

ABSTRACT

JAEGER, FREDERICK HOWARD. Simplified Plant Sample Preparation for use in Gas Chromatography-Mass Spectrometry (GC-MS) Based Metabolomic Profiling and Targeted Analyte Quantitation. (Under the direction of Dr. Lin He.)

A simple, fast, reproducible and less laborious sample preparation protocol was developed for the analysis of *Arabidopsis thaliana* using Gas chromatography coupled with mass spectrometry (GC-MS). In particular, a semi-automated machine tool is used to replace the traditional mortar-pestle method in tissue grinding. One-pot chemical extraction-derivatization is used to provide simplified sample preparation over the conventional multi-step liquid-liquid extraction protocol. Wild-type and transgenic *Arabidopsis thaliana* seedlings were used as the model system to evaluate performance of this newly developed method for use in metabolic profiling and also targeted quantitative analysis of salicylic acid for the study of systemic acquired resistance.

.

Simplified Plant Sample Preparation for use in Gas Chromatography-Mass Spectrometry
(GC-MS) Based Metabolomic Profiling and
Targeted Analyte Quantitation

By
Frederick Howard Jaeger

A thesis submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the degree of
Master of Science

Chemistry
Raleigh, North Carolina
2008

APPROVED BY:

Assistant Professor Lin He
Chair of Advisory Committee
Chemistry

Professor David Muddiman
Chemistry

Professor Edmond Bowden
Chemistry

DEDICATION

I would like to dedicate this work to my family. Without the understanding of a loving wife or the motivation of the three smiling faces that think Daddy can do anything, this work would not have been possible.

BIOGRAPHY

The author was born in Illinois in 1975. The author moved throughout the country to live in Nebraska and New Jersey before finally settling in North Carolina in 1986. After finishing High School he received a Bachelor Degree in Chemistry from UNCW. The author then went on to get married to his high school sweetheart and a few years later start a family with the birth of their first son. After working for several years, he officially joined the Chemistry Department of NCSU in 2005 in pursuit of a Master of Science degree, on a part-time basis. During his studies at NCSU the author has worked fulltime for Reichhold, Inc. as an Analytical Mass Spectrometrists and found time to add two more children to his growing family, another son and a daughter.

ACKNOWLEDGMENTS

I would like to first and foremost thank my advisor Dr. Lin He for giving me the opportunity to pursue my studies at NCSU. I would also like to thank Professors Imara Perera and Wendy Boss along with Chiu-Yueh Hung and Yang Ju Im for supplying the plant tissue used in this study. I would also like to thank Reichhold Inc. for sponsoring me in my pursuit of further education, with tuition assistance and use of analytical instrumentation. I would like to thank my supervisor Dave Grandy for his advice, words of encouragement and allowing flexibility in my work schedule so I could attend classes and perform school based research. I would also like to thank Dr. Ping Zhang for his invaluable assistance with the microscopy work and kind words of encouragement. Last but not least, I would like to thank Dr. Robert Bereman for starting me on my journey towards higher education.

TABLE OF CONTENTS

LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
LIST OF SCHEMES.....	ix

CHAPTER 1: General Introduction

1.1 Introduction.....	1
-----------------------	---

CHAPTER 2: Simplified and Improved Sample Preparation for Analysis of *Arabidopsis Thaliana*

2.1. Introduction.....	3
2.2. Experimental.....	4
2.2.1. Materials.....	4
2.2.2. Tissue Preparation.....	5
2.2.3. Analyte Derivatization.....	5
2.2.4. Instrumentation.....	6
2.3. Results and Discussion.....	7
2.4. Conclusions.....	11
2.5. References.....	12

CHAPTER 3: Gas Chromatography-Mass Spectrometry (GC-MS)-based Metabolomic Profiling

3.1. Introduction.....	26
3.2. Experimental.....	27
3.2.1. Materials.....	27
3.2.2. Tissue Preparation.....	28
3.2.3. Analyte Derivatization.....	28
3.2.4. Instrumentation.....	28
3.3. Results and Discussion.....	29
3.4. Conclusions.....	32
3.5. References.....	33

CHAPTER 4: Salicylic Acid Determination for the Study of Systemic Acquired Resistance

4.1. Introduction.....	44
4.2. Experimental.....	46
4.2.1. Materials.....	46

4.2.2. SA Extraction from Plant Tissues	46
4.2.3. Establishment of the SA Calibration Curve	47
4.2.4. Instrumentation	48
4.3. Results and Discussion.....	48
4.4. Conclusions.....	51
4.5. References.....	52

LIST OF TABLES

CHAPTER 2: Simplified and Improved sample preparation for analysis of	page
<i>Arabidopsis thaliana</i>	
Table 2.1	A list of identified chemical compounds in <i>Arabidopsis</i> seedling extracts against the NIST MS library..... 19
Table 2.2	A list of partially identified chemical compounds in <i>Arabidopsis</i> seedling extracts against the NIST MS library.....20
Table 2.3	A list of GC elution peaks with unidentified chemical structures.....21
Table 2.4	Quantitative calculation of reproducibility of the one-pot extraction/derivatization method for four representative analytes..... 22
CHAPTER 3: Gas Chromatography-Mass Spectrometry (GC-MS)-based Metabolic Profiling	
Table 3.1	Quantitative measurement of Stigmasterol in HS9-7.....39
Table 3.2	Quantitative measurement of Stigmasterol across different genetic strains.....40
Table 3.3	t-Test performed on wild-type and HS5-8 data for Stigmasterol..... 41
Table 3.4	t-Test performed on wild-type and HS9-7 data for Stigmasterol..... 42
CHAPTER 4: Salicylic Acid Determination for the Study of Systemic Acquired Resistance	
Table 4.1	Examination of method reproducibility using SA as the standard molecules.....57
Table 4.2	Evaluation of instrument reproducibility using SA as the standard molecule.....58
Table 4.3	Quantitation of SA concentration in wild-type and mutated <i>Arabidopsis</i> plants..... 59

LIST OF FIGURES

CHAPTER 2: Simplified and Improved Sample Preparation for Analysis of	page
<i>Arabidopsis Thaliana</i>	
Figure 2.1	(A) A typical mortar-and-pestle tool used in conventional grinding of plant tissue. (B) A mechanic reciprocating saw with attachment and a 20-mL polypropylene scintillation vial used in the newly developed method. The ground tissue pieces after (C) using mortar-and-pestle or (D) using the reciprocating saw. The scale bars in the images shows 1 mm length major unit with a 0.1mm increment..... 23
Figure 2.2	GC chromatograms of <i>Arabidopsis</i> seedlings extracts prepared using (A) the conventional liquid-liquid extraction, the polar fraction and (B) the non-polar fraction, and (C) the newly developed one-pot extraction/derivatization..... 24
Figure 2.3	Representative GC chromatograms of several derivatizing reagents (A) TMSI (B) HMDS (C) MSTFA (D) TMSDMA, that contain several chemical components, which could potentially overlap with plant extracts: TMS-DMA showed the least background beyond initial 4 min..... 25
CHAPTER 3: Gas Chromatography-Mass Spectrometry (GC-MS)-based Metabolic Profiling	
Figure 3.1	(A) A typical TIC of wild-type <i>Arabidopsis</i> seedlings; (B) A zoom-in of RT where stigmasterol-TMS was expected; (C) The background subtracted mass spectrum of stigmasterol-TMS; and (D) An EIC of stigmasterol-TMS for the ion $m/z = 484$ 43
CHAPTER 4: Salicylic Acid Determination for the Study of Systemic Acquired Resistance	
Figure 4.1	A nine-point calibration curve of SA spiked in <i>Arabidopsis</i> plant extracts..... 60
Figure 4.2	(A) Overlay of total ion chromatograms of uninfected (dash) and infected Samples (solid) (B) Overlays of extracted ion chromatograms of uninfected (dash) and infected samples (solid)..... 61

LIST OF SCHEMES

CHAPTER 2: Simplified and Improved Sample Preparation for Analysis of *Arabidopsis Thaliana* page

Scheme 2.1	Sample preparation flow chart using the conventional methods reported in the literature.....	17
Scheme 2.2	Modified sample preparation flow chart using the newly developed one-pot scheme.....	18

CHAPTER 3: Gas Chromatography-Mass Spectrometry (GC-MS)-Based Metabolic Profiling

Scheme 3.1	Sample preparation flow chart.....	37
Scheme 3.2	Chemical Structure of Stigmasterol, a major plant sterol.....	38

CHAPTER 4: Salicylic Acid Determination for the Study of Systemic Acquired Resistance

Scheme 4.1	Sample preparation flow chart.....	55
Scheme 4.2	TMS-DMA derivatization of salicylic acid in a one-pot format.....	56

Chapter 1

General Introduction

1.1 General Introduction

The goals of this research were to 1. Develop a simple, fast, reproducible and less laborious sample preparation method for GC-MS based analysis of *Arabidopsis thaliana* 2. The developed method must be able to perform metabolic profiling with the ability to analyze for a broad range of molecules including sugars, amino acids, sterols, fatty acids and others using a one-pot method. 3. The developed method must be able to perform a quantitative analysis of individual targeted molecules without compromising the profiling of other molecules.

Chapter 2 describes the development of a low-cost semi-automated mechanic grinding apparatus that is able to decrease tissue particle sizes and increase tissue size uniformity when compared to the conventional mortar-pestle approach. This chapter goes on to further describe the development of a one-pot, multi-solvent extraction protocol to simplify the time and labor intensive conventional liquid-liquid fractionation approach.

Chapter 3 describes the application of the method developed in Chapter 2 to metabolic profiling. Four *Arabidopsis thaliana* mutants were compared to a wild-type “standard” to look for differences in non-stressed plants. A broad range of molecules were successfully analyzed including sugars, amino acids, sterols, fatty acids and others. The expression differences were analyzed in a semi-quantitative manner.

Chapter 4 describes the characterization of the analytical figures of merit for the method developed in Chapter 2 in targeted quantitative analysis. Specifically, salicylic acid was quantified for the study of systemic acquired resistance (SAR) in wild-type and transgenic plants that were either uninfected or infected with the bacterial pathogen *Pseudomonas syringae maculicola*. A limit of detection (LOD) of 2 ng/mL and a linear dynamic range of 10 ng/mL-1000 ng/mL were established.

Chapter 2

Simplified Sample Preparation for Analysis of *Arabidopsis Thaliana*

2.1 Introduction

Development of an effective and reproducible sample preparation protocol has been one of the major tasks undertaken in GC-MS-based analysis of plants. Current methodologies for sample preparation rely upon performing mortar-pestle grinding of plant tissues, followed by liquid-liquid extractions and chemical derivatization of plant extracts with a variety of silylating reagents.¹⁻¹³ Regardless of the reported success, three major drawbacks limit broader applications of these methods, (a) the inconsistency in sample grinding; (b) the irreproducibility and slow process in liquid-liquid extraction; and (c) the high background introduced by the use of certain silylating reagents. The initial grinding of harvested tissue is a crucial step to ensure reproducible analysis downstream. In addition to the most commonly used mortar-pestle-based grinding method, several mechanic grinding approaches have also been reported with improved performance.¹⁴⁻¹⁷ For example, Fiehn et al. has introduced the use of a ball mill to produce a more consistent and reproducible grind by reducing manual labor and eliminating operational inconsistency from the process.^{1, 18-20} A high speed homogenizer such as an Ultraturrax device has also been reportedly used.²¹ Nonetheless, the requirement for an expensive equipment setup or the inability of handling sample sizes of 100 mg or less restrains their applicability in daily operations. In addition to the sample grinding step, the conventional approach to analyte extraction, which is mostly based on

liquid-liquid phase extraction, is also known to be problematic for its reproducibility in day-to-day extraction and for the biased extraction for analytes of different concentrations and/or classes. Furthermore, the laborious extraction protocol makes it less favorable for high throughput analysis (Scheme 2.1). Last but not the least, certain silylating reagents used in chemical derivatization are also known to introduce reaction artifacts that can co-elute with analytes of interest.²²⁻²⁴ Overall, despite extensive research efforts in the field, technologies that allow simple, fast, reproducible, low-cost analysis of small molecule contents in complex biological samples that do not consume prohibitive man hours or require access to expensive custom equipment have yet to be materialized.

