METABOLOMICS FOR RAPID IDENTIFICATION OF HUANGLONGBING IN ORANGE TREES AND RAPID DETECTION OF ESCHERICHIA COLI O157:H7, SALMONELLA TYPHIMURIUM, SALMONELLA HARTFORD, AND SALMONELLA MUENCHEN IN GROUND BEEF AND CHICKEN

By

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To my Mom, Dad, and Family in Ecuador

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LIST OF ABBREVIATIONS

BGE	Background electrolyte
CE	Capillary Electrophoresis
CFU	Colony forming unit
CW	Cold water
CZE	Capillary zone electrophoresis
EOF	Electro-osmotic flow
ESI	Electrospray ionization
GC	Gas chromatography
HLB	Huanglongbing
HPLC	High Performance Liquid Chromatography
HW	Hot water
IS	Internal Standard
MEECK	Micro-emulsion electrokinetic chromatography
MS	Mass Spectrometry
MWC	Methanol water chloroform
NACE	Non-aqueous capillary electrophoresis
PCA	Principal components analysis
PCR	Polymerase chain reaction
PDA	Photo diode array detector
PLS	Partial least squares
SPME	Solid Phase Micro Extraction
TSB	Tryptic soy broth

Abstract of Dissertation Presented to the Graduate School
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Rapid and reliable detection methods are of critical importance in preventing the spread of both plant diseases and foodborne pathogens. Citrus Huanglongbing (HLB) is the most destructive disease of citrus worldwide. Metabolomic techniques based on extraction, separation, and quantification methods were developed to find potential HLB biomarkers in leaves from 'Valencia' orange trees from commercial groves. Flavonoids and their derivatives such as naringenin, hesperidin, and quercetin, as well as the amino acid L-proline were significantly ($P < 0.05$) up-regulated in HLB-infected trees. Conversely, sesquiterpenes β-elemene, (-)trans-caryophyllene, and α-humulene were significantly down-regulated in HLB samples when compared to healthy and zinc deficient trees.

Foodborne pathogens were also studied using metabolomic techniques. *Escherichia coli* O157:H7, *Salmonella* Hartford, *Salmonella* Typhimurium, and *Salmonella* Muenchen were grown in tryptic soy broth (TSB) at 37 °C followed by metabolite quantification at two-hour intervals for 24 h. Results were compared to the metabolite profile similarly obtained with *E. coli* K12, *Pseudomonas aeruginosa, Staphylococcus aureus, Saccharomyces cerevisiae,* and

Aspergillus oryzae grown individually and as a cocktail under the same conditions described. Principal component analysis (PCA) achieved sample discrimination of the microorganisms grown in TSB. Metabolites responsible for PCA classification were dextrose, cadaverine, the aminoacids L-histidine, glycine, and L-tyrosine, as well as the volatiles 1-octanol, 1-propanol, 1butanol, 2-ethyl-1-hexanol, and 2,5-dimethyl-pyrazine. Partial least square (PLS) models based on the overall metabolite profile of each bacteria group were created to predict the presence of *E. coli* O157:H7 and *Salmonella* spp. in food samples. The models were tested in ground beef and chicken and were able to detect the presence of the pathogens at levels as low as 1 CFU/ g within 18 h.

CHAPTER 1
INTRODUCTION

Metabolomics

Metabolomics, the study of as many small metabolites as possible in a system, has become an important tool in many areas. Recent reviews and perspectives in the areas of human diseases (Kaddurah-Daouk and Krishnan 2009), drug discovery (Wishart 2008a), plant analysis (Hall and others 2008), nutrition (Wishart 2008b), and others has shown the broad impact and rapid growth of metabolomics. Metabolomics analyses have been generally classified as targeted or untargeted (Figure 1-1).

Targeted analyses focus on a specific group of metabolites whit most cases requiring compound identification and quantification (Ramautar and others 2006). Targeted analyses are important for assessing the behavior of a specific group of compounds in the sample under determined conditions. Targeted analyses typically require higher level of purification and a selective extraction of metabolites. In contrast, untargeted (aka comprehensive) metabolomics focuses on the detection of as many groups of metabolites as possible to obtain patterns or fingerprints without necessarily identifying nor quantifying compounds (Monton and Soga 2007). Untargeted analyses have been important for discovery of possible fingerprints of biological phenomena such as plant diseases (Cevallos-Cevallos and others 2009a). A secondary classification can be done by the criteria used for data treatment. Most metabolomics analyses have been discriminative, predictive, and/or informative (Figure 1-1). Discriminative analyses have been aimed to find differences between sample populations without necessarily creating statistical models or evaluating possible pathways that may elucidate such differences. This is achieved by the use of multivariate data analysis tools (MVDA) described in other reviews (van

der Werf and others 2005; Kemsley and others 2007). In contrast, informative metabolomics is aimed to produce sample-intrinsic information such as possible pathways, discovery of novel bioactive compounds, biomarker discovery, creation of specialized metabolite databases, and metabolites functionality.

Informative metabolomics research has been successfully applied for the development of metabolite databases such as the human metabolome database (Wishart and others 2007). Compound identification and quantification are usually important for this class of metabolomics. Finally, metabolomics reports have also been predictive. In this case, statistical models based on metabolite profile and abundances are usually created to predict a variable that was not directly measured. Models in predictive metabolomics are usually produced by partial least square (PLS) regression as discussed in the data treatment section.

In food science, metabolomics has shown to have the potential for solving major problems worldwide and is considered as a tool for addressing future needs in agriculture (Green and others 2007) and nutrition (Green and others 2007; Hall and others 2008). Moreover, metabolomics has been considered for many food research programs such as the Metabolomics for Plants, Health and OutReach (METHA-PHOR) initiative (Hall 2007).
Discriminative, informative, and predictive metabolomics have been recently used for quality, nutrition, and food components analysis (Wishart 2008b) with a significant expansion to other food applications in the last two years. This section critically reviews recent metabolomics studies in food from the perspective of the extraction, separation, detection, and data treatment as well as the application of discriminative, informative, and predictive metabolomics in the areas of food quality, safety, regulations, and microbiology, and processing. Main findings of this

section were published in Trends in Food Science and Technology (Cevallos-Cevallos and others 2009b).

The Process of Metabolomics Analysis

Metabolomics analyses consist of a sequence of methods including sample preparation, metabolite extraction, derivatization, metabolite separation, detection, and data treatment (Figure 1- 2). However, not always the complete set of steps is needed. Only detection and data analysis have been mandatory steps for the reported metabolomics studies. The selection of the steps depends on the type of study (untargeted versus targeted), kind of sample (e.g. solids vs. liquids), instrumentation to be used for separation (e.g. GC vs. LC) and detection method (e.g. MS vs. NMR). Table 1-1 summarizes recent metabolomics studies used for food analysis.

The first step in food metabolomics analysis is usually sample preparation. Solid samples such as apple peel (Rudell and others 2008) and potatoes (Dobson and others 2008) are typically ground under liquid nitrogen or after freeze-drying. Proper grinding may enhance the release of metabolites during extraction. Freeze-drying acts as a concentration step and minimizes possible differences in metabolites due to dissimilarities in moisture content between groups of sample. Other concentrated liquid samples such as honey can be diluted as a preliminary step (Donarski and others 2008). However, to maximize the amount of information to be collected, concentration steps are more appealing. Metabolites in wine (Son and others 2008) and volatiles in olive oil (Cavaliere and others 2007) have been concentrated by lyophilization and solid phase microextraction (SPME) respectively.

The following step is extraction. This step is aimed to maximize the amount and concentration of the compounds of interest. For this reason, extraction is probably the most critical step in metabolomics. In untargeted metabolomics, the nature of compounds of interest is

mostly unknown. Hence, several solvents and extraction methods should be tested and compared between the groups of samples. Most reports on untargeted food analysis do not describe preliminary comparisons among extraction solvents tested. However, the extraction methods used in foods have been similar to those found optimal in comparable research fields such as non-food plant metabolomics. For instance, the combination methanol-water-chloroform (MeOH-H_2O-$CHCl_3$) was shown to be superior to other solvents for untargeted studies in plants such as *Arabidopsis thaliana* (Gullberg and others 2004) because of its capacity of extracting both hydrophilic and hydrophobic compounds. Therefore, the effectiveness of MeOH-H_2O-$CHCl_3$ in green tea (Pongsuwan and others 2008), potatoes (Dobson and others 2008) and other foods was anticipated. For untargeted analysis is the use of sequential and selective extractions followed by metabolite analysis of each extract has been recommended (Dixon and others 2006). Usually, an initial hydrophilic extraction (typically with MeOH-H_2O) followed by centrifugation and hydrophobic extraction (typically with $CHCl_3$) of the pellet are done. Sequential extraction maximized the amount of metabolites from tomato paste (Capanoglu and others 2008) finding discriminating compounds in both hydrophilic and hydrophobic fractions. Conversely, analysis of other food stuff such as potato (Dobson and others 2008) and mushrooms (Cho and others 2007) has shown low or no sample discrimination in the hydrophobic fractions. Similar observations made in other areas such as non-food plant analysis (Cevallos-Cevallos and others 2009a) suggests a higher suitability of hydrophilic extracts for discriminative metabolomics analyses. Hydrophilic extractions in untargeted food analysis such as apple (Rudell and others 2008) and broccoli (Luthria and others 2008) have usually been made by MeOH or MeOH-H_2O. Other extractions based on deuterium oxide (D_2O) for NMR analysis are also common. Novel methods for extraction of metabolites from frozen meat where a desorption gas hit the meat

surface extracting metabolites further carried to the ionization and detection chambers have been reported (Chen and others 2007). Extraction for targeted analysis relies on the knowledge of the analytes nature. Polyphenols have been extracted from berries by a water-acetic acid combination (McDougall and others 2008) and hot water was used for targeted analysis of glucosinolates in broccoli and mustard seeds (Rochfort and others 2008). To maximize the number and amount of metabolites to be obtained and reduce extraction time, disruption methods such as ultrasonic treatments are usually part of both untargeted and targeted extractions.

After extraction, derivatization can be done. In food metabolomics, derivatization has mainly been used previous GC analysis to increase volatility of analytes. Derivatization is usually a two steps process starting with oximation of the sample to reduce tautomerism (especially from monosaccharide), followed by silylation to increase volatility by reducing hidrophilicity of functional groups OH, SH or NH (Gullberg and others 2004). Several oximation and silylation reagents have been tested in the past. Gullberg et al. (2004) reviewed previous reagents comparisons and reported that methoxiamine hydrochloride in pyridine and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide were the most appropriate reagents for oximation and silylation respectively. In food analyses, these reagents have shown to improve GC separation of metabolites in potato (Beckmann and others 2007) and other products. Derivatization times and temperatures affect each metabolite independently with major changes at the beginning of the reaction (Ma and others 2008). Therefore, preliminary experiments should be done to determine optimum derivatization times and temperatures that maximize the detection of compounds of interest. In food metabolomics analysis, several silylation reactions have been done for 90 min at 37 ˚C (Beckmann and others 2007; Dobson and others 2008) with good results.

Other key steps in metabolomics are separation and detection. Separation and detection of the metabolites have been considered the key steps in metabolic profiling. In the metabolomics literature, particular attention has been given to separation techniques such as liquid chromatography (LC) and its high performance (HPLC) or ultra performance (UPLC) types; gas chromatography (GC), capillary electrophoresis (CE); coupled to detection techniques such as mass spectrometry (MS), nuclear magnetic resonance (NMR), and near infrared spectrometry (NIR). Working principle as well as individual and hyphenated suitability of these techniques in metabolomics have been broadly discussed (Rochfort 2005; Bedair and Sumner 2008; Toyo'oka 2008; Wishart 2008b).

In food metabolomics most analysis have been done by GC, CE, and LC as seen in Table 1-1. Comparison and suitability of these techniques in food analysis have been discussed in other reviews (Wishart 2008b). Among non conventional techniques, ion mobility spectrometry (IMS) where food metabolites are carried in a flow of inert gas, ionized, and separated by a drift gas flowing in the opposite direction has been applied to metabolomics analysis of cheese, beer, and food packaging material (Vautz and others 2006).

Detection methods are mostly based on UV, NIR, MS, or NMR techniques. In food metabolomics MS and NMR have been used the most (Table 1-1). A greater amount of data is generally obtained by using MS accompanied by high throughput separation techniques such as HPLC or UPLC as shown in Table 1-2. For instance, green tea quality has been evaluated by NMR (Tarachiwin and others 2007) and UPLC-MS (Pongsuwan and others 2008). Partial least square (PLS) models from UPLC-MS yielded a higher prediction coefficient than models from NMR, probably due to the higher number of peaks detected by UPLC-MS. However, other factors such as sample variability should also be considered. Although not as sensitive as the

other detection techniques, NIR has provided a fast non-destructive fingerprint in several metabolomics analysis such as strain differentiation of wine yeast (Cozzolino and others 2006). Another technique, direct infusion mass spectrometry (DIMS) methods do not require a previous separation step achieving faster results as applied for broccoli (Luthria and others 2008).

Compound identification has been mainly achieved by database matching (mostly in GC-MS methods) or by comparison with pure standards run under same conditions. Data analysis in food metabolomics is done by several chemometrics tools. Typically, metabolomics data has been aligned before comparison. Alignment has been shown to drastically improve data classification and discrimination analysis (Son and others 2008). Discriminative metabolomics usually relies on multivariate methods such as principal components analysis (PCA) for sample classification. PCA creates new variables (principal components) by linear combinations of the metabolites detected while maximizing sample variation. Classification occurs when comparing the values of two or more principal components of each sample as used for discrimination of broccoli varieties (Luthria and others 2008). On the other hand, PLS is a MVDA technique that allows sample discrimination by reduction of dimensionality while maximizing correlation between variables. PLS has been the main technique used for predictive metabolomics studies such as the creation of a metabolite based model for sensory evaluation of watermelon (Tarachiwin and others 2008). Similarly, linear discriminant analysis (LDA) with a priori classification hypothesis has been used for discrimination of olive oil according to origin (Cavaliere and others 2007). PCA, PLS, and LD have been widely reviewed (van der Werf and others 2005; Kemsley and others 2007). Also, correlation techniques such as correlation network (CN) analysis have been used to determine the link between metabolites and establish possible reactions or pathways during a determined phenomenon. This has been an important tool in

informative metabolomics. CN creates nodes representing the metabolites detected, connected by edges that indicate the correlation as used in tomatoes (Ursem and others 2008). Genetic programming (GP) is another classificatory tool that has been used to improve the sensitivity and selectivity of the PLS models for honey origin determination (Donarski and others 2008). Most of the MVDA tools such as PCA and PLS reduce dimensionality of the data by linear combination of the original variables. In contrast, random forest (RF) analysis permits multivariate data comparison without dimensionality reduction. RF has allowed classification of potato varieties by pairwise comparisons with accuracy values greater than 92%. Also creation of a Mastermix potato model allowed discrimination of a larger number of potato varieties through RF (Beckmann and others 2007). Other common statistical tools have been used in food metabolomics and are summarized in Table 1-1.

Metabolomics in Food Quality

Targeted metabolomics focused on volatiles has shown to have the potential to assess pre-harvest issues that may affect quality. Pre-harvest fungal diseases in mango (Moalemiyan and others 2007), post-harvest bacterial contamination of onions (Vikram and others 2005) and McIntosh apples (Vikram and others 2004), as well as diseases of stored carrots (Vikram and others 2006) have been assessed by sampling headspace metabolites and analyzed by GC-MS. In each case, the volatile profile was found to disease-specific, and several compounds were tentatively identified by GC-MS databases. Changes in polyphenolic compounds during berries breeding (Stewart and others 2007) have been detected by informative metabolomics. In addition, post-harvest metabolomics analysis have the potential for detection and understanding food spoilage as reviewed by Kushalappa and others (2008).

The development of novel metabolomics techniques such as IMS has allowed monitoring of quality attributes during process. Because IMS allows *in situ* automatic sampling, it can be used for determining the completion of certain processes assuring standard quality based in a group of metabolites. This type of analysis fits the needs of biotechnological food processes in which metabolites are changing with time. Targeted informative (concentration aimed) IMS has been applied for the detection of diacetyl and 2,3 pentadione compounds in beer to determine the endpoint of the fermentation (Vautz and others 2006). Quality of health supplements has also been evaluated using metabolomics techniques (Kooy and others 2008; Liu and others 2008).

Future trends will involve the use of discriminative and predictive metabolomics as the ultimate tool for quality control analysis. Metabolite baseline of products meeting minimum quality standards can be developed. Individual samples obtained during processing can be analyzed and compared to the baseline trough MVDA techniques to determine acceptability of the batch produced. Moreover, accidental adulteration of food (e.g. allergenic inclusion or microbial contamination) can be detected by appearance of uncommon peaks in the sample metabolic profile. Informative metabolomics can elucidate the nature of the peaks of interest. In addition, combinations of predictive and informative metabolomics have the potential to become the single all-parameter analysis tool. Quality parameters are usually measured individually in the industry. Many of these parameters can be quantified in a single run of informative metabolomics. Additionally, the metabolite profile obtained can be put in a statistical model obtained by predictive analysis to estimate the parameters that are not detectable by the metabolomics analysis process (e.g. sensory attributes). Predictive models have been created to estimate sensory attributes of green tea (Ikeda and others 2007; Tarachiwin and others 2007; Pongsuwan and others 2008) , watermelon (Tarachiwin and others 2008) and mushrooms (Cho

and others 2007). Similarly, metabolomics have the potential of identifying compounds that dictate consumer taste preferences. Products preferences can be obtained by taste panels whereas discriminating compounds can be identified and correlated by metabolomics techniques. Sensory evaluations with various concentrations of the chosen compounds will confirm their impact on consumer preferences (Figure 1-3).

Metabolomics in Food Safety

Untargeted discriminative metabolomics has been applied in food safety. Neutral desorption extractive electrospray ionization MS (EESI-MS) was able to discriminate *E. coli*-contaminated spinach trough the presence of unidentified high molecular weight peaks (Chen and others 2007). The same technique discriminated spoiled fish trough the presence of putrescine, cadaverine, and the toxic compound histamine, showing a great potential of this type of analysis in food safety. Informative and predictive metabolomics in fresh raw fish has been suggested as tools to provide evidence of water contamination, temperature stress, and the fish health conditions at the moment of the catch (Samuelsson and Larsson 2008).

Metabolomics has the potential to assess the safety of novel pre and post-harvest technologies. It has been proposed that unintended effects of genetic modification of foods can been assessed by untargeted discriminative analyses (Zdunczyk 2006; Chao and Krewski 2008). Catchpole et al. (2005) utilized untargeted discriminative metabolomics to differentiate genetically modified (GM) potatoes. After removing the intended effect variable (fructans derivatives) no discrimination was observed, suggesting that GM potatoes are similar in composition to the original ones. Similarly, intended increase in flavonoid concentration in GM tomatoes have been reported trough targeted informative metabolomics (Le Gall and others 2003b) whereas small non-intended variations were detected by untargeted analysis (Le Gall and

others 2003a) concluding that no major unintended changes occurred after genetic modification. Future trends would involve the use of informative metabolomics to assess the safety of new or controversial processing technologies such as irradiation.

Metabolomics for Compliance of Food Regulations

Compliance with country of origin regulations can be verified by discriminative and predictive metabolomics. Origins of honey (Donarski and others 2008), olive oil (Cavaliere and others 2007) and wine (Son and others 2008) have been determined by discriminative and predictive metabolomics. Regulations in many countries do not allow the use of GM foods. Compliance with this regulation can be verified by metabolomics. Discriminative and predictive analysis have been used to differentiate genetic modification in maize (Levandi and others 2008), soybean (Garcia-Villalba and others 2008), potatoes (Le Gall and others 2003a; Catchpole and others 2005), and wheat (Shewry and others 2007). Metabolomics can be used for compliance verification of labeled ingredients. These analyses have relied in the use of discriminative metabolomics to differentiate among varieties of several fruits and vegetables. For instance, cherry tomato has been separated by MVDA techniques from other varieties such as beef and round tomatoes by SPME-GC-MS (Tikunov and others 2005), LC-MS and NMR (Moco and others 2008). Variety differentiation has also been applied to broccoli (Luthria and others 2008), wines (Pereira and others 2007; Son and others 2008), ginseng variety (Kang and others 2008) and age (Shin and others 2007) differentiation, and potatoes (Parr and others 2005; Dobson and others 2008).

Metabolomics in Food Microbiology

Several metabolomics application to microbial research have been suggested (van der Werf and others 2005). Bacteria identification and confirmation has been traditionally done by

complex numerous biochemical tests. In contrast, discriminative and predictive analyses have the potential for rapid and accurate bacteria identification and confirmation. This analysis are mostly MS based (Ecker and others 2008). Microorganisms are grown in culture media then concentrated (typically by centrifugation) and internal metabolites are extracted through cell disruption processes such as ultrasound or bead beating processes before separation or detection occurs. By following this method and the use of a matrix assisted laser desorption/ionization time of flight mass spectrometry MALDI-TOF-MS to detect high molecular weight compounds, 12 species of *Aspergillus* and 5 strains of *Aspergillus flavus* have been classified with 95 to 100% accuracy (Hettick and others 2008). Similar methods have been used to classify *Escherichia coli* and *Yersinia* according to growing culture media, species, and strain (Parisi and others 2008). Metabolomics can also be used for understanding microbial metabolism. Dynamics of glycolisis in *E. coli* have been assessed under systemic variation of growth rate and different glucose availability (Schaub and Reuss 2008) generating information on how glycolisis is affected under these conditions. Wine and baking yeasts have been differentiated from medical strains by using DIMS and GC-TOF-MS (MacKenzie and others 2008). In addition, exo-metabolites of several wine yeast strains were analyzed by HPLC and GC-FID to compare aroma relevant compounds to gene expression (Rossouw and others 2008) showing the potential of metabolomics for assessing gene expressions. Current methods for quantification of bacteria in food still rely on lengthy plate counts and most probable number procedures. Metabolomics analysis coupled to sensor development can have a big impact in the detection and quantification of bacteria. Metabolomics has been successfully used for biomarker discovery in other areas such as plant physiology (Glauser and others 2008). The discovery of bacteria biomarkers and their monitoring throughout growth phases has the potential to be related to the final pathogen

colony forming units (CFU). Sensors may be developed to monitor the formation of the biomarker in the culture broth and incorporate the rate of biomarker production to an algorithm that predicts the expected CFU. Metabolomics studies during *E. coli* growth have shown the time-related progression of several metabolites (Koek and others 2006). Moreover, metabolomics has the potential to find new antimicrobial compounds and to determine the analytes responsible of the antimicrobial characteristics of certain plants and food. Zhi, Yu, and Yi (2008) utilized discriminative metabolomics based on HPLC to identified dihydrocucurbitacin F-25-O-acetate as the major antimicrobial component of the herb *Hemsleya pengxianensis*. PCA data showed that *Staphylococcus aureus* treated with dihydrocucurbitacin F-25-O-acetate grouped with those treated with the herb extract.

