

Advanced data processing software enables autonomous metabolite identification on the X500R QTOF System

Using XCMS^{plus} Software

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Metabolomics is the scientific study of the chemical fingerprint which is left behind by cellular processes¹. Untargeted metabolomics studies are valuable to biomarker researchers for studying disease effects over time and to identify novel biomarkers to track disease progression. Mass Spectrometry tends to be the analytical tool of choice given its sensitivity to other techniques such as ¹H NMR Spectroscopy. The X500R QTOF System was developed for routine, robust workflows and requires minimal MS expertise. Data from a previously acquired from a prostate cancer study was processed as proof of concept, through the XCMS^{plus} software pipeline to highlight advanced data processing features required of untargeted metabolomics studies. XCMS software is the “World’s most cited metabolomics software” as it has over 1000+ citations in literature and very trusted in the metabolomics community. The software allows you to load, process and analyze their data in one interactive workspace. Peaks are annotated and matched against the METLIN database for metabolite identification.



Key benefits of the untargeted metabolomics workflow on the X500R QTOF System with XCMS^{plus} software

- Simplified operation with intuitive interface and workflow setup powered by SCIEX OS Software with minimal MS expertise.
- Get to actionable results quickly with user-friendly processing software and libraries
- Maximize lab efficiency using a robust and integrated system with automated calibration and a benchtop footprint
- Data can be loaded, processed and reviewed in a single interactive workspace
- Submit and monitor the status of multiple jobs simultaneously
- Statistical analysis including paired/unpaired t-tests, parametric and non-parametric testing (including FDR), and multivariate data analysis techniques.
- Simplified metabolite identification by linking your data to the METLIN database



Figure 1: Metabolomic profiles can clearly differentiate diseased (cancer) from normal samples. Using the untargeted metabolomics strategy described here, samples from a prostate cancer study were analyzed, as well as QC samples (spiked with known compounds). Shown in the above Scores plot, PCA analysis clearly differentiates healthy from disease and samples, confirming the original sample classification. Samples within the same group cluster together highlighting the reproducibility of the technique. The related loadings plot is used to determine which peaks are responsible for this differentiation.

Methods Sample preparation: Urine samples were obtained with disease classifications that were previously determined using accepted clinical techniques. The specific gravity of the samples was measured by testing 15 μL of urine samples with a refractometer prism. A 50 μL volume of the thawed urine samples was then transferred to a clean, labeled microcentrifuge-filter tube. An isotopically labeled internal standard mixture (20 μL) was added. The urine sample was then diluted with 400 μL 98:2 acetonitrile/water with 0.1% sodium azide, then vortexed. The sample was then centrifuged, and the supernatant was isolated. This was then dried down and reconstituted in 50 μL of 0.1% formic acid in water. The samples were transferred to glass vial and loaded into the autosampler.

Chromatography: The reverse phase HPLC separation was performed using a Shimadzu LC System, operating at a flow rate of 350 $\mu\text{L}/\text{min}$. The column used was an Ace Excel C18-PFP column (100 x 1mm, 2 μm) from ACE, maintained at 30 $^{\circ}\text{C}$. A standard reverse phase gradient was used employing mobile phase A as 0.1% formic acid in water and mobile phase B as acetonitrile. The injection volume was 3 μL in positive ion mode and 5 μL in negative ion mode.

Mass spectrometry: The data was collected using information dependent acquisition (IDA) on the X500R QTOF System (SCIEX). Using optimized source conditions, the MS mass range analyzed was 50-600 m/z and the MS/MS was acquired with a mass range of 40-600 m/z with a 25 msec accumulation time.

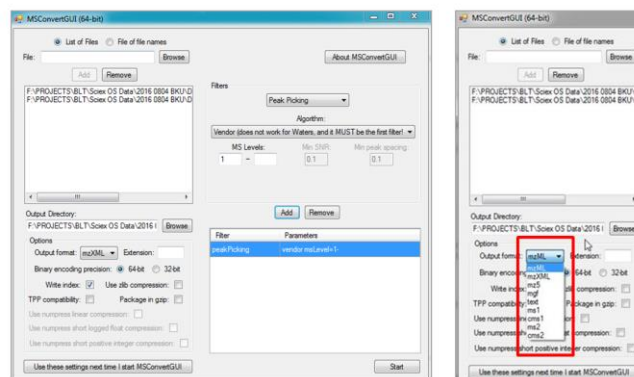


Figure 2: Data conversion parameters. Using Proteowizard wiff2 files can be converted using the above parameters to mzxml which can be loaded into XCMS^{plus}.

