Development And Evaluation Of An Accurate Mass LC/MS/MS Spectral Library For Metabolomics

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Cindy Lai; Theodore Sana; Stefan Jenkins; Steven M. Fischer, Agilent Technologies, Santa Clara, CA

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Introduction

The determination of compound identities is a crucial analytical problem for metabolomics scientists using LC/MS detection. Without compound identities it is impossible to make biological sense of study results. Many compounds observed to be differentially expressed in metabolomics studies are known primary metabolites. Easily identifying these known metabolites would facilitate metabolomics analysis. One accepted way to identify compounds is to compare observed MS/MS spectra of the unknown to a library of MS/MS spectra of metabolite standards. In this poster we present the creation of an accurate mass MS/MS library of common metabolites using three different collision energies and test the library's utility by analyzing MS/MS spectra of unknown metabolites in urine and yeast sample extracts.

Experimental

Standards: approximately 2500 metabolite standards of a number of different classes from a number of sources

Samples: human urine and yeast Saccharomyces cerevisiae.
Instrumentation: An Agilent 1200SL Series HPLC system with binary pump and degasser, well plate autosampler with thermostat, thermostatted column compartment, and an Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with dual ESI source operated in the positive or negative ion modes was used. Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution using an isocratic pump connected to a dual sprayer electrospray ionization source. Reference ions: negative ion mode 119.0363, 980.016375; positive ion mode 121.0509, 922.0098
Acquisition Conditions: LC methodology: Flow rate 0.6 ml/min, column temperature 60°C. Solvent A is Water + 0.2% Acetic Acid, solvent B is Methanol + 0.2% Acetic Acid. A 13 minute gradient from 2% B to 98% B and a 6 minute hold at 98% B with a stop time at 19 minutes, with 5 minutes post-time for column re-equilibration. An Agilent Zorbax SB-Aq (2.1 X 50mm, 1.8 um) analytical column and an Agilent Zorbax-SB-C8 Rapid Resolution Cartridge (2.1X 30mm, 3.5 um) guard column was used for separation

Results and Discussion

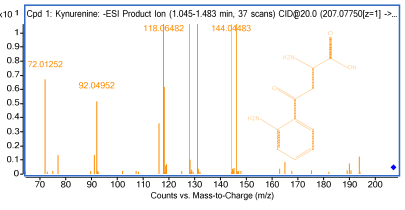
Library creation – data collection: The MS/MS spectral library was created by analyzing metabolite standards via flow injection or chromatographic methods, with detection on a Q-TOF mass spectrometer operated in positive and negative modes, at collision energies 10, 20, and 40 eV. Only singly charged positive M+H+ or negative M+H- ions are used to produce targeted MS/MS spectra. The quadrupole filter was set to transmit a peak width of 1 amu, so that only the isotope selected is transmitted, not the adjacent naturally occurring isotopes . Mass range was set to detect 50-1600 amu for MS only data, and 25-1600 amu for MS/MS data. Chromatographic peaks are found, and a spectra is generated by averaging the peak and presented to the library building tool.

Data clean-up: The first step in the library importation and spectral clean-up process filters the spectra using an absolute threshold of 100 counts and a relative threshold of 0.5% of the largest peak in a spectrum. This is to filter out peaks that are either insignificant or too weak to be of use for library matching. The ions that passed step one are then determined to be related to the precursor ion. Ions within a set mass tolerance that are related with the precursor ion were accepted, and the mass saved in the library was corrected to its calculated accurate mass. A custom program was written for this purpose.

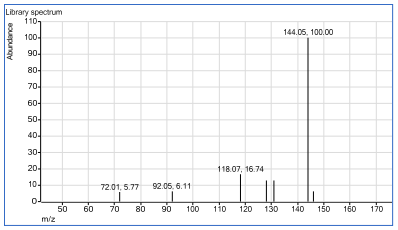
Figure 1. Comparison of raw data and post clean-up library spectra of kynurenine at ESI negative, CE = 20 eV

raw data peak list		library peak list	
m/z	counts	m/z	abund
146.0923	300		
146.0729	300		
146.0606	1600	146.0611	15.2%
144.1073	200		
144.0724	2500		
144.0618	1300		
144.0448	10500	144.0455	100.0%
131.0666	500		
131.0561	500		
131.0384	1400	131.0377	13.3%
131.0307	200		
128.0750	300		
128.0665	300		
128.0499	1600	128.0506	15.2%
118.0936	600		
118.0769	400		
118.0648	1700	118.0662	16.2%
116.0499	400		
92.0541	400		
92.0495	500	92.0506	4.8%
72.0326	200		
72.0281	300		
72.0125	700	72.0091	6.7%

