Application Note: 550

Metabolite Profiling and Identification Employing High Resolution MS Strategies and Dedicated Software

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Key Words

- Orbitrap hybrid
- SIEVE software
- Mass Frontier software
- Plant metabolomics
- Structural elucidation

Introduction

Food nutritional value, quality, resistance to pathogens, and flavor are some of the traits monitored by governments and the food industry in an attempt to promote the creation of robust, healthy, nutrition-rich cultivars that contribute to sustained agricultural development. Metabolomics has been identified as a key mass spectrometry-based approach in the analysis of such characteristics. Here, results from metabolite profiling and identification experiments on tomato samples are presented.

Goal

To highlight metabolic differences between different cultivars of tomato, through development and ripening, as part of a study to understand the metabolic and genetic basis of ripening. Metabolite profiling of wild-type (WT) and of three ripening-inhibited tomato varieties was performed in tomato fruits selected at 13 development stages.

Experimental

Metabolite profiling and fragmentation were performed on a high-resolution, high mass accuracy platform. Figure 1 displays the data processing workflow.

Sample Preparation

Four varieties of tomato were grown and sampled over the period of flowering, fruiting, and ripening as part of an Exploiting Systems Biology project of the Biotechnology and Biological Sciences Research Council of the United Kingdom. Polar and non-polar extracts were obtained from tomatoes and subjected to an untargeted metabolomics study using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The study compared three non-ripening varieties with normal (WT) tomato during fruit development. Development stages were 15, 20, 25, 30, and 40 days after flowering; the breaker stage (BR), when fruit starts to first turn color (approx 47 days +/- 1 day); and 1, 2, 3, 4, 5, 6, and 7 days after BR.

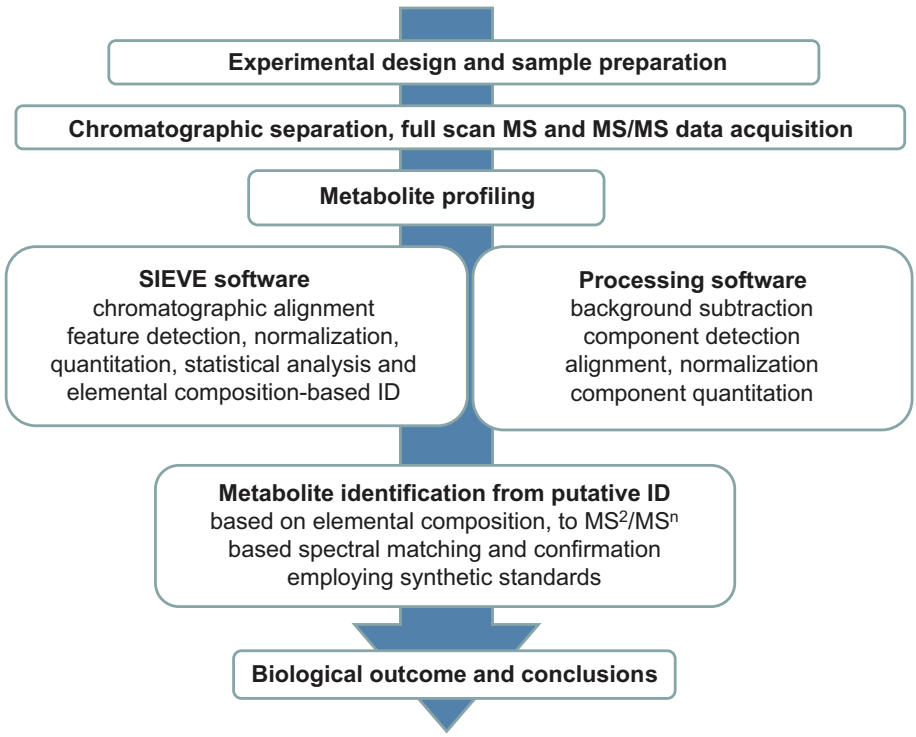

Figure 1. Data processing workflow.