The collision energy was set to 35 V with a 15 V collision energy spread to ensure high quality MS/MS on most metabolites.

Data processing: The data was processed in XCMS^{plus} software, a desktop version of XCMS Online. The wiff2 data were converted using the msconvert tool of Proteowizard (3.0.9992) to mzXML format and pushed through XCMS^{plus} to pick peaks, align features, normalize data and subsequently aid metabolite identification of the significantly extracted features.

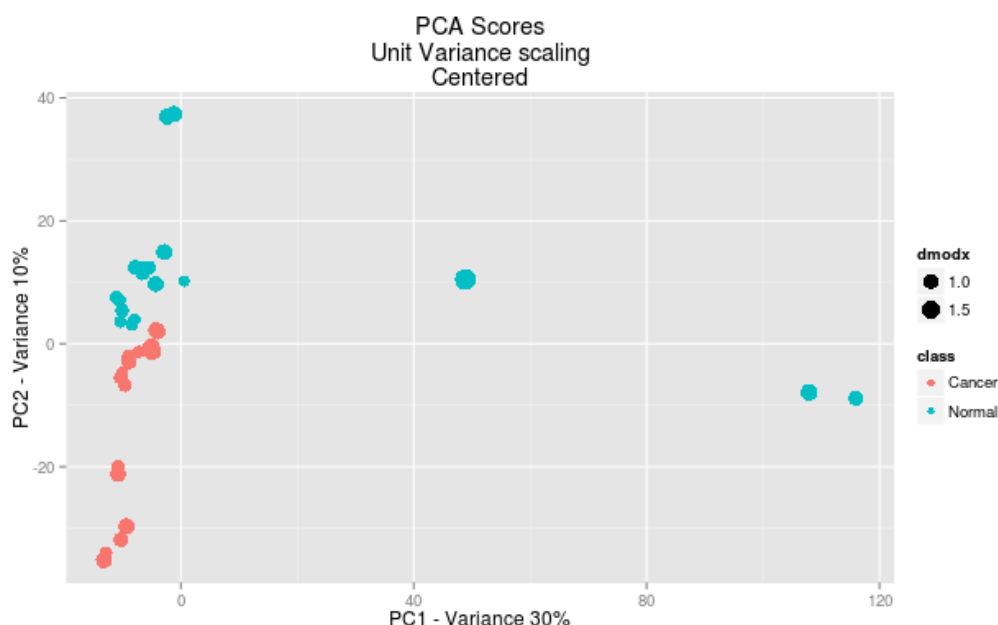


Figure 3: Scores plot from XCMS^{plus} software. The QC samples were removed from the same dataset as in Figure 1, leaving only a comparison of the healthy and diseased samples. There is a differentiation between the known sample groups highlighting that the metabolic profile is quite different in the diseased samples.

Statistical analysis

From the scores plot (Figure 3) one can see a difference along the y-axis between the healthy and diseased classified samples. From here the user can “view results table” and view a table of extracted features. The table can be sorted based on p-value significance or q-value significance (Figure 4). The q-value is the false discovery rate adjusted p-value (not featured in this figure). The feature table identifies the retention time at which a particular feature was found as well as the intensity. The fold changes between any sample groups of that particular feature are also listed in the column labeled “fold” (Figure 4). Other observations (as columns) of interest can be added by the user and used for sorting (such as m/z value or retention time (RT), Corr Var = correlation variation, Max Int = maximum intensity,

Feature Gp = feature group). Any isotopes and adducts are also identified as well as feature grouping which groups together any related ions.

