Identification and confirmation of potential markers in rose and sunflower leaf extracts

Using the Accurate Mass Metabolite Spectral Library and TripleTOF® 6600 System

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Leaves and petals of plants such as rose and sunflowers have long been used for medicinal and aesthetic purposes around the world. Recently, investigators found that phenolic antioxidants especially those levels present in rose petal extracts of deep color (intense red to mauve) may be responsible for decreasing oxidative stress which plays a significant role in many metabolic diseases, thus justifying their use in traditional medicines.[1] In addition, sunflower leaf tea is used in traditional medicine to reduce high fevers and has also been found to have astringent properties. Sunflower leaf poultice may be used on snakebites and insect bites. The leaves are diuretic and expectorant, as are the seeds, so determining what compounds are contributing to the activity and which metabolic pathways they belong to is of interest to many scientists. High resolution, accurate mass systems are the mass spectrometers of choice for global metabolomics applications as they employ a high degree of selectivity over nominal mass systems.

Unbiased targeted profiling on TripleTOF® Systems allows for the collection of MS and MS/MS data in a single-injection workflow. This discovery data can be searched with a targeted list of metabolites, from many chemical classes, pathways and species. Metabolite identification with a high resolution, accurate mass MS/MS library ensures increased confidence in assignment and purity scores of unknowns from a discovery experiment. Recently, a metabolite library has been developed to facilitate this process.[2]

Here, a study was performed to see if three separate rose leaf extracts and sunflower leaf extracts would produce variances in their profiles in comparison to one another and identify compounds associated with these changes perhaps leading to metabolic pathways for further investigation. Results illustrate the power of the technology as well as the software to drive the data processing and metabolite identification.

Figure 1. Workflow for metabolic profiling using the TripleTOF System. Data is taken from the TripleTOF 6600 System and processed through XCMS[Plus] software for feature finding. The metabolite identifications are confirmed by taking the metabolites of interest and passing them through the accurate mass metabolite library in SCIEX OS Software or MasterView™ Software.

Key features of workflow

- Unbiased profiling using the TripleTOF System allows for the collection of MS and MS/MS data in a single-injection workflow.
- Differential feature extraction using XCMS[Plus] software.
- Metabolite identification and confirmation using an accurate mass metabolite spectral library (AMMSL) ensures increased confidence in assignment and purity scores of unknowns from a discovery experiment.

Methods

Sample preparation: Extractions of rose petals from 3 different lots and an extraction of sunflower leaves were provided by University of Hohenheim, Stuttgart, Germany. An aliquot of all extracts was also combined into another "pooled" lot to serve as a quality control. The five different sample groups (3 x rose, 1 x sunflower and a pooled QC) were then injected in triplicate for analysis.

LC-MS acquisition: The TripleTOF 6600 System (SCIEX) was used to acquire data in both positive and negative ion mode using SWATH® acquisition and IDA (information dependent acquisition). The HPLC used was an Agilent 1290 (pump, column oven at 40 °C, autosampler). The column was a Phenomenex Kinetex XB-C18 column (2.6µm, 2.1 x 100mm) running at a flow rate of 300 µL/min. Injection volume of 5 µL was used. A linear gradient was formed using mobile phase A (H_2O with 5mM NH_4OAc) and B (acetonitrile with 5mM NH_4OAc).

Workflow:

1. Collection of MS and MS/MS data in a single-injection experiment.
2. Examination of the sample batch to ensure reproducibility and integrity injection to injection.
3. Detection of differential features between the different lots using multivariate statistical analysis in XCMS^{Plus} Software.
4. Generate a non-targeted "interest" list of candidates expressing the most significant differences, i.e., lowest p-values and highest log fold changes for investigation from XCMS^{Plus} Software
5. Confirm the differential features from XCMS^{Plus} with the Accurate Mass Metabolite Spectral Library in SCIEX OS Software or MasterView Software for the presence of known metabolites. (In this study, compound standards were purchased and added to the existing AMMSL library (Phlorizin and Phloretin) to allow confirmation of these specific plant metabolites.)

Profiling of metabolite features

The data files were opened in SCIEX OS Software or PeakView® Software and the chromatograms examined for reproducibility and integrity, injection to injection (Figure 2). This is always good practice to QC the data this way before starting to process it to identify any issues encountered during sample collection.