Metabolomics in Food Processing

Food processing involves the combination of physical and chemical events that may cause important changes in food components that can be detected by metabolomics. The production of cheonggukjang (a soybean and rice straw fermented drink) has been monitored by informative and discriminative untargeted analysis using NMR (Choi and others 2007). The method showed the expected time-related reduction of sugars (e.g. sucrose and fructose) and increase of acetic acid, tyrosine, phenylalanine and others. Final products were differentiated as a function of fermentation time by PCA. In addition, Capanoglu et al. (2008) utilized both targeted and untargeted informative metabolomics analyses to show that several flavonoids such as rutin, naringenin and derivates, as well as some alkaloids increased significantly after the breaking step (fruit chopping). This was explained by the possible activation of pertinent enzymes after wounding. In addition, reduction of these compounds after the pulping step was observed because of the high presence of these analytes in the removed skin and seeds. Metabolomics can

also be used to understand the suitability of certain varieties for processing. For instance, several potatoes varieties are preferred for frying whereas other for baking. To assess differences, potato varieties have been analyzed by flow infusion electrospray-ionization MS (FI-ESI-MS) and compound identification was aided by GC-MS (Beckmann and others 2007). Cultivars Salara and Agria were low in tyrosine (major substrate for polyphenol oxidases) making them suitable for slicing and frying. Tyrosine is also a precursor of aroma and flavor compounds in boiled potatoes by Strecker degradation. Cultivars found to be high in tyrosine (Désirée and Granola) are more suitable for baking (Beckmann and others 2007).

This type of analysis has shown the potential for providing valuable information to food product and process development industry. Informative metabolomics has the potential to assess unintended effects during processing and pre-processing such as changes in nutrient composition, degradation of health-related compounds, and formation of new compounds like toxins. In addition informative and discriminative metabolomics have the potential to study other pre-processing scenarios such organic food production, and denominations such as "cage free" or "grade A".

Citrus Huanglongbing

Characteristics and Significance

Citrus Huanglongbing (HLB) is one of the most important plant diseases affecting citrus. The causal agent of HLB is *Candidatus* Liberibacter, a gram negative bacteria not yet cultured to Koch's postulates. Symptoms include marked yellow regions on leaves and poor quality, small, inedible, and misshapen fruits (Halbert and Manjunath 2004). HLB has had serious damaging impact in the citrus industry in many Asian countries, wiped out a great number of trees in Brazil, and as of 2009, has seriously affected groves in 33 Florida counties making *Candidatus*

Liberibacter the most dangerous citrus pathogen in the world (Callaway 2008) and the most significant threat for the citrus industry worldwide (Bove 2006). HLB originated in Asia and was first detected in the Americas in 2004 (Brazil), reaching the United States in 2005 (Chung and Brlansky 2005). The devastating effect of HLB on citrus production in Africa, Asia, and the Americas has been well documented (Chung and Brlansky 2005), and the importance of this disease has triggered several reviews of HLB in countries such as Pakistan (Batool and others 2007), India (Das 2008), Malaysia (Hajivand and others 2009), as well as Brazil and US (Gottwald and others 2007). HLB affects most citrus varieties including 'Valencia' sweet orange. 'Valencia' orange is the most widely cultivated citrus variety in the world (Papadakis et al., 2008). Therefore HLB effects in this variety have the potential of causing serious economical loses. Since the appearance of HLB, many efforts have been directed towards controlling this disease including frequent pesticide spray to reduce psyllid population, the use of pheromone traps, and psyllid repellents. Early detection of HLB-infected trees is crucial to managing HLB's spread by reducing inoculums through tree elimination. Present methods for its quantification based on real time polymerase chain reaction are currently being optimized (Wen and others 2008). The disease is transmitted through a psyllid (*Diaphorina citri*) vector that feeds on citrus and other tropical and sub-tropical plants making vector eradication impossible.

Current Research on Detection Methods

Currently, PCR is the only approved method for diagnosis of HLB; however, this is an expensive, laborious, and time consuming alternative that does not allow in-field analysis. Other limitations such as the low concentration and uneven distribution of the bacteria in the tree (McClean 1970) make PCR detection very difficult, especially at early stages. Research on new methods such as the isothermal and chimeric primer-initiated amplification of nucleic acids

combined with cycling probe technology (Urasaki and others 2008), application of nested PCR (Kawai and others 2007), comparisons of primers for PCR and nested PCR (Ding and others 2007), along with the improvement of DNA isolation for conventional PCR (Gopal and others 2007a) has been reported. However, PCR amplification of the bacteria is very weak during spring and summer seasons (Gopal and others 2007b) increasing the probability of obtaining false negatives during these periods. Therefore, methods that do not rely on the presence of the bacteria in the sample are sought as more reliable throughout the year. To the best of our knowledge, only two non-PCR methods have been comprehensively researched. One relies on the presence of excessive amounts of starch (Takushi and others 2007a) whereas, the other on overproduction of gentisic acid (Hooker and others 1993) in infected trees. The disadvantages of these methods are that they are not HLB-specific, since excessive amounts of starch are noticed with other stress situations such as girdling (Li and others 2003) and gentisic acid overproduction is caused by other infections in several plants (Belles and others 2006). Taba et al., (2006) showed that the starch method has a 75% agreement with PCR in leaves and 95% agreement for other parts of the trees. Current research is focused on the quantification of the starch accumulation in HLB trees by using spectrophotometric methods (Taba and others 2006). This new approach may help to distinguish the starch accumulation due to HLB as opposed to starch accumulation due to other factors such as stress, zinc deficiency, and girdling (Li et al., 2003). These factors are very common and make starch detection by itself a less useful method.

Escherichia coli and *Salmonella* spp.

Escherichia coli and *Salmonella* spp. are Gram negative, facultative anaerobe bacteria. Some strains have flagella and are motile. Both are considered amongst the most important foodborne pathogens worldwide. *Escherichia coli* O157:H7 is considered a serious hazard to

public health in North America and Europe. Symptoms from *E. coli* O157:H7 infection range from mild, watery diarrhea to hemolytic uremic syndrome and hemorrhagic colitis. Several *E. coli* O157:H7 outbreaks in food have being reported over the years. Salmonellosis is also one of the most important food-borne diseases and causes substantial medical and economic burdens worldwide. Eggs, egg products, poultry meat, and pork are the most important sources of salmonellosis in humans. Implementation of hazard analysis and critical control points (HACCP) programs and detection methods are crucial for controlling food outbreaks. In conventional detection methods the contaminated food sample is suspended in an enriched media for 6–18 h and a portion of the broth is then plated on agar media and analyzed by biochemical tests and serological reactions which take 1–3 days. Another conventional technique is the most-probable-number (MPN). MPN is particularly useful for determination of low concentrations of bacteria. Here, triplicate samples or five replicates are prepared from 10-fold serial dilutions. The ratio of positive results to negative results is in relation to the concentration results in a MPN/g value. The MPN method assumes that bacteria are distributed randomly within the sample and separated from each other. The growth medium and incubation conditions have been chosen so that one viable cell multiplies and can be detected.

Objectives

The overall objective of this research was to determine the suitability of GC-MS, CE, and HPLC-MS based metabolomic techniques for the rapid diagnosis of HLB and detection of *E. coli* O157:H7, *Salmonella* Hartford, *Salmonella* Muenchen, and *Salmonella* Typhimurium. The central hypothesis was that HLB, *E. coli* O157:H7, *Salmonella* Hartford, *Salmonella* Muenchen, and *Salmonella* Typhimurium produce metabolites that can be used for their specific detection. The specific objectives are as follows:

1. To characterize the differences in the metabolite profile of HLB infected citrus leaves trough HPLC coupled to MS. The working hypothesis was that changes in the metabolite profile that take place during HLB infection can be quantified to determine the main compounds that are being created or consumed during either the tree's physiological response or during bacterial metabolism.

2. To use CE-PDA for untargeted analysis of plant metabolites and develop a CE method for characterization of possible citrus HLB biomarkers. The working hypothesis was that biomarkers that can be measured by CE are produced during HLB infection.

3. To find metabolic differences between leaves from HLB-infected, zinc-deficient, and healthy 'Valencia' orange trees from commercial groves as a first step to identify potential HLB biomarkers by combined GC-MS analysis of headspace and derivatized liquid extracts. The working hypothesis was that specific changes in metabolite profile of HLB-infected trees can be quantified by GC-MS.

4. To determine the suitability of CE, HPLC-MS, and GC-MS based metabolomic techniques for rapid and simultaneous detection of *E. coli* O157:H7, *Salmonella* Typhimurium, *Salmonella* Muenchen, and *Salmonella* Hartford in beef and chicken. The working hypothesis was that each pathogen produces a specific metabolite profile that can be used for rapid detection trough multivariate analysis.

Table 1-1. Most common metabolomics processes in food analysis

Sample: Purpose of analysis	Type	Extraction and preparation	Separation -Detection	Data treatment	Reference
Apples: light induced changes in peel	Untargeted/ discriminative	MeOH Derivatization for GC-MS	GC-MS LC-MS	PCA	(Rudell and others 2008)
Berries: polyphenol composition	Targeted/ informative	Acetic acid + water C18 and Sephadex LH 20 columns	LC-MS DIMS	Compound identification	(McDougall and others 2008)
Broccoli, mustard, and brassica: glucosinolates composition	Targeted/ informative	Hot water (90 ˚C) + sonication	LC-MS^n	Compound identification	(Rochfort and others 2008)
Broccoli: variety differentiation	Untargeted/ discriminative	Freeze dried MeOH + H_2O	LC-UV-MS DIMS	PCA, ANOVA	(Luthria and others 2008)
Cheese: Production control	Untargeted/ informative	-	IMS	Compound identification	(Vautz and others 2006)
E. coli: glycolisis metabolites	Targeted/ informative	Indirect thermal treatment	LC-MS	Compound identification	(Schaub and Reuss 2008)
Ginseng: variety Differentiation	Untargeted/ discriminative	Deuterated MeOH + buffered water	NMR	PCA	(Kang and others 2008)
Green tea quality	Untargeted/ predictive	Freeze dried MeOH +H_2O + $CHCl_3$	UPLC-TOF-MS	PCA, PLS	(Pongsuwan and others 2008)
Honey: Origen verification	Untargeted/ discriminative / predictive	Buffered water	NMR	PLS-GP	(Donarski and others 2008)
Maize: GMO identification	Untargeted/ discriminative	MeOH + Water + Ultrasonic	CE-TOF-MS	Student's t, PCA	(Levandi and others 2008)
Meat: Quality/safety	Untargeted/ discriminative	Neutral desorption	EESI-MS	PCA	(Chen and others 2007)

Table 1-1. Continued

Sample: Purpose of analysis	Type	Extraction and preparation	Separation -Detection	Data treatment	Reference
Olive oil: Origen differentiation	Targeted/ discriminative	SPME	GC-CI-MS	LDA Kruskal-Wallis and Wald-Wolfowitz tests	(Cavaliere and others 2007)
Pine mushrooms	Untargeted/ discriminative	MeOH +H_2O + $CHCl_3$	NMR	PCA	(Cho and others 2007)
Potato: GM differentiation	Untargeted/ discriminative	MeOH +H_2O + $CHCl_3$ Derivatization for GC-MS	GC-MS DIMS	PCA	(Catchpole and others 2005)
Potato: Identification of cultivars	Untargeted/ discriminative / informative	Freeze dried + MeOH + Water + Chloroform + Derivatization	GC-TOF-MS	ANOVA, PCA	(Dobson and others 2008)
Potato: Variety differentiation	Untargeted/ discriminative / informative	MeOH +H_2O + $CHCl_3$ Derivatization for GC-MS	GC-MS DIMS	RF	(Beckmann and others 2007)
Soybean: GMO differentiation	Untargeted/ informative	MeOH-EtOH-H2O	CE-TOF-MS	Compound identification	(Garcia-Villalba and others 2008)
Spinach: *E-coli* contamination	Untargeted/ discriminative	Neutral desorption	EESI-MS	PCA	(Chen and others 2007)
Tomato paste: changes during production	Targeted to antioxidants/ informative Untargeted/ informative	Targeted: H_2O-MeOH and MeOH-$CHCl_3$ Untargeted: Formic acid-MeOH-H_2O	LC-Antioxidant detector LC-TOF-MS	ANOVA, PCA	(Capanoglu and others 2008)
Tomato: metabolite correlations	Untargeted/ predictive	Volatiles: EDTA-NaOH-H_2O + SPME Sugars and organic acids: MeOH + Derivatization	GC-MS	PCA, LDA, CN	(Ursem and others 2008)

Table 1-1. Continued

Sample: Purpose of analysis	Type	Extraction and preparation	Separation -Detection	Data treatment	Reference
Tomato: variety Differentiation	Untargeted/ discriminative	Liophylizatio + MeOH + Sonication	LC –TOF-MS NMR	PCA	(Moco and others 2008)
Tomato: volatiles analysis	Targeted/ discriminative	EDTA-NaOH-H_2O + SPME	GC-MS	PCA, HCA	(Tikunov and others 2005)
Watermelon: Quality evaluation	Untargeted/ predictive	Buffered D_2O	NMR	PLS-LDA	(Tarachiwin and others 2008)
Wine: metabolite characterizat	Untargeted/ discriminative	Liophilized + buffered D_2O	NMR	PCA, PLS	(Son and others 2008)
Yeast: Aroma compounds production	Targeted/ discriminative	Diethyl ether	GC-FID	PCA, PLS	(Rossouw and others 2008)
Yeast: strain differentiation	Untargeted/ discriminative	Liophylizati + derivatization	GC-TOF-MS	PCA, HCA	(MacKenzie and others 2008)
Yeast: strain differentiation	Untargeted/ discriminative	-	NIR	PCA LDA	(Cozzolino and others 2006)

Table 1-2. Common number of peaks reported in food metabolomics[1]

Technique	Peaks reported	Main references
HPLC-UV	40 detected	(Defernez and others 2004)
UPLC-MS	1560 detected	(Pongsuwan and others 2008)
GC-MS	91 – 142 detected	(Beckmann and others 2007; MacKenzie and others 2008)
CE-MS	27 – 45 detected	(Garcia-Villalba and others 2008; Levandi and others 2008)
NMR	16 -20 identified	(Jahangir and others 2008; Son and others 2008)

[1] Table intended to provide general idea, since food matrix and extraction methods greatly influence the number of detected peaks.

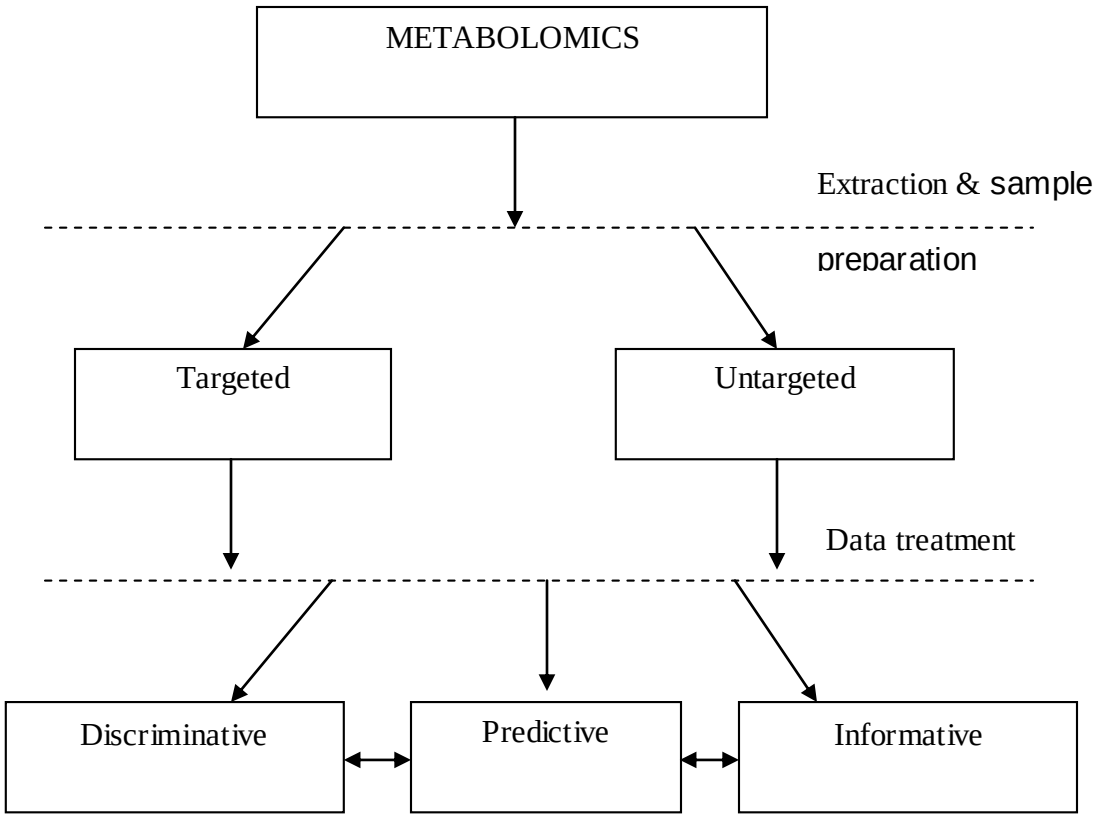

Figure 1-1. General metabolomics classification according to purpose of analysis.

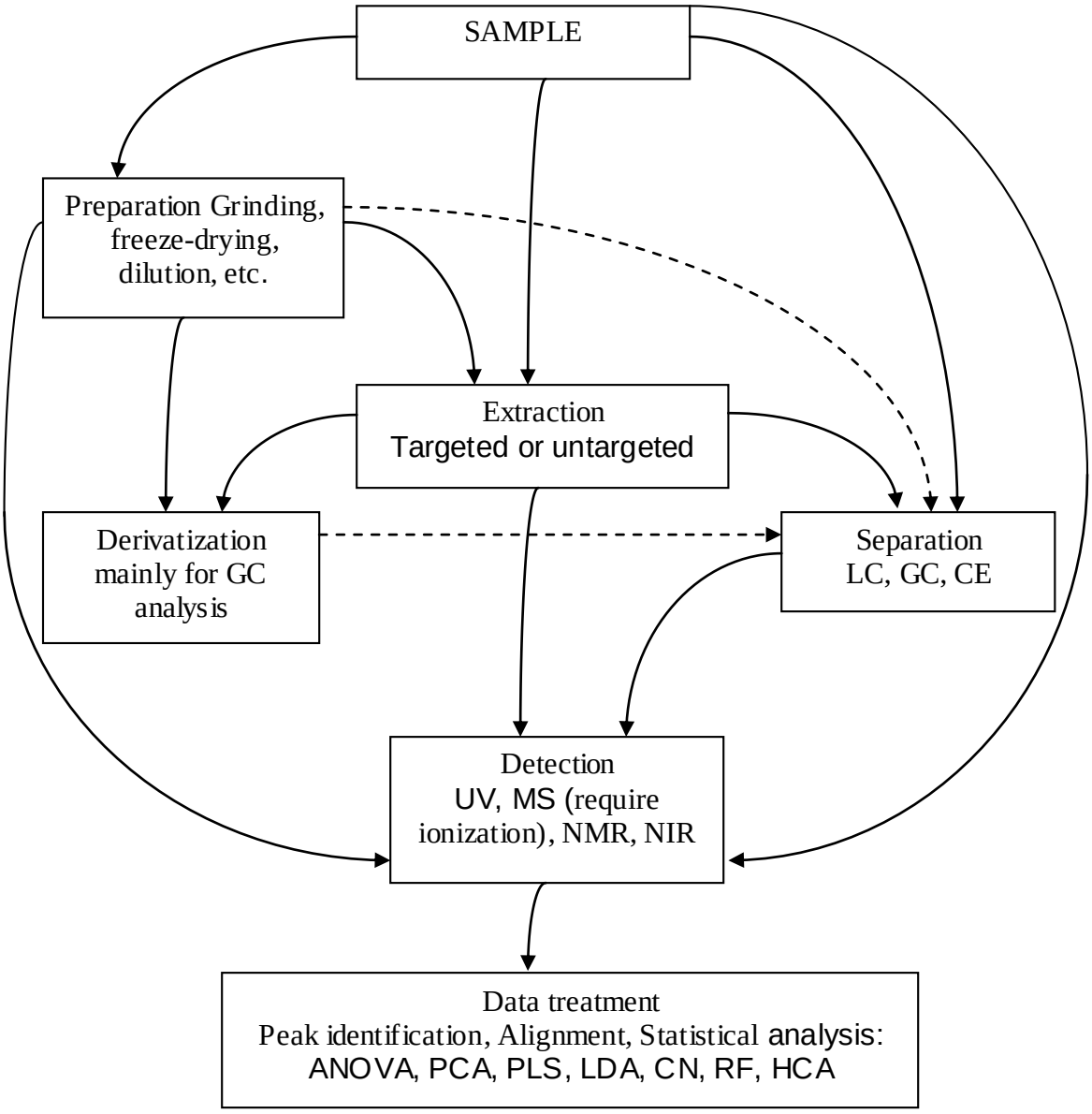

Figure 1-2. Metabolomics analysis process.