Data review

From the feature table, a feature can be highlighted and the XIC can be visualized as an overlay from all the samples processed (Figure 4 top right). One can then review the raw data and see if this is an actual feature or just noise or an irrelevant peak eluting in the solvent front. Retention time information as well as the accurate mass is displayed in this XIC plot. The mass spectrum for that XIC can be viewed as well as a simple box and whisker plot highlighting the differences in intensities between the groups (including the average and upper and lower limits of the intensity

Quick Compound Search:

JOB#134 : UNI FLORIDA PAIR

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featureid	fold	pvalue	updown	mzmed	rtmed	maxint	dataset1_r	dataset2_r	isotopes	adducts	peakgrot	usernotes
1	22.4	1.15383e-4	DOWN	133.0799	0.88	429	889	40			41	
2	3.0	7.23746e-4	UP	252.1314	10.14	60	39	118			36	
3	10.7	9.73895e-4	UP	240.1091	1.92	87	23	242			333	
4	8.0	1.07427e-4	UP	262.5754	10.15	57	12	95			36	
5	3.4	4.05188e-4	DOWN	176.0703	9.17	1,534	2,312	678	[9][M]+	[M+H]+ 175	30	
6	3.4	4.83074e-4	DOWN	130.0651	9.18	367	600	175		[M+H-HCO	30	
7	3.7	1.12008e-4	UP	342.1484	1.92	41	33	122			327	
8	2.8	1.92050e-4	UP	268.0858	8.58	71	58	162		[M+Na]+ 24	136	
9	5.1	2.49077e-4	UP	485.1375	1.13	104	15	74		[M+K]+ 446	206	
10	2.9	3.81856e-4	UP	314.0850	0.95	115	59	171			203	
11	6.6	4.55813e-4	UP	589.1487	1.05	106	14	91		[M+K+CF3	269	
12	7.3	7.27676e-4	UP	538.1277	6.33	71	6	46		[M+K+CF3	100	
13	7.3	8.30882e-4	UP	197.0536	1.04	84	10	77		[M+K]+ 156	18	
14	11.0	9.16787e-4	UP	240.1091	3.15	66	15	168			353	
15	12.5	1.70454e-4	DOWN	516.2929	9.17	169	109	9		[M+Na+K+H	30	
16	6.1	1.83140e-4	DOWN	290.1390	8.82	275	395	65		[3M+Na+2H	57	
17	8.1	2.54503e-4	UP	377.1128	1.01	87	13	108			218	
18	10.2	3.43520e-4	DOWN	346.0100	1.18	63	243	24			164	
19	15.1	3.43689e-4	DOWN	545.3281	9.95	245	203	13			37	
20	4.3	6.26140e-4	DOWN	221.0785	8.39	350	410	95		[M+2H-H2	237	
21	5.5	7.05177e-4	DOWN	560.3193	9.26	146	153	28			91	
22	2.4	7.72774e-4	DOWN	380.1922	8.32	222	240	99		[3M+2Na]2	157	
23	285.3	8.21585e-4	DOWN	294.1014	1.18	932	1,601	6			164	
24	2.3	1.03781e-4	DOWN	177.0740	9.16	202	289	123	[9][M+1]+		30	
25	2.0	1.05230e-4	DOWN	154.0691	4.94	114	580	289			317	
26	27.1	1.13210e-4	DOWN	415.2118	11.93	304	497	18	[91][M]+	[M+H]+ 414	246	
27	4.8	1.24831e-4	UP	426.0640	1.73	68	24	116			348	
28	167.0	1.34319e-4	UP	638.0246	7.90	188	1	117			112	
29	7.8	1.79796e-4	DOWN	235.0926	0.99	119	411	53		[M+H-CO]+	170	
30	144.1	1.81163e-4	DOWN	331.0531	1.02	202	452	3		[M+K+CF3	18	
31	35.9	2.30494e-4	DOWN	295.1030	1.15	140	163	5		[M+Na+CF	80	

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[Return to Job Summary](#)

