A RAPID SHOTGUN LC/MS APPROACH FOR TRADITIONAL CHINESE MEDICINE ANALYSIS USING UPLC/TOF MS^E COUPLED WITH MULTI-VARIANT STATISTICAL DATA ANALYSIS

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INTRODUCTION

Traditional Chinese Medicine (TCM) is a medicinal system that utilizes naturally occurring resources such as plants and animals for treatment of diseases. In recently years, there are increasing interests to better understand the working mechanisms of TCMs. There is a hope that with a comprehensive understanding of how TCM works, eventually, new medicines using only the effective ingredients from the relevant plants can be created so that Modern Chinese Medicine (MCM) is systematically developed with more effective remedies that are with known mechanism.

The very first step of migrating from TCM to MCM would be a comprehensive ingredient analysis of the TCM samples. However, it is well known that the ingredient analysis for TCM sample is extremely challenging for a few reasons. First, same type of plants contain different ingredients if the plant location, harvest time and method are different. Second, identical plants may have different final ingredients after harvest with different storage conditions. Third, and the most critically, most TCM plants have to go through some specific processing procedures prior to their medicinal use. Therefore, the actual ingredients taken by people from the processed plant extracts are different from the freshly harvested plant extracts. It is the ingredients from the processed plant extracts that will be actively offering physiological effects, not the ingredients from the freshly harvested plant extracts.

For this reason, there have been huge efforts by variety of people over the years trying to gain better understanding of the ingredient changes before and after the processing. However, this is a difficult task since plants typically contain hundreds of ingredients at variety concentration levels and they may belong to many different chemical classes. The most effective way to gain a comprehensive understanding of what happened to plant ingredients before and after the processing procedure at the compound level is to use a systematic sample profiling protocol so that the differences for different groups of samples can be profiled.

In this work, we propose an UPLC/TOF MS^E/MSA workflow (Figure 1) to systematically profile TCM samples and compare the ingredient differences for before and after the sample processing. The application example that was used here is the different extracts from *Polygonatum kingianum*.

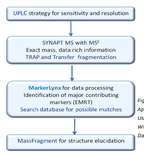

Figure 1. Generic Systematic Approach for Sample Profiling Using UPLC/TOF MS^E Coupled With Multi-Variant Statistical Data Analysis.

BACKGROUND

Polygonatum kingianum

Polygonatum kingianum is one of the original plants known as Huangjing. The *Polygonatum kingianum* roots were used as a tonic remedy for lung troubles and ringworm. Traditionally, the fresh Huangjing root is cut to thin slices upon harvest for storage. However, prior to medicinal use, these slices are soaked in alcohol then steamed to a point when all the slices are turned black in color. Figure 2 shows the pictures of the Huangjing root before and after the processing procedure. Over the years, there have been different efforts made at different levels trying to gain an understanding of the ingredient changes after the processing procedure. However, because the lack of a systematic approach, only handful ingredients have been observed, and it was unclear that whether these are the major contributors for the sample differences.

Figure 2. Huang Jing samples before and after the processing procedure

UPLC/oaTOF MS^E/MultiVariate Statistical Sample Profiling Strategy

This strategy takes the advantages of the UPLC for high resolution, high sensitivity and high speed separation, as well as the oaTOF exact mass measurement capability. The oaTOF MS^E data acquisition strategy (Figure 3) allows MS to obtain data at two distinct but parallel functions via rapid switching. First scan function sets at low collision energy (CE) and the second scan function sets at high CE. The resulted raw data file contains two distinct chromatograms, one contains intact m/z information, the other contains fragment m/z information. From a single LC injection, MS full scan and fragment ion information can be accomplished simultaneously. Once data acquisition is complete, the 3D LC/MS data can be converted into a 2D matrix by MarkerLynx XS. Each data point in the 2D matrix represents an exact mass/retention time pair (EMRT), i.e.: a marker. This data set is used for a Principal Component Analysis (PCA) to observe the grouping patterns. From the PCA plot, any two groups of samples can be paired for Orthogonal Partial Least Square-Data Analysis (OPLS-DA) . The resulted Scatter plot (S-Plot) from the OPLS-DA analysis can clearly display leading contributing markers that differentiate the two sample groups. Once identified, the markers can be searched for their elemental compositions as well as search against database for identity. The result can be further confirmed from the MS^E spectrum and use the MassFragment for structural elucidation.

ANALYTICAL METHOD

Sample Preparation:

Fresh root slice:

The fresh Huangjing root was cut to thin slices and dried for 48 h at 50°C. The slices were then cooled to room temperature prior to be sealed for storage.

Processed root slice:

Processed products: The fresh Huangjing roots were soaked in rice wine (root/wine ratio was 5/1) and were let sit till the wine was all absorbed. The wine absorbed roots were then steamed and sampled at 5 Hr, 10 Hr, 15 Hr time interval. All samples were cut to thin slices prior to being baked at 50°C for 48 hours. The samples were then cooled to room temperature and sealed for storage. The 15 Hr time point sample was used for this study.

