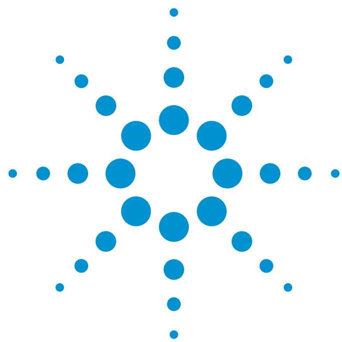

Enhanced Metabolite Profiling from Bark of *Alangium Salviifolium* Using LC/MS and GC/Q-TOF Techniques

Application Note

Metabolomics

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Abstract

Traditional herbal remedies are used as alternative medicines for a number of diseases. *Alangium salviifolium* is one such plant, used as traditional medicinal plant. Several investigations have been performed using this plant extract to demonstrate its therapeutic value. However, very few attempts have been made to identify the extensive metabolite composition of this plant. In this Application Note, we performed metabolite profiling and identification from the bark of *A. salviifolium* by extracting the sample in organic and aqueous solvents. The organic and aqueous extracts were fraction-collected using the Agilent 1260 Analytical Scale Fraction Collection System. Each of the fractions was analyzed by LC/MS and GC/Q-TOF techniques. The LC/MS/MS analyses were performed using HILIC chromatography, as well as three separate, orthogonal reverse phase columns. Data were collected using AJS source in both positive and negative ionization modes, followed by METLIN database or MS/MS library searches. Compounds from *Alangium* that could not be identified by database or library matching were subsequently searched against the ChemSpider (http://www.chemspider.com/) database of over 30 million structures using Agilent MSC software. To identify compounds generated by GC/Q-TOF, the data were searched against the Agilent-Fiehn GC/MS Metabolomics Library and Wiley/NIST libraries. The results of the combined GC libraries searches identified 62 compounds with a matching score > 70.

Using both techniques, a total of 1,016 compounds were detected, of which 511 were identified. A literature search revealed 81 out of 511 compounds had therapeutic properties against traditionally reported diseases such as cancer, microbial infections, and so forth. Our study suggests that the use of fraction collection for metabolite enrichment, biphasic solvent extraction, and orthogonal column chemistries for metabolite separation, as well as complementary LC/MS and GC/MS detection, leads to greater metabolite detection coverage in medicinal plants.

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Introduction

Alangium salviifolium is a medicinal plant reported in Ayruveda and Chinese medicine. This plant is used traditionally against several diseases such as cancer, leprosy, diabetes, paralysis, microbial infection, and others. Every part of the plant is used, either consumed orally, or applied dermally, depending on the type of disease that is treated. Experiments correlating this medicinal plant with specific diseases or activities have been done previously[1,2].

A comprehensive evaluation of untargeted metabolomics is an unbiased analysis of biochemical intermediates in a sample achieved by using complementary universal analytical techniques such as LC/MS, GC/MS, and NMR. The factors that can affect the comprehensive evaluation of a metabolome depend on:

- The method used for sample harvesting/extraction procedures
- Fractionation
- Chromatographic separation chemistry
- Ionization techniques/modes
- Acquisition parameters
- Data processing/analysis
- Identification databases/libraries[5]

We used orthogonal LC/MS and GC/Q-TOF techniques for a comprehensive metabolite analysis in the stem bark of this plant, including identification of the metabolites using recent libraries and databases.

Experimental

Workflow

Table 1 shows an outline of the workflow used in this study.

Table 1. Summary of the workflow for biphasic solvent extraction followed by analysis using LC/MS and GC/MS platforms.

Collect → Flash freeze → Store	
Aqueous extract	Organic extract
Fraction collection	Fraction collection
LC/MS and LC/MS/MS Agilent Poroshell 120 HILIC Plus and Agilent ZORBAX RRHD SB-Aq columns	LC/MS and LC/MS/MS Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl and Agilent ZORBAX RRHD Eclipse Plus C18 columns
LC/MS/MS analysis METLIN database/library	LC/MS/MS analysis METLIN database/library
GC/MS Agilent DB-5ms column	GC/MS Agilent DB-5ms column
GC/MS analysis Fiehn/Wiley/NIST library	GC/MS analysis Fiehn/Wiley/NIST library

Reagents and materials

LC/MS grade isopropanol, methanol, and acetonitrile were purchased from Fluka (Germany). Milli Q water (Millipore Elix 10 model, USA) was used for mobile phase preparation. The additives, ammonium fluoride, acetic acid, ammonium formate, formic acid, and ammonium acetate, were procured from Fluka (Germany).

