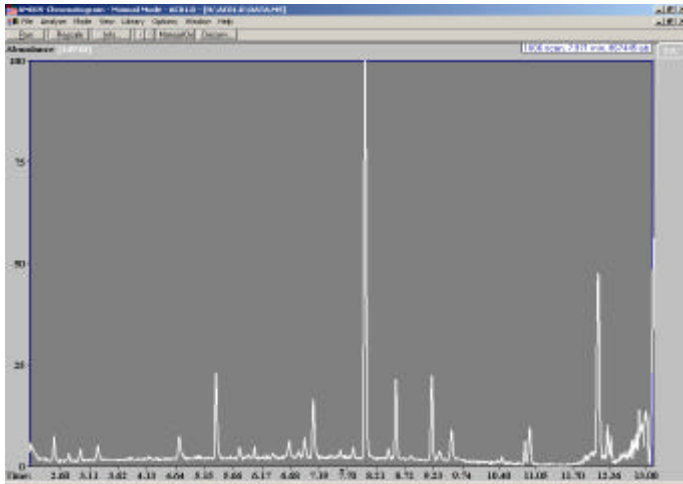


FIGURE 2. LIBRARY MATCH FOR LINALOOL

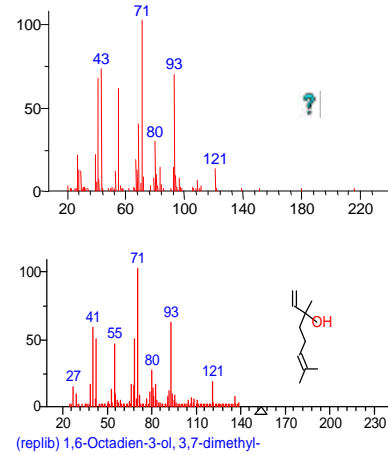


FIGURE 3. LIBRARY MATCH FOR ASARONE

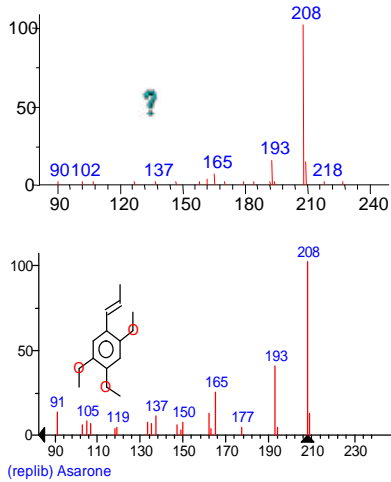


FIGURE 4. TOTAL ION CHROMATOGRAPH FROM PB-LCMS

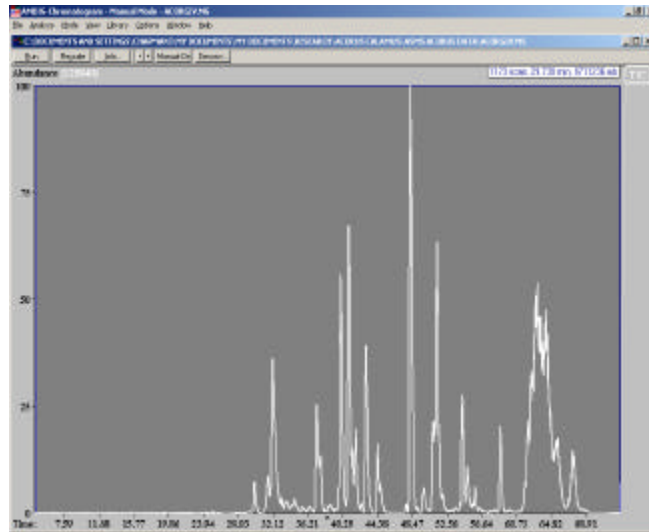


FIGURE 5. TOTAL ION CHROMATOGRAPH FROM ESI-MS

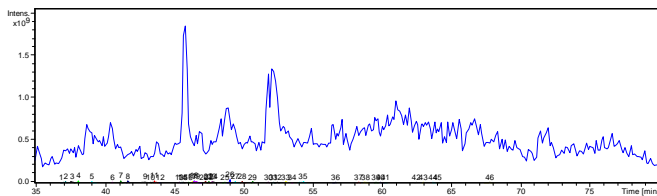
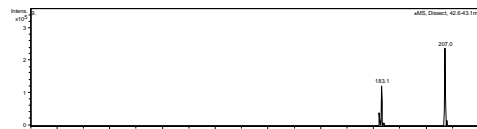