This chapter describes a simplified solution for sample preparation in GC-MS based analysis of *Arabidopsis thaliana* (Scheme 2.2) with equivalent analytical performances.⁵ The method uses off-the-shelf reagents and equipment that offers mechanical consistency and reproducibility during sample grinding. The subsequent employment of the one-pot extraction/derivatization scheme eliminates most of the aforementioned problems.

2.2 Experimental

2.2.1 Materials

Acetonitrile (ACN), tetrahydrofuran (THF), methanol (MeOH), chloroform and benzene of HPLC grade or better were purchased from EMD Chemicals. N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), trimethylsilyl dimethylamine (TMSDMA),

pyridine, pentadecane and methoxyamine hydrochloride were purchased from Sigma-Aldrich, Inc., and used as received. Zirconium oxide grinding media were purchased from Zircoa, Inc.

2.2.2 Tissue Preparation

Arabidopsis thaliana seedlings (250+ seedlings) were grown and harvested at development stage 1.04 for a plate based platform.²⁵ The samples were immersed in liquid nitrogen and ground for 30-60 sec using the traditional mortar and pestle technique.²⁶ Alternatively, the seedlings were vacuum desiccated prior to mechanic grinding, during which seedlings from one batch were placed into a 20-mL polypropylene scintillation vial. Zirconium oxide grinding media were then added to the vial and the cap was tightened securely. A 14.4V reciprocating saw (Black & Decker, Hunt Valley, MD) with a custom vial holder was used to homogenize the tissue for approximately 60 sec.

2.2.3 Analyte Derivatization

Two methods were used for analyte derivatization: in the conventional approach (Scheme 2.1), a solution of MeOH:chloroform:water (2 mL, 2.5:1:1, -20 °C) was added to 30 to 100 mg of tissue of previously homogenized tissue then mixed thoroughly at 4 °C for 30 min. Sample was then centrifuged for 10 min at 9,000×g. The supernatant was then separated and saved. The pellet at the bottom was further extracted using a 1:1 MeOH: chloroform mixture (1 mL, -20 °C). After mixing at 4 °C for 15 min, the mixture

was centrifuged at 9000×g for another 10 min. The supernatant collected was then combined with the previous collection. The addition of 500 µL of water was used to separate the water: MeOH layer from the chloroform layer where different fractions were collected in separate tubes. All solutions were dried using a Speed Vac (Savant, Waltham, MA) overnight. Methoxyamine hydrochloride (20 mg/mL in pyridine) was added to each tube and mixed at 30 °C for 90 min on a shaker. 80 µL of MSTFA was then introduced and the mixture was heated at 37 °C for 30 min. Sample was then stored at room temperature for 120 min prior to injection.³

Alternatively, 200-400 µL of a solvent mixture (3:1:1, ACN: THF: Benzene) containing 20% TMS-DMA extraction-derivatization solution (internal standard included) was added to 5-15mg of ground tissue (Scheme 2.2).⁴ After vortexing the solution for 10 sec and heating at 60 °C for 30 min, the mixture was centrifuged for 10 min at 9500×g. The supernatant was analyzed directly.

2.2.4 Instrumentation

All mixtures were analyzed using a 6890 Plus Gas Chromatograph coupled to an Agilent 5973N quadrupole Mass Spectrometer (Agilent, Palo Alto, CA), upgraded with an inert ion source and enhanced electronics package. Chromatographic separations were achieved with a BPX-50 Phenyl polysilphenylene-siloxane GC capillary column 30 m × 0.25 mm × 0.25 µm (SGE, Australia). The helium carrier gas was set to 1.2 mL per minute. One microliter of derivatized sample was injected into the instrument in splitless mode. The injection port was set at 280 °C with an initial oven temperature of 50 °C

along with a 20°C per minute ramp to 320 °C, hold for 5 min. The mass spectrometer was set in the scan mode from 30 amu to 550 amu. Data were gathered using Agilent's Chemstation software. The data gathered was then entered into Excel (Microsoft, Redmond, WA) for all statistical analyses.

Pictures of tissue grinding products were taken using an SZ-PT optical microscope (Olympus, Japan). The microscope was outfitted with a MD35 digital camera (Jincheng, China). Samples were illuminated using a Fiber-Lite model 190 fiber optic illuminator.

2.3 Results and Discussion

There are three key steps in sample preparation during comparative profiling of complex plant samples: tissue grinding, analyte extraction, and analyte derivatization to allow GC-MS analysis.

An effective and reliable sample grinding procedure is the first key element in ensuring reproducible comparative profiling of complex biological samples. Two parameters are used in evaluating the grinding performance: the size and the uniformity of the final tissue particulates. Specifically, the smaller the particulate size, the more effective the analyte extraction is expected from the maximal exposure to the extracting solvent and derivatizing reagents. A good uniformity across particulate sizes is also important to ensure reproducible extraction. Figures 2.1 shows a direct comparison between the conventional mortar-pestle method to our approach in which a reciprocating saw along with ceramic grinding media were used in tissue break-down. The

conventional mortar-pestle method resulted in particle sizes up to 1,000 μm with the most common sizes in the 200 to 250 μm range (Figure 2.1C). The use of a reciprocating saw which operates at 50 Hz, on the other hand, enabled break-down of larger tissue pieces by increasing the mechanical force imposed. The addition of ceramic spherical particles reduced the amount of empty space in the grinding vial which allowed a higher collision rate and force between tissues and a hard surface. As a result, the tissue ground by the reciprocating saw shows particle sizes consistently in the sub-50 micron range (Figure 2.1D).

The second step of an effective and reliable sample preparation procedure is to achieve extraction of molecules across many different classes in a simple and reproducible manner. The conventional method uses a multi-step liquid-liquid fractionation coupled to analyte derivatization (Scheme 2.2).³ This methodology, while effective, is laborious and time consuming, taking up to 36 h to produce a single sample set. In addition, the multiple liquid-liquid fractionation steps introduce chances for operator errors and inconsistency that could be fatal to day-to-day comparative analysis. While the biased extraction depending on solvent selection could be advantageous to simplify the complexity of the plant extract mixture, it inevitably results in information loss on the analytes that are not extracted with high efficiency. Consequently, complementary extraction and analysis has to be carried out to provide a complete picture of plant contents. A one-pot, multi-solvent extraction protocol was developed here to overcome these limitations by: 1) reducing the loss of materials and possible errors by decreasing the total solvent volume and extraction steps used, and 2) increasing the

amount of information obtained by using a mixture of solvents of different polarities simultaneously. ACN, THF, and benzene were mixed at the 3:1:1 ratio and added to the ground tissue directly, followed by a rapid analyte extraction using vortexing.

The third improvement in sample preparation was carried out by mixing TMSDMA as the derivatization reagent with the extraction media to allow concurrence of analyte extraction and derivatization. The silylating moiety makes many polar compound classes less polar, more volatile and also more thermally stable, rendering these classes more compatible to GC analysis. Many silylating reagents are available to the analyst for metabolic profiling experiments, including hexamethyldisilazane (HMDS), N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), and trimethylsilylimidazole (TMSI).¹⁴

TMSDMA was chosen in this study because it exhibits fewer reaction byproducts that interfere with the analyte elution chromatogram (Figure 2.3A-D) and it has a high silyl donor strength, providing a high derivatization yield for most analytes of interest.²⁷

The benefits of combining analyte extraction and derivatization steps are clearly seen in Figures 2.2A-C, where the Total Ion Chromatograms (TIC) of extracts from *Arabidopsis* seedlings prepared with both methods were compared. Figure 2.2A and 2.2B shows the use of the conventional methodology where two separate fractions (i.e. polar and non-polar fractions) were prepared and analyzed by GC-MS. The total sample preparation time was approximately 1.5 days. The instrument analysis time was also quadrupled in order to examine both solutions. Approximately 150 individual MS peaks were distinguishable from chromatograms of both fractions. Using the one-pot method (Figure 2.2C), ~200 peaks were distinguishable in the single chromatogram. Without

compromising the chemical information obtained, the total sample preparation time was shortened to less than one hour. Furthermore, using a single chromatographic separation instead of the two used by the previous methodology, showed sufficient peak capacity for these complex samples.

The molecules identified using MS span across a broad range of molecular classes, including small organic acids, amino acids, sugars, fatty acids and sterols. Table 2.1 lists the identified chemical components in the Total Ion Chromatogram (TIC) of the plant samples by comparing with the reference spectra found in the NIST library. The identification threshold was set at a match quality of ≥ 90 , which is obtained using the probability-based matching (PBM) algorithm developed by Fred McLafferty and co-workers.²⁸ If a match quality of ≥ 60 but < 90 was obtained then a compound was considered partially identified. The identification was incomplete often due to co-elution of other components (Table 2.2). Compounds were also considered partially identified if spectra for a particular class of molecules were confirmed but absolute identification of exact molecular structure was ambiguous due to their similar NIST MS signatures. For example, many mono-saccharides give identical fragmentation patterns according to the NIST library. When a match quality of ≤ 60 was obtained, no match was labeled, this occurred for several elution peaks due to the limited coverage inherent to the NIST library (Table 2.3). Specific standards would be required to unveil the structures of the partially identified and unknown compounds listed in Table 2.2 and 2.3.

In addition to streamlined sample processing, the detection reproducibility using the newly developed sample preparation protocol was also improved. After normalizing

the chromatogram peaks with the different amounts of tissue weighted in different rounds and injection variations by the internal standard (Pentadecane), a <6% mean Relative Standard Deviation (RSD) value was observed for most identified compounds whereas in the literature the mean RSD for the conventional sample preparation method was reported at ~10% (Table 2.4).³

2.4 Conclusions

This chapter reports the development of a simple, fast, reproducible, and less laborious sample preparation method for GC-MS based analysis of *Arabidopsis thaliana*. The use of reciprocating saw-assisted tissue grinding significantly reduced final particle sizes and improved grinding uniformity. The one-pot analyte extraction/chemical derivatization approach reduced sample preparation time from 36+ h to less than one hour and replaced the more sample-consuming less-sensitive multi-step liquid-liquid extraction. Method reproducibility of <6% was achieved, which is similar if not better than the reported RSD of 10% using conventional approaches. This method is most attractive for its universal adaptability for a wide range of biologically significant organic chemicals, including small organic acids, amino acids, sugars, fatty acids and sterols.