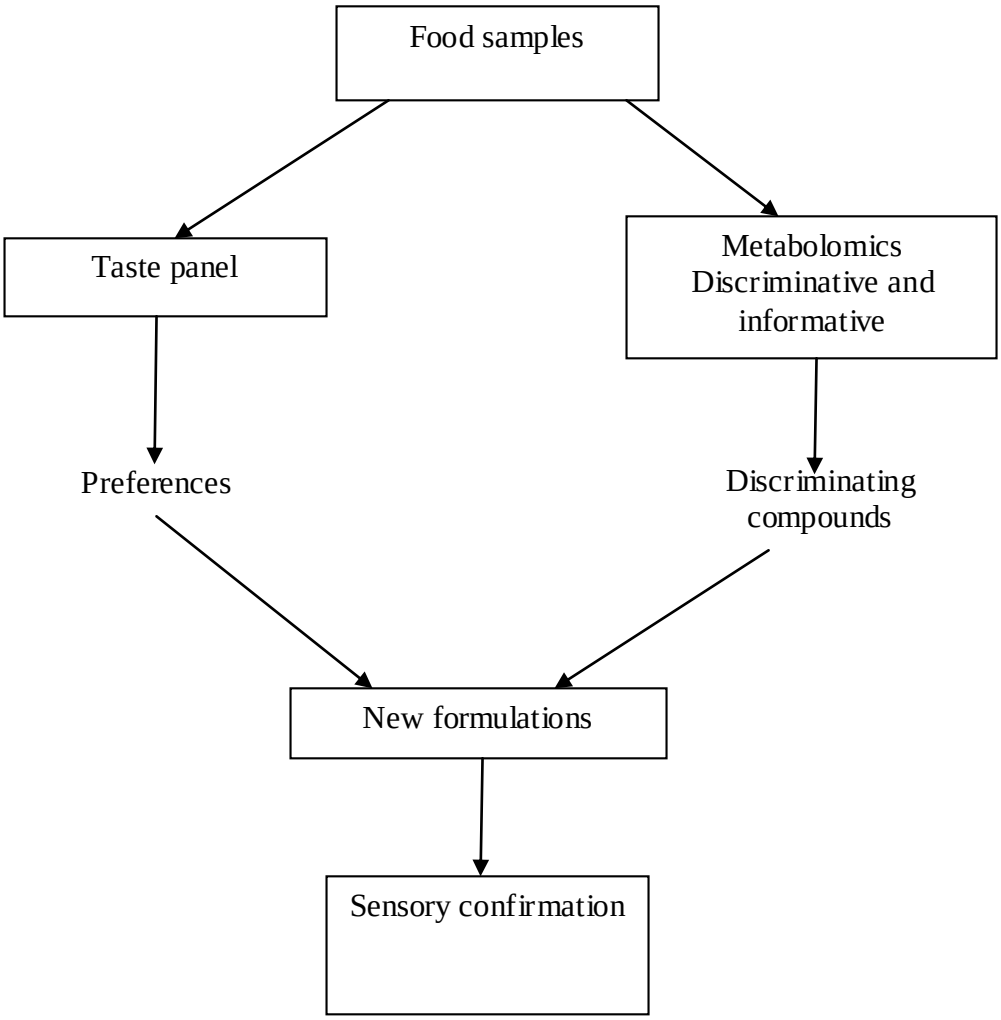

Figure 1-3. Metabolomics potential for understanding consumer preferences.

CHAPTER 2
HLB FINGERPRINTING BY HPLC-MS

Overview

Metabolite profiling is becoming a good alternative to consider when dealing with specific biomarkers in plants. Kell and other (2005) summarized this technique by stating the importance of their use for a better understanding of plant metabolites and disease diagnosis. Abu-Nada and others (2007) used GC-MS as a Metabolomics tool for the assessment of the effect of the *Phytophthora infestans* infection on the metabolites normally present in potato leaves finding that the concentration of approximately 42 metabolites significantly changed after bacterial inoculation. Glauser and others (2008) successfully used HPLC coupled to Nuclear Magnetic Resonance (NMR) as a Metabolomics tool for the detection of stress biomarkers in plant extracts; however, no research has been done regarding effects of HLB on citrus metabolites.

This part of the research characterized differences in the metabolite profile of HLB infected leaves trough HPLC- Mass Spectroscopy (MS) based on the hypothesis that changes in the metabolite profile occurring during HLB infection can be quantified to determine the main compounds that are being created or consumed during either the tree's physiological response or during bacterial metabolism. The identification of these key compounds will serve as a baseline for the development of a diagnosis technique. Main findings of this section were published in Proceedings of the Florida State Horticultural Society (Cevallos Cevallos and others 2008).

Materials and Methods

Equipment, Software, and Reagents

Methanol, chloroform, acetonitrile, and acetic acid were from Fisher (Fisher Scientific Inc., Miami, OK). The water bath was an Isotemp 3016s also from Fisher Sci (Miami, OK); the

HPLC system was composed of a Surveyor HPLC, autosampler, and PDA detector; the MS was a LCQ Advantage Ion Trap with ESI (electrospray ionization) as ion source, and the data was processed by using the Xcalibur 2.0 software.

The whole HPLC-MS-software system was from Thermo Scientific Inc. (Waltham, Ma). Color characterization was done by a Chroma Meter CR-331 from Minolta Co. (Osaka, Japan). PCR analysis was performed by the plant pathology group at the IFAS Citrus Research and Education Center (Lake Alfred, FL). Tandem MS was done at the Department of Chemistry at the University of Florida, Gainesville, FL.

Sampling and Experimental Design

Healthy (PCR negative) and diseased leaves (PCR positive) were sampled from 'Valencia' orange trees of the same age and same shoot (mostly summer and spring shoots from 10 year old trees) in a grove at Plant City, FL. Only three PCR positive trees were kept in the grove for analysis during that period of time. At least 3 leaves from different symptom intensities (infection value) per each infected tree were sampled each month from December 2007 to April 2008 and analyzed individually by HPLC – MS. The same sampling amount and frequency was applied for three healthy (PCR negative) trees.

For PCR analysis at least 3 extra leaves from various parts of the tree were sampled each month to make sure that all the healthy trees remained healthy during the sampling period. Samples were stored on dry ice during transportation (45 minutes), analyzed for color characterization, and then stored at -80 °C until processed (approximately 1 month). PCR analysis was also performed on individual leaves. Statistical analysis were performed by using SAS 9.0 from SAS Institute Inc. (Cary, North Carolina) to compare chromatograms from each infection value and significance was reported at levels of P lower than 0.05.

Infection Value and Color Analysis

An infection scale was created based on the appearance of diseased leaves. Large yellow areas and roughness are the usual sensory parameters that characterize the intensity of the symptoms in a leaf. The degree of infection was assigned by giving each leaf a number from zero to four in which zero represents a healthy looking leaf. The scale goes up if the yellowness and roughness of the leaves become more noticeable. Color characterization was performed by setting the colorimeter to perform averages of three different measurements in different parts of a leaf. The runs were consistently done in the middle, left, and right front parts of each individual leaf.

HPLC-MS Analysis

The solvent chosen for extraction was methanol due to its great extraction power. Chloroform was also used in small amounts to increase the extraction of more lipophillic compounds. Individual leaves removed from the storage were immediately weighed and ground under liquid nitrogen and an equal weight of chloroform was added and stirred in the water bath at 0 °C for one hour. Then an 80% methanol solution was added to achieve a 5:1 methanol: chloroform mixture. The solution was continuously stirred at 0 °C for 12 hours, filtered in a 0.45 µm nylon filter, and injected in the HPLC-MS system operating with a stationary phase C-18 column and a mobile phase consisting of 80% of an acetic acid solution (0.05% in water) and a 20% of acetonitrile (with 0.05% acetic acid also) during the first 12 minutes, then a gradient phase during the next 47 minutes was applied to reach a final concentration of 90% acetonitrile and 10% acetic acid solution which was held during the final 11 minutes. The MS worked with an electrospray ionization source and was operated in the 80 – 1000 m/z range.

Results and Discussion

Individual healthy leaves were analyzed monthly from December 2007 till April 2008 to determine seasonal variations in their metabolic profile. Disease suspected leaves were confirmed by PCR and positive samples were analyzed under the HPLC – MS method. Control (PCR negative) samples were also run under described conditions.

Infection Scale

An infection evaluation (IE) scale was created to determine the intensity of the symptoms and correlate it with the metabolites detected during the HPLC- MS analysis. The scale consisted of an integer number from 0 (healthy looking leaf PCR negative) to 4 (highest symptomatic level of a PCR positive leaf) and was assigned by agreement of the observers after a visual inspection. The scale goes from 0 to 4 and all the samples were PCR positive except for IE= 0. Figure 2-1 shows the IE values created for this experiment.

PCR positive leaves with symptoms more intense than an IE=4 (tentative IE=5) were also found; however these were not considered in this research due to their high similarity to characteristic zinc deficient leaves and the rarity of this type of sample. This may be due to the fact that some times HLB induces zinc deficiency, thus producing symptoms indistinguishable from genuine zinc deficiency (Bove 2006). HLB induced zinc deficiency should be further investigated. As we can see in Figure 2-1 the scale is mainly dictated by the amount of yellow and green tonalities, so the use of a color measurement technique might be helpful in order to more precisely quantify the scale. Table 2-1 shows the L* (lightness), a*(greenness), and b*(yellowness) values from the colorimeter.

As we can see in Table 2-1, no trend was found between the IE value and the color values. This may be due to the fact that different yellow and green tonalities can be seen in

leaves from the same infection scale value. Area values instead of L*, a*, and b* values may give a better correlation (we are currently working on this hypothesis by using machine vision techniques). For now the L*, a*, and b* values can only be used as a rough reference of the degree of infection of a certain leaf.

HPLC – MS Analysis

Approximately 900 compounds were detected in both, healthy and diseased leaves. Figure 2-2 shows typical chromatograms of healthy (Figure 2-2 A) and diseased leaves from different infection scale values (Figure 2-2 B, C, and D). We can also distinguish three zones of interest: The zone marked as 1 goes from the 5^{th} to the 14^{th} minutes, the zone marked as 2 goes from 20 to 30. Both zones show a significant increase ($P < 0.05$) in their peak areas with increasing IE value. Finally the zone marked as 3 goes from 50 to 70 minutes and shows a significant ($P < 0.05$) decrease in the peak area with increasing IE.

Samples of IE greater than 0 were PCR positive, whereas IE = 0 was PCR negative. No samples from IE = 1 were reported at this time, since these extreme value of IE is the most difficult to find during that time of the year. The variation of the total area of each of the zones of interest strongly correlates with the IE value. The correlation coefficients obtained were 0.92, 0.96, and -0.91 for the zones of interest 1, 2, and 3 respectively. Figure 2-3 shows these relationships. Future research will include the identification of the compounds shown in the zones of interest one and two and the comparison of the diseased metabolic profile with the ones from stressed PCR negative trees. Only some compounds from the zone of interest number three have been identified so far through library matching. These compounds are Chlorophyll (eluting at 55 minutes, especially noticed at IE=0) with molecular weight of 892.3 and its derivates such as chlorophyll a, b, and c with molecular weights of 892.3, 906.3, and 610.3 respectively eluting

at 55, 57, and 58 minutes. Mass spectrometry data is shown in Figure 2-4. The full MS scan is presented for samples of IE= 0 (Figure 2-4 A) and IE=4 (Figure 2-4 B). As we can see in Figure 2-4 most of the compounds show an increase in intensity when increasing the IE value whereas some others are being reduced. This correlates with the findings observed in Figure 2-2.

Another important issue is that HPLC-MS is not suitable for the detection of big molecules such as proteins and non-ionizable molecules such as sugars. Future research will include protein determination through 2D-electrophoresis and sugar characterization through derivatization followed by GC-MS or HPLC. Also mineral analysis performed by capillary electrophoresis is needed to determine if HLB causes deficiencies and in which stage.

In conclusion several metabolites showed a very strong correlation between their peak area and the infection scale. This shows a strong potential of the use of metabolomics for the development of a technique for detecting citrus Huanglongbing. More work is needed to establish the sugars and proteins profile of diseased trees and to compare these profiles with the ones obtained under other stress conditions. Also, more work is needed to match the spectra profile of each compound for identification.

Table 2-1. Color values of leaves at different perceived infection scale values. Each value represents the average and standard error of four different leaves

IE value	L*	a*	b*
0	34.82 ± 5.2	-6.81 ± 1.8	11.78 ± 1.2
1	53.19 ± 4.3	-19.35 ± 7.2	38.92 ± 6.2
2	37.90 ± 2.8	-7.76 ± 2.8	13.16 ± 3.8
3	47.11 ± 4.3	-14.14 ± 5.2	35.08 ± 5.9
4	45.78 ± 5.5	-10.12 ± 3.8	35.12 ± 5.2

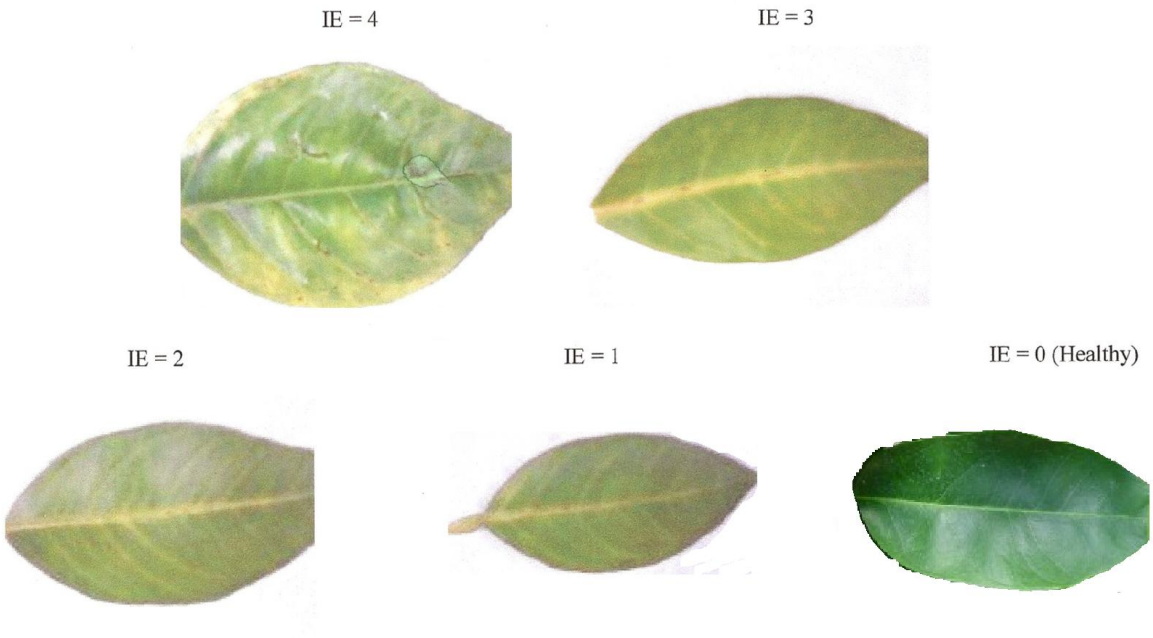

Figure 2-1. Infection evaluation values for citrus Huanglongbing samples.

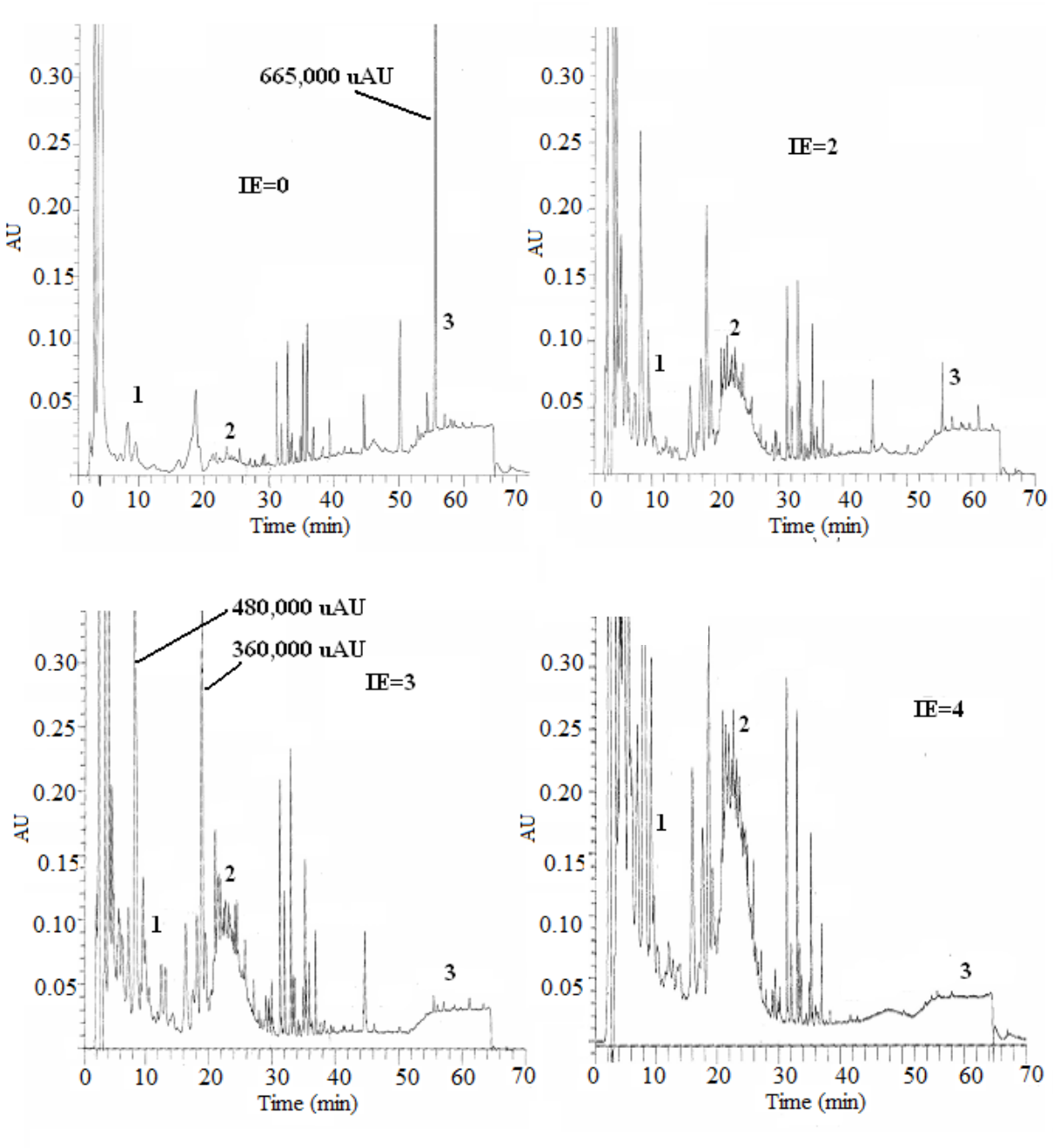

Figure 2-2. Chromatograms of orange leaves. A) Healthy leaf with IE=0, B) symptomatic leaf with IE= 2, C) Symptomatic leaf with IE=3, and D) symptomatic leaf with IE= 4. Numbers 1, 2, and 3 represent the zones of interest.

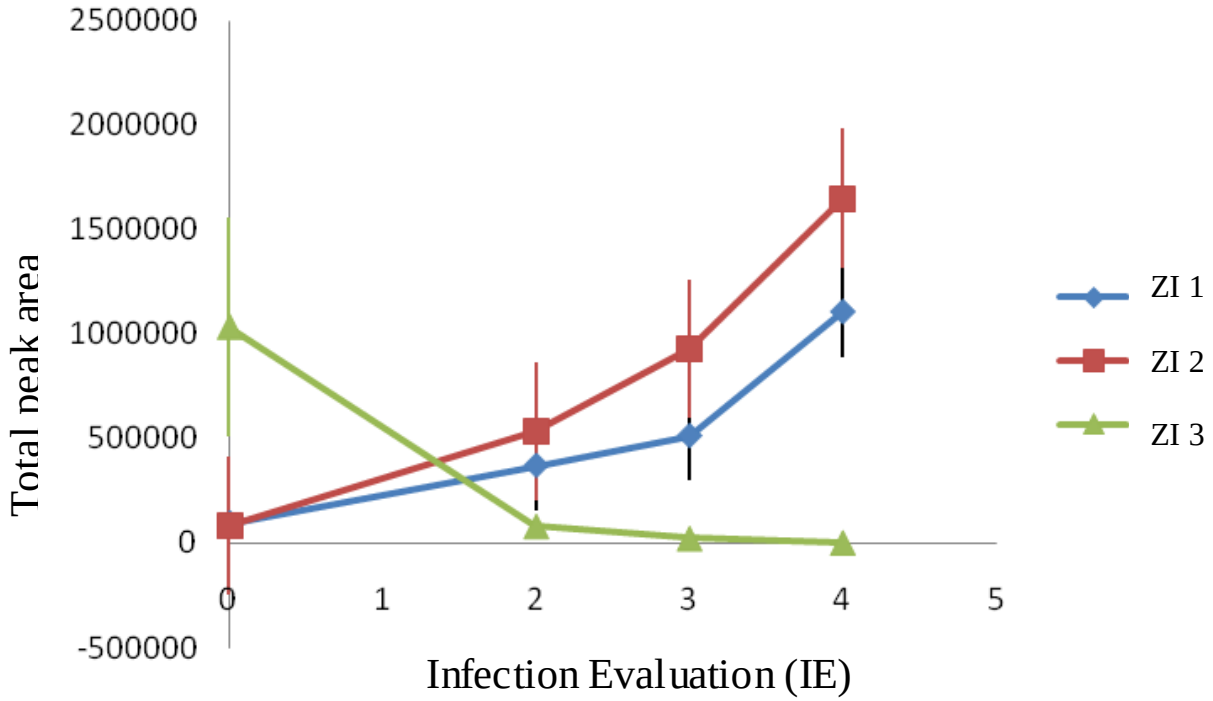

Figure 2-3. Cumulative peak areas per zone of interest (ZI) versus infection scale. The area values were obtained by adding all the peak areas within a zone of interest.

