Evaluation of Automated Sample Preparation, Retention Time Locked GC-MS and Automated Data Analysis for the Metabolomic Study of *Arabidopsis* Species

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INTRODUCTION

Success rate in metabolomic studies largely depends on the quality of sample preparation, the stability and reproducibility of the separation and detection and quality of data handling/interpretation.

⇨ The reproducibility of the sample preparation step is a critical parameter. For metabolomic profiling of biological samples using GC-MS, often a combination of oximation and silylation is used and this derivatisation method is applicable to acids (e.g. fatty acids), sterols, amines, amino acids, sugars, etc. Automation of the derivatisation process enhances repeatability and the time between sample preparation and analysis can be kept short and constant, which is important since TMS-derivatives have a limited stability.

⇨ GC-MS analysis using retention time locked conditions results in stable retention times in function of time and allows comparison of large data sets, even acquired on different (but same nominal) columns.

In this study, *Arabidopsis thaliana*, a popular model organism in plant biology and genetics, was used for testing an automated sample preparation approach upfront GC-MS analysis. Several features that were up- or down regulated in the different species were revealed by means of PCA and ANOVA.

EXPERIMENTAL

Plant tissue samples from 2 genotypes (Wassilewskija and Columbia) and two ages (young, old) were extracted by chloroform/methanol/water. The aqueous phase was dried under nitrogen. Oximation and silylation were carried on automatically on an Agilent 7693A automatic Liquid Sampler.

Fig.1 Automated sample preparation system

Table 1. Automated Sample Preparation Sequence

No.	Operations
1.	Return all vials
2.	Move vial from tray vial #49 to back turret position #2
3.	Move vial from tray vial #50 to back turret position #3
4.	Move vial from front sequence vial to back turret position #1
5.	Wash syringe in back tower, drawing from wash A1 dispensing into waste A1 2 times (wash with pyridine)
6.	Dispense 70 µL from vial sample 2 to vial sample 1 on back tower (70 µL of 20 mg/mL solution of methoxyamine hydrochloride in pyridine)
7.	Wash syringe in back tower, drawing from wash A2 dispensing into waste A2 3 times (wash with pyridine)
8.	Move vial from back turret position #2 to tray vial #49
9.	Move vial from back turret position #1 to mixer
10.	Mix at 2000 rpm 2 times for 30 seconds
11.	Move vial from mixer to heater
12.	Heat vial at 30 degrees C for 3600 seconds
13.	Move vial from heater to back turret position #1
14.	Dispense 70 µL from vial sample 3 to vial sample 1 on back tower (70 µL MSTFA + 1 % TMCS)
15.	Wash syringe in back tower, drawing from wash A3 dispensing into waste A3 3 times (wash with toluene)
16.	Move vial from back turret position #3 to tray vial #50
17.	Move vial from back turret position #1 to mixer
18.	Mix at 2000 rpm 2 times for 30 seconds
19.	Move vial from mixer to heater
20.	Heat vial at 37 degrees C for 1800 seconds
21.	Move vial from heater to front sequence vial

Analytical parameters

Instrument: Agilent 7890A GC & 5975 MSD

Inlet: SSL at 250 °C, 1 µL, split ratio 10:1
Carrier gas: Helium, constant flow, 0.92 mL/min, retention time locked
Column: DB-5MS, 30 m x 0.25 mm x 0.25 µm + 10 m Duraguard capillary column
Oven: 60 °C (1 min) - 10 ºC /min - 325 °C (10 min)
Detection: MS (scan range *m/z* 50 - 600)

RESULTS

A typical GC-MS profile obtained for the hydrophobic fraction of *Arabidopsis thaliana* is shown below in Figure 2.

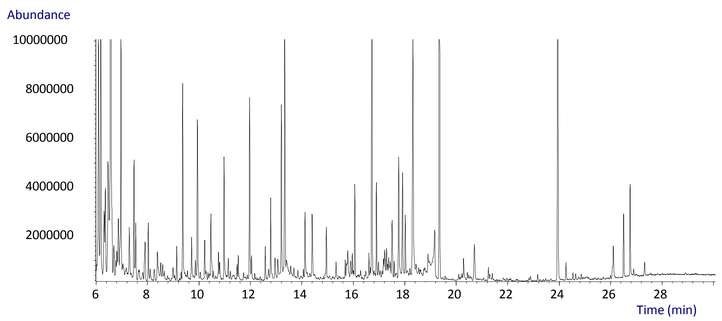

Fig.2 A typical GC-MS profile obtained for hydrophobic fraction of *Arabidopsis thaliana*

RSDs

The repeatability of the sample preparation was tested by analysing aliquots of one sample in six-fold. RSD% on peak areas are given below. Typically the values are < 10%, which is more than satisfactory.