2.5 References

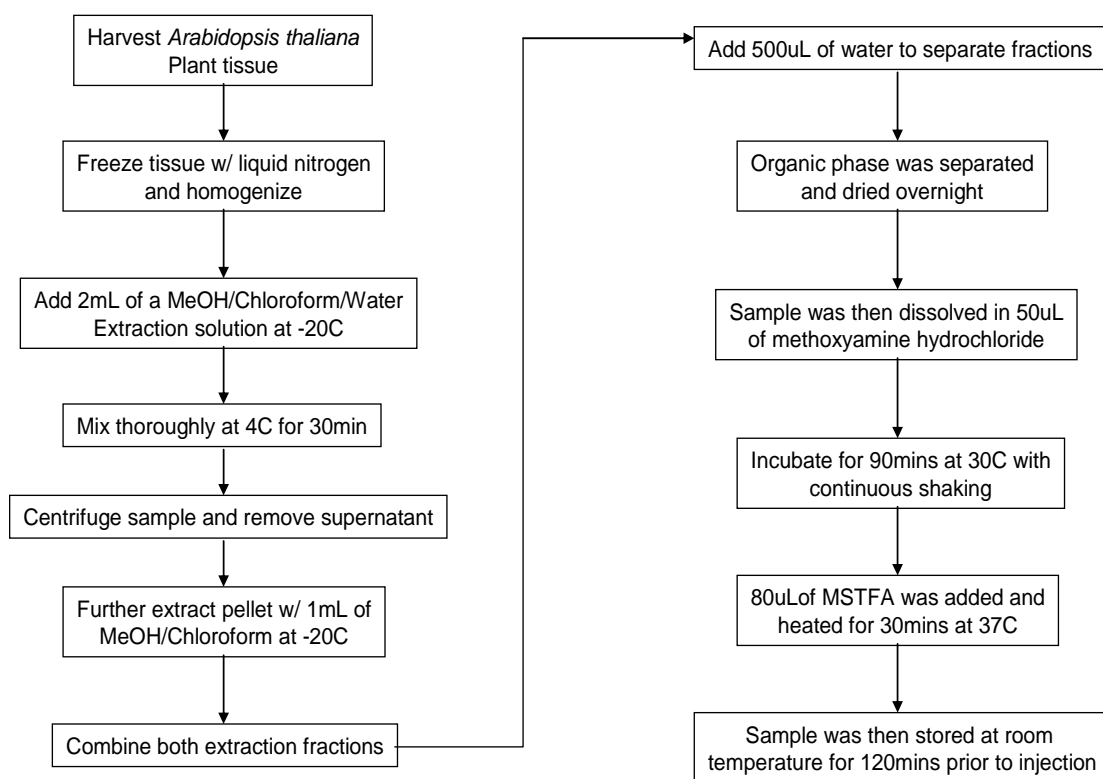
1. Fiehn, O.; Kopka, J.; Trethewey, R.N.; Willmitzer, L. Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry. *Anal. Chem.* **72**, 3573-3580 (2000)
2. Fiehn, O.; Kopka, J.; Dormann, P.; Altmann, T.; Trethewey, R.N.; Willmitzer, L. Metabolite profiling for plant functional genomics. *Nat. Biotechnol.* **18**, 1157-1161 (2000)
3. Weckwerth, W.; Wenzel, K.; Fiehn, O. Process for the integrated extraction, identification, and quantification of metabolites, proteins and RNA to reveal their co-regulation in biochemical networks. *Proteomics* **4**, 78-83 (2004)
4. Velez, H.; Glassbrook, N.J.; Daub, M. Mannitol metabolism in the phytopathogenic fungus *Alternaria alternata*. *Fung. Genet. Biol.* **44**, 258-268 (2007)
5. De Hoog, C.L., Mann, M. Proteomics. *Annu. Rev. Genom. Human Genet.* **5**, 267-293 (2004)
6. Borner, J., Buchinger, S., Schomburg, D. A high-throughput method for microbial metabolome analysis using gas chromatography/ mass spectrometry. *Anal. Biochem.*, **367**, 143-151 (2007)
7. Beckmann, M., Enot, D.P., Overy, D.P., Draper, J. Representation, comparison, and interpretation of Metabolome fingerprint data for total composition analysis

- and quality trait investigation in potato cultivars. *J. Agric. Food Chem.* **55**: 3444-3451 (2007)
8. Villas-Boas, S.G., Mas, S., Akesson, M., Smedsgaard, J. Nielsen, J. Mass Spectrometry in Metabolome analysis. *Mass Spectrom Rev* **24**: 613-646 (2005)
 9. Qiu, Y., Su, M., Liu, Y., Chen, M., Gu, J., Zhang, J., Jia, W. Application of ethyl chloroformate derivatization for gas chromatography-mass spectrometry bases metabonomic profiling. *Anal. Chim. Acta*, **583**, 277-283 (2007)
 10. Gullberg, J., Jonsson, P., Nordstrom, A., Sjostrom, M., Moritz, T. Design of experiments: an efficient strategy to identify factors influencing extraction and derivatization of *Arabidopsis thaliana* samples in metabolomic studies with gas chromatography/ mass spectrometry. *Anal. Biochem.*, **331**, 283-295 (2004)
 11. Jonsson, P., Johansson, A.I., Gullberg, J., Trygg, J., A, J., Grung, B., Markland, S., Sjostrom, M., Antti, H., Moritz, T. High-throughput data analysis for detecting and identifying differences between samples in GC/MS-based metabolomic analyses. *Anal. Chem.*, **77**, 5635-5642 (2005)
 12. Shellie, R.A., Welthagen, W., Zrostlikova, J., Spranger, J., Ristow, M., Fiehn, O., Zimmermann, R. Statistical methods for comparing comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry results: Metabolomic analysis of mouse tissue extracts. *J. Chromatogr., A*, **1086**, 83-90 (2005)

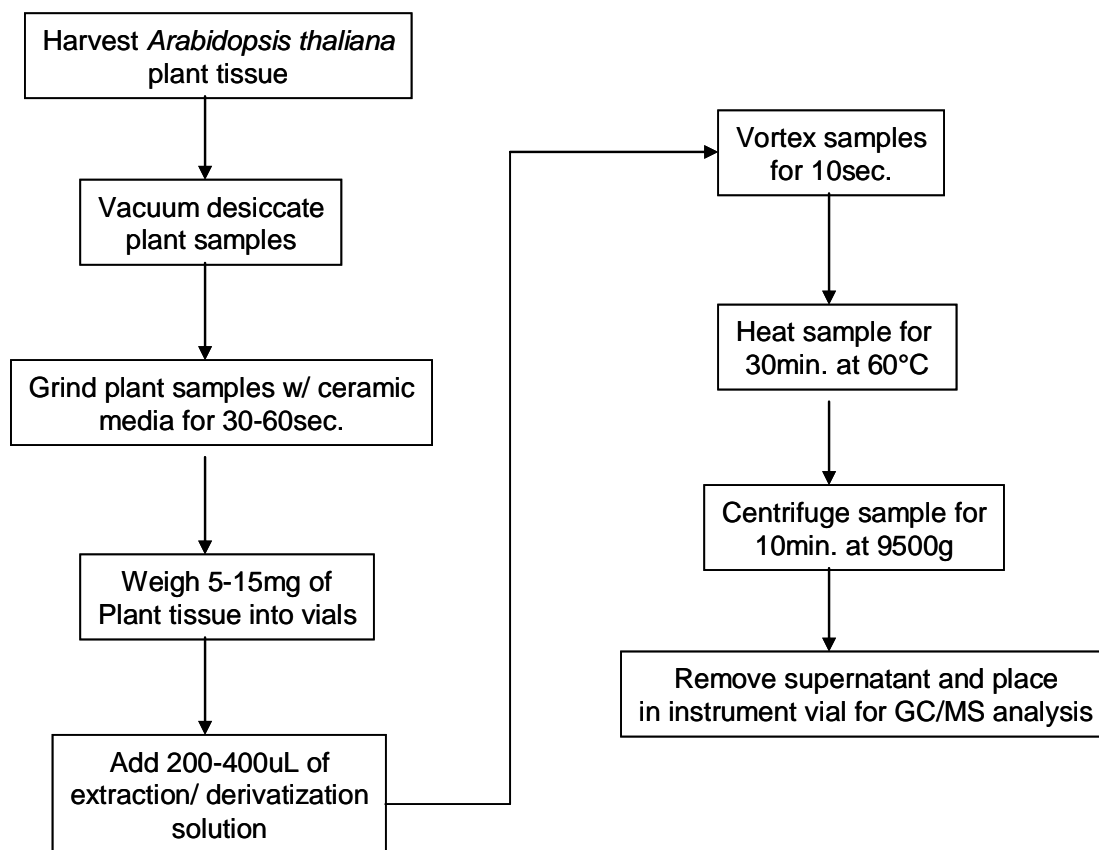
13. Miura, D., Tanaka, H., Wariishi, H. Metabolomic differential display analysis of the white-rot basidiomycete *Phanerochaete chrysosporium* grown under air and 100% oxygen. *FEMS Microbiol. Lett.*, **234**, 111-116 (2004)
14. Jobic, C., Boisson, A.M., Gout, E., Rascle, C., Fevre, M., Cotton, P., Bligny, R. Metabolic processes and carbon nutrient exchanges between host and pathogen sustain the disease development during sunflower infection by *Sclerotinia sclerotiorum*, *Planta*, **226**, 251-265 (2007)
15. Jaki, B.U., Franzblau, S.G., Cho, S.H., Pauli, G.F. Development of an extraction method for mycobacterial metabolome analysis. *J. Pharm. Biomed. Anal.*, **41**, 196-200 (2006)
16. Morgenthal, K., Wienkoop, S., Scholz, M., Selbig, J., Weckwerth, W. Correlative GC-TOF-MS-based metabolite profiling and LC-MS-based protein profiling reveal time-related systemic regulation of metabolite-protein networks and improve pattern recognition for multiple biomarker selection. *Metabolomics*, **1**(2), (2005)
17. Noctor, G., Bergot, G., Mauve, C., Thominet, D., Lelarge-Trouverie, C., Prioul, J. A comparative study of amino acid measurement in leaf extracts by gas chromatography-time of flight-mass spectrometry and high performance liquid chromatography with fluorescence detection. *Metabolomics*, **3**(2), (2007)
18. Tohge, T., Nishiyama, Y., Hirai, M.Y., Yano, M., Nakajima, J., Awazuhara, M., Inoue, E., Takahashi, H., Goodenowe, D.B., Kitayama, M., Yamazaki, M., Saito, K. Functional genomics by integrated analysis of Metabolome and transcriptome

- of *Arabidopsis* plants over-expressing an MYB transcription factor. *Plant J.*, **42**, 218-235 (2005)
19. Hirai, M., Klein, M., Fujikawa, Y., Yano, M., Goodenowe, D.B., Yamazaki, Y., Kanaya, S., Nakamura, Y., Kitayama, M., Suzuki, H., Sakurai, N., Shibata, D., Tokuhisa, J., Reichelt, M., Gershenzon, J., Papenbrock, J., Saito, K. Elucidation of gene-to-gene and metabolite-to-gene networks in *arabidopsis* by integration of metabolomics and transcriptomics. *J. Biol. Chem.*, **280**(27), 25590-25595 (2005)
20. Stobiecki, M., Skirycz, A., Kerhoas, L., Kachlicki, P., Muth, D., Einhorn, J., Mueller-Roeber, B. Profiling of phenolic glycosidic conjugates in leaves of *Arabidopsis thaliana* using LC/MS. *Metabolomics* **2**(4) (2007)
21. Orth, H.C.J., Rentel, C. and Schmidt, P.C. Isolation, purity analysis and stability of hyperforin as a standard material from *Hypericum perforatum* L.J. *Pharm. Pharmacol.* **51**: 193-200 (1999)
22. Little, J.L. Artifacts in trimethylsilyl derivatization reactions and ways to avoid them. *J. Chromatogr., A*, **844**, 1-22 (1999)
23. Riddick, L., Gentry, E.L., McDaniel, M., Brumley, W.C. Evaluation of N-methyl-N-tert-butyldimethylsilyl trifluoroacetamide for environmental analysis under both EIMS and electron capture NICIMS conditions and comparison to trimethylsilyl reagents under EIMS. *Intern. J. Environ. Anal. Chem.* **86**(5), 299-312, (2006)
24. Brumley, W.C., Grange, A.H., Kelliher, V., Patterson, D.B., Montcalm, A, Farley, J.W. Environmental screening of acidic compounds based on capillary zone

- electrophoresis/laser-induced fluorescence detection with identification by gas chromatography/mass spectrometry and gas chromatography/high-resolution mass spectrometry. *J. AOAC Int.*, **83**(5), 1059-67 (2000)
25. Boyes, D., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R., Gorlach, J. Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. *Plant Cell* **13**, 1499-1510 (2001)
26. Eggermont, K; Goderis, I.J., Broekaert, W.F. High-throughput RNA extraction from plant samples based on homogenization by reciprocal shaking in the presence of a mixture of sand and glass beads. *Plant Molec. Biol. Repor.* **14**, 3, 273-279 (1996)
27. Knapp, D.R. *Handbook of Analytical Derivatization Reactions*, John Wiley & Sons, Inc., New York, pp 9, (1979)
28. McLafferty, F.W., Hertel, R.H. and Villwock, R.D. Probability Based Matching of Mass Spectra. *Org. Mass Spectrom.*, **9**, 690-702 (1974)



Scheme 2.1 Sample preparation flow chart using the conventional methods reported in the literature.