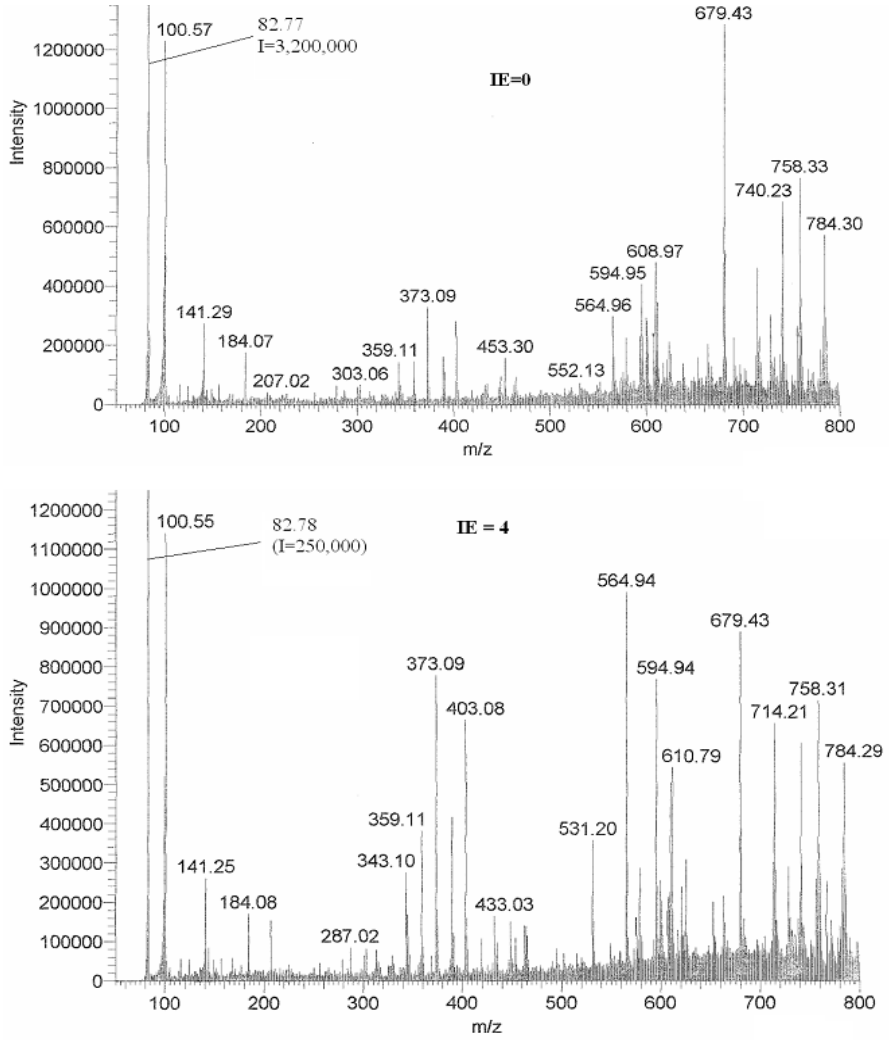

Figure 2-4. Full MS scan for a leaf with IE=0 (A) and IE=4 (B).

CHAPTER 3
HLB FINGERPRINTING BY CAPILLARY ELECTROPHORESIS

Overview

Capillary electrophoresis (CE) is one of the most used techniques for metabolomics-based fingerprinting. Several forms of capillary electrophoresis have been successfully used in targeted analysis (J. W. Jorgenson and Lukacs 1981; Negro and others 2003; Chen-Wen Chiu and others 2007; Erny and others 2007; Herrero and others 2007; Ravelo-Perez and others 2007; García-Villalba and others 2008). Untargeted metabolomics studies are very limited (Monton and Soga 2007), and in most cases involve the use of CE coupled to electrospray ionization-mass spectrometry (ESI-MS) system (Ramautar and others 2006). However, background electrolyte (BGE) formulations capable of being used in an ESI system are limited to low salts, and volatile components which do not necessarily provide the best separation efficiency (Monton and Soga 2007). Another limitation is the extra cost of the ESI-MS system.

Capillary electrophoresis coupled to photodiode array detector (CE-PDA) is a powerful and inexpensive separation tool. It has been successfully applied in untargeted analysis in mice (Vallejo and others 2008). For instance, Garcia-Perez and colleagues (2008), demonstrated the effectiveness of CE-PDA for non-targeted metabolic differentiation of *Schistosoma mansoni* infection in mice. Recently, untargeted CE-PDA analysis was successfully used for fingerprinting *Siraitia grosvenorii* for quality monitoring purposes (Hu and others 2008). However, to the best of our knowledge, there is no report on untargeted metabolomics analysis for fingerprinting of plant diseases using CE-PDA. The objective of this research was to use CE-PDA for untargeted analysis of plant metabolites and develop a CE-PDA method for

characterization of possible citrus Huanglongbing (HLB) biomarkers. Main findings of this section were published in Electrophoresis (Cevallos-Cevallos and others 2009a).

Materials and Methods

Reagents

HPLC-grade reagents (methanol, chloroform, and acetonitrile), sodium tetraborate decahydrate, 1- butanol, ethanolamide, sodium acetate, sodium phosphate, hexane, sodium dodecyl sulfate, naringenin, narirutin, hesperidin, tangeritin, synephrine, quercetin, gentisic acid, and ferulic acid were purchased from Fisher Scientific Inc. (Pittsburg, PA). Deionized, ultrafiltered water was used for all experiments. Pure reagents were run to determine their UV-Vis spectra in CE analysis.

Equipment and Software

The water bath used for all the experiments was an Isotemp 3016s from Fisher Scientific (Pittsburg, PA). The capillary electrophoresis system model P/ACE MDQ with PDA, the data acquisition and analysis software Karat 32 version 5.0 was from Beckman Coulter (Fullerton, CA). The capillary was bare fused silica from Polymicro Technologies (Phoenix, AZ) 50 µm ID, 56 cm total length (48 cm to the detector). Analysis of variance was carried out using SAS 9.0 from SAS Institute Inc. (Cary, NC) and significant differences were reported at 95% confidence level.

Sampling and Experimental Design

Healthy and diseased leaves were sampled from ‘Valencia’ orange trees of the same age and type of shoot (summer and spring shoots from 10-year-old trees) in a commercial grove in Plant City, FL. Sampling started 4 weeks after symptoms were discovered. However, as of 2008 there was no way to estimate when the trees were initially infected by HLB. Polymerase chain

reaction (PCR) analyses were outsourced to the Plant Pathology Laboratory at the University of Florida's Citrus Research and Education Center in Lake Alfred, FL. At least six leaves from three different PCR-positive and PCR-negative trees were sampled monthly from November 2007 to April 2008 to assess changes in metabolite profiles caused by uncontrolled changes in climate and other seasonal stress. After using some of the samples for PCR and CE optimization experiments, 36 HLB-infected and 18 healthy leaves were individually run under CE-PDA optimized conditions and statistically compared. Samples were stored on dry ice during transportation (45 min), and then stored at -80°C until analyzed (≈ 1 month).

Extraction, Sample Preparation, and CE Conditions Tested

Solvents with different polarities were tested in both healthy and diseased samples to maximize extraction of the compounds that showed significant differences between control and infected samples. Individual leaves (≈ 0.45 g) were ground to a fine powder under liquid nitrogen. Solvent was added to reach a final concentration of 4% w/v of ground tissue. Pure water, methanol, and chloroform were tested as solvents for extraction. The combination methanol/water/chloroform (MWC) used by Gullberg and others (Gullberg and others 2004) was also tested in an 8:1:1 ratio since a variety of compounds with different polarities was expected in this type of sample. The mixture was sonicated on ice for 30 min in a sonicator model FS20H from Fisher Scientific (Pittsburg, PA). Three extraction time-temperature combinations were tested: 12 h at 4°C, 12 h at 0°C, and 2 h at 60°C in the temperature-controlled water bath. After extraction samples were filtered using 0.45 µm nylon syringe filters and ferulic acid was added to a final concentration of 100 mg/L as internal standard (IS). Endogenous ferulic acid was not detected in either healthy or infected samples under tested conditions, and did not interfered with any peaks in the electropherograms. The effect of pH was tested by adjusting the aqueous borate

solution with 1 N NaOH or 1 N HCl as needed to reach pH values of 10.91, 9.30, 8.08, 6.51, 5.2, and 3.81. Sodium phosphate adjusted to pH 6.51 with 1 N HCl and sodium acetate adjusted to pH values of 5.2 and 3.81 with 1 N HCl were also tested as BGE to evaluate the influence of a buffering system at those low pH in which borate does not have buffering capacity. Phosphate and acetate buffers were prepared at half and three times the borate concentration, respectively, to keep ionic strength constant. Adjustment of pH was done before the addition of the organic BGE components. Organic modifier tested were acetonitrile (ACN) in the 0-30% range, and 1-butanol in the 0-15% range.

Selected Extraction and CE Analysis Conditions

Of the extraction and CE analysis conditions tested the following provided the best results. Extraction conditions required the use of MWC in an 8:1:1 ratio as solvent, followed by 30-min sonication on ice, and 12-h extraction at 0°C. Optimum BGE for capillary zone electrophoresis (CZE) consisted of 76% 11.2 mM sodium borate solution at pH 9.3, 15% ACN, and 9% 1-butanol (after sample addition sodium borate concentration was 8.5 mM). For non-aqueous capillary electrophoresis (NACE) BGE was composed of 15% ACN and 20 mM sodium acetate in methanol with no pH adjustment. For micro-emulsion electrokinetic chromatography (MEEKC) BGE contained 15% ACN, 0.8% hexane, 15% SDS, and 20% 1-butanol in a 10 mM Sodium borate solution pH 9.3 at 20 kV and 25°C. In all cases, separation was run at 20 kV and 25°C.

All solutions were prepared fresh weekly and kept at 4°C. Before the first use the capillary was conditioned with methanol for 10 min, 0.1 M HCl for 5 min, 1 M NaOH for 5 min, and BGE solution for 20 min with 2-min water rinsing between each solution. For CZE analysis the capillary was rinsed between runs with 0.1M NaOH for 2 min and BGE for 3min. For NACE

and MEEKC analysis rinsing was with deionized, ultrafiltered water (Ω > 18 MΩ.cm) for 2 min followed by BGE for 3 min.

Compound Identification

Compound identification was done by comparing UV spectra and migration time of each peak to those of pure standards. UV spectra of each peak were obtained by PDA analysis from 190 to 600 nm wavelength. Identification of compounds was done using the tool 'library search' from the 32 Karat software. This tool uses an algorithm that compares the UV spectra at the apex of each detected peak with the spectra of pure standards run under same conditions and stored in our internal library. A similarity value in the scale from 0 to 1 was obtained with each library match. Compound identity was obtained and reported in Table 3-1 only when the similarity value of the spectra comparison was 0.90 or higher and an increase in the size of the peak was observed when spiking the sample with the corresponding pure standard. Mobility of each peak was calculated by using synephrine and ferulic acid (IS) as reference analytes as described by Erny and Cifuentes (2007) because mobility values obtained by this approach are independent of the voltage and capillary dimensions (Erny and Cifuentes 2007)

Results and Discussion

Extraction

Extraction with water at 4°C and 60°C for 12 and 2 h, respectively, were compared to methanol and chloroform extracts at 0°C. Cold water (CW), hot water (HW), methanol, and MWC extracts were run under CZE conditions. HW and CW showed the lowest extraction efficiency since they each were only able to yield 10 peaks. Based on migration time and UV spectra comparison, the same compounds and 14 others were extracted with pure methanol.

However, methanol alone was not effective at extracting non-polar compounds. Typical electropherograms are shown in Figure 3-1.

Chloroform extracts were run under MEEKC conditions. Extraction with chloroform yielded nine peaks of which five were not detected with water and methanol based extractions according to UV spectra matching. No significant difference ($P > 0.05$) was found when comparing healthy and infected chloroform extracts. Therefore, pure chloroform as extraction solvent was not further considered. MWC extraction was selected for the remaining experiments because it showed the best extraction efficiency since the analytes detected included those obtained from water, methanol, and chloroform extracts alone based on UV spectra comparison.

Capillary Zone Electrophoresis (CZE)

Several CZE separation parameters were tested. Increasing pH values from 3.61 to 8.08 increased the analyte mobility as shown in Figure 3-2. This is due to the increase in the EOF caused by greater ionization of silanol groups in the capillary wall at high pH (Rabanal and others 2001). In contrast pH values of 9.3 and above decreased the mobility of most of analytes. This may be explained by the high flavonoids content in plant extracts, which usually have pKa-values above 8.5 (Herrero-Martínez and others 2005). Therefore, the net negative charge at those pH values may cause attraction to the opposite pole. The use of phosphate as BGE was also tested at pH 6.51. Phosphate concentrations tested were half borate concentration in order to test under same ionic strength. In the same manner acetate was also tested as BGE at pH values of 5.2 and 3.8. Acetate concentration was three times greater than borate concentration but equal ionic strength. Both buffers yielded separation and peak intensity very similar to that obtained by borate BGE at the same pH. As shown in Figure 3-2, sodium borate pH 9.3 resulted in the best

resolution and therefore, was chosen for further experiments. A similar effect was observed when changing the BGE concentration.

Sodium borate concentrations were tested from 5 to 75 mM. The best compromise between peak resolution and analysis time was with 8.5 mM sodium borate. High concentrations of sodium borate improved separation at the expense of the electro-osmotic flow (EOF) which decreased with increasing ionic strength as previously reported (Edwards and others 2006). However borate concentrations greater than 8.5 mM caused peak broadening and even disappearance of the late migrating peaks, possibly due to higher longitudinal diffusion and stronger interaction of analytes with the capillary at low EOF (Bohuslav Gas and others 1997). The use of ACN as an organic modifier in CZE and NACE has been widely studied (Loranelle L. Lockyear and others 2002; Belin and Gülaçarla 2007). However, optimal ACN concentration depends on the type of analyte and BGE used. Increase in ACN concentrations in the range of 0 to 30% caused increments in the migration time as shown in Figure 3-3. EOF reduction by ACN in aqueous BGE has been reported before (Loranelle L. Lockyear and others 2002; Belin and Gülaçarla 2007). According to Smoluchowski's equation, a decrease in permittivity and zeta potential will decrease EOF.

$$\mu_{eo} = \zeta\varepsilon/\eta \qquad (3\text{-}1)$$

where μ_{eo} is the electrosmotic mobility, ζ is the zeta potential, ε is the relative permittivity constant, and η is viscosity. A reduction of the zeta potential has been observed when increasing ACN. This is due to ACN absorption to the capillary wall which changes the electrical properties of the Stern layer and reduces the capillary surface charge (Schwer and Kenndler 1991). ACN has a low permittivity value of 35.94 (Marja-Liisa 2002) that lowered EOF. Also the lower permittivity constant of ACN may have caused changes in the dissociation equilibrium of the

dissolved compounds, thus modifying their electrophoretic properties. In contrast, ACN has a low viscosity of 0.341 MPa·s (Marja-Liisa 2002) accounting for a theoretical increase of EOF. However, the effects of permittivity and zeta potential changes are greater as indicated by the decrease in EOF shown in Figure 3-3. ACN also allowed a better separation between compounds and detection of a higher number of baseline resolved peaks, likely due to the increase in solubility of the organic analytes. The most efficient ACN concentration was 15%, as higher concentrations did not improve separation but increased total analysis time and baseline instability.

To further improve separation, MEKC was also evaluated by adding SDS from 5 to 120 mM (data not shown). SDS micelles did not improve separation at concentrations below 20 mM. Above 20 mM resolution, baseline stability, and repeatability were compromised. This same effect was also reported in previous MEEKC studies (Yin and others 2008). Because the addition of SDS did not enhance separation of our sample's compounds, addition of 1-butanol as second organic modifier was evaluated due to its lower polarity that may dissolve low polar compounds. Increasing 1-butanol increased analyte migration time but improved resolution and increased the number of detected peaks by four compared to BGE with no 1-butanol as shown in Figure 3-4. Decreased mobility was probably due to a decrease in EOF related to the higher viscosity of 1-butanol (Marja-Liisa 2002). Increase in the number of peaks may be due to additional partitioning of the analytes in the different zones of the BGE. 1-butanol has also been used in NACE and MEEKC modes (Yin and others 2008). However, its use in CZE is not very common. Up to 15% 1-butanol was tested but 9% was chosen because higher concentrations increased peak broadening probably due to longitudinal diffusion at low EOF (Herrero-Martínez and others 2005).

Non Aqueous Capillary Electrophoresis (NACE)

NACE was also tested because the nature of our analytes of interest was unknown. In addition, NACE is particularly suitable for analytes that are not readily soluble in aqueous BGE (Cunhong Li 2005). Concentrations of ACN ranging from 5 to 50 % in methanol were tested along with ethanol amide to increase the pH of the organic solution. Fifteen percent ACN and 20 mM ethanol amide provided the best results in NACE mode. Higher amounts of these compounds did not improve separation. EOF was reduced by increasing ionic strength with sodium acetate 20 to 50 mM to provide better separation. Sodium acetate resolved the peaks at 20 mM or higher as shown in Figure 3-5. However, fewer peaks were detected by NACE in a 90-min run compared to CZE and none of the compounds detected in NACE were absent in CZE (compared by UV spectra of standard compounds run under both conditions). This suggests that presence of water in the BGE improves the solubility of some important analytes. Also, water increases the permittivity constant of the BGE. Higher relative permittivity facilitates ionic dissociation and increases EOF (Marja-Liisa 2002). In NACE systems ion-ion interactions are stronger and, in the cases where the relative permittivity of the BGE is below 10, ions tend to pair to neutral species making separation more difficult (Marja-Liisa 2002).

Micro Emulsion Electro Kinetic Chromatography (MEEKC)

A common MEEKC BGE consists of a micro emulsion of hexane in a sodium borate aqueous solution aided by high concentrations of SDS and 1-butanol (Yin and others 2008). Optimum proportion of these reagents varies with the type of analyte. In this case no separation was observed with SDS concentrations below 15%. Higher concentrations increased analysis time to over 90 min. Concentrations of 1-butanol below 20% yielded poorer emulsion stability and lower repeatability. Therefore, 15% SDS and 20% 1-butanol were chosen to form the

microemulsion with a 10 ppm sodium borate solution. Higher concentrations of sodium borate increased migration time and did not provide additional resolution. Hexane concentration was 0.8%, and as previously reported (Yin and others 2008) its variation did not cause significant changes (data not shown). No significant differences ($P > 0.05$) were found in chloroform extracts. Therefore, all further analyses were run using MWC extracts for which CZE gave the largest number of peaks and highest resolution.

Selection of UV Wavelength

For comparison purposes 190 nm was chosen because most analytes of interest showed one maximum absorbance peak at this wavelength. Higher wavelengths are usually more specific (Monton and Soga 2007), and thus, very useful for targeted metabolomics analysis. However, assessing untargeted differences in biological systems require the detection of as many metabolites as possible. For NACE experiments the wavelength used was 205 nm because this is the UV cutoff of methanol in the BGE.

Compound Identification and Statistical Analysis

A series of standards compounds were dissolved in the MCW mixture to simulate the sample preparation and run under the selected CZE conditions. A total of 36 HLB-infected and 18 healthy samples were run and peaks were automatically identified by the tool 'library search' based on UV spectra and confirmed with migration time and UV spectra of pure standards run individually and by spiking the sample. Electropherograms were aligned before performing statistical analysis as recommended by Garcia-Perez (García-Pérez and others 2008). Alignment was performed using the 'Align' tool in the 32 Karat software. Reference peaks for alignment were selected based on peak identity (migration time and UV spectra). We found this tool to be particularly suitable when a low number (e.g. 35 or lower) of peaks are detected with no major

deviations in migration times. Normalization was done to the IS since other normalization methods usually give comparable results (García-Pérez and others 2008). ANOVA of 36 PCR-confirmed infected and 18 PCR-confirmed healthy samples revealed six compounds present in significantly higher ($P < 0.05$) concentrations in HLB-infected samples. Three of these compounds were identified by mobility and UV spectra as hesperidin, naringenin, and quercetin by our internal database and using pure standards. However, unlike in orange juice, quercetin has not been previously detected in orange leaves by other methods. These six compounds were always in significantly ($P < 0.05$) higher concentrations (154% or higher increase) in the infected samples as shown in Figure 3-7. Similar results have been reported for other plants since Abu-Nada and others (2007) found a much higher proportion of compounds being up-regulated than those down-regulated in potato infected with *Phytophtera infestans*. In this study, we provide evidence that HLB induces production of hesperidin, quercetin, and naringenin. Hesperidin has been reported to increase in orange leaf during blight induced zinc deficiency (Manthey and others 2000) suggesting its participation in the plant response-to-stress mechanism. Also HLB may possibly be causing mineral deficiency trough phloem obstruction in infected trees. However, this type of HLB secondary effects need to be further researched. Naringenin (Aziz and others 1962) and quercetin (Mamani-Matsuda and others 2004) have been reported to have microbial inhibition properties. Therefore, the plant probably synthesizes these compounds as a defense mechanism against pathogens. Gentisic acid (free form) concentration was not significantly ($P > 0.05$) different as shown in Table 3-1 suggesting that it may not be an effective biomarker. However, the glycoside form of gentisic acid previously reported to increase in HLB infected trees (Hooker and others 1993) was not detected in this analysis probably because of its low UV-Vis absorbance. Table 3-1 summarizes the migration time, mobility, wavelengths of

maximum absorbance, and the potential identity of each detected metabolite. While spectra allowed tentative identification of hesperidin, naringenin and quercetin, three other potential biomarkers remain unknown. Other analytical tools are required for their positive identification.