Next the data was processed the using XCMS^{Plus} Software[3] for multivariate statistical analysis. Peaks are picked, aligned and differential features reported based on p-value significance.

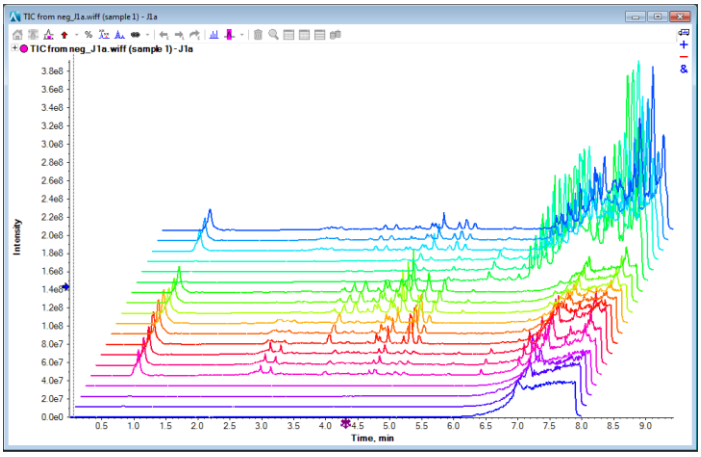


Figure 2. Stacked total ion chromatograms (TICs) of the negative mode plant data. Overlaid TICs highlight the injection to injection reproducibility across the batch. Retention time reproducibility had CV's of <1.6%.

Figure 3 shows the Scores plot from a Principal Component Analysis, which shows the differences between the three rose lots and sunflowers extracts as well as the pooled QC's. The "pooled" QC samples are shown at the center of the scores plot as expected since they are a composite of all samples. The replicate injections in each group also cluster together well this is a good indication that high quality data was collected for this study.

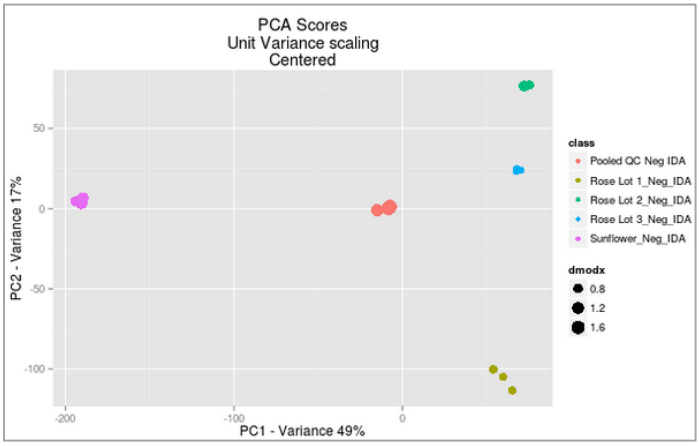
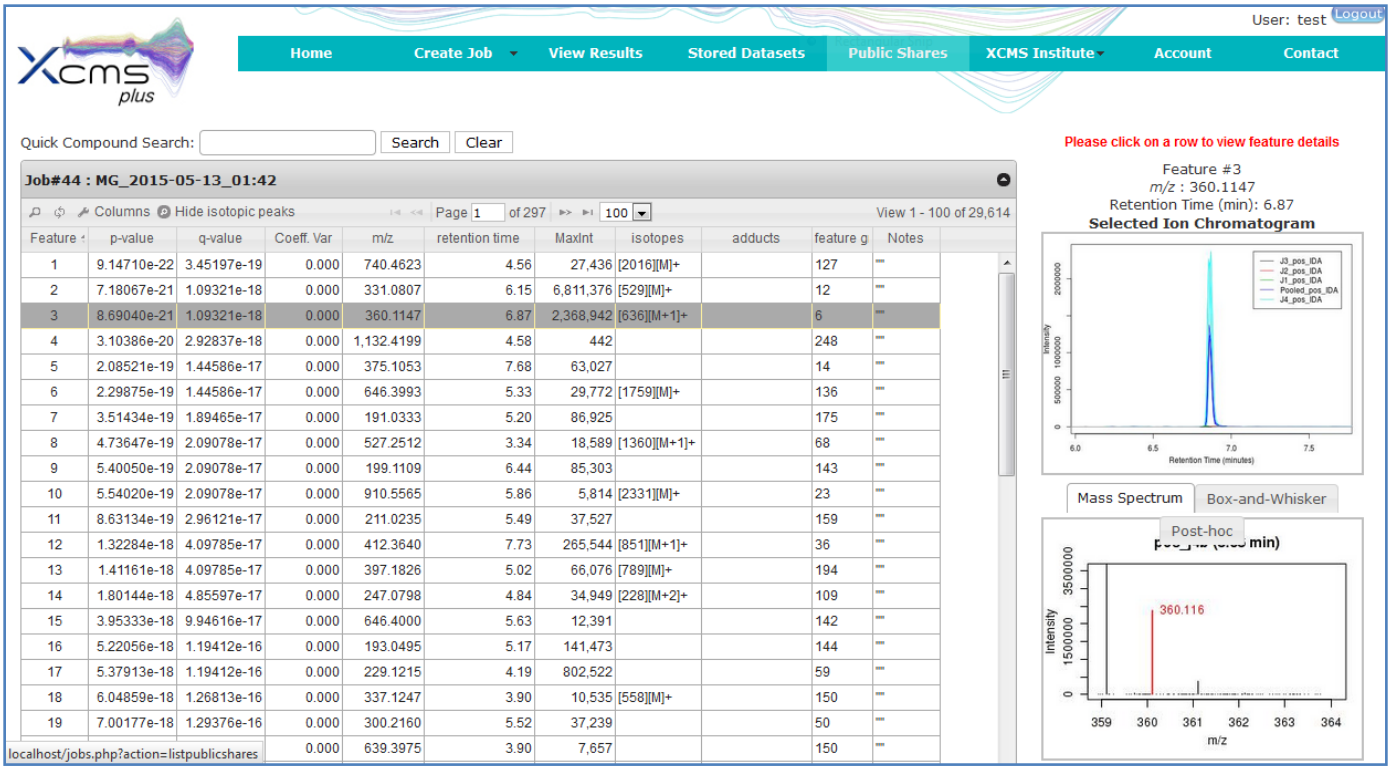