FIGURE 6. ESI-MS FOR ASARONE



RESULTS

The GCMS was able to separate 47 constituents from the fraction AC01 under the conditions previously described. We were able to successfully develop a chromatographic method using a reverse-phase column with UV detection at 254 nm that could resolve ~35 peaks from the Acorus calamus leaf extract. When this separation was coupled with the ESI and Particle Beam mass spectrometers we were able to obtain the Total Ion Chromatographs shown in FIGURES 4 and 5. The collected data for the GCMS and Particle Beam LCMS was submitted for deconvolution and extracted ion analysis using the AMDIS program. The deconvolution program found 189 components in the Particle Beam TIC for the organic extract of the leaves. We were able to obtain EI data on all 189 of the Acorus calamus leaf constituents from the Particle Beam LCMS. Using this combined information from the GCMS and the Particle Beam LCMS, we were able to identify 29 constituents conclusively and these were confirmed 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.

To this point we have identified only a fraction of the chemical constituents of the Acorus calamus leaves. While we were able to match several of these as previously identified, we did identify several constituents not previously reported in the literature. 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/uses of Acorus calamus leaves or rhizomes and its presence in the leaves would implicate it as the medicinally active agent. 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 a psychoactive agent and teas containing this compound are used as inflammatories and analgesics and to act as a purgative, laxative, and cathartic. Another lignan, galgravin, was one of several lignans used for the treatment of malaria and rheumatism.

TABLE 1. IDENTIFIED CONSTITUENTS

(-)-4-Terpineol* (GC)	2-Allyl-5-ethoxy-4-methoxyphenol** (PB)	Epieudesmin** (PB)	Lysidine* (GC)
(-)-Spathulenol* (GC)	Borneol* (GC)	Furyl ethyl ketone** (GC)	Nonanoic Acid** (GC)
2,2,5,5-Tetramethyl-3-hexanol (GC)	Bornyl acetate* (GC)	Galgravin** (PB)	Retusin** (PB)
(9E,12E,15E)-9,12,15-Octadecatrien-1-ol** (PB)	Butyl Butanoate* (GC)	Geranylacetate* (GC)	Sakuranin** (PB)
Acetic Acid* (GC)	Camphor* (GC)	Isoelemicin* (PB)	á-Ursolic acid
Acetophenone* (GC)	Dehydroabietic acid** (PB)	Isoeugenol Methylether* (PB)	
Apigenin 4',7-dimethyl ether** (PB)	Dehydrodiisoeugenol* (PB)	Linalool* (GC)	
?-Asarone* (PB)	Elemicin* (PB)	Linolenic acid** (PB)	

*Previously identified

**Not previously identified in Acorus calamus

CONCLUSIONS

Almost all new commercial LC/MS systems today are atmospheric pressure ionization instruments. 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. Nothing helps more than the direction that can be obtained from a standardized database of mass spectra with which to match. Commercial and standardized libraries are not yet prevalent for CI and API-ES. CI and API-ES are typically used to obtain molecular weight information. For this reason CI and API-ES are commonly used as a tool to confirm an identity obtained from matching an unknown spectrum with a mass spectral database. In summary, an evaluation of our experiments showed the following:

1. The Particle Beam LCMS works well for the organic extract obtained from the leaves and eliminates the need for any preparatory pre-purification steps.
2. The ability to search EI spectral libraries makes the identification of the constituents much less tedious and allows the correlation to the ESI-LCMS.
3. The ESI works well for obtaining mass spectral data, but working with true unknowns and the absence of an ESI library limits identifications.
4. LCMS with Particle Beam ionization is capable of providing fragmentation information on molecules inaccessible to other techniques.

FUTURE WORK

Our intentions are to utilize the Particle Beam ionization in combination with Chemical Ionization and Electrospray Ionization to identify all volatile and non-volatile polar and non-polar constituents by the same chromatographic separation. We plan to develop an ESI search library for the chemical constituents of the plant by correlating the HPLC elution profiles from the ESI and Particle Beam LCMS separations, identifying the constituents utilizing the electron impact NIST library, and identifying the corresponding constituents and their ESI fragmentation patterns.