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Whole fruit pericarp was ground in liquid nitrogen and the resulting powdered fruit tissue was used for subsequent extractions. Triplicate analytical replicates of all four tomato varieties at each of the 13 development stages were analyzed. Comparative analyses were carried out using previously published methods.[1-4]

Liquid Chromatography

Separations were performed on a reversed-phase C18 column (2.1 x 150 mm, 1.7 µm particle size) and a UHPLC system. Solvents used were 0.2% formic acid in water (Solvent A) and 98:2:0.2 acetonitrile/water/formic acid (Solvent B). The gradient started at 100% A, was held for 2.5 minutes at 0.25 mL/min, and then was gently ramped to 10% B. After 7.5 minutes, the flow rate was increased to 0.40 mL/min. After 10 minutes, the gradient was switched to 100% B, held for 2 minutes, and then equilibrated back to the starting conditions after 18 minutes.

Mass Spectrometry

MS detection was carried out using a Thermo Scientific LTQ Orbitrap Velos hybrid mass spectrometer operated in positive mode. The mass range analyzed was *m/z* 85-900 at 30,000 FWHM resolving power. MS/MS spectra were acquired using data dependent analysis (DDA) for metabolite MS^2 confirmatory purposes. The top 3 precursors were selected for HCD fragmentation with a normalized collision energy of 50. The mass spectrometer was mass calibrated prior to starting the sequence of injections. All data was acquired using external calibration.

Data Processing

A technology preview prototype version of Thermo Scientific SIEVE software 2.0 was employed for data processing, specifically for alignment, peak detection, and metabolite identification with ChemSpider™ search via elemental composition. The software was employed for the generation of a full list of components quantified across the entire data set. Umetrics SIMCA-P™ software was used for statistical analysis. Profiling was performed in a pair-wise fashion, either in a direct comparison of the metabolite profiles of two tomato varieties or for the same tomato variety, comparing two development stages. Thermo Scientific Mass Frontier software was used for confirmatory MS/MS metabolite structural determination.

Results and Discussion

Metabolite identification was based on a two-pronged approach. First, accurate mass was used to infer elemental composition, leading to preliminary metabolite identification (Figure 2). Secondly, CID and HCD MS^2 product ion data were compared against theoretical fragmentation derived with Mass Frontier™ software. The first used MS^2 product ion data matching against theoretical fragmentation patterns derived with Mass Frontier software (Figure 3).

Good chromatographic performance was obtained by employing short separations whereby hundreds of components were profiled. In conjunction with robust external mass calibration, metabolites were measured with small retention time differences at high mass accuracy, leading to strongly suggestive identifications made by elemental composition (Figures 2 - 5).

Where theoretical matches were made to more than one elemental composition, additional validation of metabolite identification was provided by matching the *in silico* MS^2 spectrum of the putatively identified metabolite to the observed spectrum (Figure 3). In some instances, standards were available for confirmatory identification of both MS^2 fragmentation patterns as well as for matching of chromatographic retention times.

Figure 2A shows glutamic acid was detected with the processing software in a tomato QC sample. The top panel is a TIC chromatographic profile; the middle panel displays the full MS acquired at 1.53 minutes. In Figure 2B, using the complementary peak detection method available in SIEVE™ software, a comparison was made between wild-type samples at BR vs. BR +7 days. The top panel represents the integrated intensities measured across triplicates in the two groups. The middle panel is a Volcano plot of the log ratio between the two groups (ratio 3.67) vs. the log p-value. The lower panel represents putative metabolite identification in the ChemSpider search, specifically by searching the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (http://www.genome.jp/kegg/).

Multivariate analysis differences in metabolic trajectories were observed for the tomato cultivars, and some of the key metabolite differences have been identified. Figure 4 and Table 1 show metabolite profiling results of WT tomatoes, BR vs. BR +7 days, and some of the differences detected between the samples. Figure 5 and Table 2 show the metabolite profiling results of WT versus ripening-inhibited tomato varieties at BR and the differences detected.

Principal components analysis (PCA) was carried out using SIMCA-P software. The data was Pareto scaled and points were colored by tomato genotype. Before ripening, the MU2, MU3 and WT varieties overlap; at the onset of ripening the four genotypes diverge (Figure 6).