Scheme 2.2 Modified sample preparation flow chart using the newly developed one-pot scheme.

Table 2.1 A list of identified chemical compounds in *Arabidopsis* seedling extracts against the NIST MS library.

Compound Name	Retention Time (min.)	Molecular Weight (Da)	Quant. Ion	Qualifier Ions
Alanine	4.30	233	116	73, 147, 218
Glycine	4.60	219	102	73, 147, 219
Valine	5.00	261	144	73, 218, 246
Glycerol	5.25	308	205	73, 147, 218
Leucine	5.47	275	158	73, 232, 260
Iso-Leucine	5.60	275	158	73, 218, 260
Phosphate	5.87	314	299	73, 314, 207
Proline	5.97	259	142	73, 216, 244
Serine	6.05	321	204	73, 218, 306
Threonine	6.17	335	291	73, 218, 320
Fumaric Acid	6.37	260	245	73, 147, 45
2-Furanone, 3,4-dihydro	6.90	262	247	73, 147, 262
Malic acid	7.10	350	233	73, 335, 190
Aspartic acid	7.30	349	232	73, 349, 306
3-Aminopiperidin-2-one	7.40	258	128	73, 243, 258
Ornithine	7.66	420	142	73, 174, 200
Glutamine	7.95	363	246	73, 348, 128
Asparagine	8.50	348	231	73, 188, 348
Citric acid	8.94	480	273	73, 465, 347
Myo-Inositol	9.47	612	305	73, 367, 432
Ascorbic acid	9.60	464	332	73, 449, 205
Scyllo-Inositol	9.73	612	305	73, 367, 432
Hexadecanoic acid	10.20	328	313	73, 328, 145
Phytol	10.57	368	143	73, 353, 123
Myo-Inositol phosphate peak 1	10.97	764	299	73, 387, 315
Octadecanoic acid	11.09	356	341	73, 356, 145
Myo-Inositol phosphate peak 2	11.16	762	299	73, 387, 315
9,12-Octadecadienoic acid	11.20	352	337	73, 262, 129
9,12,15-Octadecatrienoic acid	11.34	350	335	73, 350, 108
3,5-Dimethoxycinnamic acid	11.96	368	368	73, 353, 338
2-Monopalmitin	12.25	474	313	73, 218, 460
1-Monopalmitin	12.35	474	371	73, 239, 459
2-Monostearin	12.94	502	341	73, 218, 487
1-Monostearin	13.06	502	399	73, 487, 267
Campesterol	15.20	472	472	73, 343, 382
Stigmasterol	15.29	484	484	73, 394, 255
Sitosterol	15.64	486	396	73, 486, 357

Table 2.2 A list of partially identified chemical compounds in *Arabidopsis* seedling extracts against the NIST MS library.

Compound Name	Retention Time (min.)	Molecular Weight(Da)	Quant. Ion	Qualifier Ions
Sugar-TMS 1	8.21	N/A	437	73, 217, 257
Sugar-TMS 2	8.39	N/A	437	73, 217, 257
Sugar-TMS 3	8.57	N/A	217	73, 204, 147
Sugar-TMS 4	8.70	N/A	217	73, 204, 147
Sugar-TMS 5	8.76	N/A	217	73, 204, 147
Sugar-TMS 6	8.82	N/A	306	73, 204, 217
Sugar-TMS 7	8.87	N/A	204	73, 306, 319
Sugar-TMS 8	9.00	N/A	204	73, 217, 191
Sugar-TMS 9	9.24	N/A	204	73, 217, 191
Sugar-TMS 10	9.31	N/A	217	73, 305, 318
Sugar-TMS 11	11.58	N/A	361	73, 437, 217
Sugar-TMS 12	11.75	N/A	361	73, 437, 217
Sugar-TMS 13	11.77	N/A	361	73, 437, 217
Sugar-TMS 14	11.85	N/A	361	73, 437, 217
Sugar-TMS 15	11.98	N/A	361	73, 437, 217
Sugar-TMS 16	13.45	N/A	204	73, 361, 217
Phosphate sugar-TMS 1	8.73	N/A	299	73, 357, 445
Phosphate sugar-TMS 2	10.63	N/A	299	73, 357, 445
Phosphate sugar-TMS 3	10.73	N/A	299	73, 357, 445
Phosphate sugar-TMS 4	10.86	N/A	299	73, 315, 387
Phosphate sugar-TMS 5	11.29	N/A	299	73, 217, 305
Phosphate sugar-TMS 6	11.40	N/A	299	73, 387, 357
Phosphate sugar-TMS 7	11.43	N/A	299	73, 387, 357

Table 2.3 A list of GC elution peaks with unidentified chemical structures.

Compound Name	Retention Time (min.)	Molecular Weight (Da)	Quant. Ion	Qualifier Ions
Unknown 1	9.34	N/A	173	73, 156, 275
Unknown 2	16.22	N/A	441	57, 308, 147
Unknown 3	16.40	N/A	133	73, 119, 177
Unknown 4	17.36	N/A	219	57, 219, 278
Unknown 5	17.70	N/A	316	57, 191, 291
Unknown 6	17.77	N/A	385	145, 239, 313
Unknown 7	18.07	N/A	371	57, 399, 385
Unknown 8	19.34	N/A	145	129, 413, 385
Unknown 9	19.76	N/A	399	371, 413, 457
Unknown 10	19.86	N/A	408	385, 262, 145
Unknown 11	20.34	N/A	129	145, 313, 260

Table 2.4 Quantitative calculation of reproducibility of the one-pot extraction/derivatization method for four representative analytes.

Sample	Identified Compound	Normalized MS	SD	RSD%
		Intensity		
At1	L-Valine	4473.0	214.5	4.6
At2	L-Valine	4865.7		
At3	L-Valine	4519.5		
		4619.4		
At1	GABA	7366.8	261.7	3.4
At2	GABA	7890.0		
At3	GABA	7641.5		
		7632.8		
At1	Hexadecanoic acid	15738.6	597.8	3.7
At2	Hexadecanoic acid	16840.4		
At3	Hexadecanoic acid	15887.4		
		16155.4		
At1	Sitosterol	7179.4	461.2	6.0
At2	Sitosterol	7962.1		
At3	Sitosterol	7993.4		
		7711.6		

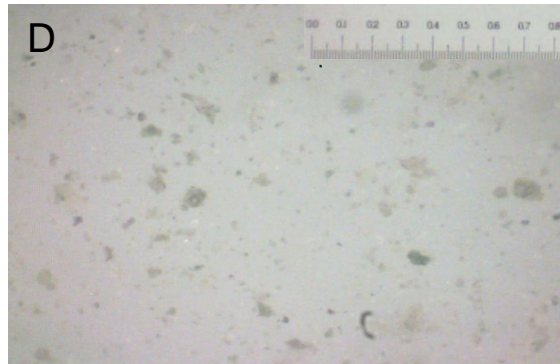
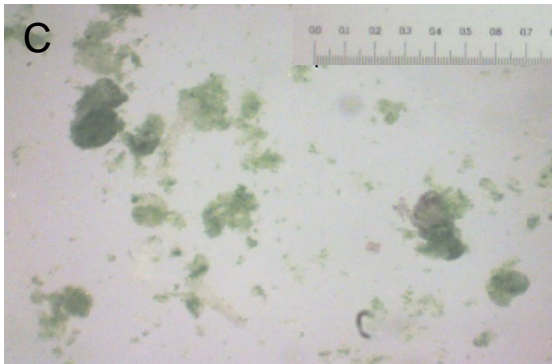


Figure 2.1 (A) A typical mortar-and-pestle tool used in conventional grinding of plant tissue. (B) A mechanic reciprocating saw with attachment and a 20-mL polypropylene scintillation vial used in the newly developed method. The ground tissue pieces after (C) using mortar-and-pestle or (D) using the reciprocating saw. The scale bars in the images shows 1 mm length major unit with a 0.1mm increment.

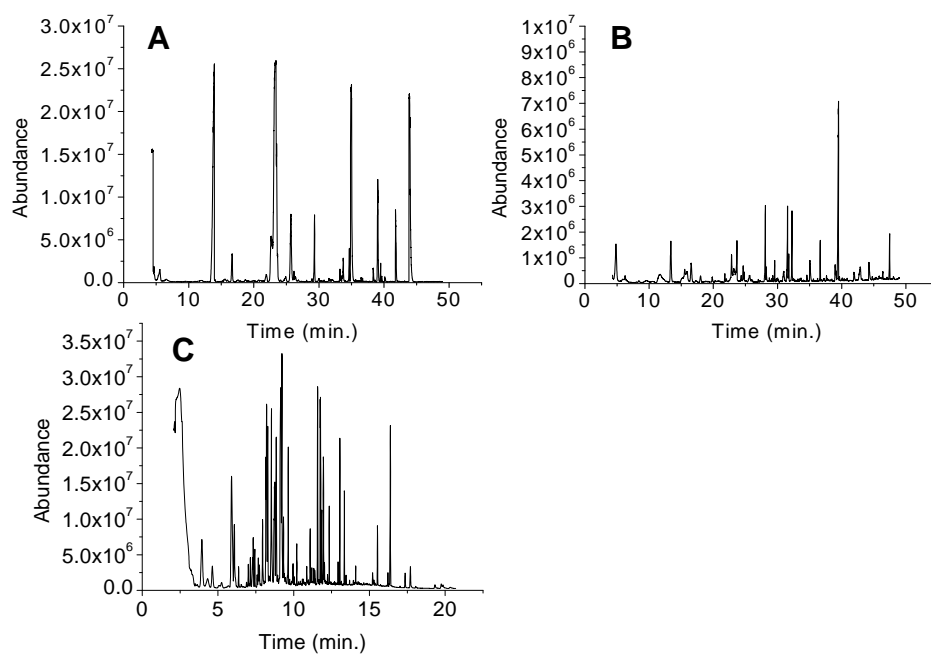


Figure 2.2 GC chromatograms of *Arabidopsis* seedlings extracts prepared using (A) the conventional liquid-liquid extraction, the polar fraction and (B) the non-polar fraction, and (C) the newly developed one-pot extraction/derivatization.

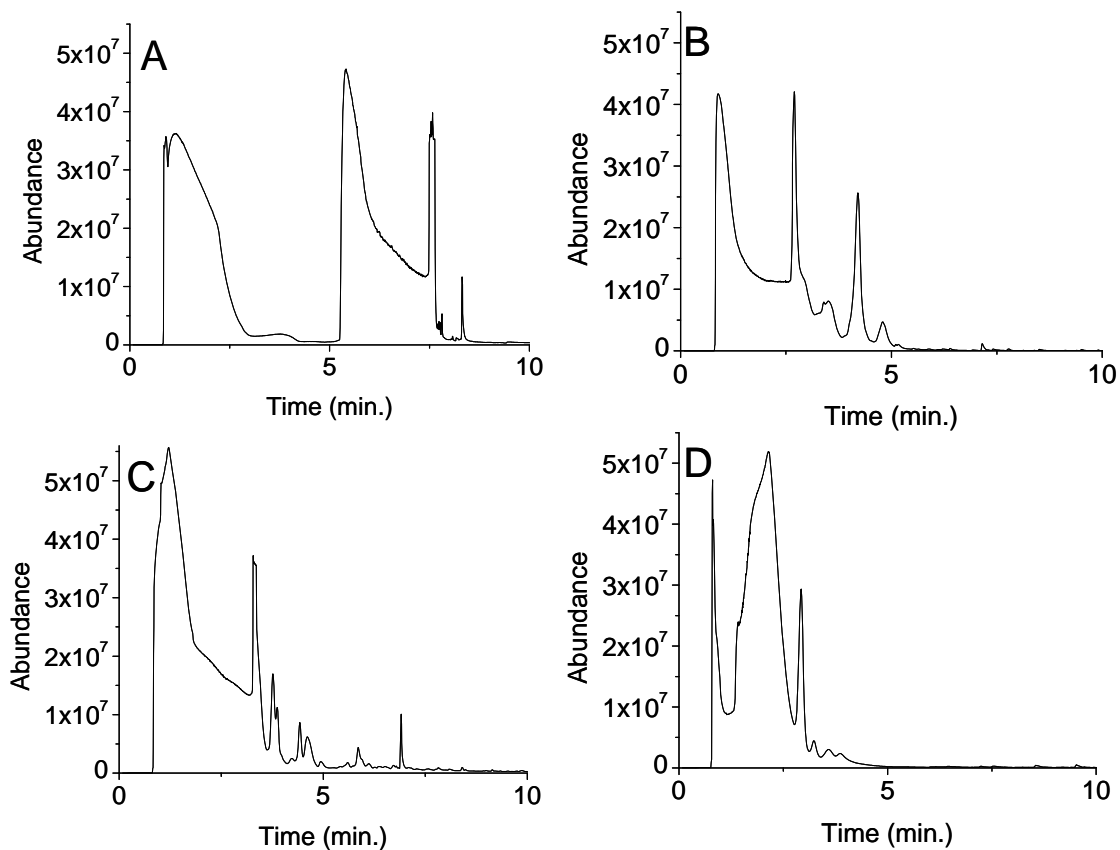


Figure 2.3 Representative GC chromatograms of several derivatizing reagents, (A) TMSI (B) HMDS (C) MSTFA (D) TMSDMA, that contain several chemical components, which could potential overlap with plant extracts: TMS-DMA showed the least background beyond initial 4 min.