Figure 3. Principle component analysis (PCA) in XCMS^{Plus} Software. The different lots of plants are differentiated by their metabolite profiles. The PCA plot highlights that 49% of the variation in our dataset is being explained in the first principle component. PC2 explains another 17% of the variation (or difference) in our data. This is in fact true when you see that the biggest difference is left to right between the sunflower and rose extracts. The second biggest difference is between the three different rose lots.



Feature	p-value	q-value	Coeff. Var	m/z	retention time	MaxInt	isotopes	adducts	feature g	Notes
1	9.14710e-22	3.45197e-19	0.000	740.4623	4.56	27,436	[2016][M]+		127	
2	7.18067e-21	1.09321e-18	0.000	331.0807	6.15	6,811,376	[529][M]+		12	
3	8.69040e-21	1.09321e-18	0.000	360.1147	6.87	2,368,942	[636][M+1]+		6	
4	3.10386e-20	2.92837e-18	0.000	1,132.4199	4.58	442			248	
5	2.08521e-19	1.44586e-17	0.000	375.1053	7.68	63,027			14	
6	2.29875e-19	1.44586e-17	0.000	646.3993	5.33	29,772	[1759][M]+		136	
7	3.51434e-19	1.89465e-17	0.000	191.0333	5.20	86,925			175	
8	4.73647e-19	2.09078e-17	0.000	527.2512	3.34	18,589	[1360][M+1]+		68	
9	5.40050e-19	2.09078e-17	0.000	199.1109	6.44	85,303			143	
10	5.54020e-19	2.09078e-17	0.000	910.5565	5.86	5,814	[2331][M]+		23	
11	8.63134e-19	2.96121e-17	0.000	211.0235	5.49	37,527			159	
12	1.32284e-18	4.09785e-17	0.000	412.3640	7.73	265,544	[851][M+1]+		36	
13	1.41161e-18	4.09785e-17	0.000	397.1826	5.02	66,076	[789][M]+		194	
14	1.80144e-18	4.85597e-17	0.000	247.0798	4.84	34,949	[228][M+2]+		109	
15	3.95333e-18	9.94616e-17	0.000	646.4000	5.63	12,391			142	
16	5.22056e-18	1.19412e-16	0.000	193.0495	5.17	141,473			144	
17	5.37913e-18	1.19412e-16	0.000	229.1215	4.19	802,522			59	
18	6.04859e-18	1.26813e-16	0.000	337.1247	3.90	10,535	[558][M]+		150	
19	7.00177e-18	1.29376e-16	0.000	300.2160	5.52	37,239			50	
			0.000	639.3975	3.90	7,657			150	