(A)

(B)

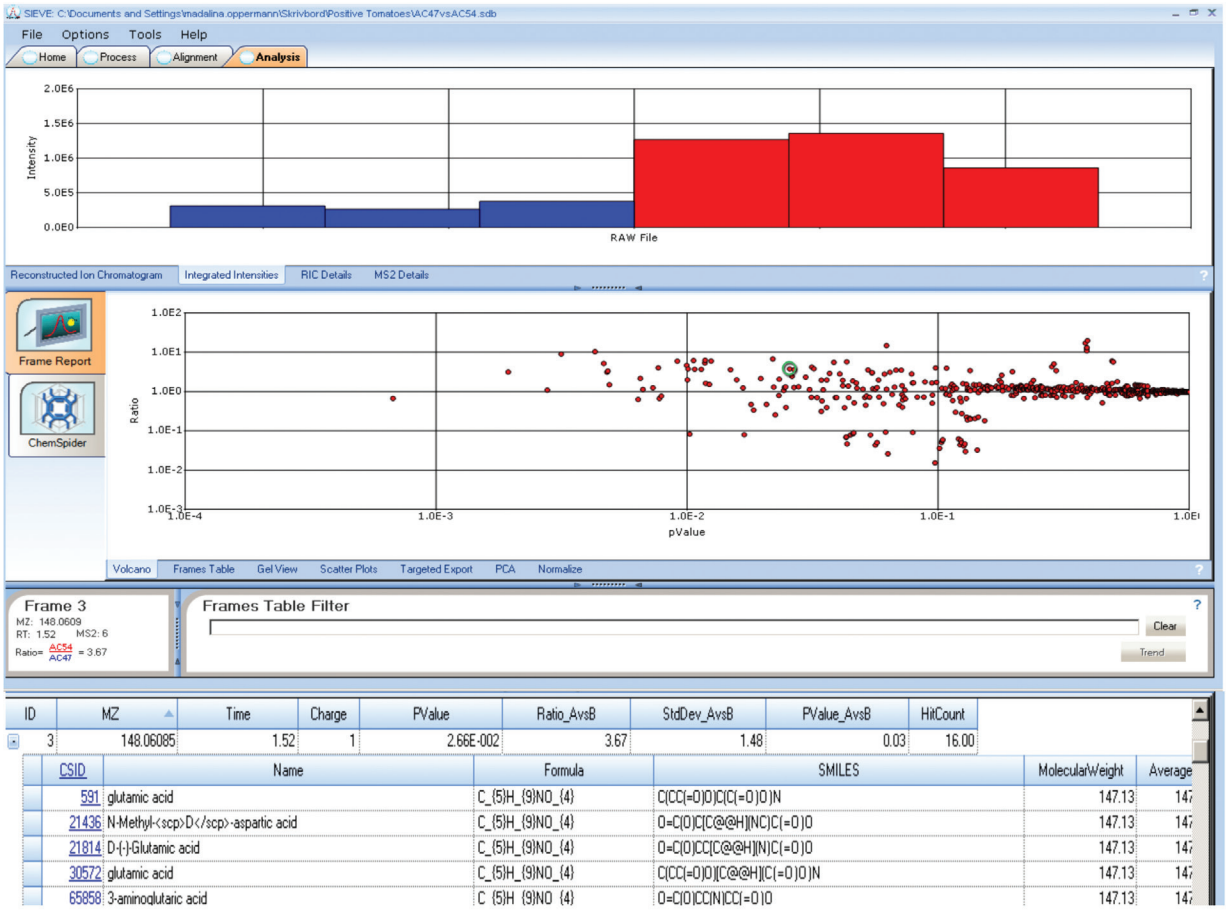


Figure 2. Glutamic acid profiling, quantification and ID. (A) Glutamic acid was detected by the processing software in a tomato QC sample. (B) Comparison between wild-type samples at BR vs. BR +7 days.