Chapter 3

Gas Chromatography-Mass Spectrometry (GC-MS)-based Metabolomic Profiling

3.1 Introduction

Performing comparative studies of transgenic plant species is becoming more and more common in academic and industrial laboratories to monitor genetic impacts on plant phenotypes and to elucidate the mechanisms of various signal transduction pathways in plants.^{1, 2} Consequently, a comprehensive collection of genomic, proteomic and metabolomic data from the same system is desired to allow complete characterization and comparison between transgenic species and wild-types.³⁻⁵ While the use of sequenced model organisms and DNA microarray technology has made genomic data readily accessible, gaining access to proteomic and metabolomic data has been challenging.

Current methodologies for metabolic profiling include the use of many different analytical techniques including Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). FTIR along with cluster analysis has been used in a recent study where ‘holistic’ metabolic fingerprints were obtained to differentiate species, population, single plant genotype and chromosomal differences.⁶ NMR has also been used for plant metabolic profiling for its capability of monitoring a broad range of metabolites without prior target labeling.⁷ A recent study has shown the ability of ¹H NMR to successfully differentiate between genetic variants.⁸ MS has proven to be a very powerful technique for metabolic profiling. Early work that coupled MS with GC has demonstrated the ability to differentiate

hundred of analytes from a single analytical methodology.⁹ Recent MALDI-MS work has successfully profiled metabolites in tomatoes.¹⁰ Additional work for in vivo analysis of *sinomenium acutum* stems has been demonstrated to profile phytochemicals/secondary metabolites.¹¹ HPLC-MS analysis of *Arabidopsis thaliana* to profile phenolic glycosidic conjugates in leaves has been performed, as well as in systematic identification of flavonoids and isoflavonoids in roots and cell suspension cultures of *Medicago truncatula* and *Cyanothece* species ATCC 51142.¹²⁻¹⁴

In this chapter the one-pot sample preparation methodology described in Chapter 2 is applied to metabolic profiling. Metabolite extracts from several *Arabidopsis thaliana* transgenic plants are compared to that of a wild-type standard under normal growth conditions.

3.2 Experimental

3.2.1 Materials

Acetonitrile (ACN), tetrahydrofuran (THF), methanol (MeOH), chloroform and benzene of HPLC grade or better were purchased from EMD Chemicals. N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), trimethylsilyl dimethylamine (TMSDMA), pyridine, pentadecane and methoxyamine hydrochloride were purchased from Sigma-Aldrich, Inc., and used as received. Zirconium oxide grinding media were purchased from Zircoa, Inc.

3.2.2 Tissue Preparation

Arabidopsis thaliana seedlings (250+ seedlings) were grown and harvested at development stage 1.04 for a plate based platform.¹⁵ The samples were immersed in liquid nitrogen and ground for 30-60 sec using the traditional mortar and pestle technique.¹⁶ Alternatively, the seedlings were vacuum desiccated prior to mechanic grinding, during which seedlings from one batch were placed into a 20-mL polypropylene scintillation vial. Zirconium oxide grinding media were then added to the vial and the cap was tightened securely. A 14.4V reciprocating saw (Black & Decker, Hunt Valley, MD) with a custom vial holder was used to homogenize the tissue for approximately 60 sec.

3.2.3 Analyte Derivatization

200-400 μ L of an 80:20 solvent mixture (3:1:1, ACN: THF: Benzene): TMS-DMA extraction-derivatization solution (internal standard included) was added to 5-15mg of ground tissue (Scheme 3.1).¹⁷ After vortexing the solution for 10 sec and heating at 60 °C for 30 min using a digital heat block (VWR, Germany) along with an aluminum heat plate (Mosaic Analytical, Chapel Hill, NC), the mixture was centrifuged for 10 mins at 9500 \times g. The supernatant was analyzed directly.

3.2.4 Instrumentation

All mixtures were analyzed using a 6890 Plus Gas Chromatograph coupled to an Agilent 5973N quadrupole Mass Spectrometer (Agilent, Palo Alto, CA), upgraded with

an inert ion source and enhanced electronics package. Chromatographic separations were achieved with a BPX-50 Phenyl polysilphenylene-siloxane GC capillary column 30 m \times 0.25 mm \times 0.25 μ m (SGE, Australia). The helium carrier gas was set to 1.2 mL per minute. One microliter of derivatized sample was injected into the instrument in splitless mode. The injection port was 280°C with an initial oven temperature of 50°C along with a 20°C per minute ramp to 320°C, hold for 5 min. The mass spectrometer was set in the scan mode from 30 amu to 550 amu. Data were collected using Agilent's Chemstation software and analyzed in Microsoft excel.

3.3 Results and Discussion

The employment of our newly developed sample preparation protocol (Chapter 2) in metabolite profiling was examined in a small scale *Arabidopsis thaliana* seedling experiment where the differences between wild-type and four transgenic species were investigated. The natural genetic variation of plant samples is known to cause a certain amount of fluctuation in metabolite contents; therefore large sample sets, i.e. 250+ seedlings per batch, were purposely used in this study to reduce the impact of genetic variations and improve measurement reproducibility. Nevertheless, it is well-known that small fluctuations in growing conditions, such as small differences in moisture levels, nutrient levels and light levels, manifest into large variations in the plant samples with differences up to 30% have been routinely cited.¹⁸

In this study four lines of transgenic *Arabidopsis thaliana* were compared for differences from a wild-type “standard”, with labels of 2-6, 2-8, HS5-8 and HS9-7. The

first two transgenic lines were inserted with the coding region of the human type I Inositol Polyphosphate 5-phosphatase cDNA to express the human type I inositol polyphosphate 5-phosphatase enzyme.¹⁹ This enzyme specifically hydrolyzes inositol-1,4,5-trisphosphate, which has been linked to signaling events involved in gravitropic responses.²⁰ The transgenic lines HS5-8 and HS9-7 instead express the human type I alpha phosphatidylinositol 4-phosphate 5 kinase enzyme, which catalyzes the synthesis of phosphatidylinositol-4,5-bisphosphate and results in an increase in phosphatidylinositol-4,5-bisphosphate and also an increase in inositol-1,4,5-trisphosphate.¹⁹ (Yang Ju Im, et.al unpublished results). Phenotypically these four transgenic lines did not exhibit any significant changes in plant morphology when compared to wild-type plants under normal growing conditions.¹⁹ However, it is predicted that the changes in the level of phosphatidylinositol-4,5-bisphosphate and inositol-1,4,5-trisphosphate could be expected “to affect lipid and protein interactions and alter cell structure and membrane biosynthesis”.^{20, 21}

Chemical components present in the total ion chromatogram (TIC) of the plant samples were identified by comparing with the reference spectra found in the NIST library. The labeled peaks were then compared across the five genetic variants. Differences found in individual molecules between wild-type and transgenic lines are only considered significant if it passes the t-test evaluation. For example, stigmasterol, a phytosterol, was identified as one of the species whose concentrations in the seedling extracts varied across different strains. Figure 3.1 shows a typical TIC of the extract from wild-type *Arabidopsis thaliana* seedlings. A zoom-in of the elution window where

stigmasterol (Scheme 3.2) trimethylsilyl ether (TMS) was expected shows no distinct peak due to the presence of several molecules that have partially co-eluted with stigmasterol-TMS at the same retention time. A high background was also noted in the TIC at this retention time, which further complicated identification and quantification of stigmasterol. Selective analysis was therefore used to subtract the background (Figure 3.1C). Comparing the fragments to the mass spectrum contained in the NIST library confirmed the presence of stigmasterol-TMS with a match quality of 98. The ion at $m/z = 484$ was then used in quantitation in an extracted ion chromatogram (EIC) (Figure 3.1D). After normalization against the sample weight and the internal standard responses, the average amount of stigmasterol in strain HS9-7 was calculated and listed in Table 3.1. Differences in the amount of stigmasterol extracted from seedlings of different strains are shown in Table 3.2: the wild-type sample had a calculated intra-harvest percent difference of 11.6%. For the transgenic lines, the intra-harvest variations ranged from 9.6% to 21%. The variations for the HS5-8 line and the HS9-7 line comparing to the wild type were 52.5% and 74.6%, respectively. A T-test at a 95% confidence level was performed on the data that confirmed the differences between the wild-type and transgenic lines HS5-8 and HS9-7 were sufficiently significant beyond biological fluctuation, but not with the 2-6 and 2-8 lines (Table 3.3 and Table 3.4). The lower amounts of sterols in HS5-8 and HS9-7 are consistent with the expected influence to membrane biosynthesis with the increase in the levels of phosphatidylinositol-4,5-bisphosphate and inositol-1,4,5-trisphosphate.^{20, 21}

3.4 Conclusions

In this chapter we described the application of our one-pot sample preparation method for GC-MS based analysis of *Arabidopsis thaliana* to the task of metabolic profiling. This method was able to track multiple analytes across multiple classes of molecules. Several transgenic plants were compared to a wild-type standard under normal growing conditions and a potentially significant difference was observed and compared in a semi-quantitative manner.