Collection of plant material and extraction procedure

A. salviifolium bark was collected from the plants near Mysore, India and immediately transferred to liquid nitrogen and stored at –80 °C until further use. Two grams of bark tissue were powdered using a mortar and pestle in liquid nitrogen. For extraction, 40 mL of degassed solution containing chloroform:methanol:water in the ratio of 1:2.5:1 (v/v/v) was added. The undissolved sample was crushed for 5 minutes using mortar and pestle, transferred to 1.5 mL eppendorf tubes, and vortexed for 5 minutes at 4 °C. The tubes were centrifuged at 20,800 rpm for 2 minutes and the supernatant was pooled from all the tubes into a glass vial. A 1-mL amount of the supernatant was transferred to an eppendorf tube and 400 µL of water was added. The tubes were vortexed for 10 seconds followed by centrifugation at 20,800 rpm for 2 minutes. The aqueous (upper) and organic (lower) layers were separated and dried separately in a speed vac (Eppendorf).

Fraction collection

To the dried aqueous and organic layers, 200 µL of 50:50 and 30:70 mobile phase A and B of respective fractionation method (Table 2) were added. The vials were sonicated to resuspend the compounds. HPLC separation was performed by injecting the resuspended mixtures from multiple vials of each extract to an Agilent 1260 Infinity analytical purification system equipped with a 1-mL Manual FL-Injection valve (p/n 5067-4191) and the fractions were collected in 45 wells of a 96-well plate and dried in a speed vac.

Dual AJS-ESI-Q-TOF MS conditions

The dried aqueous fractions were resuspended in 250 µL of 50:50 methanol:water containing 0.2 % acetic acid and sonicated for 10 seconds, whereas the organic fractions were suspended in 30:70 mobile phase A:B (Table 3 – organic) followed by centrifugation at 3,000 rpm for 10 minutes. Then, 5 µL of the resuspended fractions were injected onto an Agilent 1260 Infinity LC System interfaced to an Agilent 6540 Accurate mass Q-TOF LC/MS system. Reference solution was prepared using an API-TOF Reference Mass Solution Kit (p/n G1969-85001). A 10-µL amount of HP921 and 5 µL of purine was dissolved in 1 L of methanol:acetonitrile:water (750:200:50) containing 0.1 % acetic acid, and was sprayed using an isocratic pump at a flow rate of 0.4 mL/min. The MS and chromatographic parameters are shown in Table 3A and 3B.

Table 2. Chromatographic parameters for fractionation.

Parameter	Aqueous extract		Organic extract	
Mobile phases	A) Water + 10 mM ammonium acetate B) 100 % acetonitrile		A) 95:5 water:methanol with 0.1 % formic acid and 5 mM ammonium formate B) 65:30:5 isopropanol:methanol:water with 0.1 % formic acid and 5 mM ammonium formate	
Flow rate	1.2 mL/min		1.2 mL/min	
Injection volume	1 mL		0.3 mL	
Autosampler thermostat	4 °C		4 °C	
TCC temperature	25 °C		25 °C	
DAD	210 and 254 nm		210 and 254 nm	
Peak width	> 0.05 minutes		> 0.05 minutes	
Fraction collection mode	Time-based		Time-based	
Total time	13 minutes		13 minutes	
Column	Agilent ZORBAX SB-C18, 9.4 × 50 mm, 5 µm (p/n 846975-202)			
Time slices	0.292 min/well		0.292 min/well	
Gradient	Time (min)	% Solvent B	Time (min)	% Solvent B
	0.0	5	0.0	60
	1.0	5	1.0	60
	8.0	35	8.0	100
	8.1	95	11.0	100
	10.0	95	11.1	60
	10.1	5	12.0	60
	12.0	5		

Table 3A. The MS source and chromatographic parameters used in LC/MS and LC/MS/MS analysis.

LC/MS parameters	
Injection volume	5 µL
Flow rate	0.4 mL/min
Thermostated column temperature	40 °C
Gas temperature	250 °C
Drying gas flow	10 L/min
Nebulizer	30 psig
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
VCap	3,500 V
Nozzle voltage	1,000 V
Fragmentor	100 V

Table 3B. Chromatographic parameters used in the LC/MS and LC/MS/MS analysis.