Please click on a row to view feature details

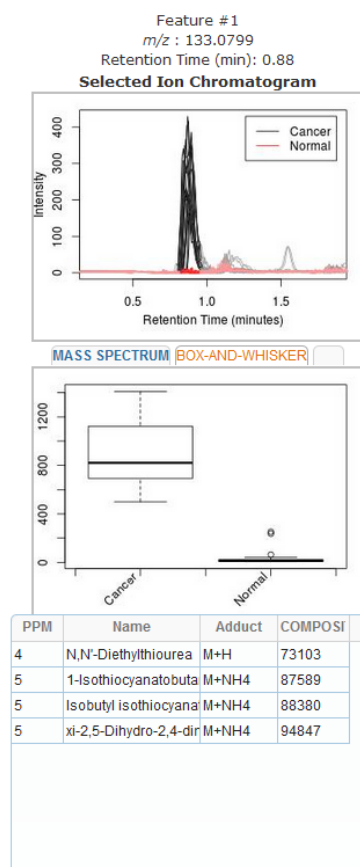


Figure 4: Table of extracted features from XCMS^{plus} software. Showing all features extracted from this dataset (a total of 2532 features). These can be sorted by p value, fold change or any other parameter in the feature table. The highlighted feature (#1 in the table) then allows the user to view that particular extracted feature from the total ion chromatogram (TIC, top right). Here one can see the huge concentrations of this feature in the cancer group vs the normal group (where there is virtually no signal). The related box and whisker plot can be visualized (middle, right). Lastly any matches to the METLIN database can be highlighted in the table (bottom, right). Identifications are ordered from highest match to the accurate mass of the MS1 parent ion. One can click directly on the composite ID number to be directed to the METLIN database webpage regarding that particular compound (Figure 5).

per group) (Figure 4, middle right). Finally each feature is matched for identification to the METLIN database. If MS/MS spectra are available then the column labelled “MS/MS” is populated with “y” meaning MS/MS is available (not shown here). The database identifications are listed in a table (Figure 4 bottom, right) and ranked by m/z error (ppm) and then alpha/numerically. Then using composite ID column one can click the METLIN ID and be referred to the METLIN website (<https://metlin.scripps.edu>) where any MS/MS confirmation could be compared to the experimental spectra (Figure 5). A range of MSMS spectra are available at varying collision energies.

The most compelling visualization tool in XCMS^{plus} software is the interactive cloud plot (Figure 6). Key visualization features include:

- P-value is represented by how dark or light the color is.
- Fold change is represented by the radius of each feature.
- Retention time is represented by position on the x-axis.
- Mass-to-Charge ratio is represented by position on y-axis.
- Sliders for p-value and fold change are in the Main Panel.
- Sliders for intensity, retention time, and mass-to-charge are in the Advanced Tab. A link to the table representation of the graph is displayed at the bottom along with the settings used to generate the graph.

The cloud plot is completely interactive and can be filtered on any function listed above. So you can be more stringent for the p-value and also the mean fold change allowing for only the more highly significant features to be displayed.

Conclusions

The X500R QTOF System is a robust, easy to operate, benchtop system that requires minimal MS expertise to perform untargeted metabolomics analyses. As metabolomics continues to expand in disease research, robust easy-to-use solutions that provide quality answers will be increasingly important.

In this study, samples from a pilot prostate cancer study was analyzed and a clear difference between healthy and disease urine samples were detected using this untargeted metabolomics approach, confirming the original disease classifications.

XCMS^{plus} Software was used to extract the features from the dataset and simultaneously identify them against a large well-known database. All of the data review and any statistical data analysis could be completed within a single software package.

The software allows advanced data processing and data analysis which are often required by researchers for untargeted metabolomics studies.

This pilot study provided confidence in the approach and the next phase of the study analyzing a much larger set of samples is underway.

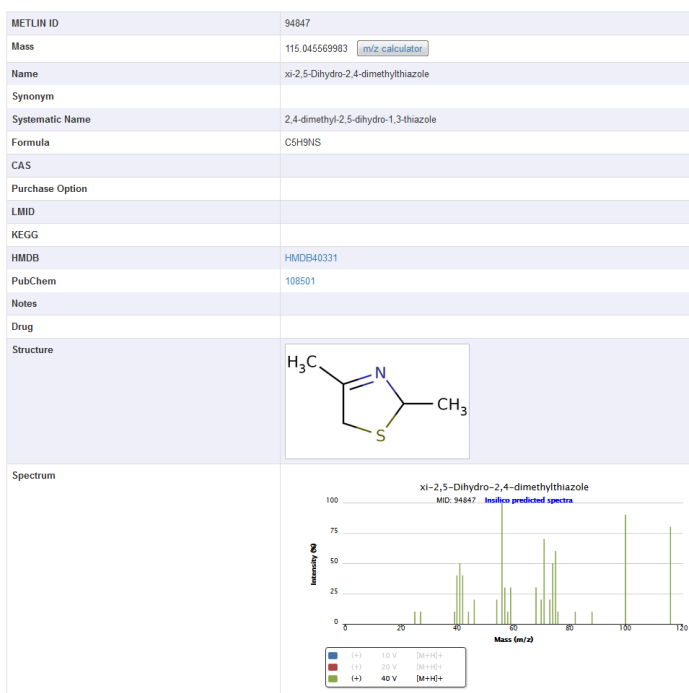


Figure 5: METLIN database entry. Highlighting information relevant to the metabolite selected from the table of extracted features. One can view the METLIN ID number and other details such as other names for the compound as well as molecular formula. Most importantly the structural and MSMS spectra are available to compare with any experimental data collected. Fragmentation spectra are collected at multiple collision energies and in both positive and negative ionization modes (only if applicable in both modes).