Magnified view of raw data for MS/MS spectra of kynurenine at CE =20 eV



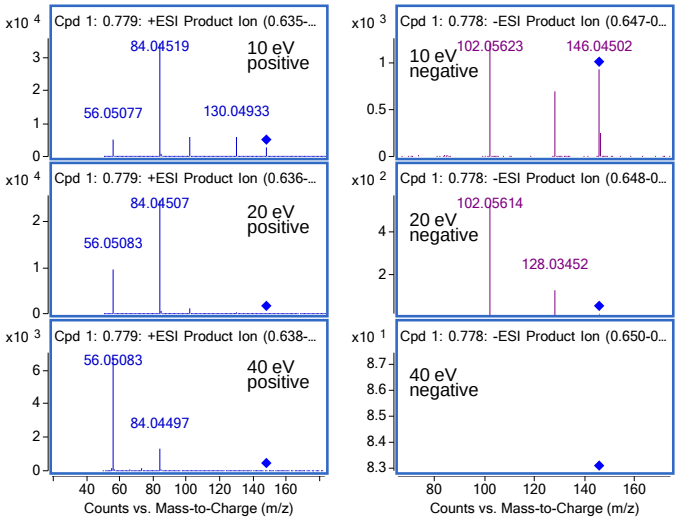
Library entry for MS/MS spectra of kynurenine at ESI negative, CE = 20 eV



Results and Discussion

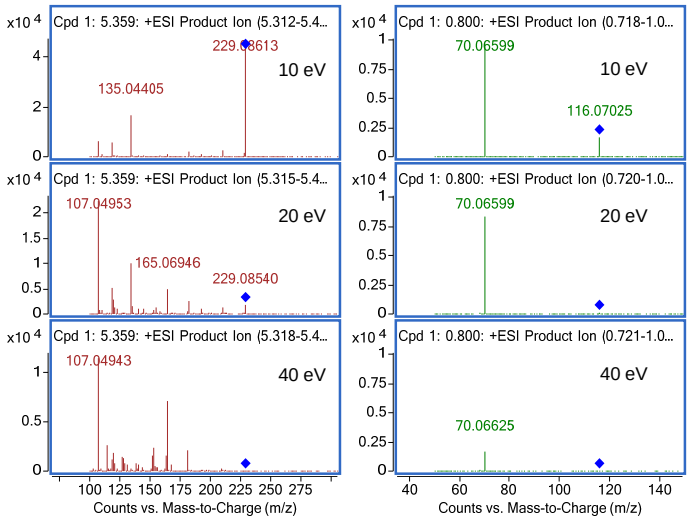
Fragmentation of positive and negative ions: Ionization polarity has significant impact on compound detection and fragmentation patterns. Even at the same collision energy, bond dissociation may vary with positive and negative ionization. Fragments observed in one polarity will not necessarily be observed in the opposite polarity, even after adjusting for the charge difference. In Figure 2 Glutamic Acid is analyzed by ESI positive and ESI negative mode. At CE=10 eV, the 56.05077 and 84.04519 fragments present in positive mode are non-existent in the negative mode. Both positive and negative ions are valuable and can be used for metabolite identification.

Figure 2. Positive and negative ionization of Glutamic Acid at ESI, CE=10, 20, and 40 eV.



Multiple collision energies: Compounds have different fragmentation behavior because of their varying chemical and physical properties. Not every compound dissociates at the same collision energy, and no single collision energy is able to provide valuable MS/MS fragmentation pattern for compounds across different classes. Figure 3. compares the fragmentation patterns between resveratrol and proline at different collision energies in ESI positive. All three collision energies give rich fragmentation patterns in resveratrol, with increasing fragment abundance as the energy increases. Proline produces only two major ions at 10 eV, with precursor ion minimally observable in 20 and 40 eV. Clearly, each compound has its unique fragmentation behavior and the use of multiple collision energies enhances the confidence in library searching.

Figure 3. MS/MS spectral comparison of resveratrol (left) and proline (right) at ESI positive, CE=10, 20, 40 eV.



Spectral library matching: The MS/MS library search routine is capable of forward (match all ions in unknown spectra to the library) and reverse (match only library ions to the unknown spectra) searches. Spectral matching is done by searching the corresponding peaks in library and unknown spectra within a set mass tolerance. For searching the library, a default tolerance for precursor ion is set at symmetric +/-5 ppm, and a default tolerance for product ion is set at symmetric +/-10 ppm. When a corresponding peak is found, a dot product of library peak intensity and unknown peak intensity is calculated. A matching score is then generated by summing the dot products for all the peaks in a given spectra, then normalized to produce a score of 0 to 100 with 100 being a perfect match.

Library matching for unknowns in urine: To test the utility of the MS/MS library, a targeted analysis of caffeine in human urine sample was performed. Figure 4 shows the TIC of urine sample analyzed chromatographically in positive ESI, and the caffeine extracted compound chromatogram (ECC) detected at retention time 4.102 minute. A targeted MS/MS analysis at 10, 20, and 40 eV collision energy for caffeine ion (195.08730) was acquired. Figure 5 compares the 20 eV spectra of caffeine in urine, to the corresponding 20 eV spectra of caffeine in the spectral library as mirror images to each other.