Parameter	Aqueous fractions analyzed using an Agilent ZORBAX RRHD SB-Aq, 2.1 × 50 mm, 1.8 µm column (p/n 857700-914)		Aqueous fractions analyzed using an Agilent Poroshell 120 HILIC Plus, 3.0 × 50 mm, 2.7 µm column (p/n 699975-301)	
Ionization mode	Positive MS and positive AutoMSMS	Negative MS and Negative AutoMSMS	Positive MS and positive AutoMSMS	Negative MS and negative AutoMSMS
Mobile phases	A) Water with 0.2 % acetic acid B) Methanol with 0.2 % acetic acid	A) Water with 1 mM ammonium fluoride B) 100% acetonitrile	A) (90:10) acetonitrile:water with 50 mM ammonium acetate B) (50:40:10) acetonitrile:100% water:water with 50 mM ammonium acetate	
LC gradient	Time (min)	% mobile phase B	Time (min)	% of mobile phase B
	1.00	5.0	3.00	0.0
	10.0	35.0	10.00	100.0
	11.0	95.0	13.00	100.0
	13.0	95.0	13.10	0
	13.1	5.0	17.00	0
	15.0	5.0		
Parameter	**Organic fractions analyzed using an Agilent ZORBAX Eclipse Plus C18, 3.0 × 50 mm, 1.8 µm column (p/n 959757-302)**		**Organic fractions analyzed using an Agilent ZORBAX Eclipse Plus Phenyl-Hexyl 3.0 × 50 mm, 1.8 µm column (p/n 959757-312)**	
Ionization mode	Positive MS and positive AutoMSMS	Negative MS and negative AutoMSMS	Positive MS and positive AutoMSMS	Negative MS and negative AutoMSMS
Mobile phase	A) 95:5 water:methanol with 0.1 % formic acid and 5 mM ammonium formate B) 65:30:5 isopropanol:methanol:water with 0.1 % formic acid and 5 mM ammonium formate			
LC gradient		Time (min)	% of mobile phase B	
		1.00	60.0	
		8.00	100.0	
		11.00	100.0	
		11.10	60.0	
		14.00	60.0	

GC/Q-TOF conditions

The derivatization and experimental parameters for both aqueous and organic fractions were performed as described elsewhere[6]. An Agilent 7200 GC/Q-TOF was used for acquisition with absolute retention times, which was locked to the internal standard d27 myristic acid from the Agilent Fiehn GC/MS Metabolomics Standards Kit (p/n 400505) with retention time locking (RTL) software system. The GC/Q-TOF conditions used are provided in Table 4.

Table 4. Conditions used for GC/Q-TOF.

GC conditions	
Column	Agilent DB-5ms, 30 m × 0.25 mm, 0.25 µm, Guard length 10 m (p/n 122-5532G)
Injection volume	1 µL
Split mode and ratio	Split 10:1
Split/Splitless inlet temperature	250 °C
Oven temperature program	60 °C for 1 minute, 10 °C/min to 325 °C, 10 minutes hold
Carrier gas	Helium at 1.2798 mL/min constant flow
Transfer line temperature	290 °C
Q-TOF conditions	
Ionization mode	EI
Source temperature	230 °C
Quadrupole temperature	150 °C
m/z scan	50 to 600 *m/z*
Spectral acquisition rate	5 spectra/s, 2,679 transients/spectrum, collecting both in centroid and profile modes

Data analysis

Agilent MassHunter Qualitative Analysis (v. B.06.00 SP1) software was used for processing MS, AutoMSMS data. The accurate mass MS data were processed using the **Find by Molecular Feature** tool to export the compounds to Agilent Mass Profiler Professional (MPP) Software. To remove the molecular features arising from the background, the data obtained from each fraction were background subtracted using the blank data in MPP. The ID browser was used to identify putative compounds by searching against the METLIN database comprising 64,092 compounds.

The LC/MS/MS data were processed using the **Find by AutoMSMS** tool, and the spectral pattern generated was compared against the Metlin metabolite library comprising accurate mass MS/MS information for 19,714 compounds. A few selected compounds found in *Alangium* species detected in the METLIN database, but not in Metlin library, were processed using Molecular Structure Correlation Software (MSC). The MSC software (version B.05.00 Build 19) performed the systematic bond breaking for the proposed structure or from a database, and matched the observed fragment ions, followed by the assignment of an overall score. The interface provided the formula and overall score, which was the combined MS and MS/MS score, and the molecular formulas for the fragment ions with ppm *m/z* error.

