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Non-Targeted Metabolomics of Rice Profiling Analysis by Agilent 1290UHPLC/6550 iFunnel Q-TOF System

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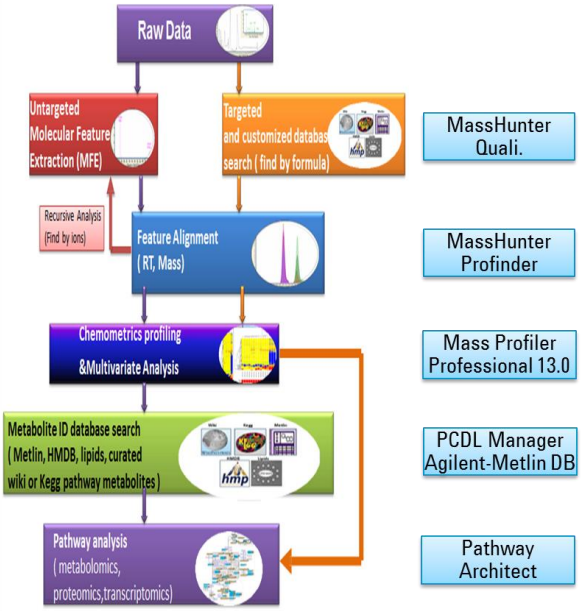
Introduction

Metabolomics is a comparative analysis of metabolites, the intermediate or final products of plant metabolism, found in sets of similar biological samples that it investigated for the difference of small molecular (molecular weight < 1500), often of highly complex samples, in which the chemical identify is often unknown.

Rice (*Oryza sative* L.) is an ideal candidate to study traits related to health due to its importance as both a global staple food and a model system for cereal crops. Evaluating metabolite profiles can be a high throughput method to identify variation in health properties of dietary components. Metabolomics is a useful approach to assess the influence of genetics on total metabolite variation in the rice.

Extraction methods have a vital impact on non-targeted metabolomics. In this study, We evaluated the effect of three extraction methods on rice seed metabolic profiling and provided selection strategy for future rice metabolomics studies. The three extraction buffers and two rice cultivars were used for metabolites analysis using Agilent 1290 UHPLC coupled to a 6550 iFunnel Q-TOF.

Figure 1. Agilent metabolomics workflow



Experimental

Agilent 1290 Infinity UHPLC/ 6550 iFunnel Q-TOF system

A 1290 ultra high performance system containing a binary pump and degasser, well-plate autosampler with thermostate, thermostatted column compartment, and an Agilent 6550 iFunnel Q-TOF mass spectrometer equipped with Agilent Jet-stream sources was used to analyze samples. A C18 column (Agilent Eclipse-plus C18, 3.0 x 150 mm, 1.8 µm) was used for the metabolites separation with 0.1% formic acid in water (A) and acetonitrile (B) as mobile phase. A linear gradient was used as shown in the table below . The flow-rate was 0.4 ml/min and injection volume was 2ul.

Time (min)	0	1	5	12	15	20
A (%)	98	98	60	30	5	5
B (%)	2	2	40	70	95	95

Post Time: 5.0 min

Mass spectral data of rice samples (leaf and grain) were acquired in extended dynamic range and TOF only mode. Data were acquired using the following settings: ESI capillary voltage was set at 3500 V for both ion modes, nozzle voltage at 250 V (+) and 1500V (-) and fragmentor at 150V. The nebulizer was 25 psi and the nitrogen sheath and drying gas were set at the flow rate of 12 L/min and 16 L/min, respectively.

Sample preparation

Samples were grounded into fine powder and three extraction solvents (1) water : acetonitrile : isopropanol=2 : 3 : 3 (v : v : v); (2) water : methanol=2 : 8 (v : v); (3) methanol : chloroform : water=4 : 5 : 3 (v : v : v), were used to extract tissue-specific metabolites from 20 mg grain and leaf, the extracts were then analyzed by LC/MS.

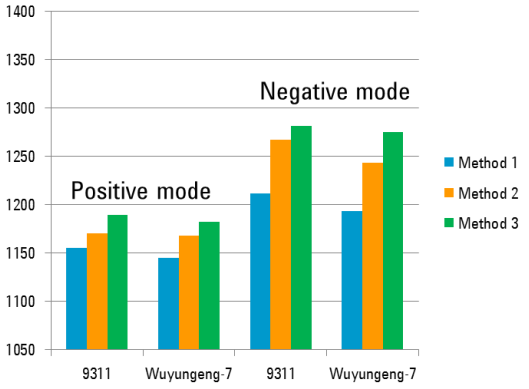
Data processing

LC/MS data files were processed by Agilent MassHunter Profinder software. Feature finding was achieved using the batch recursive feature extraction , which locates the groups of co-variant in each chromatogram. Each of these groups represented a unique features. Processing of raw data files resulted in features, which are defined as time-aligned ions (isotopes, adducts and dimers) summarized to the calculated neutral mass, an abundance and a retention time. Finally, the calculated neutral mass and RT and summed ion abundances were stored with Agilent cef. file and generic file for subsequent comparative analysis in Agilent Mass Profiler Professional 13.0 software.