Table 3-1. List of the 24 peaks and IS detected by optimum CZE conditions as described in Figure 3-6. Compounds marked with a * showed significant difference ($P < 0.05$) between healthy and diseased samples

Compound No.	Migration time (min)	Mobility $(cm^2V^{-1}min^{-1})10^{-3}$	λ_{max} (nm)	Possible compound
1	7.01	19.17	227	Unknown
2	7.31	18.38	190, 234 low	Synephrine
3	7.51	17.89	190, 210, 330	Tangeritin
4*	8.43	15.94	204, 280	Hesperidin
5	8.63	15.57	205, 286	Narirutin
6*	9.41	14.28	190, 250, 350	Unknown
7	10.13	13.26	190, 210	Unknown
8*	10.48	12.82	196, 310	Naringenin
9	10.73	12.52	190, 210, 230	Oxalic acid
10	11.06	12.15	230, 275	Unknown
11	11.71	11.47	212, 278	Unknown
12	11.98	11.21	224, 280	Unknown
13*	12.34	10.89	220, 278, 320	Quercetin
IS	12.65	10.62	210, 300	Ferulic Acid
14	13.39	10.03	200, 310	Gentisic Acid
15	16.44	8.17	190, 220	Unknown
16*	16.62	8.08	227	Unknown
17	17.25	7.79	190, 220	Unknown
18	17.52	7.67	229	Unknown
19	18.88	7.11	228	Unknown
20	18.75	7.16	230	Unknown
21	19.54	6.87	225	Unknown
22*	20.24	6.64	231	Unknown
23	22.07	6.09	227	Unknown
24	24.75	5.43	234	Unknown

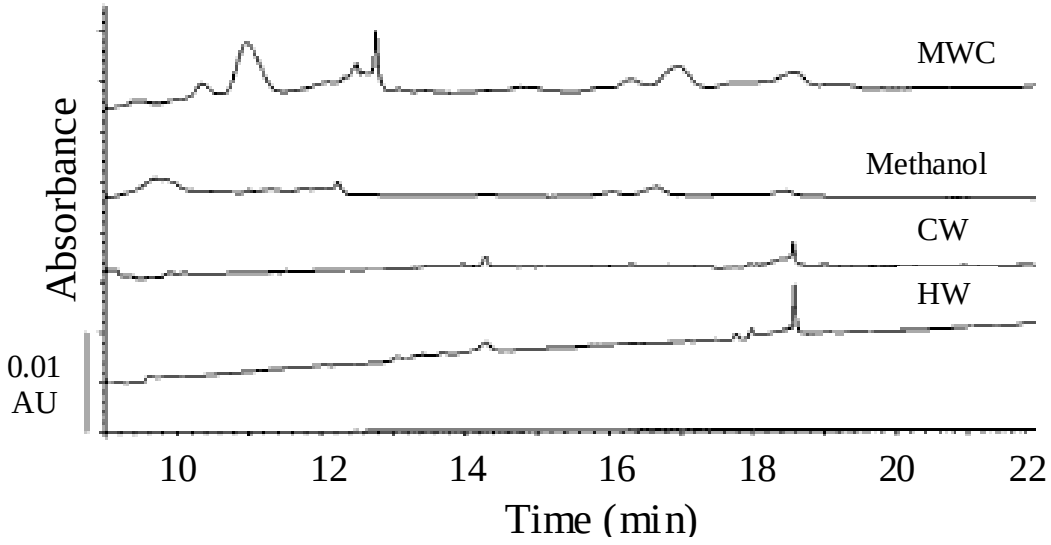

Figure 3-1. Electropherogram at 190 nm of CW, HW, methanol, and MWC extracts of 'Valencia' orange leaves. BGE: 8.5 mM sodium borate, 15% ACN, and 9% 1-butanol pH 9.3 at 20 kV, 10 s sample injection.

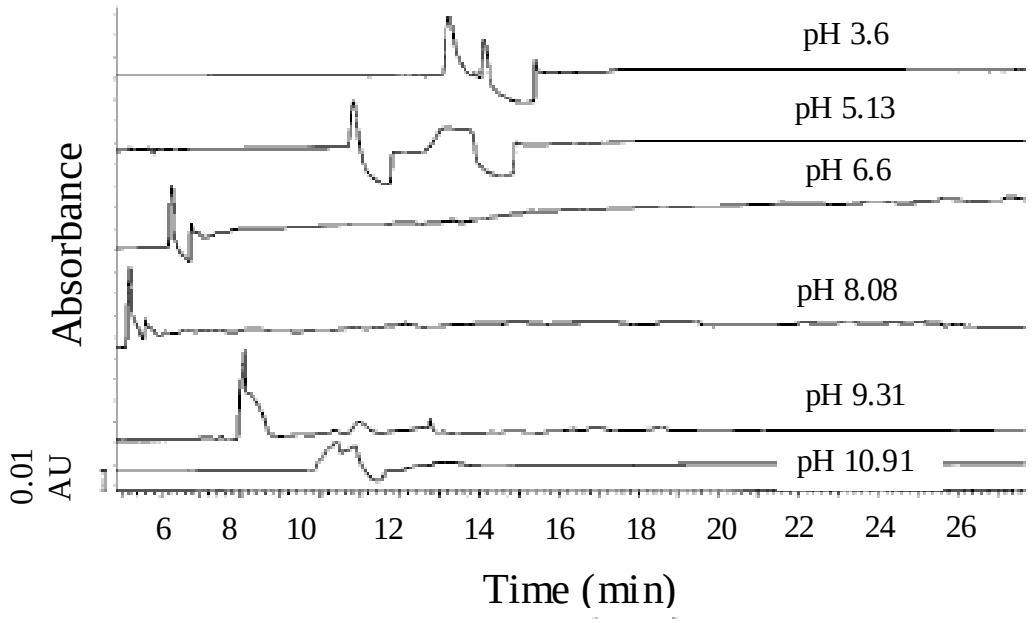

Figure 3-2. Effect of the pH in separation. Electropherogram at 190 nm of MWC extracts of 'Valencia' orange leaves. BGE: 8.5 mM sodium borate, 5% ACN, 10 s sample injection, 20 kV, 25 ˚C.

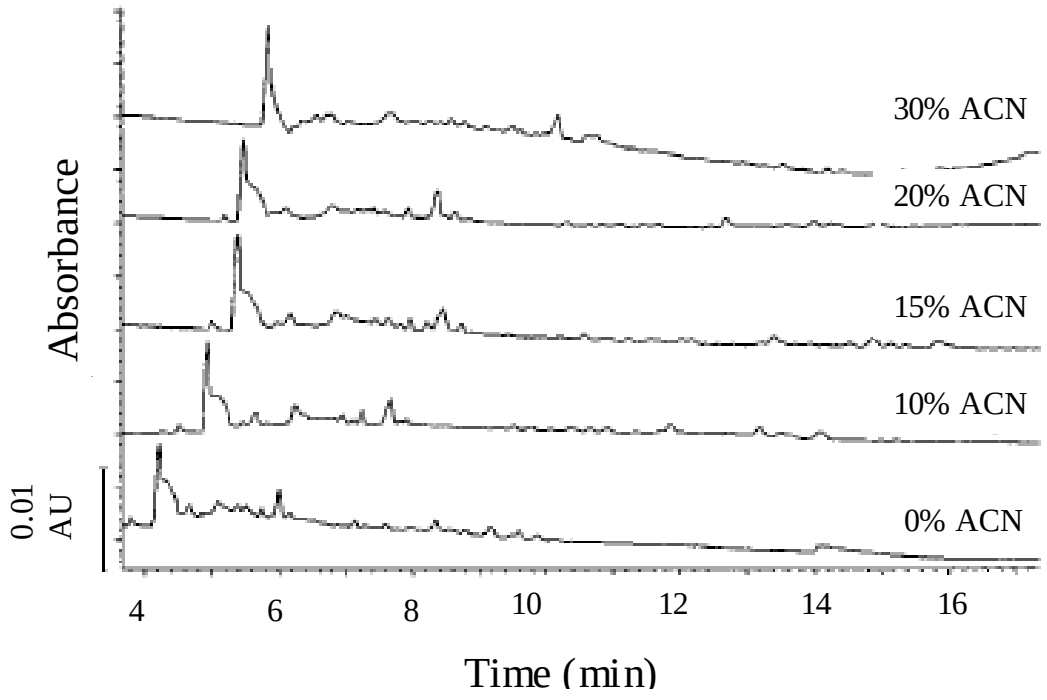

Figure 3-3. Effect of ACN in separation by CZE. Electropherogram at 190 nm of MWC extracts of 'Valencia' orange leaves. BGE: 8.5 mM sodium borate, pH 9.30 at 20 kV, 25 ˚C, and 10 s sample injection.

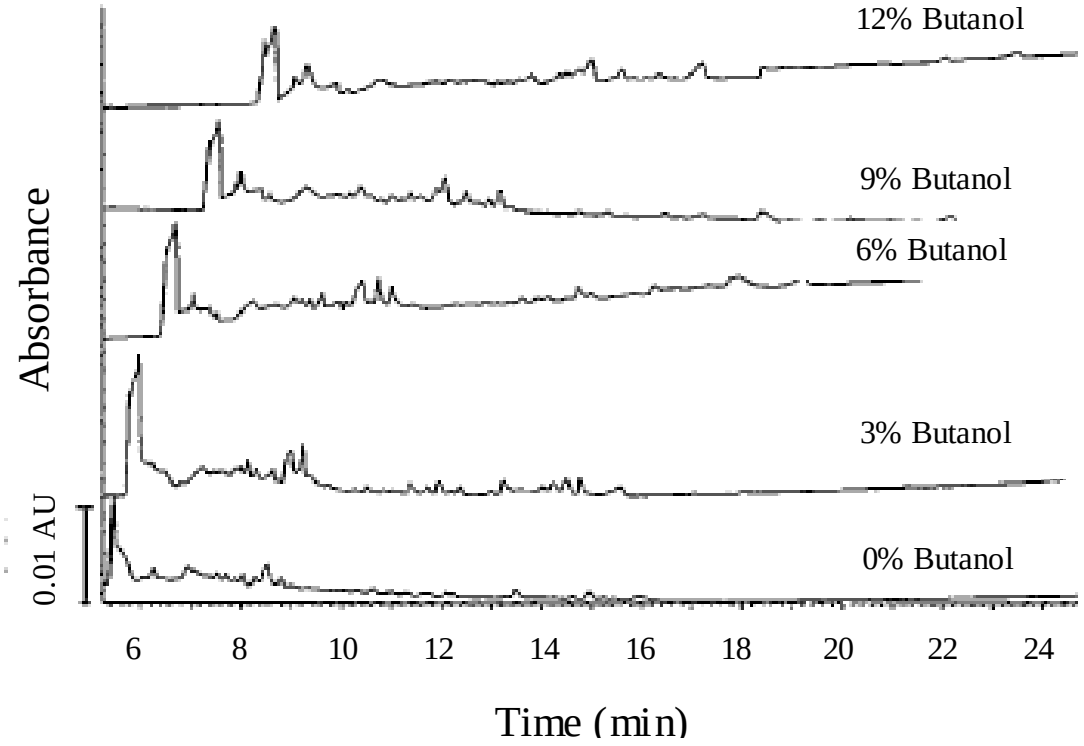

Figure 3-4. Effect of 1-butanol in separation by CZE. Electropherogram at 190 nm of MWC extracts of 'Valencia' orange leaves. BGE: 8.5mM sodium borate, 15% ACN, at 20kV, 25 ˚C, and 10 s sample injection.

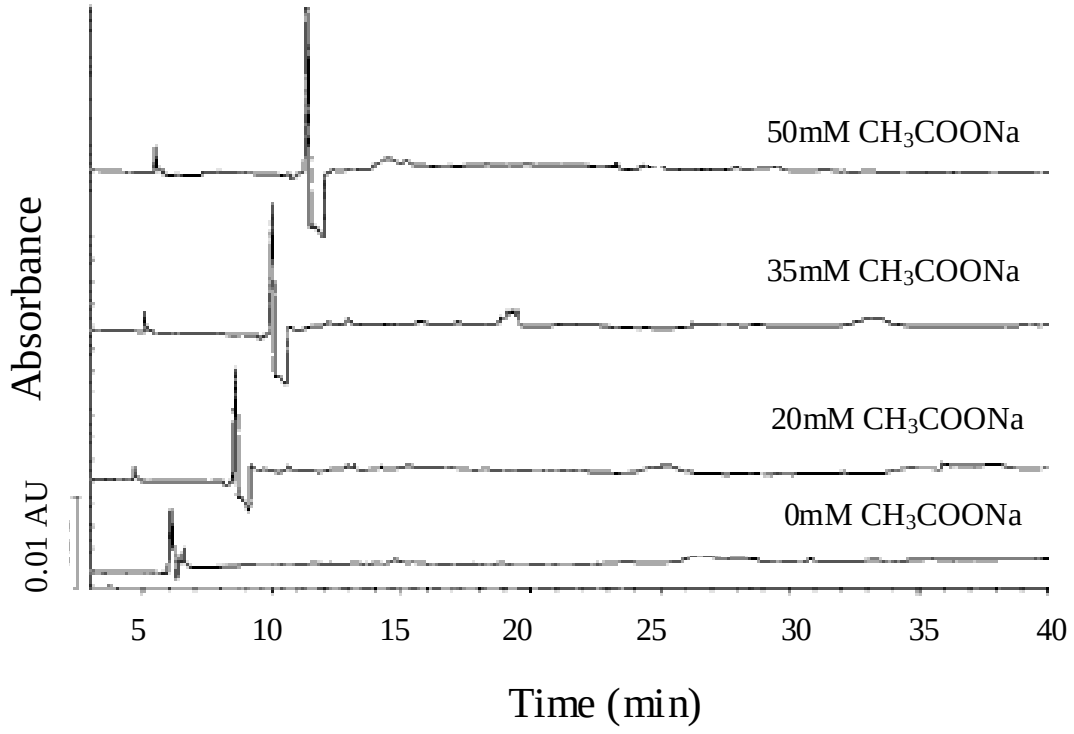

Figure 3-5. Effect of sodium acetate in separation by NACE. Electropherogram at 206 nm of MWC extracts of 'Valencia' orange leaves. BGE: 15% ACN, 20 mM ethanol amide in methanol at 20 kV, 25 ˚C, and 10 s sample injection.

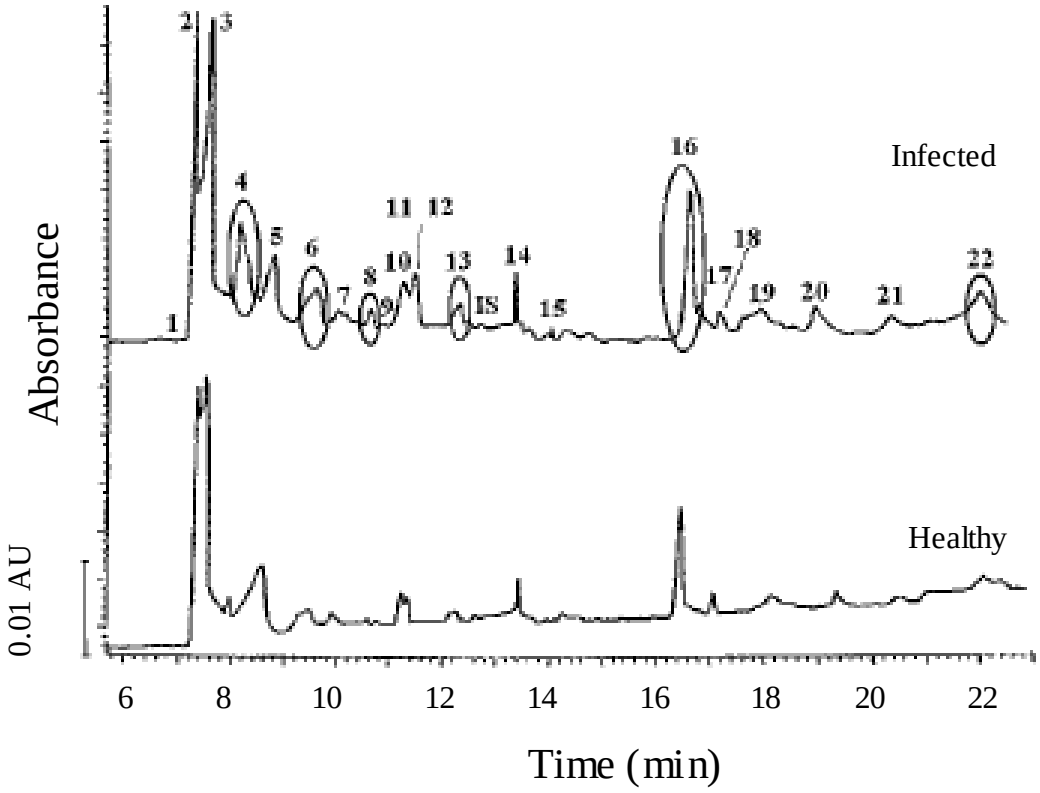

Figure 3-6. Typical electropherograms of healthy and infected leaves extracts run with BGE: 8.5mM sodium borate (pH 9.3), 15% ACN, 9% butanol at 20kV, 25 ˚C, and 10 s sample injection. Significant difference was found in the circled compounds. Peak numbers correspond to compounds in Table 3-1.

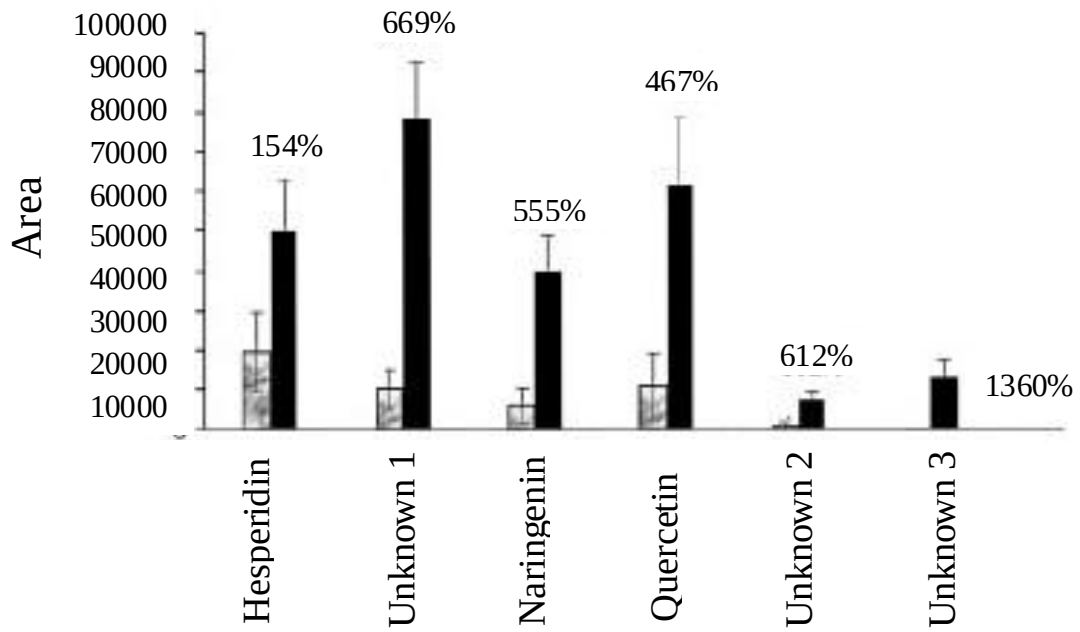

Figure 3-7. Mean concentration (in area units) of the 6 compounds found to be significantly different in healthy (□) and infected (■) samples. Error bars represent standard errors of 36 infected and 18 healthy samples extracted and run under the optimized CZE conditions. Percentages represent the mean increment of each compound in infected samples.

CHAPTER 4
HLB FINGERPRINTING BY GC-MS AND COMPARISON WITH ZINC DEFICIENCY

Overview

Previous metabolite profiling reports of healthy and Huanglongbing (HLB) infected leaves of sweet orange by techniques based on HPLC-MS (Cevallos-Cevallos and others 2008; Manthey 2008) and capillary electrophoresis-photo diode array detector (Cevallos-Cevallos and others 2009c) reported several flavonoid-type compounds and hydroxycinnamates as possible biomarkers. However, samples with zinc deficiency (ZD) were not analyzed and specificity of suggested biomarkers was not reported. Besides HPLC and capillary electrophoresis, metabolomic analyses by GC-MS provide an alternative for detecting less polar and low molecular weight compounds (Fancy and Rumpel 2008) and have been shown to be a powerful tool for metabolite profiling in plants. This kind of studies are considered as targeted metabolomics because they are aimed at those compounds detectable by GC-MS (Cevallos-Cevallos and others 2009b; Sawada and others 2009). Additionally, metabolomic discrimination in plants can be achieved without the need of compound identification (Hall and others 2002) increasing differentiation power by including unknowns compounds. Abu-Nada (2007) suggested specific metabolite variations during several stages of *Phytophthora infestans* infection in potato leaf extracts using GC-MS. Additionally, metabolite changes due to stress conditions such as drought (Semel and others 2007) and wounding (Yang and Bernards 2007) have been quantified by GC-MS analyses of tomato and potato plants, respectively. In most of the GC-MS-based metabolomics reports, liquid extracts have been dried and derivatized prior to analysis in order to increase detection of more polar compounds (Fancy and Rumpel 2008). However, drying may cause substantial losses of the highly volatile compounds usually found in

the sample's headspace. These highly volatile compounds have been shown to play an important role in metabolite profiling of several plant stresses and diseases (Tikunov and others 2007). As an example, GC-MS profiling of headspace metabolites allowed differentiation of two fungal diseases in mangoes (Moalemiyan and others 2007). Similarly, headspace analysis of tomato plants permitted determination of changes in metabolites occurring after infection with tomato mosaic virus (Deng and others 2004). In spite of the importance of these two types of analysis, no combined headspace and liquid extracts analyses of plant diseases have been reported. Additionally, no GC-MS-based metabolomic studies of HLB, as well as no comparison between HLB and zinc deficiency in sweet orange leaves have been reported. The objective of this study was to find metabolic differences between leaves from HLB-infected, zinc-deficient, and healthy 'Valencia' orange trees from commercial groves as a first step to identify potential HLB biomarkers by combined GC-MS analysis of headspace and derivatized liquid extracts. Main findings were published in Phytochemical Analysis (Cevallos-Cevallos and others 2010).

Materials and Methods

Reagents

HPLC grade reagents, L-proline, L-threonine, L-alanine, arabitol, inositol, butanedioic acid, methoxyamine hydrochloride (20mg mL^{-1}) in pyridine (Styczynski and others), and *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) were purchased from Fisher Scientific Inc. (Pittsburg, PA). Trans-Caryophyllene, isocaryophyllene, and α-humulene were from Sigma-Aldrich (Saint Louis, MO). All other reagents were from Fisher as well.