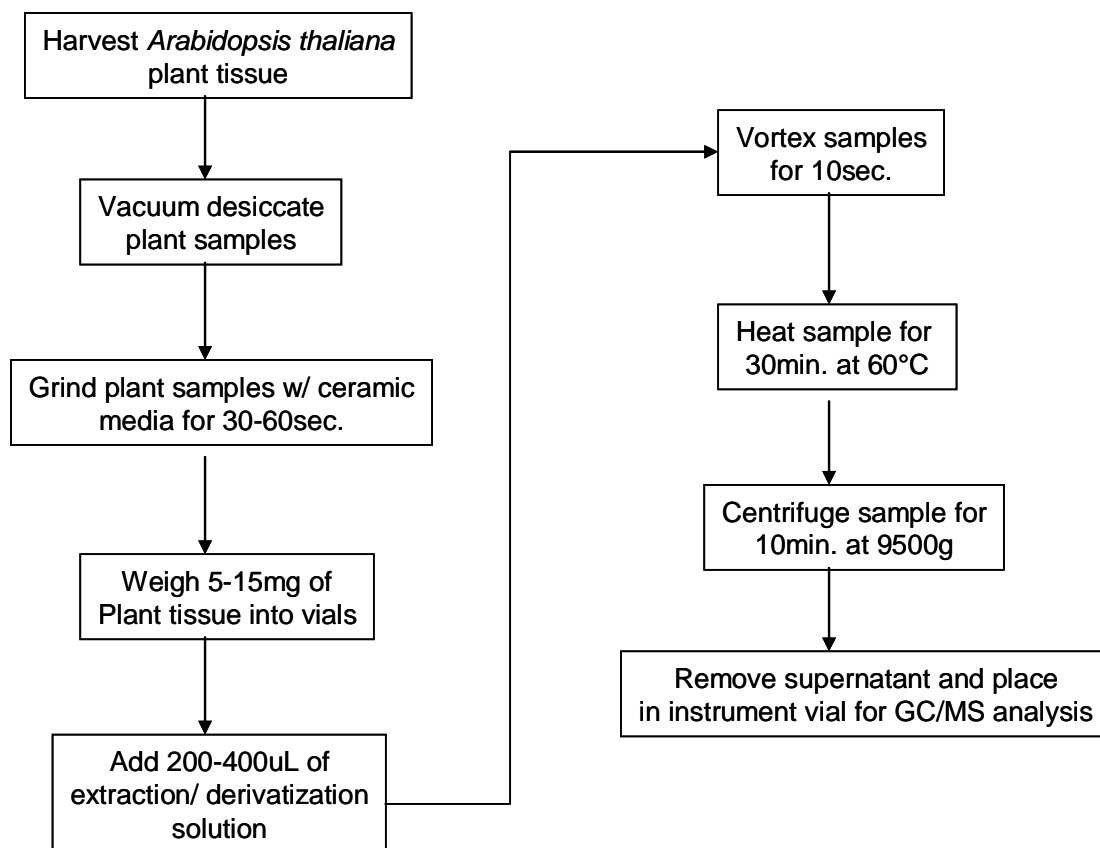
3.5 References

1. Lange, B.M., Ghassemian, M. Comprehensive post-genomic data analysis approaches integrating biochemical pathway maps. *Phytochemistry* **66**, 413-451 (2005)
2. Weckwerth, W. Metabolomics in Systems Biology. *Annu. Rev. Plant Biol.* **54**, 669-89 (2003)
3. Kultz, D., Fiol, D., Valkova, N., Gomez-Jimenez, S., Chan, S.Y., Lee, J. Functional genomics and proteomics of the cellular osmotic stress response in ‘non-model’ organisms. *J. Exp. Biol.* **210**, 1593-1601 (2007)
4. Rochfort, S. Metabolomics Reviewed: A New “Omics” Platform Technology for Systems Biology and Implications for Natural Products Research. *J. Nat. Prod.* **68**, 1813-1820, (2005)
5. Joyce, A.R., Palsson, B.O. The model organism as a system: integrating ‘omics’ data sets. *Nat. Rev. Molec. Cell Biol.* **7**(3), 198-210 (2006)
6. Goodacre, R., Roberts, L., Ellis, D.I., Thorogood, D., Reader, S.M., Ougham, H., King, I. From phenotype to genotype: whole tissue profiling for plant breeding *Metabolomics* 2007 Online First
7. Fiehn, O. Metabolomics- the Link between Genotypes and Phenotypes. *Plant Mol. Biol.* **48**, 155 (2002)
8. Moing, A., Maucourt, M., Renaud, C., Gaudillere, M., Brougisse, R., Leboutellier, B., Gousset-Dupont, A., Vidal, J., Granot, D., Denoves-Rothan, B. et.al. Quantitative metabolic profiling by 1-dimensional H-1-NMR analyses:

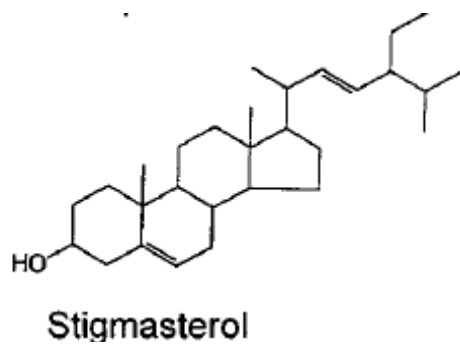
- application to plant genetics and functional genomics. *Func. Plant Biol.* **31**, 889-902 (2004)
9. Fiehn, O.; Kopka, J.; Dormann, P.; Altmann, T.; Trethewey, R.N.; Willmitzer, L. Metabolite profiling for plant functional genomics. *Nat. Biotechnol.* **18**, 1157-1161 (2000)
 10. Fraser, P.D., Enfissi, E.M.A., Goodfellow, M., Eguchi, T., Bramley, P.M. Metabolite profiling of plant carotenoids using the matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Plant J.* **49**, 552-564 (2007)
 11. Ng, K., Liang, Z., Lu, W., Tang, H., Zhao, Z., Che, C., Cheng, Y. In vivo analysis and spatial profiling of phytochemicals in herbal tissue by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* **79**, 2745-2755 (2007)
 12. Stobiecki, M., Skirycz, A., Kerhoas, L., Kachlicki, P., Muth, D., Einhorn, J. Mueller-Roeber, B. Profiling of phenolic glycosidic conjugates in leaves of *Arabidopsis thaliana* using LC/MS. *Metabolomics* **2**(4) (2007)
 13. Farag, M.A., Huhman, D.V., Lei, Z., Sumner, L.W. Metabolic profiling and systematic identification of flavonoids and isoflavonoids using HPLC-UV-ESI-MS and GC-MS. *Phytochemistry* **68**, 342-354 (2007)
 14. Ding, J., Sorenson, C.M., Zhang, Q., Jiang, H., Jaitly, N., Livesay, E.A., Shen, Y., Smith, R.D., Metz, T.O. Capillary LC coupled with high-mass measurement

- accuracy mass spectrometry for metabolic profiling. *Anal. Chem.* **79**, 6081-6093 (2007)
15. Boyes, D., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R., Gorlach, J. Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. *Plant Cell* **13**, 1499-1510 (2001)
16. Eggermaont, K; Goderis, I.J., Broekaert, W.F. High-throughput RNA extraction from plant samples based on homogenization by reciprocal shaking in the presence of a mixture of sand and glass beads. *Plant Molec. Biol. Repor.* **14**, 3, 273-279 (1996)
17. Velez, H.; Glassbrook, N.J.; Daub, M. Mannitol metabolism in the phytopathogenic fungus *Alternaria alternata*. *Fung. Genet. Biol.* **44**, 258-268 (2007)
18. Fiehn, O.; Kopka, J.; Trethewey, R.N.; Willmitzer, L. Identification of uncommon plant metabolites based on calculation of elemental compositions using gas chromatography and quadrupole mass spectrometry. *Anal. Chem.* **72**, 3573-3580 (2000)
19. Perera, I.Y., Hung, C.-Y., Brady, S., Muday, G.K. and Boss, W.F. A Universal Role for Inositol 1, 4, 5-Trisphosphate-Mediated Signaling in Plant Gravitropism. *Plant Physiol.* **140**, 746-760 (2006)
20. Im, Y.J., Perera, I.Y., Brglez, I., Davis, A.J., Stevenson-Paulik, J., Phillippy, B.Q., Johannes, E., Allen, N.S., and Boss, W.F. Increasing Plasma Membrane

21. Yeung, T., Terebiznik, M., Yu, L., Silviu, J., Abidi, W.M., Philips, M., Levine, T., Kapus, A., and Grinstein, S. Receptor activation alters inner surface potential during phagocytosis. *Science*, **313**, 347-351 (2006)



Scheme 3.1 Sample preparation flow chart.



Scheme 3.2 Chemical Structure of Stigmasterol, a major plant sterol.

Table 3.1 Quantitative measurement of stigmasterol in HS9-7.

Sample Replicates	Compound Name	R.T.	Ion	Peak Height	NF 1 (Wt)	NF 2 (ISTD)	Normalized counts	SD	RSD%
HS9-7-1A(1)	Stigmasterol	15.3	484	3581	9.0	1.03	409.3		
HS9-7-2A(1)	Stigmasterol	15.3	484	6076	14.3	1.05	444.7		
HS9-7-2B(1)	Stigmasterol	15.3	484	4960	11.4	1.22	531.6		
HS9-7-1A(2)	Stigmasterol	15.3	484	4766	9.0	0.84	442.5		
HS9-7-1B(2)	Stigmasterol	15.3	484	4388	8.2	0.96	515.4		
HS9-7-2A(2)	Stigmasterol	15.3	484	7501	14.3	0.84	441.6		
HS9-7-2B(2)	Stigmasterol	15.3	484	5590	11.4	0.91	448.5		
Average Response=							462.0	44.2	9.6

Table 3.2 Quantitative measurement of Stigmasterol across different genetic strains.

	Compound	AVG Normalized Counts	% Change from WT	% Intraharvest difference
WT	Stigmasterol	1815.5	--	11.6
"2-6"	Stigmasterol	1711.4	-5.7	10.1
"2-8"	Stigmasterol	1310.4	-27.8	17.8
HS5-8	Stigmasterol	862.3	-52.5	21.2
HS9-7	Stigmasterol	462.0	-74.6	9.6

Table 3.3 t-Test performed on wild-type and HS5-8 data for Stigmasterol.

t-Test: Paired Two Sample for Means		
Wild-type and HS5-8		
	<i>Variable 1</i>	<i>Variable 2</i>
Mean	1815.5	862.25
Variance	25402.58	8001.125
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
df	1	
t Stat	19.27704752	
P(T<=t) one-tail	0.01649759	
t Critical one-tail	6.313748599	
P(T<=t) two-tail	0.032995179	
t Critical two-tail	12.7061503	

Table 3.4 t-Test performed on wild-type and HS9-7 data for Stigmasterol.

t-Test: Paired Two Sample for Means		
Wild-type and HS9-7		
	<i>Variable 1</i>	<i>Variable 2</i>
Mean	1815.5	538.95
Variance	25402.58	11873.405
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
df	1	
t Stat	35.80785414	
P(T<=t) one-tail	0.008887077	
t Critical one-tail	6.313748599	
P(T<=t) two-tail	0.017774155	
t Critical two-tail	12.7061503	

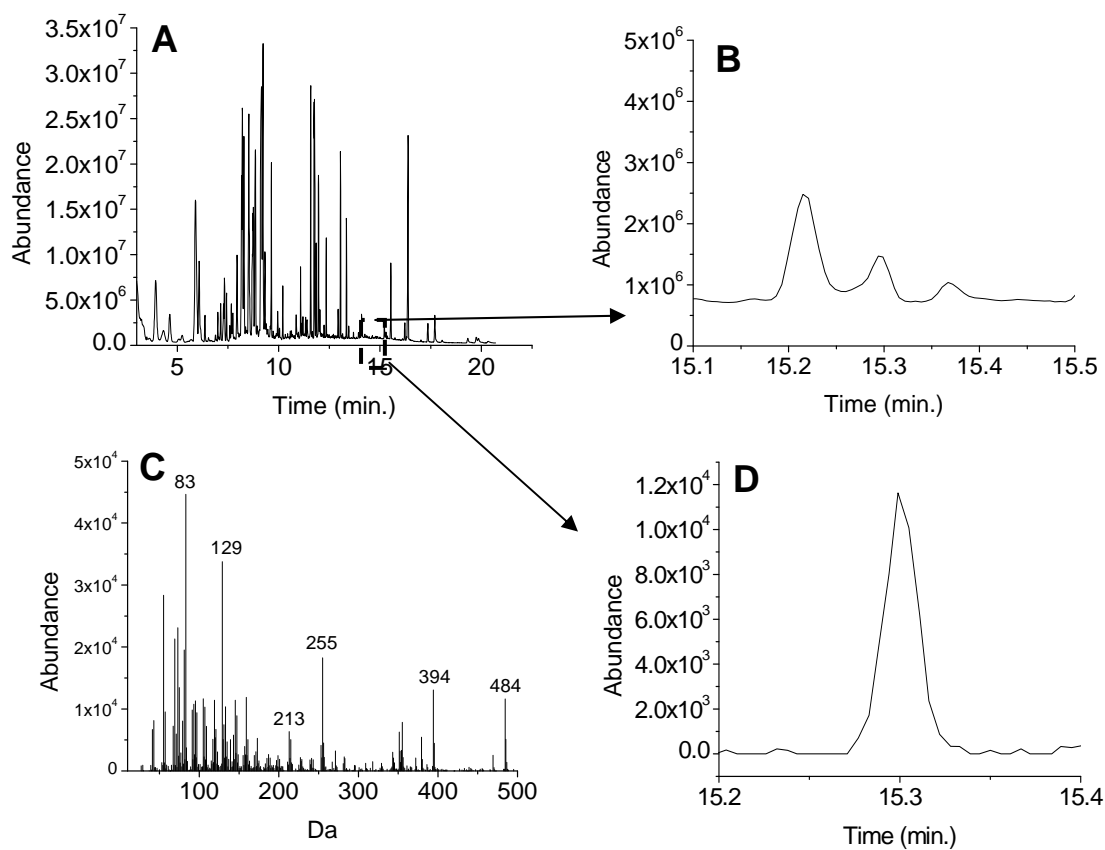


Figure 3.1 (A) A typical TIC of wild-type *Arabidopsis* seedlings; (B) A zoom-in of RT where stigmasterol-TMS was expected; (C) The background subtracted mass spectrum of stigmasterol-TMS; and (D) An EIC of stigmasterol-TMS for the ion $m/z=484$.