Results and Discussion

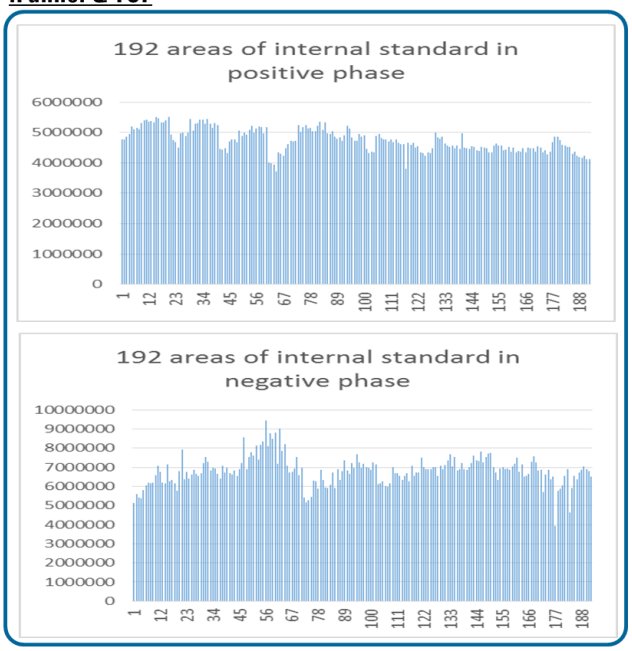
Table 1. The number of features detected from two rice seeds (9311 and Wuyungeng-7)

Extraction Method	Positive mode		Negative mode	
	9311	Wuyungeng-7	9311	Wuyungeng-7
Method 1	1155	1145	1211	1193
Method 2	1170	1168	1267	1243
Method 3	1189	1182	1281	1275



The three extraction solvents are : (Method 1) water : acetonitrile : isopropanol=2 : 3 : 3 (v : v : v); (Method 2) water : methanol=2 : 8 (v : v); (Method 3) methanol : chloroform : water=4 : 5 : 3 (v : v : v) for testing rice seeds. The data showed that the extraction method 3 had the best coverage of detection and yield at the cost of a double workload because this solvent contains chloroform, while the second method could be quite effective and increases features about 1.2-4.6% compared to the method 1.

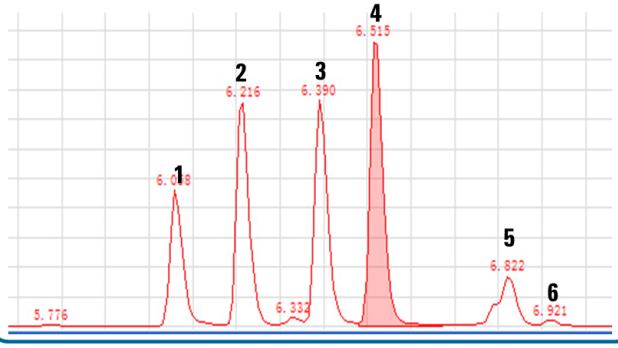
Figure 2. High Stability of Agilent 1290 UHPLC/ 6550 iFunnel Q-TOF



The relative standard deviations (RSD,%) of the 192 peak areas of the internal standard (IS) in positive and negative ion modes were 7.9% and 10.8%, respectively, indicating Agilent 6550 iFunnel Q-TOF mass spectrometer was very stable during continuously analysis of over three hundreds of samples and ten days continuously. (Figure 2) The mass accuracy of all results in both polarities was 1.01ppm and 0.61ppm, respectively. (data not shown)