Equipment and Software

The GC model HP 5890 coupled to an HP 5971 series quadrupole mass spectrometer with ChemStation B.02.02 data acquisition software and the Wiley 138K mass spectral database

were from Hewlett Packard (Palo Alto, CA). Sensitivity and reproducibility of liquid extracts and headspace volatile analyses was maintained by regular cleaning of the ion source (approx once a month) and verified by daily running known concentrations of pure standards in the GC-MS. The chromatographic column used for both headspace and liquid extract analyses was a DB5-MS 60 m x 0.25 mm x 0.25 µm (length x I.D.x film thickness) from J & W Scientific (Folsom, CA). The water bath (model Isotemp 3016s) and sonicator (model FS20H) were from Fisher Scientific (Pittsburg, PA). Data was aligned to correct deviations in retention time by using MetAlign software (www.metalign.nl) and normalized to the total area prior to principal components analysis (PCA). Compounds were tentatively identified as described in the materials and methods section prior to analysis of variance (ANOVA). PCA was run to compare the overall metabolite profile of the samples and ANOVA was run to determine significance of individual compounds. ANOVA of peak areas was performed for each detected compound in all samples and for all analyses. PCA and ANOVA were carried out using MATLAB R2008a from The MathWorks (Natick, MA) and significant differences were reported at 95% confidence level. Both PCA and ANOVA were run on the peak intensities, similar to what was done in previous studies (Abu-Nada and others 2007; Moalemiyan and others 2007; Semel and others 2007)

Sampling and Experimental Design

'Valencia' sweet orange was chosen for this study because it is the most widely cultivated citrus variety in the world. Leaves of healthy and symptomatic HLB-infected (PCR positive) 'Valencia' sweet orange trees were sampled in commercial groves located in Plant City (grove 1) and Lake Alfred (grove 2), FL and leaves from trees showing ZD were collected from research trees at a University of Florida's Citrus Research and Education Center (CREC) grove

in Lake Alfred, FL. HLB-infected samples were taken approximately 3 months and 3 weeks after symptoms were first noticed for groves 1 and 2 respectively. All sampled leaves were from trees of the same age and similar shoots (spring and summer shoots from 10-year-old trees). Samples were kept on dry ice during collection and transport (45 min approximately), and then stored at -80°C until analyzed (approximately 2 months). PCR analyses were run to confirm HLB infection and were outsourced to the Plant Pathology Laboratory at the CREC in Lake Alfred, FL. At least six leaves from three different PCR-positive, PCR-negative (healthy), and ZD-affected trees were sampled monthly from November 2007 to October 2008 to assess seasonal variability. After using some of the samples for PCR analyses and GC-MS optimization experiments, 36 HLB-infected, 28 ZD-affected, and 18 healthy leaves were individually analyzed.

Extraction Conditions

Extraction conditions were similar to those previously reported for HLB metabolite profiling in capillary electrophoresis (Cevallos-Cevallos and others 2009c) with some modifications. Briefly, individual leaves (≈ 0.45 g) were ground to a fine powder with liquid nitrogen. Approximately 15 mg of L-threonine were added as a first internal standard (IS1). Solvent was to a final concentration of 4% w/v of ground tissue. Solvent was a methanol/water/chloroform combination suggested by Gullberg et al. (2004) in a 8:1:1 ratio and was added within one minute of grinding to stop degradation reactions. The mixture was sonicated on ice for 10 min. The extraction was done overnight at 0 °C in the temperature-controlled water bath. After extraction samples were filtered using 0.45 µm nylon syringe filters and (E,E,)-2,4 nonadienal was added to a final concentration of 800 mg L^{-1} as second internal standard (IS2). IS1 and IS2 were added as quality control of the derivatization and headspace

extraction respectively as well as to assure adequate GC-MS analysis and library matching. Endogenous IS1 and IS2 were not detected in either sample category under tested conditions, and did not interfere with any peaks in the chromatograms. Extraction with pure chloroform and pure methanol overnight at 0 °C were also tested but yielded significantly less peaks than the combination methanol/water/chloroform described above and therefore were not used for this study (see results and discussion section).

Headspace Analysis

A solid phase micro-extraction (SPME) fiber 50/30 µm DVB/Carboxen ^{TM}/ PDMS StableFlex ^{TM} for manual holder 57328-U from Supelco (Bellefonte, PA) was conditioned prior to its first use at 270 ºC for 1 h, and at the start of every day at 240 ºC for 5 min. Fifteen milliliters of the samples extracted with methanol/water/chloroform 8:1:1 were transferred to a 50 mL vial and equilibrated at 40 ºC for 30 min while stirred. The pre-conditioned SPME fiber was exposed to the headspace of the equilibrated samples for 40 min at 40 ºC and then splitlessly injected into the GC-MS. The injector temperature was 240 ºC, the oven was initially held at 55 ºC for 1 min, the temperature rate was 7 ºC min^{-1}, and the final temperature was 260 ºC held for 5 min. Ultrapure hydrogen was used as the carrier gas at 1 mL min^{-1}. The MS was tuned to maximum sensitivity in electron impact mode, positive polarity, and the total ion current was recorded for a mass range of 25-650 amu. The GC-MS interface was set to 318 ºC. The scan was recorded after a solvent delay of 8 min with scan frequency of 4 s^{-1}.

Liquid Extract Analyses

For liquid extracts, 180 µL were transferred into a 1-mL GC vial and dried under a nitrogen flow. MOX was added (30 µL) to the dried extract and allowed to react for 17 h at room temperature. Other combinations of time and temperature showed lower reproducibility as

reported in similar studies (Gullberg and others 2004). After methoximation with MOX, silylation reactions were induced by adding 80 µL of MSTFA for 2 h at room temperature. Other times and amounts of MSTFA yielded lower number of detected peaks and poorer reproducibility (see results and discussion section). Volumes of 0.3 µL of derivatized sample were splitlessly injected into the GC-MS. The injector was at 250 ºC, the initial oven temperature was 70 ºC held for 1 min, the temperature rate was 10 ºC min^{-1}, and the final temperature was 315 ºC held for 10 min. After 8 min of solvent delay the total ion current of mass fragments in the range of 50 – 650 amu was recorded. Other MS conditions were identical to that used for headspace analysis.

Compound Identification

Mass spectra obtained were visually observed at the beginning, middle, and end width of each peak to detect coelution. No coelution was detected in any of the peaks. Compound identification was done by library matching of mass spectra using the Wiley library and our internal databases. Compound identity was obtained and reported in Tables 2 and 3 only when the matching value of the mass spectra comparison was 70 or higher and an increase in the size of the peak was observed when spiking the sample with the corresponding pure standard. Additionally, the linear retention index (LRI) was reported for each compound detected. A series of alkanes from C8 (LRI = 800) to C20 (LRI = 2000) were run under the GC-MS conditions set for both liquid extracts and headspace volatiles. The LRI's were estimated by direct correlation with the retention times of the alkanes.

Preliminary Validation of Possible HLB Biomarkers

Preliminary validation was done using training and validation groups of samples from each of the previously described groves, as performed in other metabolomic studies (Ikeda and

others 2007; Tarachiwin and others 2007; Pongsuwan and others 2008). The validation group consisted of ten samples of each treatment (HLB, ZD, and healthy) taken from each of the two groves and analyzed as described in previous sections. ANOVA was run on each compound to determine significance of the potential biomarkers. Peak areas as well as infected- healthy and infected-zinc deficiency ratios were compared between groves. Additionally, non symptomatic leaves from HLB-infected trees were analyzed following the methodology previously described and suitability of suggested biomarkers for pre-symptomatic HLB differentiation was proposed. PCR analyses of both symptomatic and non-symptomatic leaves were also performed.

Results and Discussion

Extraction and Derivatization Conditions

Polar extracts obtained with pure methanol were compared to chloroform and methanol/water/chloroform (8:1:1) extracts. No significant differences were found when comparing chloroform extracts from healthy and HLB-infected leaves confirming previous observations that non-polar compounds are less likely candidates for fingerprinting in plant metabolomic analyses (Cevallos-Cevallos and others 2009b). Samples from healthy and HLB-infected trees showed significant differences when extracted with pure methanol and with the combination methanol/water/chloroform (8:1:1). However, the 8:1:1 mixture yielded a higher number and greater concentration of peaks (Figure 4-2) and therefore was used as the extraction media for this study. Results were in accordance to previously reported data for HLB fingerprinting using capillary electrophoresis (Cevallos-Cevallos and others 2009c).

Amount of silylation reagent and duration of the reaction are the two known concerns that need to be addressed prior application of a derivatization methodology (Kanani and others 2008). Several derivatization conditions were tested by adding MSTFA from 40 µL to 120 µL

(20 µL intervals) during 40, 80, 120, 160, and 200 min. The amount of the silylation agent MSTFA added caused a significant effect on the 11 compounds reported in Figure 4-3 and Table 4-1. The derivatized form of glucose, galactose oxime, and mannitol (Figure 4-3) as well as L-proline, unknown 6, and inositol (Table 4-1) showed a maximum peak area after reacting with 80 µL of MSTFA. Lower and higher amounts of MSTFA caused a reduction in peak areas probably due to incomplete derivatization and dilution effect respectively. Conversely, the derivatized form of glucitol (Figure 4-3), unknown 5, glutamic acid, and glycine (Table 4-1) showed a maximum peak area when reacting with 40 µL of MSTFA. Higher levels of MSTFA caused a reduction in their peak areas, probably due to a dilution effect. All compounds were detectable at both 40 and 80 µL of MSTFA but the latter showed the lowest standard error and improved reproducibility (Figure 4-3 and Table 4-1) and was chosen for this study.

Variation in MSTFA reaction time significantly affected six compounds only (Figure 4-4). The lowest standard error and better reproducibility was observed between 80 and 150 min of reaction. Therefore, a reaction time of 120 minutes was selected for the metabolomics study. Adding 80 µL of MSTFA followed by a reaction time of 120 min yielded the best compromise between peak area and reproducibility.

GC-MS Analyses of Derivatized Samples

Figure 4-5 shows typical chromatograms of samples from healthy, HLB-infected, and ZD-affected trees from groves 1 and 2. Data was aligned and normalized to the total area prior to statistical analysis. PCA was carried out to compare the overall metabolite profile of each sample group. Figure 4-6A shows that PCA was able to classify all the samples according to their initial physiological condition, suggesting marked differences in the metabolite profile of each sample group. No classification was observed based on groves or sampling time, suggesting that

differences between HLB, ZD, and healthy samples were greater than possible differences between groves or sampling season. Principal components (PC) 1 and 2 accounted for 56.34% of the variation and sample classification occurred mostly in the PC1. Out of the 10 compounds with the highest absolute loading values in PC1 (Figure 4-6B), only L-proline, unknown 20, inositol, fructose, and arabitol showed significant differences among sample groups. ANOVA was run in each detected compound to find other possible biomarkers among the metabolites with low loading values. While all compounds detected were present in all of the samples, significant differences were found in the concentration of several compounds. Table 4-2 shows the tentative identity of the most abundant compounds detected (peak to noise ratio of 4) as well as those compounds showing significant differences among sample groups. L-Proline and unknown 22 showed significantly higher concentrations in HLB-infected leaves when compared to the healthy and ZD-affected ones (Figure 4-7A). These results are in agreement with previous reports of increased proline in citrus under physiological or biological stresses. Proline accumulation in citrus (Rivas and others 2008; Gimeno and others 2009) and other plants such as sugar cane (Suriyan and Chalermpol 2009) has been reported with stress conditions such as girdling and drought stress. Additionally, proline accumulation has also been linked to bacterial attack in other plants such as potatoes (Abu-Nada and others 2007). Therefore, L-proline alone cannot be considered as an HLB-specific biomarker. Fructose and unknown 18 were in significantly higher concentration in both HLB-infected and ZD-affected samples when compared to the healthy ones (Figure 4-7B). Fructose may be released by sucrose synthase-mediated ADPglucose synthesis, which has been reported to be an important step in starch biosynthesis in leaves (Munoz and others 2006). This observation is in agreement with previous reports on starch accumulation in HLB infected leaves (Etxeberria and others 2009; Achor and

others 2010). Starch concentration in HLB infected leaves has been reported to increase more than 10 folds (TAKUSHI and others 2007b). Mineral imbalances, such as boron deficiency has also been shown to cause fructose accumulation in citrus (Han and others 2008). Although combined accumulation of fructose and glucose in HLB-infected plants has been reported (Dagraca 1991), no significant differences in glucose content were detected in this study. Unknown compound 20 and oxo-butanedioic acid were in significantly higher concentrations in zinc deficient than in healthy and HLB-infected samples whereas arabitol, neo-inositol and unknown 15 were in significantly lower concentrations (Figure 7C).

SPME Analyses

Figure 4-8 shows typical chromatograms of headspace analyses of leaves from healthy, HLB-infected, and ZD-affected trees. Table 4-3 shows the tentative identity of the volatiles detected. None of the compounds detected by SPME analyses were detected in liquid extracts, probably due to the lost of volatiles during sample drying prior to derivatization. Data show the potential of using combined headspace and liquid extracts analysis for maximizing the number of tentative biomarkers in GC-MS based metabolomic research.

As with liquid extracts, SPME data was aligned and normalized to the total area prior to statistical analysis. PCA (Figure 4-9A) was not able to classify the analyzed samples, suggesting similarities in the headspace metabolite profile in the sample groups. A weak discrimination of the HLB-infected samples can be seen in PC2, suggesting very small differences in the compounds with the highest PC2 loading values. Figure 4-9B shows the loading plot of PC1 and PC2 as well as the 10 compounds with the highest absolute PC2-loading values. Additionally, ANOVA was run for each compound to determine significant differences and possible biomarkers. Although PCA was not able to classify samples run by SPME, significant

differences were found in the concentration of several headspace volatiles after running ANOVA. This shows the importance of ANOVA in metabolic differentiation studies. Table 4-3 shows the tentative identity of the most abundant compounds detected (peak to noise ratio of 4) as well as those compounds showing significant differences among sample groups. Significantly lower concentrations of several sesquiterpenes were observed in the non-healthy samples. β-Elemene, trans-caryophyllene, and α-humulene were significantly lower in HLB-infected samples only (Figure 4-10A), whereas isocaryophylene, α-selinene, and β-selinene were significantly lower in the HLB-infected and zinc deficient samples (Figure 4-10B). Decreased concentrations of sesquiterpenes has been reported in citrus with drought stress (Hansen and Seufert 1999). The increased amounts of sesquiterpenes such as β-elemene and trans-caryophyllene detected upon infection with several pathogens such as phytoplasmas in *Hypericum perforatum* (Bruni and Sacchetti 2005) and other plants, suggested a strong antimicrobial activity of these compounds. However, the low level of sesquiterpenes found in diseased leaves in this study, suggests an important post-infection inhibition of sesquiterpene biosynthesis upon infection, which may increase the susceptibility of 'Valencia' oranges to HLB. Recently, eight different degrees of susceptibility to this infection were characterized (Folimonova and others 2009). Further research is needed to confirm sesquiterpene inhibition in susceptible and tolerant varietes after infection with HLB.

Although the change in concentration of no single compound may be exclusively attributed to HLB, the combined use of L-proline, β-elemene, trans-caryophyllene, and α-humulene increased specificity. The combination of these biomarkers has the potential to be found with drought stress only. However, drought-affected plants don't show symptoms close to those of HLB. Hence, L-proline, β-elemene, trans-caryophyllene, and α-humulene along with

visual observation of the symptoms have the potential to differentiate trees with HLB from those healthy or with zinc deficiency, the most common and HLB-similar symptomatic condition in citrus. These results complement previous work on HPLC-MS in which flavonoids and hydroxycinnamates were observed to change in HLB infected trees (Cevallos-Cevallos and others 2008; Manthey 2008). Additionally, proposed biomarkers can be targeted by traditional chemical assays, sensors or biosensors, reducing the analysis costs and improving portability as well as rapidity. Future research is needed to determine physiological similarities between HLB and drought stress-affected plants.

Preliminary Validation of HLB Biomarkers

Ten samples from each treatment and each grove (total 30 samples per grove) were used to validate proposed HLB biomarkers among groves. Each of the two groves analyzed showed the same significant differences in the compounds reported in Tables 2 and 3. Figure 4-11 shows that the peak areas were similar between groves for each proposed biomarkers, except for Unknown 22, L-proline, and Unknown 11. Same effect can be seen when comparing ratios of HLB-infected with healthy or zinc deficient samples between groves (Table 4-4). The differences in the concentrations and ratios of these potential biomarkers might be due to dissimilarities in the severity of the infection. To further test this hypothesis, samples showing less severe or no symptoms from HLB-infected trees were analyzed and compared to healthy ones. Figure 4-12 shows reduced differences in all proposed biomarkers. Only trans-caryophyllene and α-humulene showed significant differences when comparing mildly infected with healthy leaves. Therefore, the combination of all the proposed biomarkers cannot be used for HLB detection in non-symptomatic leaves. These results are in agreement with previous reports on HPLC-MS fingerprinting of HLB showing that metabolic differences are proportional

to the intensity of the visual symptoms (Cevallos-Cevallos and others 2008), suggesting the need of sampling highly symptomatic leaves for metabolomic analysis. All PCR analysis performed in non symptomatic leaves were negative, showing agreement with metabolomic results. This suggests that the bacteria were probably not present in the sampled non-symptomatic leaves but changes in trans-caryophyllene and α-humulene were probably induced by the presence of the bacteria in the symptomatic leaves of the same tree. Further research is needed to better understand the response mechanism of citrus trees after HLB infection. Further validation studies involving different cultivars, groves, seasons, diseases, and stresses are needed to find HLB-specific biomarkers and determine possible metabolic differences among biotic and abiotic stresses. Additional research in greenhouse plants is needed to determine if pre-symptomatic and pre-PCR-positive changes in the metabolite profile occur in citrus.

Table 4-1. Effect of amount of MSTFA added on the compounds expressing the highest variation.

	Amount of MSTFA (µL)								
Compound	60			80			100		
Unknown 5	3356	+/-	553.311	2878.33	+/-	237.144	1418.33	+/-	249.843
Unknown 6	3925	+/-	488.257	3849	+/-	260.407	2845	+/-	186.551
L-proline 2TMS	13662	+/-	738.219	12120.3	+/-	153.576	10189.3	+/-	340.36
L-glutamic acid	10649	+/-	472.347	9604	+/-	344.252	7437	+/-	472.168
L- glycine,TMS	7188	+/-	1610.79	6340.33	+/-	861.635	4478.33	+/-	228.803
Inositol 6TMS	8785	+/-	2989.65	8897	+/-	2099.09	6402.33	+/-	1940.34

Table 4-2. Main compounds detected in derivatized samples*

Lin Ret ndex LRI)	Tentative identity	Relative abundance (%)		
		ZD	HLB	Healthy
967	UN1	0,177 +/- 0,005	0,192 +/-	0,121 +/-
979	Benzene, 1,2,5-trimethyl	1,400 +/- 0,026	1,198 +/- 0,022	0,593 +/- 0,016
994	Tetrasiloxane, decamethyl	0,048 +/- 0,001	0,076 +/- 0,001	0,218 +/- 0,005
1004	UN2	0,037 +/- 0,001	0,074 +/- 0,001	0,017 +/- 0,000
1020	Butanoic acid, 2-[(trimethylsilyl)oxy	0,153 +/- 0,004	0,201 +/- 0,004	0,130 +/- 0,003
1024	UN3	0,030 +/- 0,001	0,017 +/- 0,001	0,057 +/- 0,001
1030	UN4	0,006 +/- 0,000	0,002 +/- 0,000	0,000 +/- 0,000
1062	L-Alanine, N-(trimethylsilyl)-, trime	0,055 +/- 0,001	0,038 +/- 0,001	0,004 +/- 0,000
1134	UN5	0,034 +/- 0,001	0,316 +/- 0,007	0,057 +/- 0,001
1206	3,7-Dioxa-2,8-disilanonane, 2,2,8,8-t	0,025 +/- 0,001	0,038 +/- 0,001	0,100 +/- 0,001
1212	UN6	0,374 +/- 0,006	0,462 +/- 0,005	0,421 +/- 0,003
1248	L-Proline 2TMS	1,257 +/- 0,014	4,258 +/- 0,038[a]	0,928 +/- 0,008
1280	UN7	0,141 +/- 0,003	0,078 +/- 0,001	0,271 +/- 0,003
1294	L-Serine TMS	0,136 +/- 0,002	0,220 +/- 0,002	0,191 +/- 0,001
1406	Butanedioic acid, [(trimethylsilyl)ox	3,009 +/- 0,043	1,802 +/- 0,025	3,028 +/- 0,020
1451	L-glutamic acid, N-(trimethylsilyl)-	0,272 +/- 0,003	0,226 +/- 0,003	0,315 +/- 0,003
1462	Glycine, N,N-bis(trimethylsilyl)-	0,135 +/- 0,002	0,402 +/- 0,007	0,841 +/- 0,009
1468	Tetronic acid TMS	0,071 +/- 0,002	0,000 +/- 0,000	0,000 +/- 0,000
1486	UN8	0,123 +/- 0,001	0,391 +/- 0,006	0,176 +/- 0,001
1519	UN9	0,001 +/- 0,000	0,007 +/- 0,000	0,016 +/- 0,000
1599	Arabitol TMS	ND[a]	0,166 +/- 0,002	0,209 +/- 0,002
1609	UN10	0,395 +/- 0,004	1,020 +/- 0,011	0,473 +/- 0,006
1617	UN11	0,103 +/- 0,002[a]	0,397 +/- 0,005[a,b]	0,675 +/- 0,007[b]
1623	UN12	0,149 +/- 0,002[a]	0,065 +/- 0,001[a,b]	0,027 +/- 0,001[b]
1670	Citric acid 4TMS	0,096 +/- 0,002	0,015 +/- 0,000	0,008 +/- 0,000
1675	1-(4'-Trimethylsilyloxyphenyl)-1-Trime	0,194 +/- 0,003	0,472 +/- 0,007	0,228 +/- 0,002
1701	UN13	18,930 +/- 0,229	5,513 +/- 0,054	3,381 +/- 0,091
1707	Fructose 5TMS	5,141 +/- 0,058[a]	7,429 +/- 0,140[a]	0,608 +/- 0,007

Table 4-2. Continued

Lin Ret ndex LRI)	Tentative identity	Relative abundance (%)		
		ZD	HLB	Healthy
1716	D-gluco-hexodialdose, 4TMS	0,130 +/- 0,001	0,203 +/- 0,005	0,029 +/- 0,001
1721	D-Mannitol 6TMS	20,334 +/- 0,151	11,780 +/- 0,184	5,365 +/- 0,050
1740	Galactose oxime 6TMS	0,600 +/- 0,010	0,397 +/- 0,010	0,006 +/- 0,000
1764	Inositol 6TMS	0,190 +/- 0,002[a]	1,299 +/- 0,013	1,844 +/- 0,015
1789	Galactonic acid 6TMS	0,027 +/- 0,000[a]	0,088 +/- 0,02 [a,b]	0,175 +/- 0,002[b]
1811	UN14	0,118 +/- 0,004	0,173 +/- 0,002	0,282 +/- 0,002
1855	Myo-Inositol 6TMS	0,570 +/- 0,006	1,019 +/- 0,008	0,907 +/- 0,005
1864	D-Glucitol 6TMS	0,349 +/- 0,004	0,564 +/- 0,007	0,300 +/- 0,002
1894	UN15	0,012 +/- 0,000[a]	0,099 +/- 0,001	0,062 +/- 0,000
1905	Glucose 5TMS	0,260 +/- 0,003	0,734 +/- 0,011	0,544 +/- 0,009
2043	UN16	0,127 +/- 0,002	0,140 +/- 0,002	0,088 +/- 0,001
2047	UN17	0,127 +/- 0,002	0,045 +/- 0,001	0,117 +/- 0,001
2063	UN18	0,182 +/- 0,03[a]	0,128 +/- 0,002 [a]	0,009 +/- 0,000
2081	Butanedioic acid, oxo (TMS)	0,176 +/- 0,03 [a]	0,000 +/- 0,000	0,000 +/- 0,000
2091	UN19	0,125 +/- 0,002	0,067 +/- 0,001	0,170 +/- 0,002
2096	Butanedioic acid, oxo (TMS)	0,182 +/- 0,004	0,052 +/- 0,001	0,026 +/- 0,001
2147	UN20	0,181 +/- 0,03 [a]	0,051 +/- 0,001	0,006 +/- 0,000
2175	Sucrose TMS	26,443 +/- 0,254	46,378 +/- 0,354	55,111 +/- 0,262
2188	UN 21	0,002 +/- 0,000	0,034 +/- 0,001	0,000 +/- 0,000
2240	UN22	0,023 +/- 0,001	0,186 +/- 0,003 [a]	0,032 +/- 0,001

* Values are in percentage +/- standard deviation. Values in the same raw with the same superscript are not significantly different.