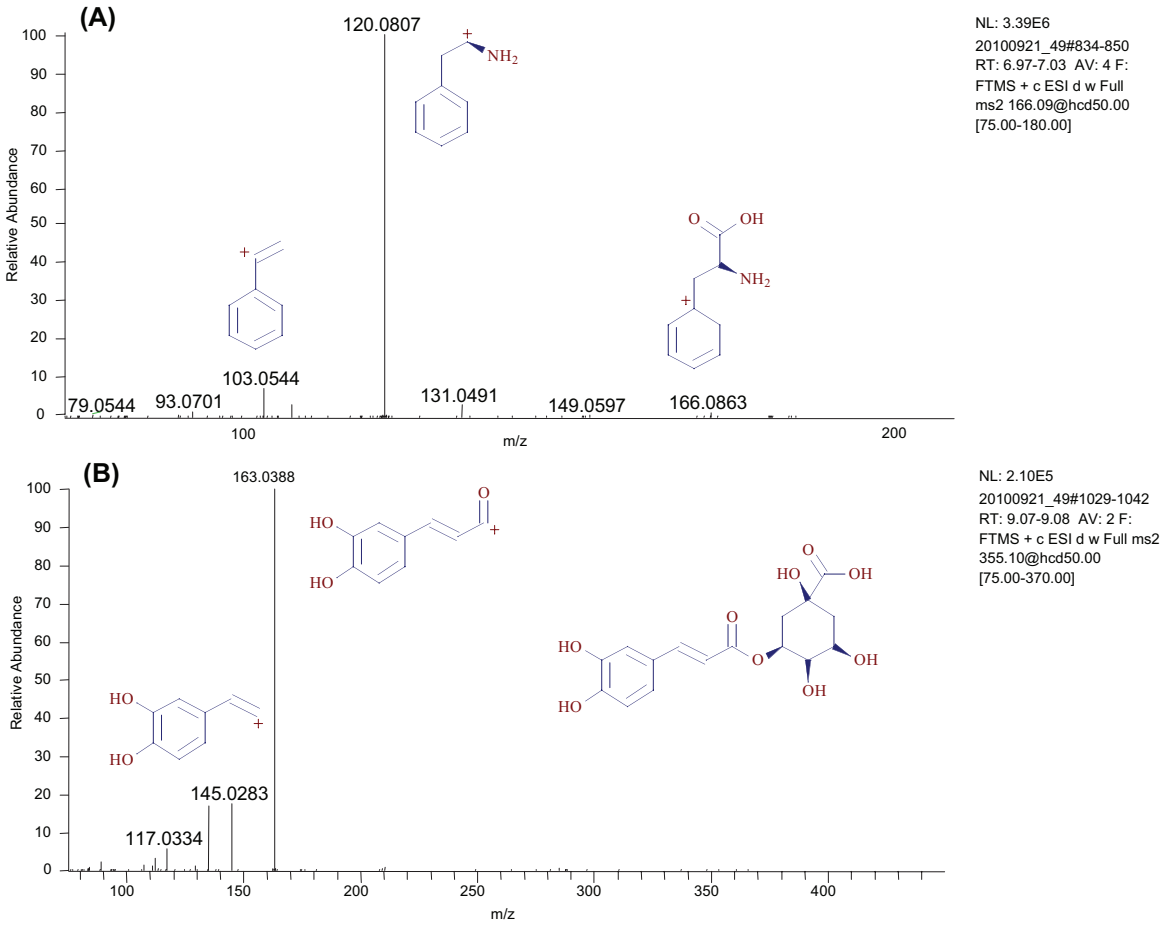


Figure 3. HCD fragmentation of: (A) phenylalanine and (B) chlorogenic acid obtained from a wild-type tomato extract. Measurements of intact precursors and fragment ions were better than 2 ppm, in accordance with preliminary identifications made with the prototype version of SIEVE software and consistent with the predicted elemental compositions.