Figure 4. Results table from XCMS^{Plus} Software. The results table highlights the top 19 candidates with the lowest p-values from the positive mode data. Each row is a feature extracted from the data. Selecting a row such as #3 (grey highlight) displays the XIC's from all samples for visual comparison. The light blue traces shown were the sunflower extracts, dark blue were the "pooled" sample traces.

The XCMS^{Plus} feature table lists all of the differentiating features by p-values and or q-value (FDR adjusted p value). Clicking on any row in the feature table of the software, extracts the ion chromatogram on the right hand side (Figure 4). Results can be explored in detail using the interactive features.

MS/MS confirmation of target list

After exploration of the profiling data, the identities of each metabolite should next be confirmed using the high resolution MS/MS data collected in the same files. A differential feature list can be exported from XCMS^{Plus} Software and imported into MasterView Software for ID confirmation using the Accurate Mass Metabolite Spectral Library.

Confirmation by MS/MS means higher confidence in the assigned metabolite. The top pane in Figure 5 shows the XIC of the highlighted row for Catechin. The center pane contains the report information and formulas, the bottom two panes show the library matches of MS and MS/MS spectra. Relative quantitative amounts of the compounds from the target list were easily reviewed and summarized. Table 1 shows that the rose extracts had high levels of Kaempferol, Quercetin, Catechin, Glutamic acid and L- Phenylalanine. Phlorizin (a flavonoid known to be present in apple and cherry), was used as an internal standard and was spiked into the rose leaf extracts at different concentrations. No Phlorizin was added to the sunflower extract; therefore it was not identified in this sample. Sunflower extracts showed high levels of Genistein in positive mode compared to lower levels in the rose extracts. The presence and relative amounts of several endogenous organic acids are also shown in Figure 5.

Table 1. Summary of differential compounds between sunflower and rose extracts.

Metabolite Name	Formula	M+H	M-H	RT (min)	Area Rose 1	Area Rose 2	Area Rose 3	Area Sunflower
Citric acid	$C_6H_8O_7$	193.0343	191.0197	0.7	24050	17200	6300	1100
L-Glutamic acid	$C_5H_9NO_4$	148.0604	146.0459	0.72 (+)	246170	464400	401424	8180
L-Phenylalanine	$C_9H_{11}NO_2$	166.0863	164.0717	1.46 (+)	25460	41900	48000	NA
Catechin	$C_{15}H_{14}O_6$	291.0863	289.0718	2.7	404440	125500	33000	NA
Genistein	$C_{15}H_{10}O_5$	271.0601	269.0456	6.16	NA	NA	NA	306980
Kaempferol	$C_{15}H_{10}O_5$	287.0551	285.0405	4.85 (+)	2490000	5000000	9000000	10000
Phlorizin	$C_{21}H_{24}O_{10}$	437.1442	435.1297	4.6	179000	265700	426000	NA
Quercetin	$C_{15}H_{10}O_7$	303.0499	301.0354	4.45 (+)	3304000	224300	2200000	NA
Quercitrin/Astragalin	$C_{21}H_{20}O_{11}$	449.1078	447.0933	4.4	1005000	420000	1003000	NA