Solute [retention time]	**RSD%**
Myristic Acid d27 [16.727]	**5.78**
2-hydroxypyridine [6.519]	**8.16**
Phosphoric acid [9.966]	**6.65**
L-threonine 1 [10.224]	**6.42**
Fumaric acid [10.94]	**5.33**
Aspartic acid 1 [12.002]	**2.03**
D-malic acid [12.794]	**3.10**
L-glutamic acid 1 [13.338]	**10.64**
Purine riboside [21.776]	**8.75**
Dehydroascorbic acid 1 [16.863]	**8.96**
L-sorbose 2 [17.235]	**9.93**
Methyl-beta-D-galactopyranoside	**7.56**
Lactulose 1 [23.867]	**3.76**
Allo-inositol [17.245]	**5.06**
D-(+) trehalose [24.752]	**8.94**
Cellobiose 1 [24.444]	**5.26**

Data Analysis

Samples were processed via two strategies:

⇨XCMS combined with PCA
⇨Peak deconvolution by means of AMDIS and data preprocessing (alignment, normalization, fitting) + statistical analysis in Mass Profiler Professional (MPP)

DATA ANALYSIS & FEATURE DETECTION

PCA – scatter plots allows the differentiation of the samples

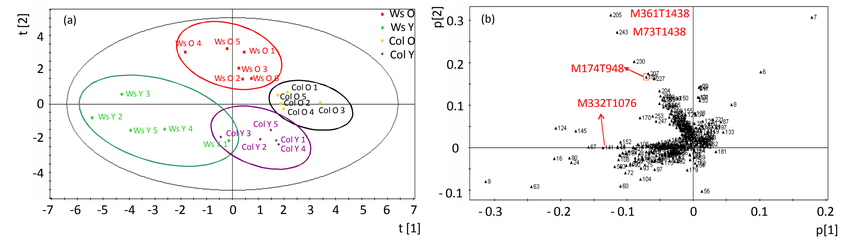

Fig. 3 PCA analysis for different types of *Arabidopsis* samples: (a) PCA score plot; (b) PCA loading plot. A=3; $R^2X = 0.764$; $R^2X[1] = 0.421$; $R^2X[2] = 0.259$.

List of detected features

Retention time (min)	ID (PCA)	PCA	MPP	Identification
6.6	6	M173T396		
6.583	7	M115T395	115.0@6.6033144	
6.6	8	M116T396		
19.333	9	M73T1160		
19.35	16	M217T1161	73.0@19.361052	
19.35	24	M147T1161		
19.367	67	M305T1162		
14.45	90	M140T867	140.0@14.4262495	
17.933	141	M332T1076	332.@17.926731	L-ascorbic acid
13.217	145	M156T793	156.0@13.211834	
13.283	124	M73T797	No	
9.967	63	M299T598	No	
15.8	189	M174T948	No	Putrescine
23.967	205	M361T1438		
23.967	207	M362T1438		
23.967	230	M217T1438	No	Sucrose
23.967	243	M73T1438		
23.967	227	M147T1438		
6.843			147.0@6.842525	
9.725			116.0@9.724838	
12.05			174.0@12.050264	Beta-alanine
12.644			266.0@12.643849	
12.972			84.0@12.972083	
13.347			84.0@13.347258	
14.131			73.0@14.130791	
14.957			73.0@14.956616	Asparagine
15.902			174.0@15.902134	
16.127			156.0@16.126585	
16.614			142.0@16.613987	
17.218			228.0@17.21756	
17.697			174.0@17.697157	
18.327			73.0@18.326788	
19.172			73.0@19.171556	
19.18			73.0@19.180017	
20.838			144.0@20.838469	
24.279			73.0@24.27918	
26.115			73.0@26.114862	

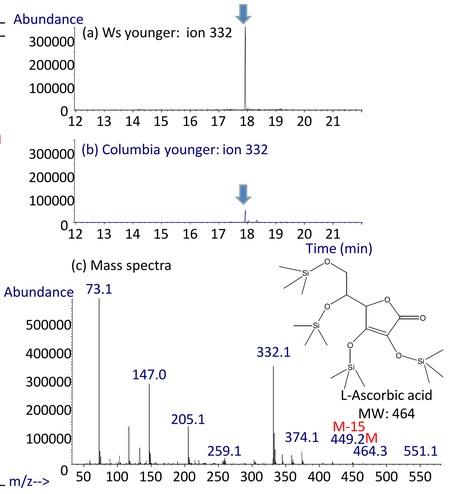

Fig.4 Extract ion chromatogram (ion 332): (a) Ws younger leaves sample; (b) Columbia younger leaves sample; (c) mass spectra and the structure of silylated L-ascorbic acid.

CONCLUSIONS

⇨Automated sample preparation results in much better reproducibility
⇨Detailed profiles are obtained under RTL-GC-MS conditions
⇨Upon analyzing different plant samples, several discriminating features could be revealed

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