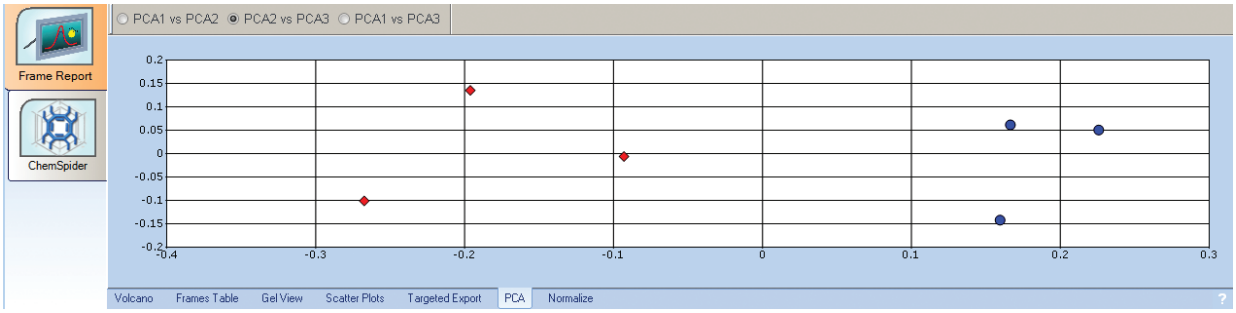
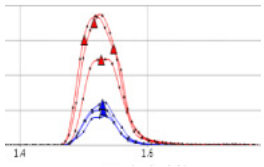
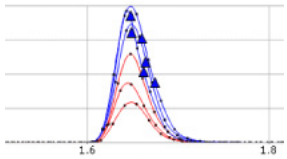
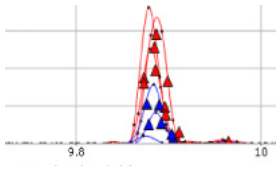


Figure 4. Metabolite profiling of WT tomatoes, BR vs. BR +7d . PCA Plot of wild-type tomatoes, breaker vs. breaker +7 days.

Table 1. Metabolite profiling of WT tomatoes, BR vs. BR +7d, showing some of the differences detected between samples.

Frame ID	m/z	RT (min)	pValue	Ratio	Std Dev	Name	Formula	Reconstructed Ion Chromatogram
3	148.06085	1.52	2.66E-02	3.67	1.48	glutamic acid	$C_5H_9NO_4$	
87	159.05128	1.64	1.79E-02	0.48	0.24	allantoin	$C_4H_6N_4O_3$	
7	273.07568	9.88	1.07E-02	3.77	3.67	naringenin	$C_{15}H_{12}O_5$	