Table 4-3. Main compounds detected by headspace SPME*

Linear Ret Index (LRI)	Tentative identity	Relative abundance (%)		
		ZD	HLB	Healthy
900	4-Octene, 2,6-dimethyl-,[S-(E)]-	0,070 +/- 0,001	0,230 +/- 0,003	0,213 +/- 0,003
908	3-Octene, 2,6-dimethyl	0,053 +/- 0,001	0,147 +/- 0,002	0,305 +/- 0,003
919	α-Thujene	0,777 +/- 0,010	1,067 +/- 0,011	1,248 +/- 0,014
922	Octane, 2,6-dimethyl	0,307 +/- 0,005	0,759 +/- 0,010	0,373 +/- 0,005
934	UN1	0,513 +/- 0,004	0,835 +/- 0,007	0,860 +/- 0,005
937	UN2	0,075 +/- 0,001	0,186 +/- 0,003	0,216 +/- 0,003
946	UN3	0,138 +/- 0,003	0,424 +/- 0,004	0,518 +/- 0,007
958	2-Octene, 2,6-dimethyl	0,546 +/- 0,005	1,081 +/- 0,007	1,250 +/- 0,009
970	Sabinene	8,429 +/- 0,080	9,042 +/- 0,086	8,715 +/- 0,082
976	1,5-Heptadiene, 2,3,6-trimethyl-	0,249 +/- 0,004	0,671 +/- 0,007	0,532 +/- 0,006
980	β-Pinene	1,407 +/- 0,010	1,692 +/- 0,008	1,276 +/- 0,011
985	Trans-carane	0,000 +/- 0,000	0,247 +/- 0,003	0,190 +/- 0,003
990	2,6-Octadiene, 2,6-dimethyl-	0,585 +/- 0,005	1,098 +/- 0,006	1,147 +/- 0,007
996	1,3-Hexadiene, 3-ethyl-2,5-dimethyl-	0,445 +/- 0,004	0,550 +/- 0,005	0,732 +/- 0,004
1008	δ-3-Carene	4,659 +/- 0,044	5,360 +/- 0,046	5,159 +/- 0,050
1016	α-Terpinene	0,283 +/- 0,004	0,504 +/- 0,003	0,478 +/- 0,004
1025	Benzene, 1-methyl-2-(1-methylethyl)-	3,041 +/- 0,024	8,168 +/- 0,067	3,717 +/- 0,030
1031	*d*-Limonene	8,130 +/- 0,048	19,208 +/- 0,137	5,980 +/- 0,061
1041	1,3,7-Octatriene, 3,7-dimethyl	5,229 +/- 0,023	3,679 +/- 0,033	5,010 +/- 0,023
1059	γ-Terpinene	0,336 +/- 0,004	0,765 +/- 0,003	0,549 +/- 0,003
1088	α-Terpinolene	1,060 +/- 0,006	1,069 +/- 0,004	1,084 +/- 0,005
1096	Undecane	1,290 +/- 0,007	2,453 +/- 0,017	0,890 +/- 0,003
1152	UN4	0,508 +/- 0,004	0,771 +/- 0,006	0,465 +/- 0,002
1274	(E,E)-2,4-nonadienal	3,287 +/- 0,002	3,287 +/- 0,002	3,287 +/- 0,002
1350	α-Cubebene	0,191 +/- 0,006	0,289 +/- 0,004	0,440 +/- 0,003
1354	UN5	0,000 +/- 0,000	0,025 +/- 0,001	0,074 +/- 0,002
1365	UN6	0,000 +/- 0,000	0,030 +/- 0,001	0,221 +/- 0,002
1372	Dihydro-neoclovene (II)	0,336 +/- 0,004	0,903 +/- 0,007	0,821 +/- 0,010
1383	UN7	2,602 +/- 0,009[a,]	2,029 +/- 0,007[a]	2,939 +/- 0,011[b]

Table 4-3. Continued

Linear Ret Index (LRI)	Tentative identity	Relative abundance (%)		
		ZD	HLB	Healthy
1391	β-Elemene	26,983 +/- 0,152	14,053 +/- 0,082[a]	21,582 +/- 0,153
1396	UN8	0,185 +/- 0,003	0,497 +/- 0,005	0,655 +/- 0,006
1404	UN9	0,400 +/- 0,005	0,978 +/- 0,009	0,900 +/- 0,010
1407	UN10	0,126 +/- 0,003	0,067 +/- 0,001	0,384 +/- 0,004
1413	Ledane	1,564 +/- 0,010	2,744 +/- 0,016	3,034 +/- 0,020
1425	Trans-caryophyllene	10,471 +/- 0,028	5,408 +/- 0,02 [a]	8,996 +/- 0,032
1431	Unknown 11	1,561 +/- 0,07	1,286 +/- 0,04 [a]	1,678 +/- 0,007
1434	Trans-β-Farnesene	0,692 +/- 0,08 [a,b]	0,382 +/- 0,04 [a]	1,573 +/- 0,019 [b]
1443	UN12	0,400 +/- 0,007	0,278 +/- 0,07	0,719 +/- 0,004
1448	Isocaryophyllene	0,548 +/- 0,008 [a]	0,927 +/- 0,09 [a]	1,442 +/- 0,008
1456	α-Humulene	3,607 +/- 0,016	1,700 +/- 0,01 [a]	2,687 +/- 0,012
1467	UN13	0,072 +/- 0,002	0,123 +/- 0,04	0,390 +/- 0,004
1481	UN14	0,354 +/- 0,006	0,289 +/- 0,05	0,527 +/- 0,007
1485	β-Selinene	0,475 +/- 0,004 [a]	0,416 +/- 0,06 [a]	0,796 +/- 0,003
1489	α-Selinene	0,765 +/- 0,005 [a]	0,692 +/- 0,08 [a]	1,095 +/- 0,003
1502	δ-Cadinene	0,373 +/- 0,004	0,100 +/- 0,02	0,432 +/- 0,003

* Values are in percentage +/- standard deviation. Values in the same raw with the same superscript are not significantly different.

Table 4-4. Ratios infected/healthy (i/h) and infected/zinc-deficient (i/z) of the proposed HLB biomarkers from two different groves*

	Grove 1						Grove 2					
	i/h			i/z			i/h			i/z		
L-proline	6.5	+/-	1.35	3.84	+/-	1.01	4.67	+/-	0.47	5.43	+/-	1.65
Unknown 22	255.64	+/-	62.41	291.40	+/-	63.02	23.20	+/-	6.46	47.82	+/-	14.35
B-Elemene	0.52	+/-	0.19	0.74	+/-	0.21	0.56	+/-	0.43	0.68	+/-	0.38
Trans caryophylene	0.48	+/-	0.14	0.63	+/-	0.15	0.28	+/-	0.04	0.48	+/-	0.20
Unknown 11	0.28	+/-	0.03	0.65	+/-	0.35	0.58	+/-	0.20	0.64	+/-	0.11
A-Humulene	0.44	+/-	0.14	0.39	+/-	0.10	0.52	+/-	0.34	0.59	+/-	0.27

*Grove 1 = grove in Plant City, Grove 2 = grove in Lake Alfred.

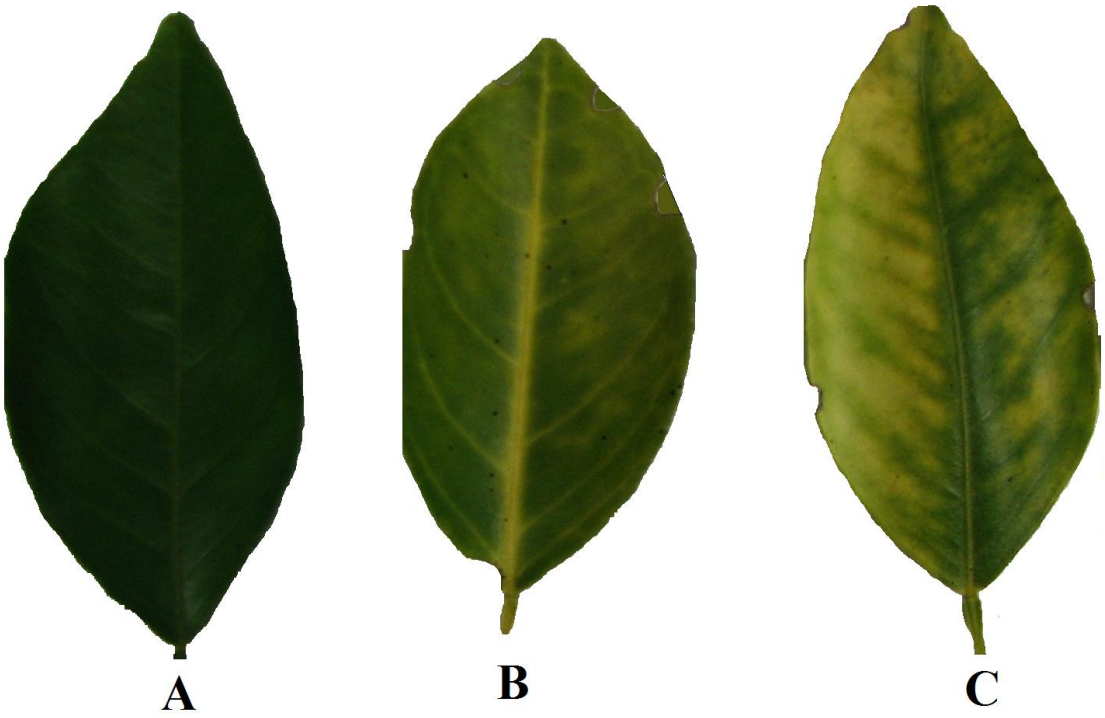

Figure 4-1. Healthy (A), HLB-infected (B), and zinc deficient leaves (C).

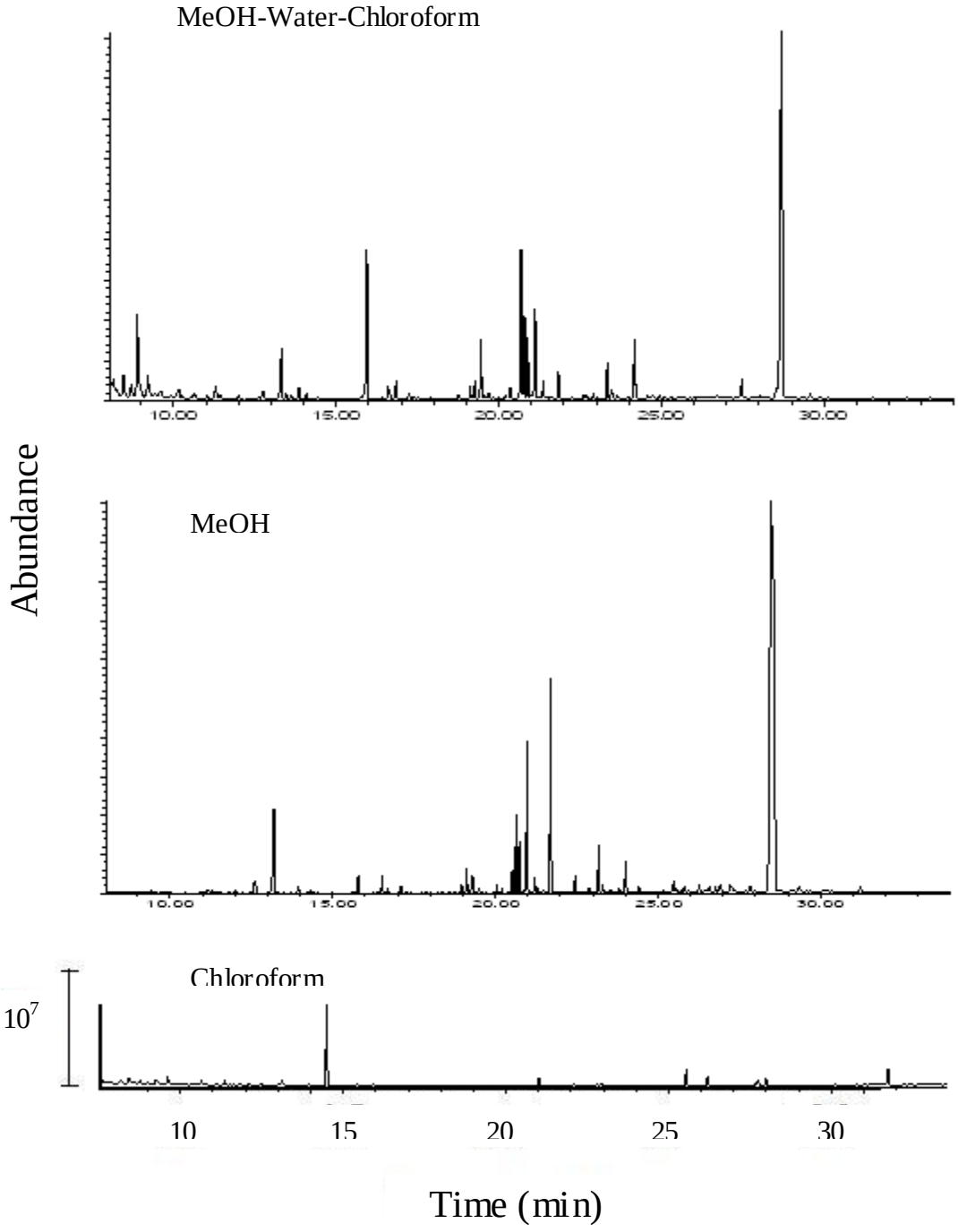

Figure 4-2. Typical chromatograms of 'Valencia' orange leaf extracted with methanol-water-chloroform 8:1:1, methanol alone, and chloroform.

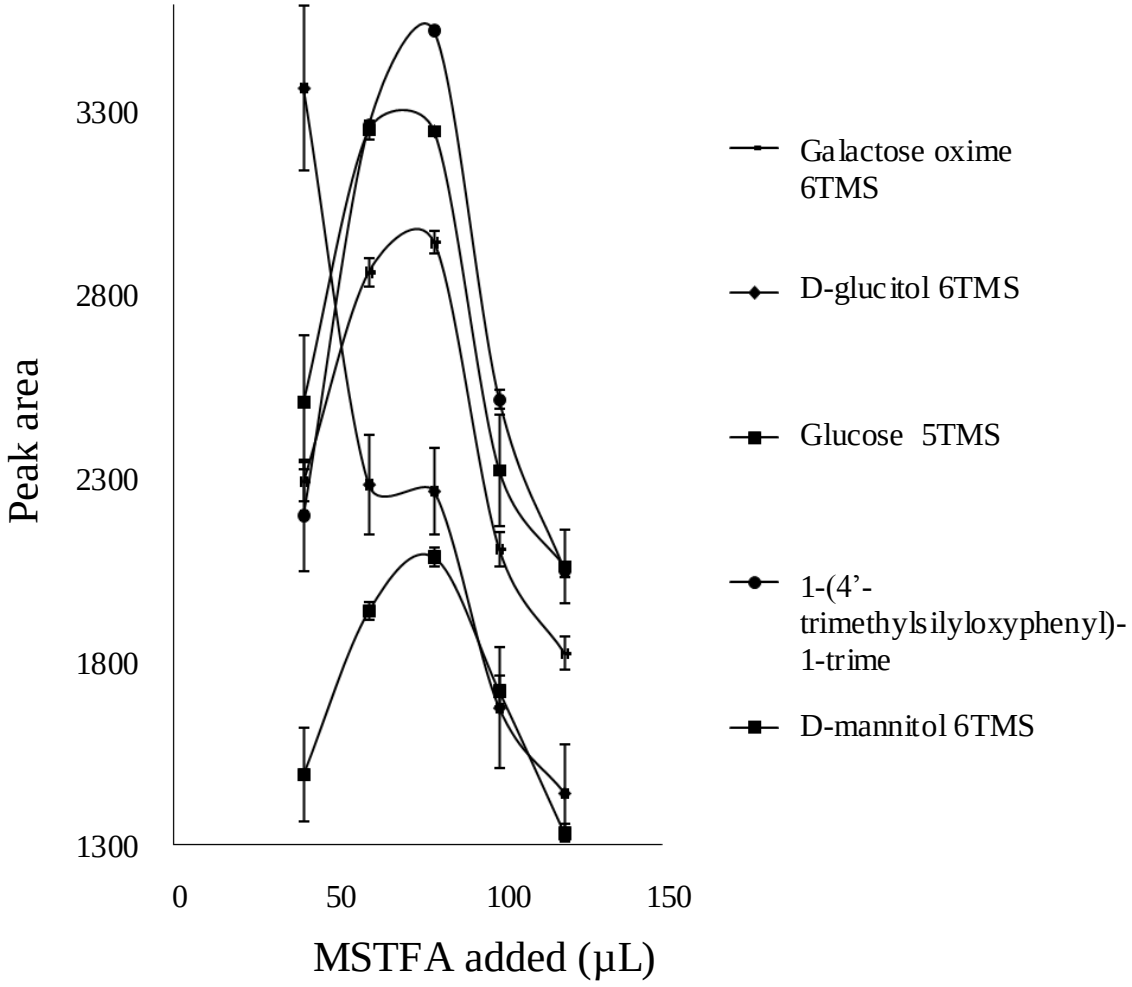

Figure 4-3. Effect of the amount of MSTFA added in the compounds showing the highest variation.

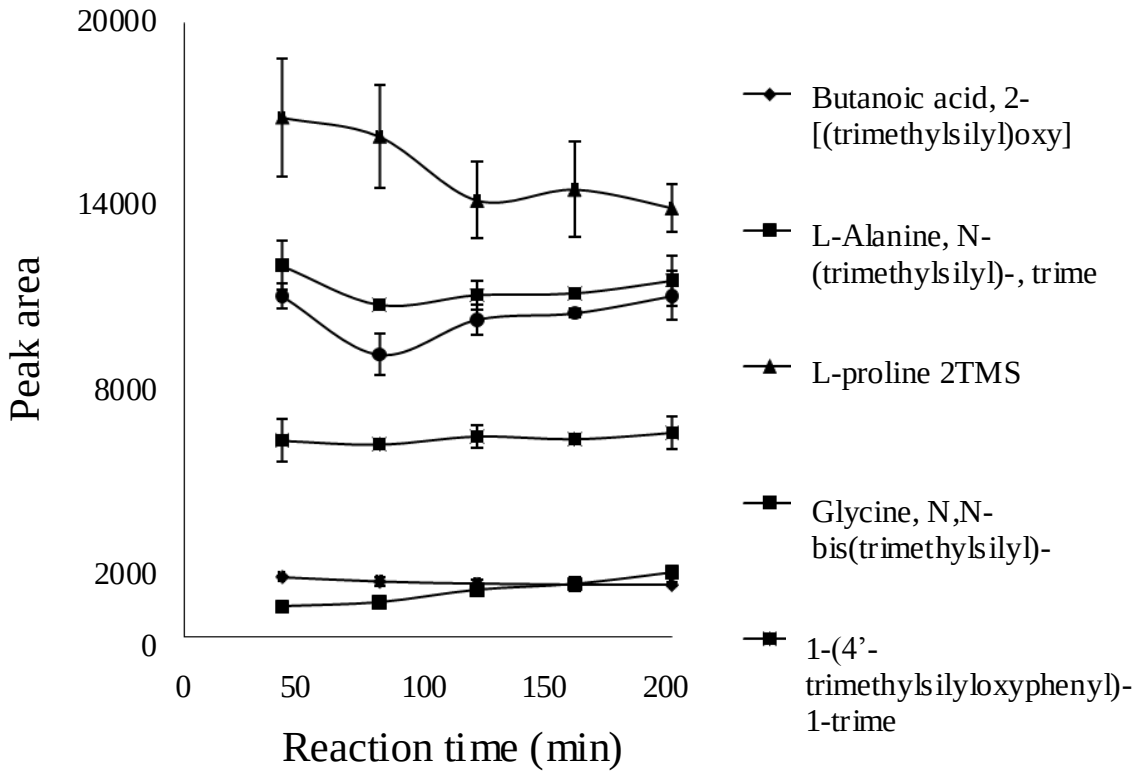

Figure 4-4. Effect of the MSTFA reaction time in the compounds showing the highest variation.