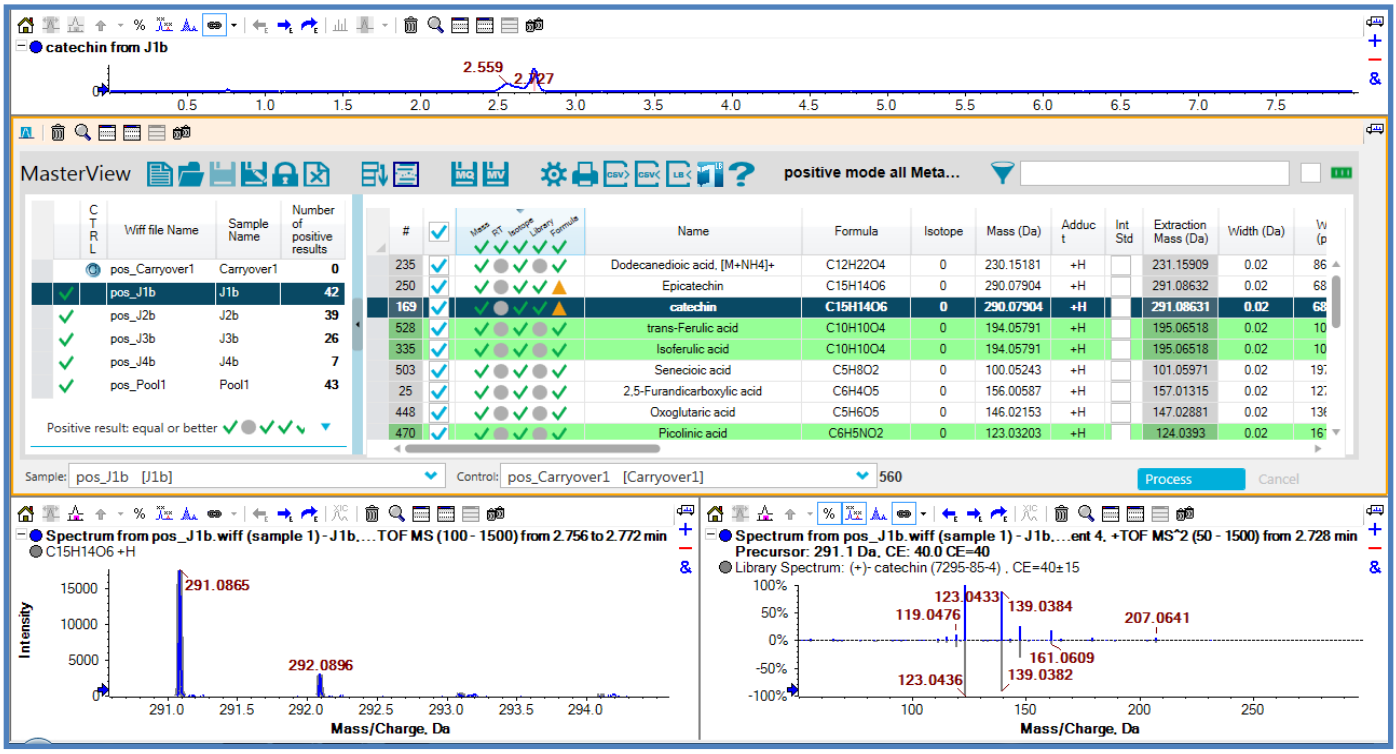


Figure 5. Accurate Mass Metabolite Spectral Library (ASSML) to confirm the identities of detected differential features. Clicking on an entry in the table (Catechin) exposes further results to explore. The bottom left pane yields the isotopic pattern and accurate mass spectra with blue (experimental) overlaid upon the theoretical isotopic pattern (gray). The bottom right pane shows the library matched MS/MS spectra (blue experimental spectra and gray reflected library spectra). The top pane shows the Extracted Ion Chromatogram (XIC) MS data for Catechin.

Conclusions

Combining XCMS^{Plus} and the Accurate Mass Metabolite Spectral Library was beneficial in confirmation of several antioxidants and endogenous metabolites present in both rose and sunflower leaf extracts. SWATH Acquisition allows collection of MS/MS of all precursors meaning no missing data when compared to traditional IDA workflows.

References

1. Cunja, V.; Mikulic-Petkovsek, M.; Stampar, F.; Schmitzer, V. Compound Identification of Selected Rose Species and Cultivars: an Insight to Petal and Leaf Phenolic Profiles JASHS March 2014 vol.139 no.2 157-166.
2. Accurate Mass Metabolite Spectral Library developed by Gerard Hopfgartner, Emmanuel Varesio, Tobias Bruderer, University of Geneva, Switzerland.
3. Developed by Gary Siuzdak and colleagues at Scripps Research Institute, La Jolla, CA.

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