The 7200 GC/Q-TOF data was processed using MassHunter Unknown Analysis Software (version B.06.00). This software uses mass spectral deconvolution, which automatically finds peaks and deconvolutes spectra from coeluting compounds using model ion traces. The spectral information was matched with the Agilent-Fiehn library with retention time index with respect to FAME mix (Agilent Fiehn GC/MS Metabolomics Standards Kit, p/n 400505). The data were also searched against NIST 11 and Wiley 9 mass spectral libraries. The compounds with a library match score > 70 % were considered. The results from LC/MS/MS and GC-QTOF were searched in the literature for their therapeutic importance.

Results and Discussion

In this Application Note, we performed a comprehensive analysis of *A. salviifolium* bark metabolites using multiseparation protocols/ionization modes and multiplatform approaches. Initially, we performed a fraction collection of the aqueous and organic extracts by injecting 1 mL of the extract for preliminary separation and enrichment of the metabolites. The Accurate Mass MS results, when matched with the METLIN database, tentatively found 954 compounds with a database match score > 90 %. Literature search revealed 81 of 954 compounds had therapeutic properties. The majority of these therapeutic compounds were secondary metabolites that are reported to have anticancer and anti-inflammatory activities (Figure 1). These compounds belonged to various plant secondary metabolite classes such as terpenoids, flavonoid, saponins, alkaloids, glycosides, and so forth. AutoMSMS analysis of all fractions resulted in identification of 449 compounds.

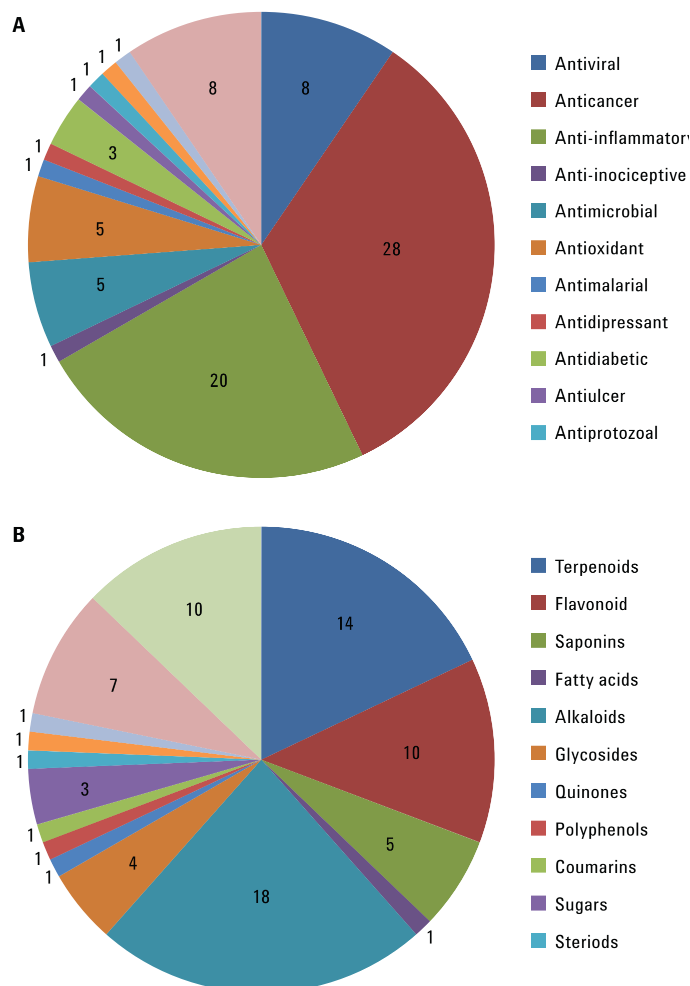

Figure 1. METLIN database matched compounds from *A. salviifolium* and grouped by therapeutic use (A) and compound class (B) based on literature reports.

Five compounds reported to be commonly present in *Alangium* species could not be identified in this study by LC/MS/MS spectral matching since the spectra for these compounds were not available in the METLIN MS/MS library. The spectral information was used to identify the compounds using Agilent MassHunter MSC software (Figure 2). The overall MSC score for all the compounds was > 97 %, except for cephaeline which was 80 %. Using the accurate mass precursor and fragment ion information for cephaeline, and the METLIN accurate mass database, we were able to identify the putative structures based on the MS/MS spectra obtained for cephaeline (Figure 3). Thus, this approach of using MSC for tentative ID confirmation can be a useful tool in shortlisting the number of compounds for subsequent confirmation using actual standards. Table 5 shows the results of *Alangium* compounds identified by MSC software.