Sample Extraction:

Weigh 30g of each sample, and add to each sample 200 mL of 35% acetone. Sonicate for 20 minutes. Repeat adding acetone and sonicate two more times for each sample. The combined supernatant of each sample was filtered prior to be loaded on to a macro porous resin SP825 column. Sample was eluted with the following solvents stepwise: water (3bv), 10% acetone (3bv), 80% acetone (3bv), and methanol (3bv). The fraction obtained from the 80% acetone elution was evaporated to dryness. Reconstitute to 10 mL with 80% acetone. Shake well prior to LC injection.

UPLC conditions:

Column:	ACQUITY UPLC HSS T3 Column 2.1 x 100 mm, 1.7 µm, 45 °C		
Flow Rate:	600 µL/min.		
Mobile Phase:	A: Water + 0.1% Formic Acid; B: ACN		
Injection Volume:	5 µ L		
Gradient:	0 min.	95%A	
	25 min.	5%A	Curve 6
	27 min.	0%A	Curve 6
	30 min.	95%A	Curve 1

MS conditions:

Ionization Mode:	ESI Negative	Acquisition Range:	50-1500 m/z
Capillary V:	3000 V	Cone V:	35 V
Desolvation T:	450 °C	Desolvation Gas:	800 L/Hr

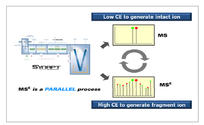

Figure 3. Schematics of the oaTOF MS^E data acquisition strategy.

RESULTS AND DISCUSSION (I)

Figure 4 shows the comparison of the base peak ion chromatograms (BPI) obtained for Huangjing fresh slice extract (4b) and for 15 Hr Huangjing alcohol steamed slice extract (4a) from the UPLC/TOF MS^E analysis. The chromatograms shown here are from the low energy scan. Superficially, a general trend about these two samples was observed. The alcohol steamed sample clearly displayed more peaks at higher intensities later in the chromatogram (especially after retention time of 10 minutes), and it showed obvious decrease for early eluters both in terms of the peak number and peak intensities. Giving the fact that the separation was performed on a reverse phase column, this trend indicated that the fresh extract contained more polar ingredients and the alcohol steam extract contained more less polar or non-polar ingredients.

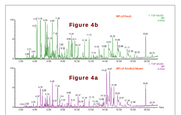

Figure 4. BPI Comparison of Fresh Extract vs Alcohol Steamed Extract .

However, for such complex samples, the best approach to chemically highlight the differences is by using a multi-variant statistical analysis (MSA) tool. One of the pre-requisition for applying MSA tool on LC-MS data is that the data has to be in 2-D format, i.e., the 3-D LC-MS dataset has to be reduced into a 2-D matrix before they can be analyzed by MSA. This is automatically accomplished by MarkerLynx XS software so that 3D LC-MS data points are converted into Exact Mass Retention Time Time (EMRT) pairs. The differences of our two groups of samples can be clearly highlighted by the S-Plot shown in Figure 5 as a result of the OPLS-DA analysis.

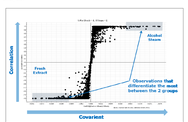

Figure 5. Scatter Plot from OPLS-DA for the Fresh Extract vs Alcohol Steamed Extract.

RESULTS AND DISCUSSION (II)

Table 1 is the list of the top 10 leading EMRT pairs for both sample groups. The top half (numbers in red) is for markers with higher concentration in the alcohol steam extract, and the bottom half (numbers in blue) is for the fresh extract. The exact mass can be used to search the elemental composition, which can then be used for further querying of existing databases to find possible chemical structures.

Table 1. Leading markers obtained from the S-Plot.

Take m/z 913.4451_7.54 min EMRT pair as an example. The elemental composition of this marker was found to be $C_{45}H_{70}O_{15}$. Possible match obtained by searching the Chemspider database was 80-Hi-(25R, 25S) *psiloside* D1 . The fragment ion information can be obtained from the high energy scan obtained from the same LC injection. MassFragment was used for structural elucidation as shown in Figure 6a. Also shown in Figure 6b is the fragment ion information by injecting the standard of this compound previously obtained from our lab.

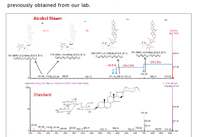

Figure 6. The high CE spectra of m/z 913 from Alcohol Steamed extract (6a). And the high CE spectrum of the HJ-16 (25R, 25S) psalioside.

CONCLUSION

- **Generic UPLC/oaTOF MS^E/MarkerLynx XS for sample profiling strategy is fast and efficient.**
- **Multi-variant statistical analysis helps to identify leading markers from each sample group effectively.**
- **MS^E data offers fragment information for identify confirmation and structural elucidation.**

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