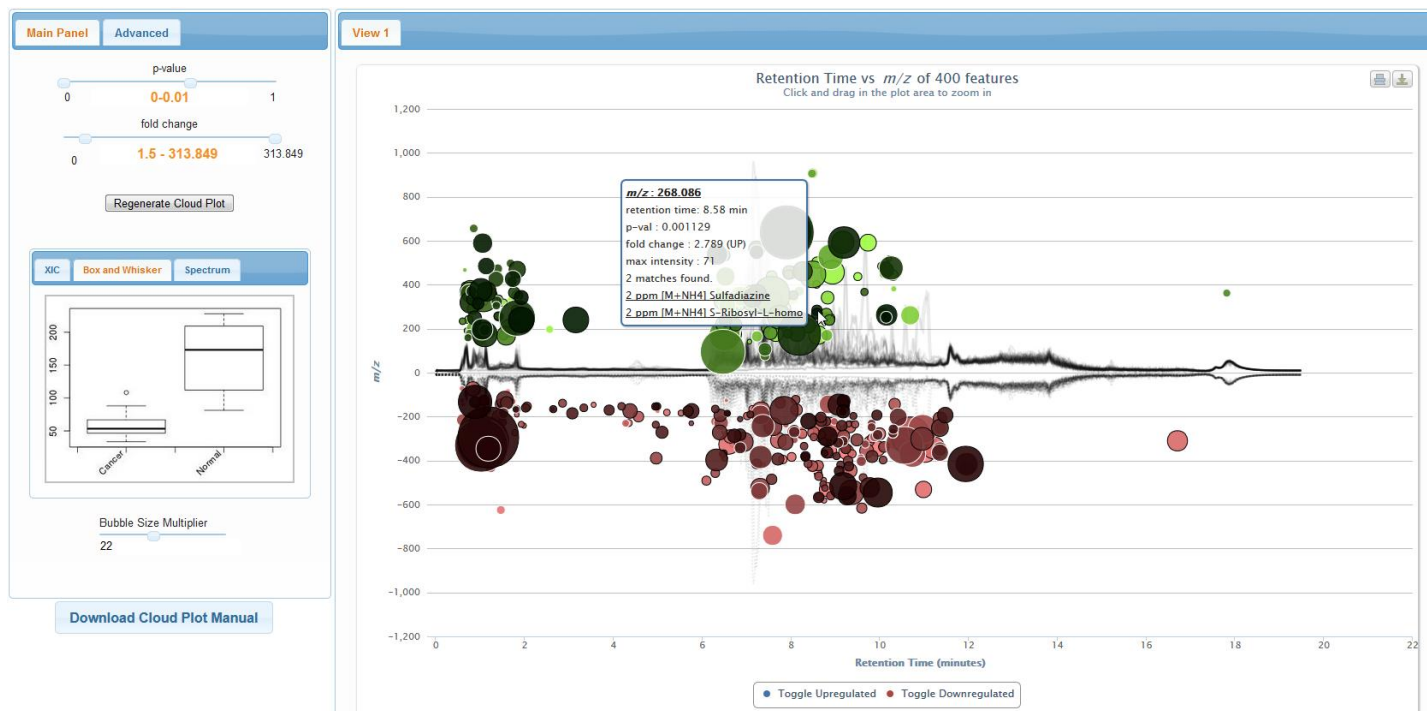


Figure 6: Interactive cloud plot. The cloud plot displays features whose intensities are altered between sample groups. In this case 400 features were highlighted as having a p-value of less than 0.01. Up-regulated features are represented as circles on the top of the plot and down-regulated features are represented as circles on the bottom of the plot, where the size and the degree of color saturation corresponds to the (log) fold change of the feature. Circles with black outlines indicate hits in the database. The lighter or darker the color of the circle relates directly to the significance of the p-value. The cloud plot is completely interactive, where you can filter p value, m/z value, as well as retention time to allow the viewer to see more/less features based on the filtering criteria.

References

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