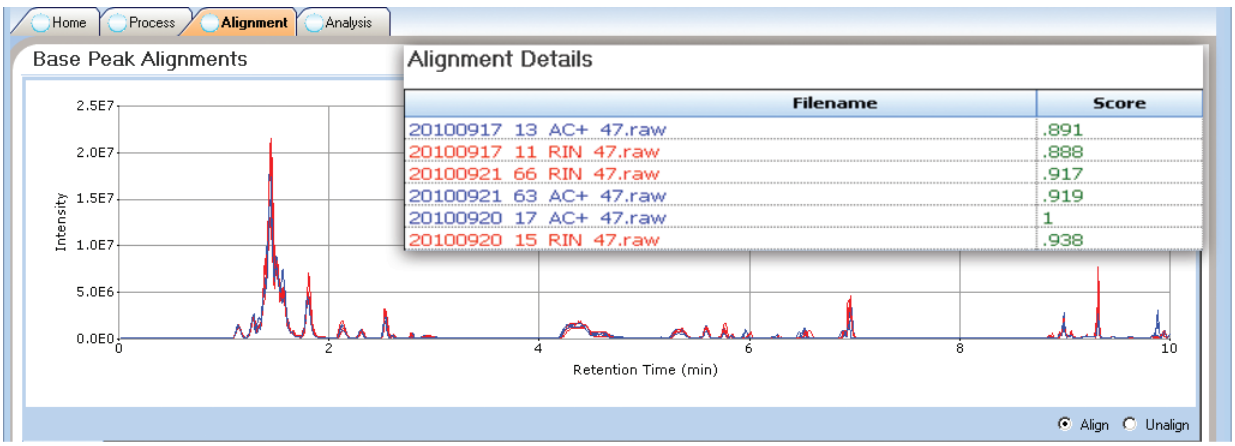
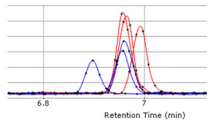
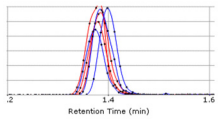
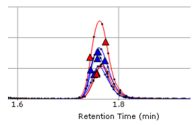
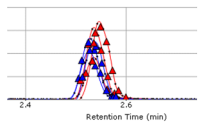
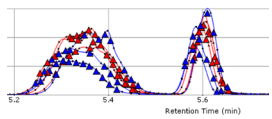
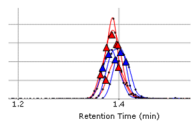
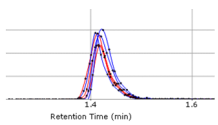
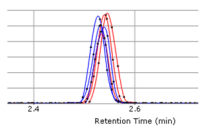
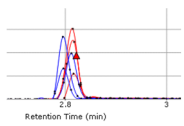
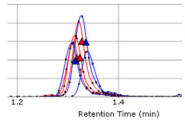
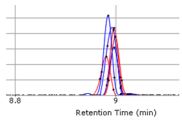
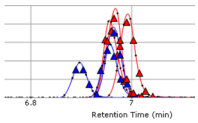
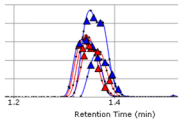
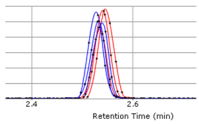
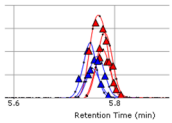
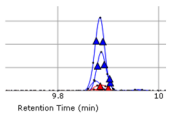
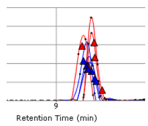
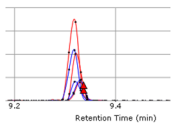
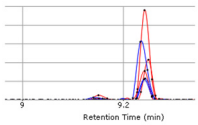


Figure 5. Metabolite profiling of wild-type versus ripening-inhibited tomato varieties at breaker stage. Chromatographic alignment and scoring (1 represents perfect alignment, 0 represents no alignment) of breaker stage of two varieties.

Table 2. Metabolite profiling of wild-type versus ripening-inhibited tomato varieties at breaker stage, showing differences detected between samples at breaker stage. Triangles in the Reconstructed Ion Chromatogram reflect MS^2 acquisitions.

Frame ID	*m/z*	RT (min)	pValue	Ratio	Std Dev	Name	Formula	Reconstructed Ion Chromatogram
463	95.0493	6.95	1.52E-01	1.2	0.21	phenol	C_6H_6O	
23	106.04992	1.38	6.59E-01	1.06	0.31	serine	$C_3H_7NO_3$	
50	116.07058	1.77	7.27E-01	1.11	0.68	proline	$C_5H_9NO_2$	
17	118.08617	2.53	5.50E-01	1.13	0.44	valine	$C_5H_{11}NO_2$	
44	132.10184	5.58	3.94E-01	1.18	0.42	isoleucine/leucine	$C_6H_{13}NO_2$	
5	133.06071	1.39	5.41E-01	1.16	0.5	asparagine	$C_4H_8N_2O_3$	
41	134.04485	1.41	3.31E-01	0.9	0.21	aspartic acid	$C_4H_7NO_4$	
477	135.02876	2.31	6.83E-01	1.14	0.86	malic acid	$C_4H_6O_5$	
187	150.05818	2.81	6.64E-01	1.18	0.93	methionine	$C_5H_{11}NO_2S$	
79	156.07672	1.31	9.57E-01	1.01	0.5	histidine	$C_6H_9N_3O_2$	