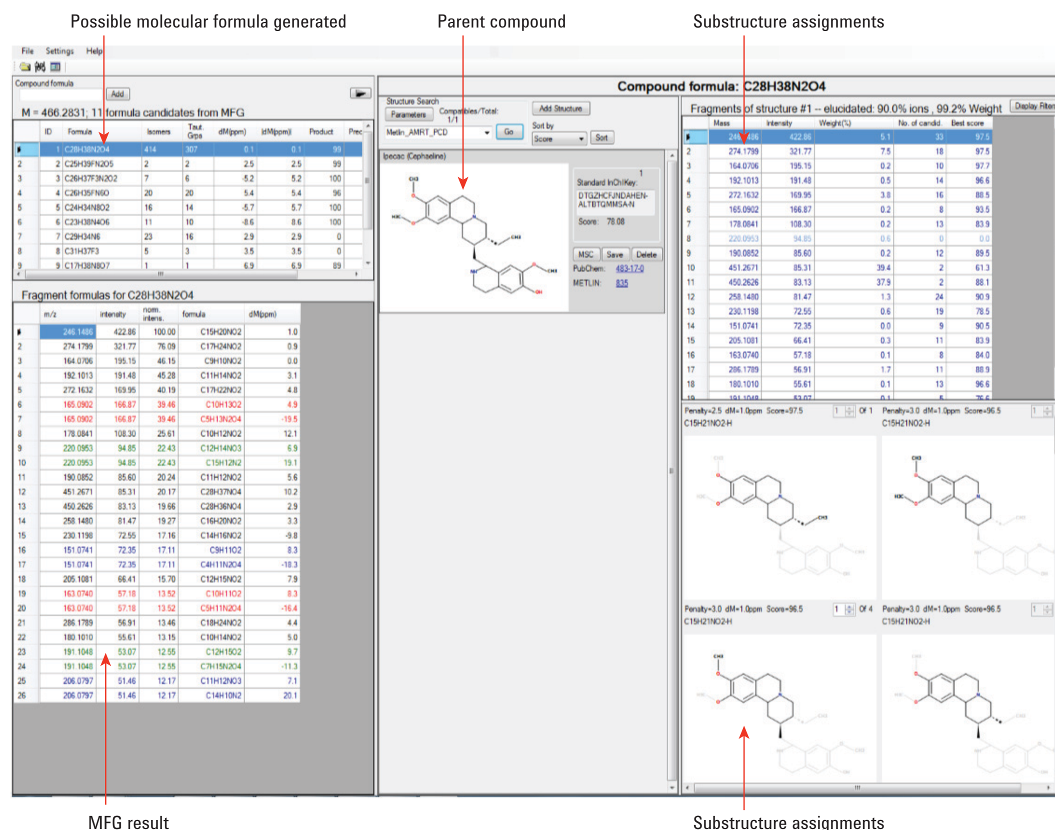

Figure 2. Results from Agilent MSC software tool for identifying the compounds that did not have a spectral match in the METLIN MS/MS library.

The separation chemistries for LC/MS/MS were performed using Poroshell HILIC Plus and three orthogonal reverse phase columns (ZORBAX Eclipse Plus C18, ZORBAX Eclipse Plus SB-Aq, and ZORBAX Eclipse Plus Phenyl Hexyl) for the separation of hydrophilic and hydrophobic compounds, respectively. The largest number of compounds were identified in ZORBAX Eclipse Plus C18 (197), followed by ZORBAX Eclipse Plus SB-Aq (187), Poroshell HILIC Plus (175), and ZORBAX Eclipse Plus Phenyl-Hexyl (139) columns (Figure 4). Significant compound overlaps were found between HILIC Plus/SB-AQ and Eclipse Plus C18/Eclipse Plus Phenyl-Hexyl columns: 53 and 59 compounds, respectively. Only 10 compounds were common to all four column types. The three different reverse phase columns, Eclipse Plus C18, Eclipse Plus SB-AQ, and Eclipse Plus Phenyl Hexyl separated 79, 73, and 28 unique compounds, respectively. Poroshell HILIC Plus revealed 80 unique compounds. Similar observations on enhanced metabolite coverage have been made earlier using HILIC and a reverse phase Eclipse Plus C18 column[7]. Our results using three different RP columns (for nonpolar and intermediate polar), along with an HILIC (for polar compounds) clearly reveal the requirement for different separation chemistries for uncompromised metabolomics study.