Figure 3. Excellent Separation with Eclipse-plus column & Gradient

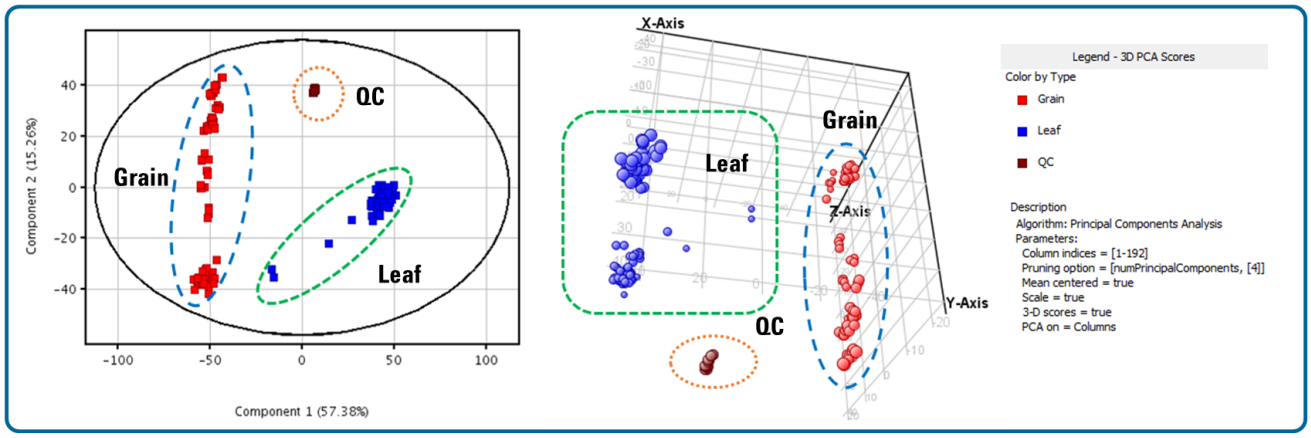
Peak Number	Formula	RT	Name
1	C33H36O16	6.06	Tricin O-glucoside O-guaiacylglyceryl ether derivant
2	C33H36O16	6.22	Tricin O-glucoside O-guaiacylglyceryl ether
3	C33H36O16	6.39	Tricin 4'-O-(erythro-β-guaiacylglyceryl) ether 7-O-β-D-glucopyra
4	C33H36O16	6.52	Tricin 4'-O-(threo-β-guaiacylglyceryl) ether 7-O-β-D-glucopyranc
5	C33H36O16	6.82	Tricin 4'-O-(erythro-β-guaiacylglyceryl) ether 9''-O-β-D-glucopyra
6	C33H36O16	6.92	Tricin 4'-O-(erythro-β-guaiacylglyceryl) ether 7''-O-β-D-glucopyra



Agilent Eclipse-plus C18, 3.0X150mm, 1.8um column shows high performance of peaks separation. Six isomers with very similar chemical structures can be completely separated. (Figure 3) Under this condition, the leucine and isoleucine were also well separated. (data not shown)

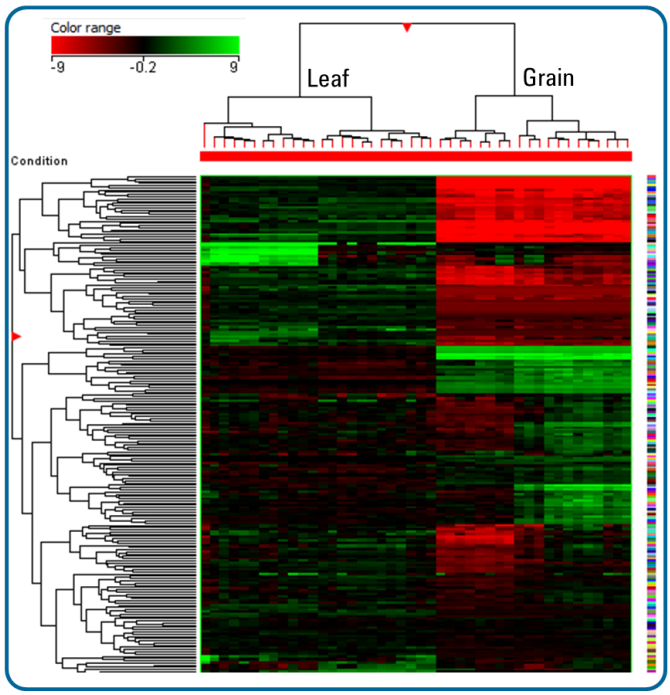
Results and Discussion

Figure 4. The results of diffferential metabolites using Principal Component Analysis (PCA) in Agilent MPP 13.0 software



In order to obtain a global view of differential metabolites between grains and leaves, PCA of about 3600 features were conducted from both polarities. The fact that 16 quality control (QC) samples, which were pooled from all experimental samples and were run after every 11 experimental samples, closely clustered together in both 2D and 3D PCA plots, indicating that the analytical reproducibility of the system is excellent and that the resulted metabolic variability among different groups of samples are highly reliable and physiologically meaningful. The first principal component (PC 1) accounted for 57.38% of the total variance, unraveled distinct tissue-specific metabolism between grains and flag leaves in rice.

Figure 5. Heat map of tissue-specific metabolism in rice



A total of 220 rice metabolites were annotated/identified by the Agilent-Metlin PCDL and *NIST* library. A heat map was constructed using the relative expression values of the metabolites on correlation distance and average linkage condition. High expression values are shown in green.

Conclusions

Our objective to evaluate an LC/MS method with the different extraction buffer for grain and leaf of rice. Agilent developed excellent separation condition for isomeric compound. (Figure 3) The good mass accuracy and relative standard deviations have be shown in Figure 2. Agilent MPP 13.0 has shown the power statistical calculation for PCA and Clustering analysis in Figure 4 and 5. This study can be data visualizations for up and down regulation of metabolites in grain and leaf with heat map. There are 220 metabolites were annotated/identified by Agilent's library.

Acknowledgements

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