Chapter 4

Salicylic Acid Determination in the Study of Systemic Acquired Resistance

4.1 Introduction

For many years scientists have recognized that plants respond to pathogen attack by inducing both a specific defense response locally as well as a non-specific defense response that gives a plant a systemic immunity.¹ Systemic immunity, also known as systemic acquired resistance (SAR), can be characterized by the increased ability of a plant to defend against future attacks by similar, or sometimes different, pathogens at the locations near the initial attack or throughout the plant.^{1, 3-5} It has been stated that “systemic acquired resistance confers quantitative protection against a broad spectrum of microorganisms in a manner comparable to immunization in mammals.”⁵

Salicylic acid (SA) is known to be one of the important signal molecules in local plant defense and in SAR.^{4, 6} A technique that allows direct quantification of SA before and after pathogenic infection is needed to understand the important biological roles it plays in SAR. Previous techniques for quantitative analysis of SA have included high performance liquid chromatography (HPLC), capillary electrophoresis (CE), and gas chromatography mass spectrometry (GC-MS). GC-MS analysis of SA in a large variety of plants was performed by Scotter *et. al.* using a Bis(trimethylsilyl)trifluoroacetamide/Trimethylchlorosilane derivatization scheme, which achieved an LOD of 0.2 µg/g using sample sizes of 1-10g.⁷ HPLC coupled with a UV detector was used for SA quantitation by Shah *et .al.*, which achieved an LOD of 1 µg/g with sample sizes of 200 mg.⁸ HPLC

coupled to a fluorimetric detector was used recently by Iriti *et. al.* to quantify SA in ripened red tomatoes based on a methodology developed by Venema *et. al.* in 1996 which an LOD of 0.2 µg/g (dried tissue) was achieved with a 1-g sample size (dried tissue).^{9, 10} Quantification of SA in *Arabidopsis thaliana* using solid phase extraction and isocratic high-performance anion exchange chromatography was used by Rozhon *et. al.* with an LOD of 5 ng/g and a 100 mg sample size.¹¹ Salicylic acid quantitation has been successfully performed using capillary electrophoresis on *Arabidopsis thaliana* by Shapiro *et. al.* with an LOD of 60 ng/g and a sample size of 20-100mg.¹² An electrochemical method based upon square wave voltammetry was also used for the determination of SA in willow barks and branches with an LOD of 1.7 ng/mL but with sample sizes from 0.5 g-2.0 g.¹³

This chapter describes the application of the method developed in the previous chapter to quantify SA in *Arabidopsis thaliana* for the study of SAR. A major advantage of the developed method is the ability of providing sufficient detection sensitivity to quantify SA at a low-ppb level, which allows studying basal amounts of SA in tissue samples that are only a few mg in size. Consequently it enables systematic studies of SAR responses by examining individual or partial leaves at adjacent positions or those with varying proximities to the initial pathogen attack site.

4.2 Experimental

4.2.1 Materials

Acetonitrile (ACN), tetrahydrofuran (THF), and benzene of HPLC grade or better were purchased from EMD Chemicals. Trimethylsilyl dimethylamine (TMS-DMA), salicylic acid (SA), and pentadecane were purchased from Sigma-Aldrich, Inc., and used as received. Zirconium oxide grinding media were purchased from Zircoa, Inc.

4.2.2 SA Extraction from Plant Tissues

Arabidopsis thaliana plants were grown to the development stage 3.9 on a soil-based platform.¹⁴ The leaves were then infected with *Pseudomonas syringae maculicola* (PSM). The leaves were harvested after 2 d, followed by submerging into liquid nitrogen and storing at -80 °C. The plants were vacuum desiccated prior to mechanic grinding, during which the leaves from each strain were placed into a 20-mL polypropylene scintillation vial. Zirconium oxide grinding media were then added to the vial and the cap was tightened securely. A 14.4V reciprocating saw (Black & Decker, Hunt Valley, MD) with a custom vial holder was used to homogenize the tissue for approximately 60 sec.

In a 1000 mL graduated cylinder 2.4 L of ACN, 800 mL of THF and 800 mL of benzene were poured into a pre-cleaned 4-L solvent bottle to reach a 3:1:1 ratio.¹⁵ This extraction solution (ES) was then mixed for 2 min or until reached thorough mixing. The final solvent mixture was stored in a sealed container and can be stored at room temperature if needed. The derivatization solution (DS) was prepared by mixing 4 mL of

TMS-DMA and 16 mL of extraction solution for 2 min. 20 μ L of pentadecane solution was added to the same mixture as the internal standard.

To quantitate the unknown concentration of SA in plant tissue, 200-400 μ L of the DS solution containing internal standard was added to 5-15mg of ground tissue (Scheme 4.1). After vortexing the solution for 10 sec, it was heated at 60°C for 30 min. The mixture was then centrifuged for 10 min at 9500 \times g, and the supernatant was analyzed in GC-MS directly.

4.2.3 Establishment of the SA Calibration Curve

A 10 mg/mL stock solution of SA was prepared by dissolving 100 mg of SA standard to 5 mL of ES. 2 mL of TMS-DMA was then added to the mixture, followed by adding ES to make the final volume of the mixture to 10 mL. The mixture was heated at 60°C for 30 min to allow for the trimethylsilyl derivatization to take place. The solution was then diluted with the DS to make a 10 μ g/mL stock solution. Note: It is important to add excess TMS-DMA to each dilution of the primary stock solution to avoid incomplete derivatization.

A 9-point analytical calibration curve for SA was established for this study. A series of dilutions to the 10 μ g/mL SA stock solution was made to prepare the standard solutions of 10-1000 ng/mL.

4.2.4 Instrumentation

All mixtures were analyzed using a 6890Plus gas chromatograph (GC) coupled to an Agilent 5973N quadrupole mass spectrometer (MS) (Agilent, Palo Alto, CA), upgraded with an inert ion source and enhanced electronics package. Chromatographic separations were achieved with a BPX-50 Phenyl polysilphenylene-siloxane GC capillary column $30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$ (SGE, Australia). The helium carrier gas was set to 1.2 mL per minute. One microliter of derivatized sample was injected into the instrument in splitless mode. The injection port was set at 280°C with an initial oven temperature of 50°C along with a 20°C per minute ramp to 320°C, hold for 5 mins. The mass spectrometer was set in the scan mode from 30 amu to 550 amu. Alternatively, the mass spectrometer can be put into selective ion monitoring (SIM) mode to maximize sensitivity or SIM/Scan mode to increase sensitivity without compromising supporting metabolic profiling information that is available with this analysis (Chapter 3). Data were gathered using Agilent's Chemstation software. The data gathered were then either calculated using Chemstation or entered into Excel for statistical analysis.

4.3 Results and Discussion

TMS-DMA was reacted with SA to allow quantitative analysis of SA in the plant extracts (Scheme 4.2). Even with a high concentration of SA in the system (1000 ng/mL) the derivatization reaction was found to have no artifacts or partially reacted species found in the chromatogram of the standard. Figure 4.1 shows a nine-point curve generated with a series of SA standard solutions of concentrations ranging from 10

ng/mL to 1000 ng/mL. The MS ion intensity peaks of SA ($m/z = 267$) were normalized against the ion intensities of pentadecane, the internal standard. A linear fitting was obtained over two orders of magnitude in the SA concentration. A coefficient of determination (R^2) of 0.9998 was achieved with the calibration equation of $y = 1.22 \times 10^3(x) - 9.83 \times 10^3$. The limit of detection (LOD) for SA was found to be 2 ng/mL, comparable to the most sensitive methods reported to date by Rozhon *et. al.* (5 ng/mL) and Petrek *et. al.* (1.7 ng/mL). It allowed the establishment of the baseline for the SAR study where the basal level of SA in *Arabidopsis thaliana* prior to infection was at a level of low-ng/mg d.w.⁷ The demonstrated detection dynamic range was also sufficiently broad to bracket the potential increase of SA in the infected species.¹⁶ The instrument reproducibility was calculated at 1.13% by injecting a 400 ng/mL standard of SA four times, and the extraction/derivatization reproducibility was calculated at <5% with four replicates (Table 4.1 and 4.2). The recovery of the developed sample processing method was estimated at 92.5% by spiking SA into 10.0mg *Arabidopsis* tissue samples with a final concentration of 400ng/mL.

Overall, the methodology developed in Chapter 2 allows detection of SA in the low ng/mg range using only 5-15 mg plant tissue. It is a drastic improvement over previous methods where 100-500 mg of tissue was routinely needed to achieve similar levels of sensitivity.^{11, 13} The significantly shortened sample preparation time (72 h vs. 1 h) and procedures (13 steps vs. 9 steps) also rendered it an attractive method in SAR studies.

The examination of SA expression during SAR responses was carried out using a genetic mutant of *Arabidopsis thaliana*, PBS3, to compare to the wild-type plants grown. PBS3 is a GH3 acyl adenylase family member that is known to regulate SA levels after pathogen attacks.¹⁷ While the exact disruption pathway to SA metabolism is unknown, Nobuta *et. al.* surmise that “PBS3-1 mutant contains two point mutations ..., resulting in nonconserved amino acid changes in highly conserved residues...Thus our results suggest that amino acid conjugation plays a critical role in SA metabolism and induced defense responses, with PBS3 acting upstream of SA, directly on SA, or on a competitive inhibitor of SA”.¹⁷ For PBS3, the overall level of SA is predicted to be much lower than that in the wild-type and a compromised accumulation of SA is expected.¹⁷ During the study, each plant batch to be analyzed was weighed in duplicate and each weighed sample was run in duplicate to reduce operator or instrument errors during the analysis. Figure 4.2 shows a fraction of the Total Ion Chromatograms (TIC) and the Extracted Ion Chromatograms (EIC) of an uninfected sample (dashed line) and an infected sample (solid line). A clear difference in the amount of SA was observed, confirming the effective accumulation of SA after pathogen infection. Table 4.3 shows the difference in SA between wild-type and transgenic *Arabidopsis thaliana* leaves harvested before infection and 2 days after infection with a PSM pathogen. A lower concentration of SA was observed for the transgenic plant and a 14 fold increase in SA 2-d after infection for the wild-type vs. a smaller, 10-fold increase for the transgenic plant. The measured results were in agreement with the predicted outcome; however, the significance of the differences can only be validated with additional sample batches.¹⁷