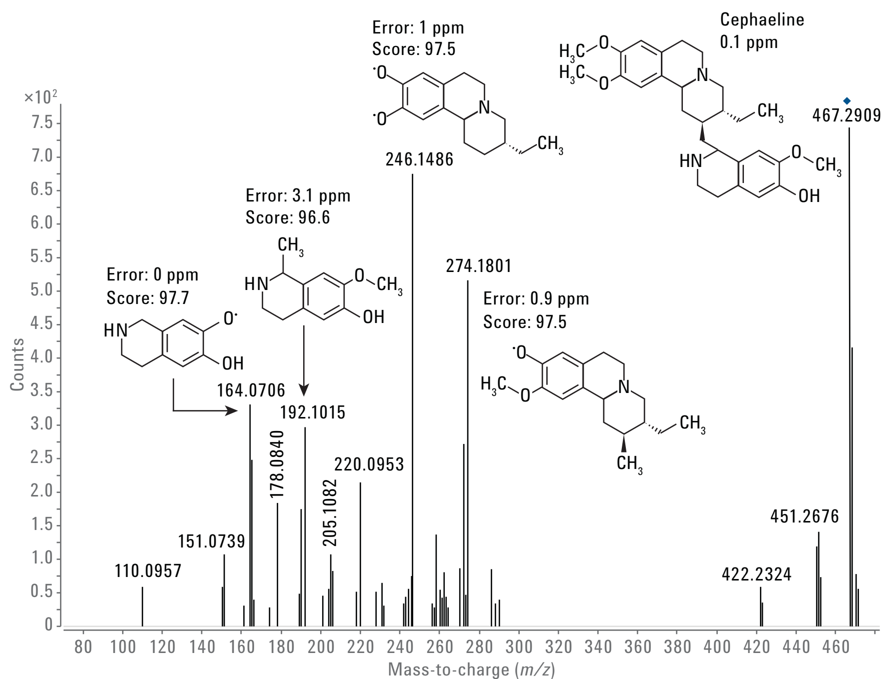

Figure 3. Proposed metabolite fragment structures for Cephaeline based on MSC analysis.

Table 5. Compounds found in Alangium species identified by Agilent MSC Software.

Metabolite	CAS/KEGG	Formula	Mass difference (ppm)	Overall score
Ankorine	13849-54-2	$C_{19}H_{29}NO_4$	-2.56	99.94
Deoxytubulosine	C11817	$C_{29}H_{37}N_3O_2$	-0.69	98.97
Ipecac (Cephaeline)	483-17-0	$C_{28}H_{38}N_2O_4$	-0.28	80.06
Lacinilene C 7-methyl ether	56362-72-2	$C_{16}H_2OO_3$	-1.84	98.95
Tubulosine	2632-29-3	$C_{29}H_{37}N_3O_3$	2.86	97.31

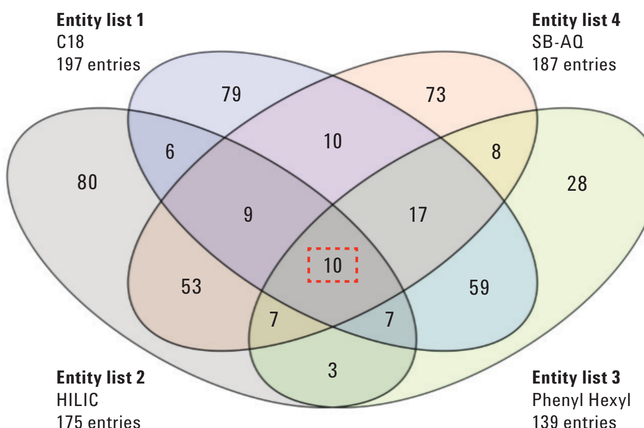

Figure 4. METLIN library matched compound distribution based on column chemistry. Figure drawn using MPP Software.

The compounds obtained from MS/MS analyses in positive and negative ionization modes are summarized in Figure 5. Clear differentiation of compounds for both ionization modes was observed for all column chemistries used in the study. More sugars and acidic amino acids were detected in negative mode ionization compared to positive mode ionization. Fewer than nine compounds were common for positive and negative ionization modes among all the column types. This shows that the use of single ionization mode could significantly reduce the coverage of metabolites.