Frame ID	m/z	RT (min)	pValue	Ratio	Std Dev	Name	Formula	Reconstructed Ion Chromatogram
118	161.10745	8.99	3.94E-01	0.82	0.4	tryptamine	$C_{10}H_{12}N_2$	
11	166.08611	6.96	9.86E-02	1.54	0.68	phenylalanine	$C_9H_{11}NO_2$	
29	175.11893	1.33	5.78E-01	0.85	0.38	arginine	$C_6H_{14}N_4O_2$	
447	176.07068	2.53	6.82E-01	1.05	0.24	heteroauxin	$C_{10}H_9NO_2$	
37	182.08109	5.77	1.53E-01	1.66	0.95	tyrosine	$C_9H_{11}NO_3$	
19	273.07574	9.88	1.84E-01	0.08	0.15	naringenin	$C_{15}H_{12}O_5$	
75	355.10248	9.06	5.41E-02	1.32	0.34	chlorogenate	$C_{16}H_{18}O_9$	
16	416.35254	9.32	4.59E-01	1.61	2.11	tomatidine	$C_{27}H_{45}NO_2$	
224	611.16083	9.24	5.38E-01	1.46	1.98	rutin	$C_{27}H_{30}O_{16}$	

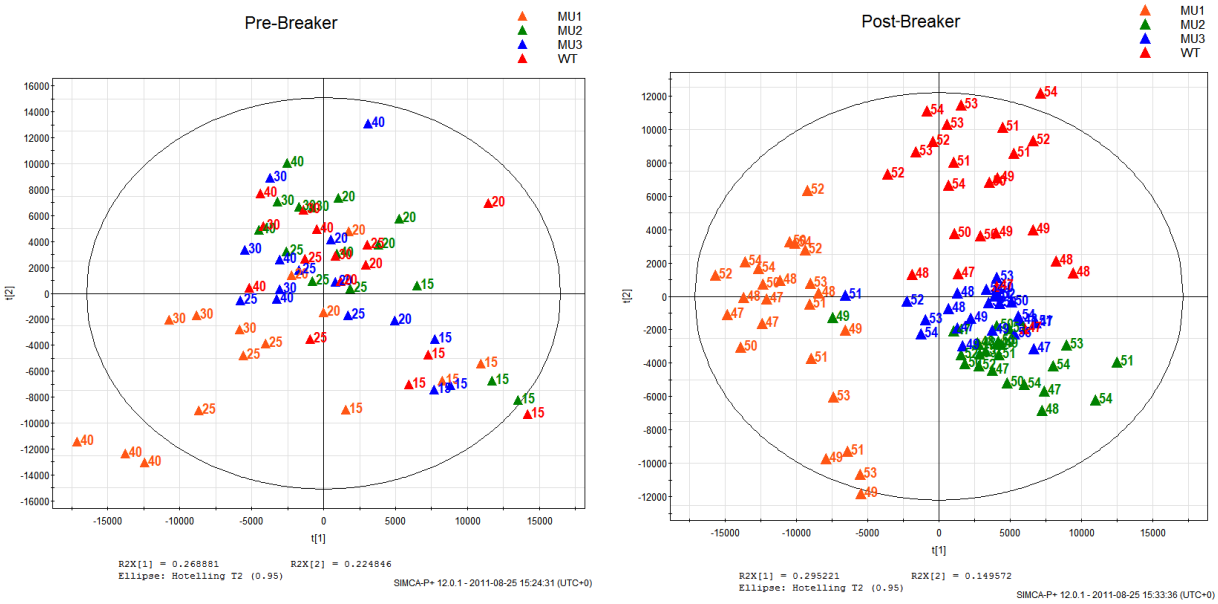


Figure 6. SIMCA-P results of tomato variety discrimination in pre-breaker and post-breaker stages.

Conclusion

The workflow employed was efficient in the study of both developmental and cultivar-related metabolic differences associated with tomato fruit ripening and led to the distinction of features that differentiate the tomato cultivars, as well as the developmental stages of the tomato fruits. The results obtained on the hybrid system from metabolite profiling and identification experiments provide evidence that the strategies selected can be successfully applied in the LC-MS based detection and identification of metabolites in plant extracts. Background subtraction in the processing software that was employed for the analysis of 9 blanks, 44 QC samples, 10 tomato pericarp mixes and 156 samples (4 tomato varieties at 13 development stages) in triplicate, led to the discovery of 319 potential metabolites.

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