4.4 Conclusions

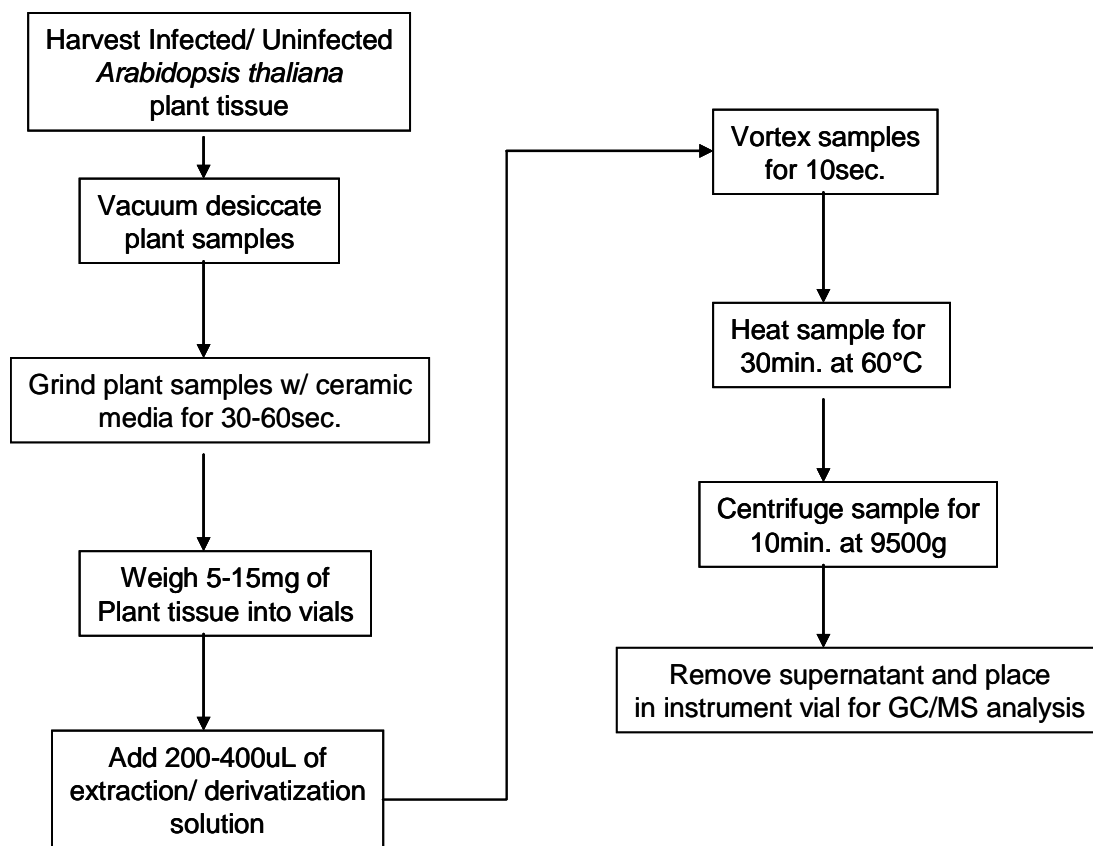
This chapter describes the analytical evaluation of the performance of the sample preparation method developed in Chapter 2. Good sensitivity, broad dynamic range, good reproducibility were all demonstrated using SA as the standard molecule. The application of this platform to quantitate SA in infected *Arabidopsis thaliana* for the study of systemic acquired resistance was concept-proofed, albeit meaningful conclusions can only be drawn with much larger sample populations to reduce the impacts of biological fluctuations.

4.5 References

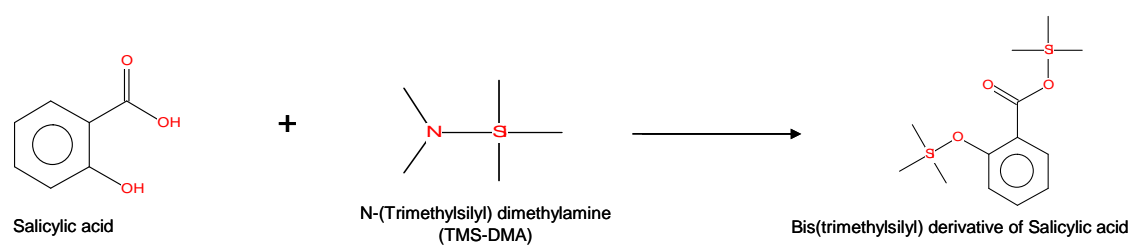
1. Chester, K.S. The problem of Acquired Physiological Immunity in Plants. *Q. Rev. Biol.* **8**, 275-324 (1933)
2. Kuc, J. Induced immunity to plant-disease. *Bioscience*, **32**, 854-860 (1982)
3. Ryals, J., Uknes, S., Ward, E. Systemic acquired-resistance. *Plant Physiol.* **104**, 1109-1112
4. Ryals, J., Lawton, K.A., Delaney, T.P., Friedrich, L., Kessmann, H., Neuenschwander, U., Uknes, S., Vernooij, B. Weymann, K. Signal transduction in systemic acquired resistance. *Proc. Natl. Acad. Sci.*, Vol. 92, 4202-4205 (1995)
5. Sticher, L., Mauch-Mani, B., Metraux, J.P. Systemic Acquired Resistance *Annu. Rev. Phytopathol.*, **35**, 235-270 (1997)
6. Dempsey, D.A., Shah, J. Klessig, D.F. Salicylic acid and disease resistance in plants. *Crit. Rev. Plant Sci.* **18**, 547-575 (1999)
7. Scotter, M.J., Roberts, D.P.T., Wilson, L.A., Howard, F.A.C., Davis, J., Mansell, N. Free salicylic acid and acetyl salicylic acid content of foods using gas chromatography-mass spectrometry. *Food Chem.*, **105**, 273-270 (2007)
8. Nandi, A., Welti, R., Shah, J. The *Arabidopsis thaliana* dihydroxyacetone phosphate reductase gene suppressor of fatty acid desaturase deficiency 1 is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *Plant Cell*, **16**, 465-477, (2004)
9. Iriti, M., Mapelli, S., Faoro, F. Chemical-induced resistance against post-harvest infection enhances tomato nutritional traits. *Food Chem.*, **105**, 1040-1046, (2007)

10. Venema, D.P., Hollman, P.C.H., Janssen, K.P.L.T.M., Katan, M.B. Determination of acetylsalicylic acid and salicylic acid in foods using HPLC fluorescence detection. *J. Agric. Food Chem.*, **44**, 1762-1767 (1996)
11. Rozhon, W., Petutschnig, E., Wrzaczek, M., Jonak, C. Quantification of free and total salicylic acid in plants by solid-phase extraction and isocratic high-performance anion-exchange chromatography. *Anal. Bioanal. Chem.*, **382**, 1620-1627, (2005)
12. Shapiro, A.D., Gutsche, A.T. Capillary electrophoresis-based profiling and quantitation of total salicylic acid and related phenolics for analysis of early signaling in Arabidopsis disease resistance. *Anal. Biochem.*, **320**, 223-233, (2003)
13. Petrek, J., Havel, L., Petrlova, J., Adam, V., Potesil, D., Babula, P., Kizek, R. Analysis of salicylic acid in willow barks and branches by an electrochemical method. *Russ. J. Plant Physiol.*, **54**(4), 553-558, (2007)
14. Boyes, D., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R., Gorlach, J. Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. *Plant Cell* **13**, 1499-1510 (2001)
15. Velez, H.; Glassbrook, N.J.; Daub, M. Mannitol metabolism in the phytopathogenic fungus *Alternaria alternata*. *Fung. Genet. Biol.* **44**, 258-268 (2007)

16. Bowling, S.A., Guo, A., Cao, H., Gordan, S., Klessig, D.F., and Dong, X. A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. *Plant Cell*, **6**, 1845-1857 (1994)
17. Nubota, K., Okrent, R.A., Stoutemyer, M., Rodibaugh, N., Kempema, L., Wildermuth, M.C., and Innes, R.W. The GH3 Acyl Adenylase Family Member PBS3 Regulates Salicylic acid-Dependent Defense Responses in Arabidopsis. *Plant Physiol.*, **144**, 1144-1156 (2007)



Scheme 4.1 Sample preparation flow chart.



Scheme 4.2 TMS-DMA derivatization of salicylic acid in a one-pot format.

Table 4.1 Examination of method reproducibility using SA as the standard molecules.

Sample	Compound Name	Normalized MS Peak Count	SD	RSD%
At1A	Salicylic acid	39429.0		
At1B	Salicylic acid	39225.0		
At1C	Salicylic acid	39654.0		
At1D	Salicylic acid	42890.0		
		40299.5	1735.9	4.31

Table 4.2 Evaluation of instrument reproducibility using SA as the standard molecule.

Sample	Compound Name	Normalized MS Peak Count	SD	RSD%
400ng/ml A	Salicylic acid	18682.0		
400ng/ml B	Salicylic acid	18961.0		
400ng/ml C	Salicylic acid	18686.0		
400ng/ml D	Salicylic acid	18446.0		
		18693.8	210.6	1.13

Table 4.3 Quantitation of SA concentration in wild-type and mutated Arabidopsis plants.

Growth Batch	Name	[SA] (ng/mg)*
At1	WT-untreated	2.07
At1	PBS 3 untreated	1.48
At1	WT-PSM	29.51
At1	PBS 3 PSM	15.09

*Concentration given is against the weight of dried tissue.

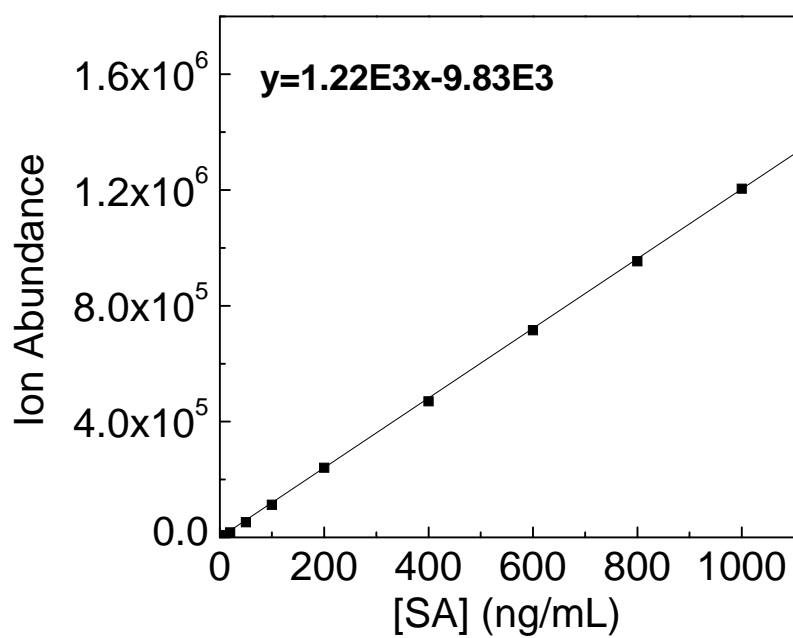


Figure 4.1 A nine-point calibration curve of SA spiked in Arabidopsis plant extracts.

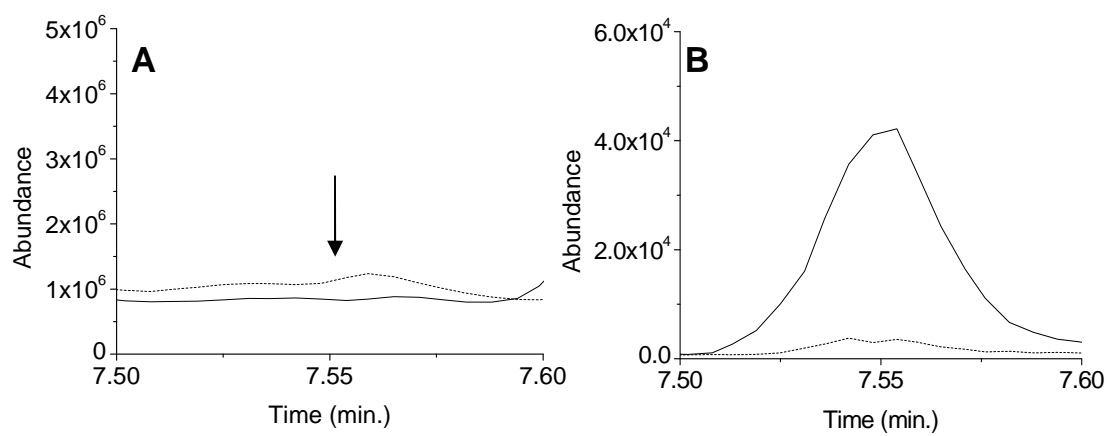


Figure 4.2 (A) Overlay of total ion chromatograms of uninfected (dash) and infected Samples (solid) (B) Overlays of extracted ion chromatograms of uninfected (dash) and infected samples (solid).