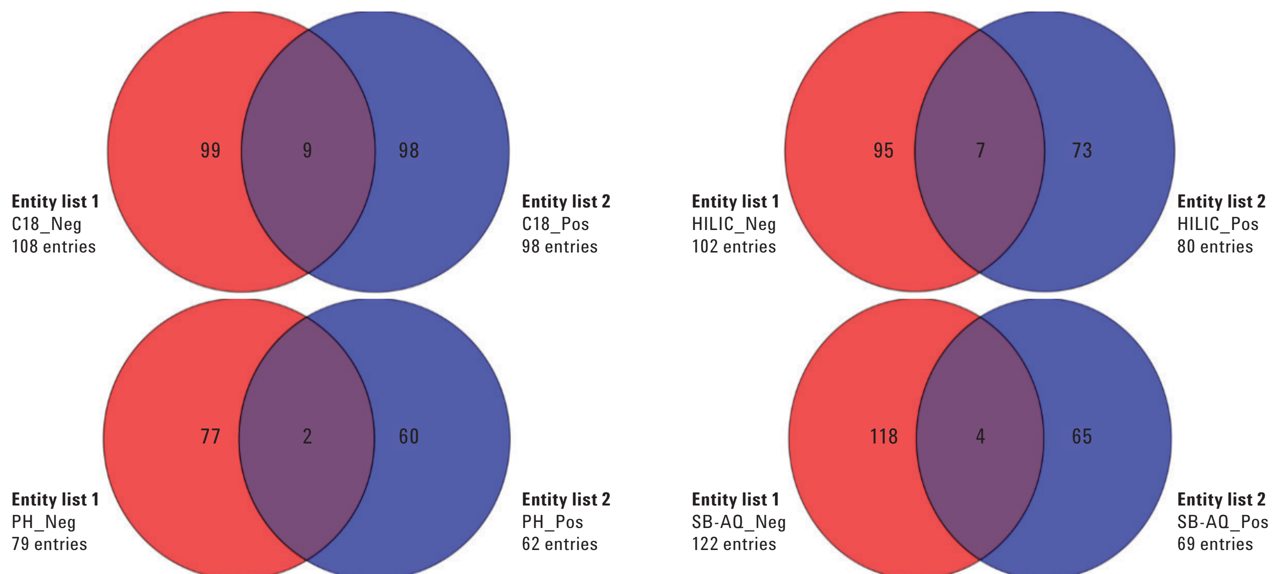

Figure 5. METLIN library matched compounds distribution based on ionization modes.

A screenshot of MassHunter Unknown Analysis Software is shown in Figure 6. This software provides the features of the chromatogramic comparison; query versus database spectrum alignment, molecular structure, and the components. The components comprise details of each compound.

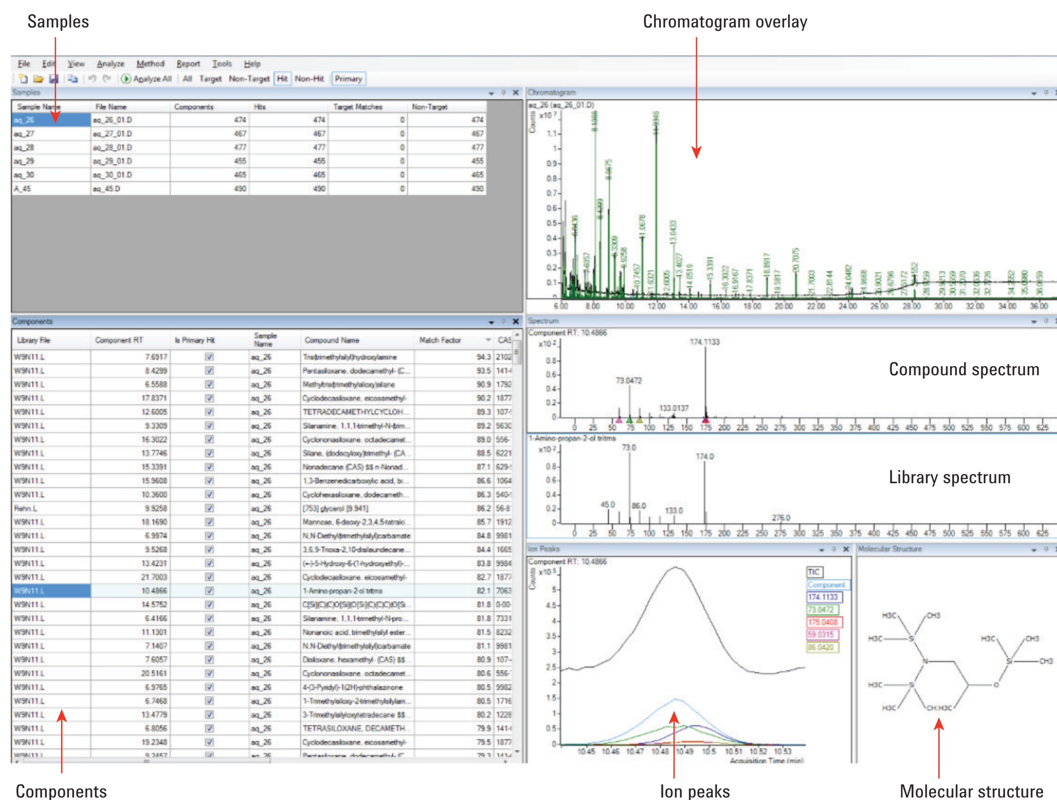

Figure 6. Fiehn/Wiley/NIST library matched analysis using Agilent MassHunter Unknown Analysis Software.