Results and Discussion

Figure 4 TIC of urine and ECC for caffeine

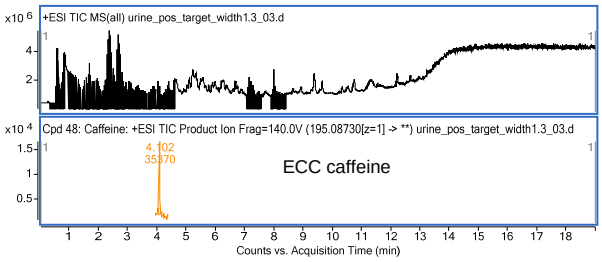
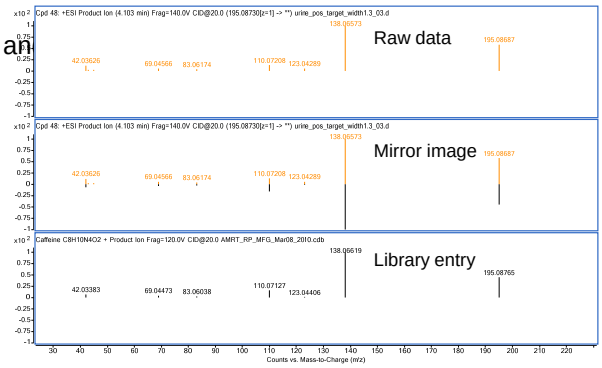
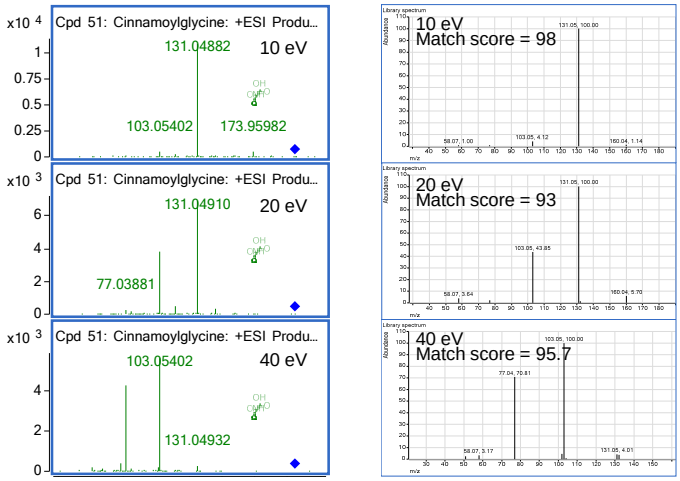


Figure 5. Mirror image of caffeine's library entry at 20 eV, an



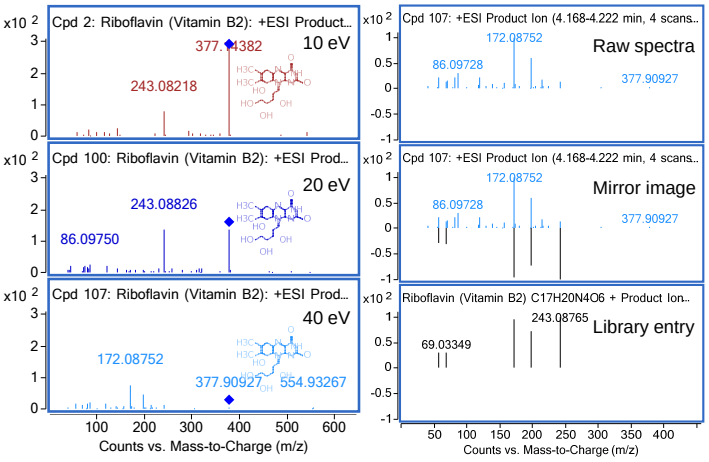
Another example of a metabolite from urine demonstrates the utility of the library search is capable of matching unknown fragment ions to library entries at all three collision energies. Figure 6 compares the raw data of cinnamoylglycine at 10, 20, and 40 eV and its corresponding library entry. Note the high match score at all energies.

Figure 6. Raw data spectra of cinnamoylglycine in urine and cinnamoylglycine in library at 10, 20, and 40 eV, ESI positive.



Metabolites in Yeast Saccharomyces cerevisiae: Extracts were analyzed by MS only. Compounds of interest with initial retention time and mass match to the database were further investigated with targeted MS/MS analysis. Collected MS/MS spectra were searched against the library entries and identified. An example of riboflavin is demonstrated at different collision energies in Figure 7. When the collision energy is increased from 20 to 40 eV, precursor ion abundance decreases in conjunction with fragment ion abundance increases. The fragmentation pattern matched the library at all collision energies, 20 eV matching is displayed.

Figure 7. MS/MS spectra for riboflavin in yeast at ESI positive 10, 20, 40 eV



Conclusions

- *An accurate mass LC/MS/MS library for over 2700 common endogenous metabolites is developed.*
- *Spectra were collected in ESI positive and ESI negative, and at 10, 20, and 40 eV collision energy*
- *We demonstrated that multiple collision energies are necessary because of the differences in fragmentation behavior of different classes of compounds*
- *We have developed library curation tools for MS/MS spectra for improving library quality and matching*
- *Library matching reproducibility and utility have been evaluated by testing complex matrices such as urine, yeast, and erythrocyte. The results demonstrate the MS/MS library is a reliable and valuable tool for confirming metabolite identity in metabolomics analysis.*