The compounds found by GC/Q-TOF were primarily flavonoids, fatty acids, sugars, terpenes, and so forth. For example, D-lyxose identified from the GC/Q-TOF analysis of aqueous extract using MassHunter Unknown Analysis Software is shown in Figure 7. The acquired GC/Q-TOF spectrum is shown in Figure 7A, while Figure 7B shows the Fiehn library spectrum. The matching score is 89.6. In addition, the retention time (RT) in the library (14.74 minutes) matches with the RT of the acquired spectra (14.75 minutes).

The LC/MS/MS and GC/Q-TOF analysis resulted in identification of 449 and 62 compounds, respectively. The enhanced number of compounds observed for LC/MS/MS was primarly due to the use of orthogonoal columns. It is well established that the LC/MS and GC/Q-TOF are complementary techniques for comprehensive metabolomics to identify nonvolatile and volatile compounds (Figure 8).

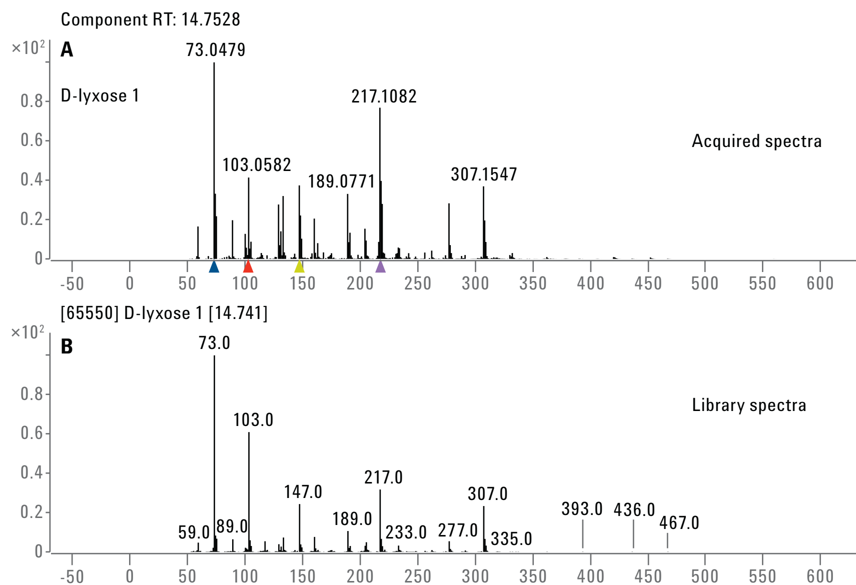

Figure 7. GC/Q-TOF spectral search results.

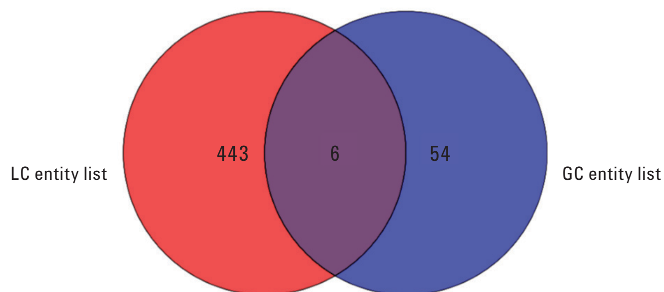

Figure 8. Compounds identified using LC/MS/MS and GC/Q-TOF analysis.

Conclusions

This study demonstrates the utility of applying a comprehensive metabolite separation and detection strategy to aid in identification of metabolites in *A. salviifolium* bark. Fractionation was used for enrichment. In addition, a multiplatform approach was used to detect compounds with different degrees of polarity. Using four different column chemistries, combined with two ionization modes increased the total number of metabolites identified. The compounds that were not found in the METLIN library were identified using MSC software. Our results show that eighty one secondary metabolites identified in this study are reported to have therapeutic value.

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