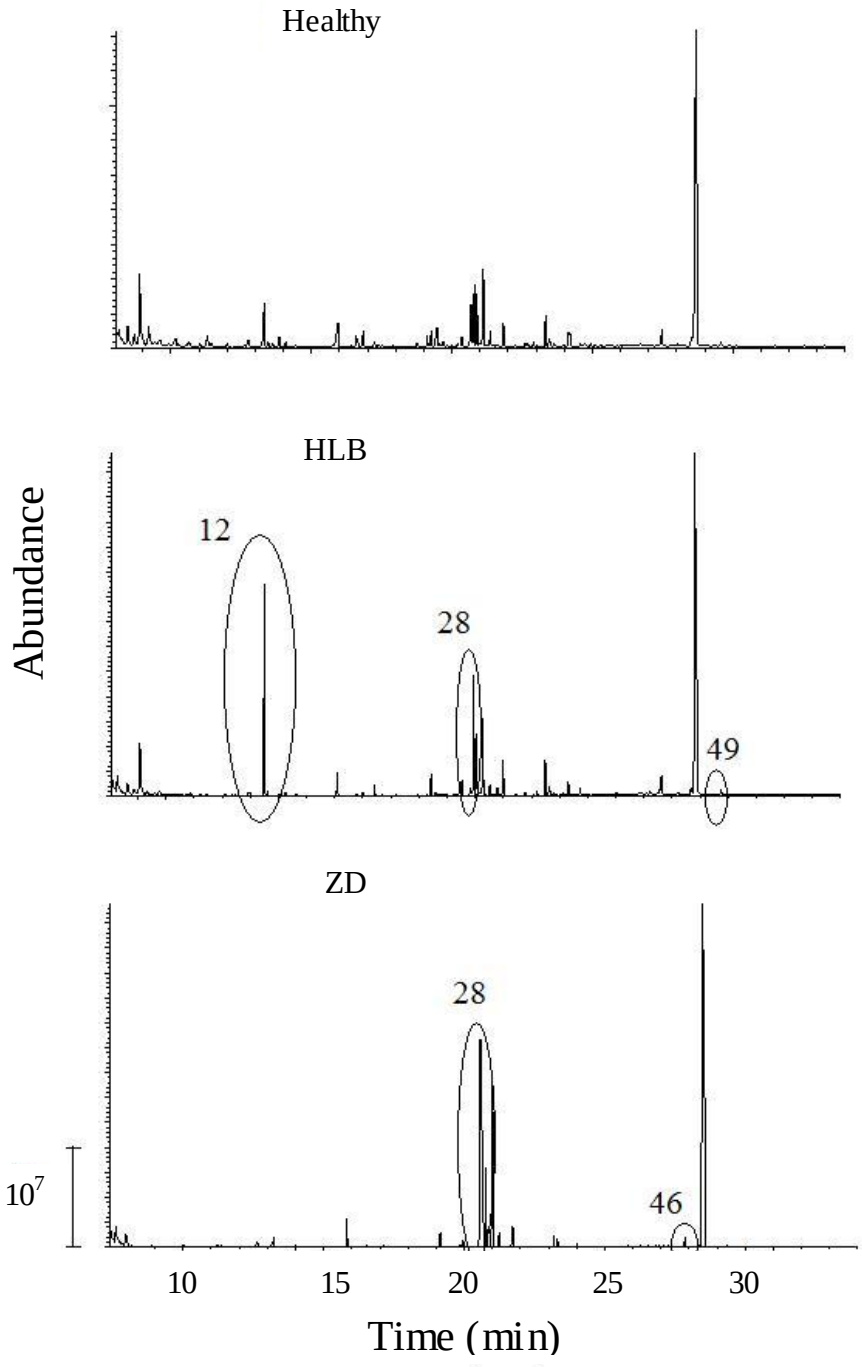

Figure 4-5. Typical chromatograms of derivatized liquid extracts of healthy, HLB-infected, and zinc deficient leaves. Circled compounds represent visible significant differences. Peak numbers correspond to Table 4-1.

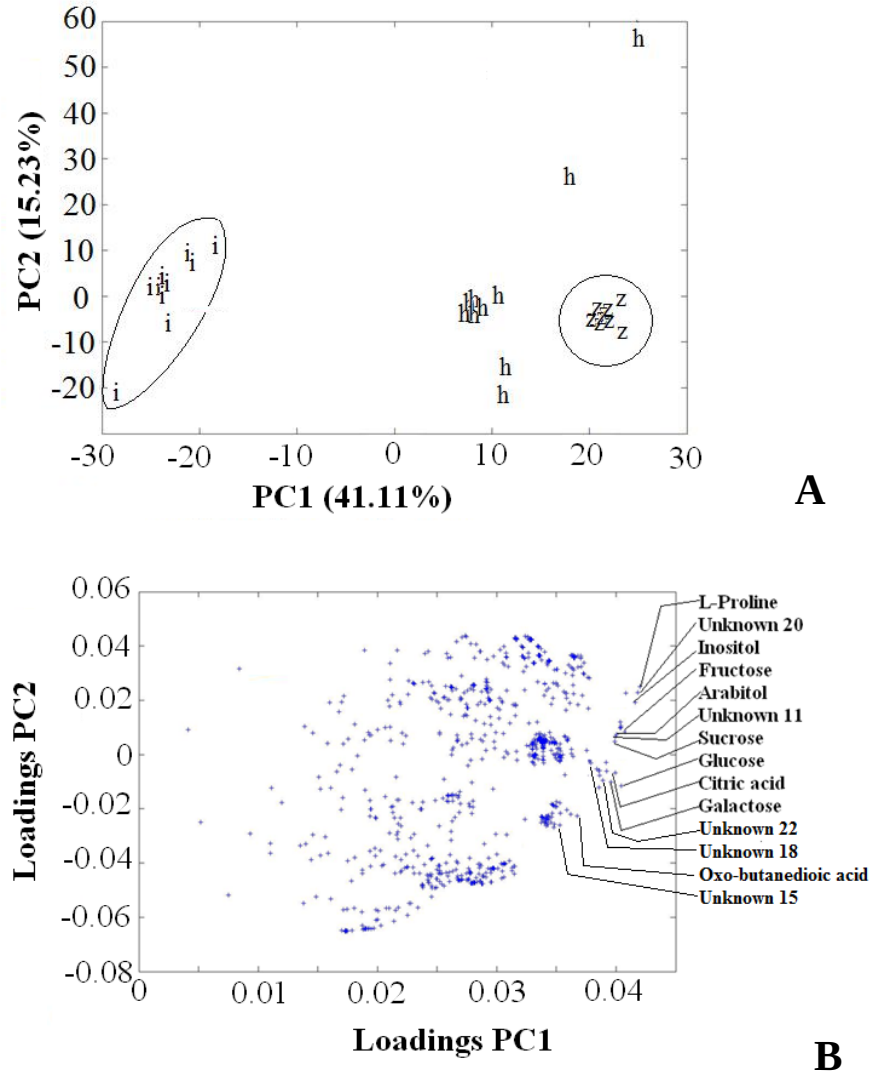

Figure 4-6. Principal components analysis of derivatized liquid extracts. Figure A: score plot of HLB-infected (i), healthy (h), and zinc deficient (z) samples illustrated in PC1 and PC2. Figure B: Loading plot of PC1 and PC2. The 10 compounds with the highest loadings on PC1 are marked

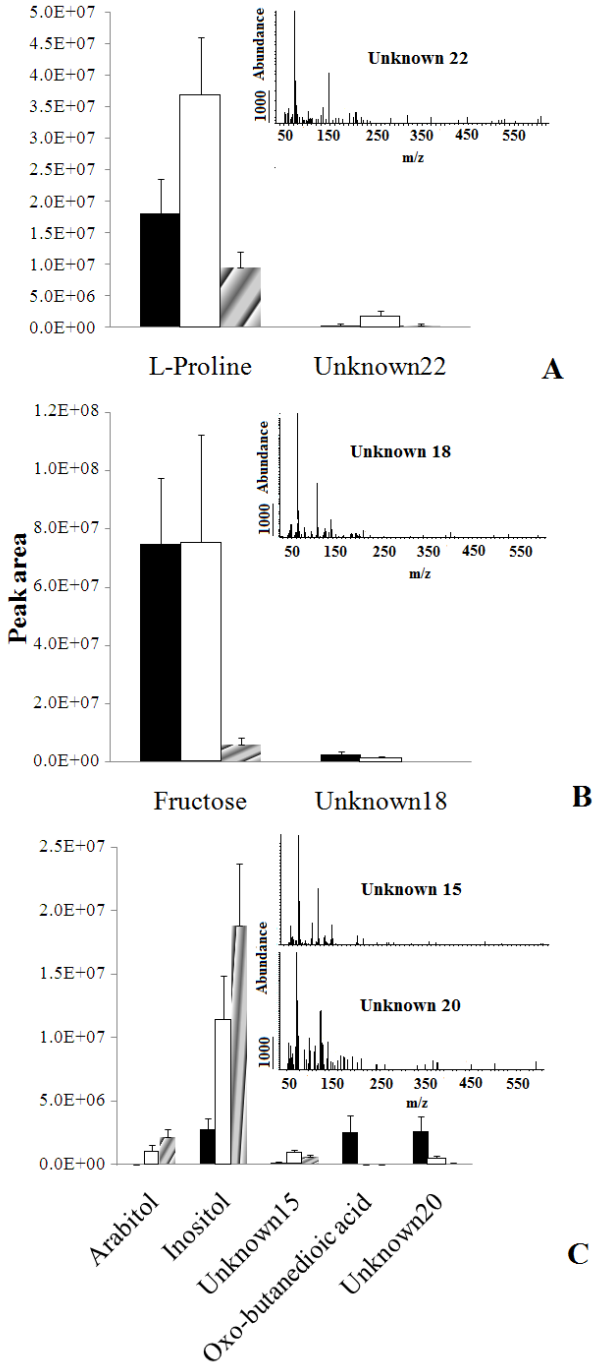

Figure 4-7. Compounds showing significant differences in derivatized liquid extracts of zinc deficient (■), HLB-infected (□), and healthy (◪) leaves. Figure A: Significantly different compounds in HLB-infected leaves only. Figure B: Significantly different compounds in HLB-infected and zinc deficient leaves. Figure C: Significantly different compounds in zinc deficient leaves only. Insets show the mass spectra of each unknown compound

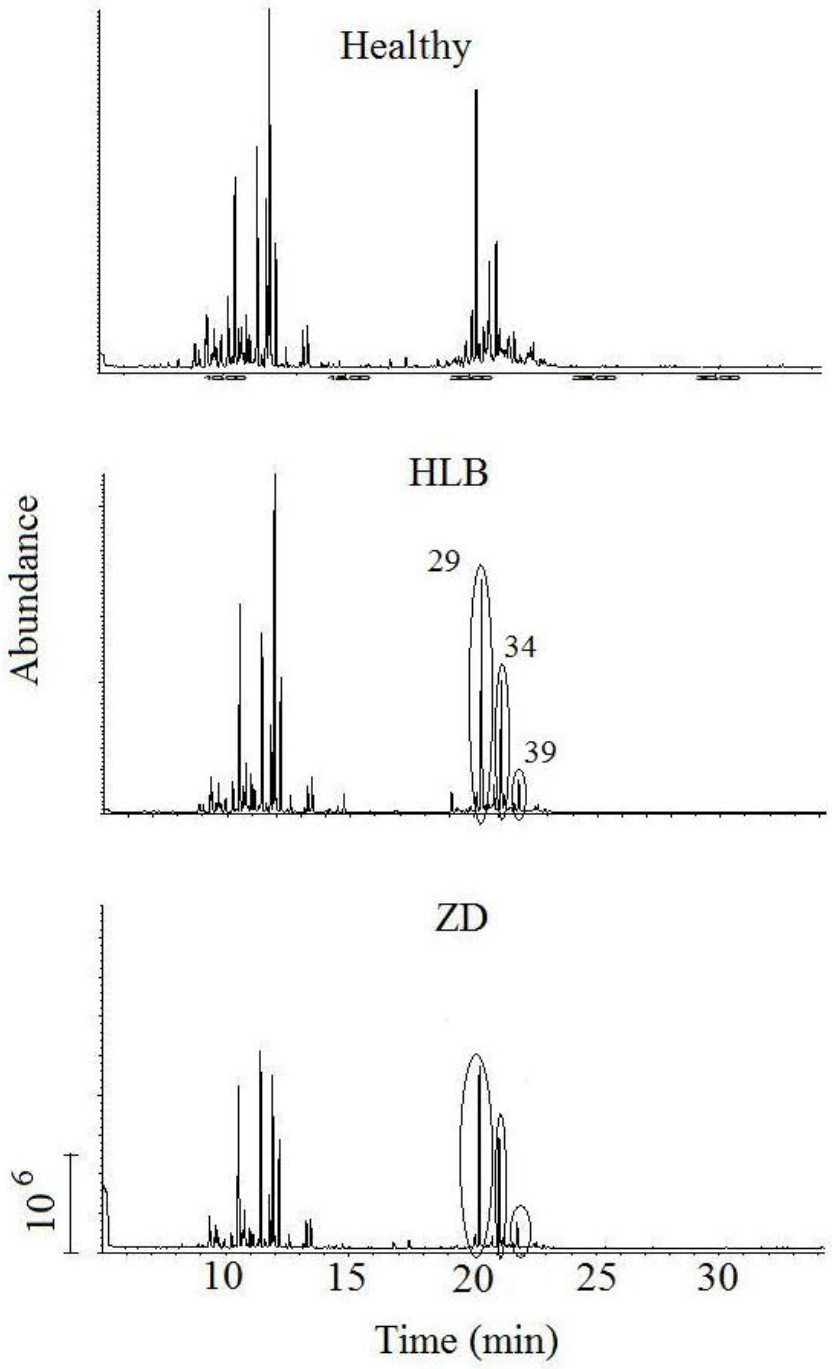

Figure 4-8. Typical chromatogram of SPME analyses of healthy, HLB-infected, and zinc deficient leaves. Circled compounds represent visible significant differences. Peak numbers correspond to Table 4-2.

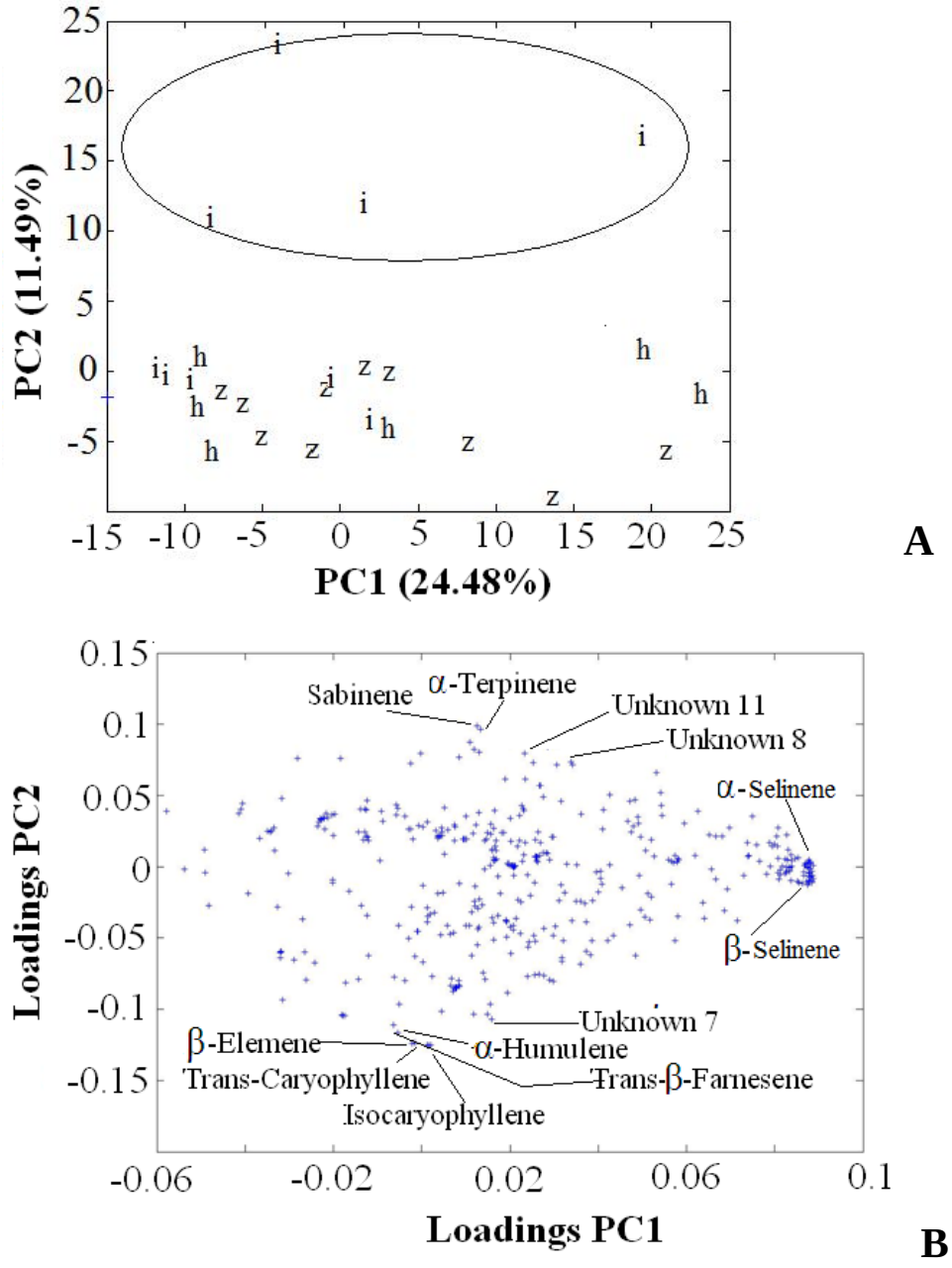

Figure 4-9. Principal components analysis of headspace metabolites. Figure A: score plot of HLB-infected (i), healthy (h), and zinc deficient (z) samples illustrated in PC1 and PC2. Figure B: Loading plot of PC1 and PC2. The 10 compounds with the highest loadings on PC2 are marked.

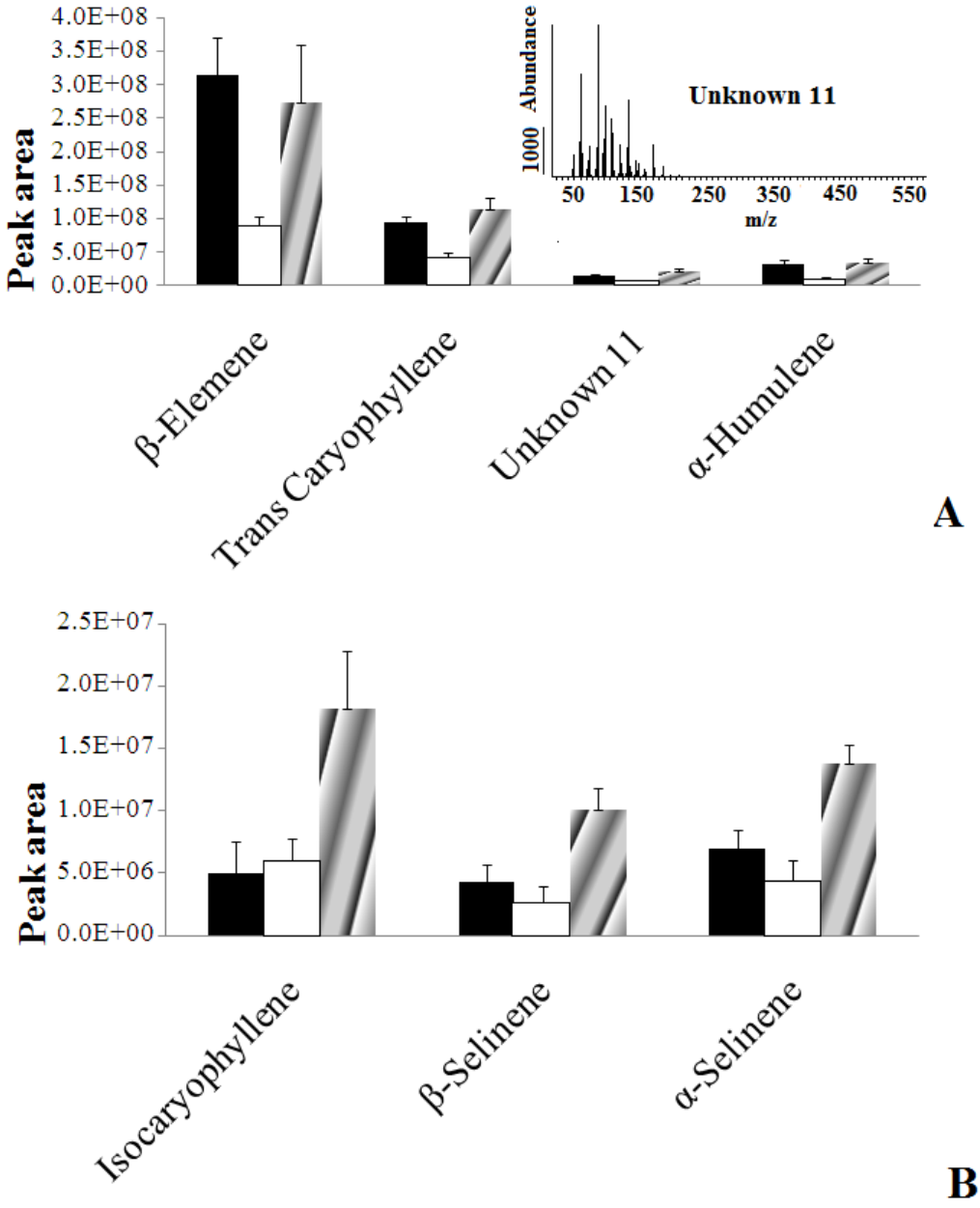

Figure 4-10. Significantly different headspace metabolites of zinc deficient (■), HLB-infected (□), and healthy (◪) leaves. Figure A: Metabolites showing significant differences in HLB-infected samples only. Figure B: Metabolites showing significant differences in both HLB-infected and zinc deficient samples when compared to healthy ones. The inset shows the mass spectra of the unknown compound.

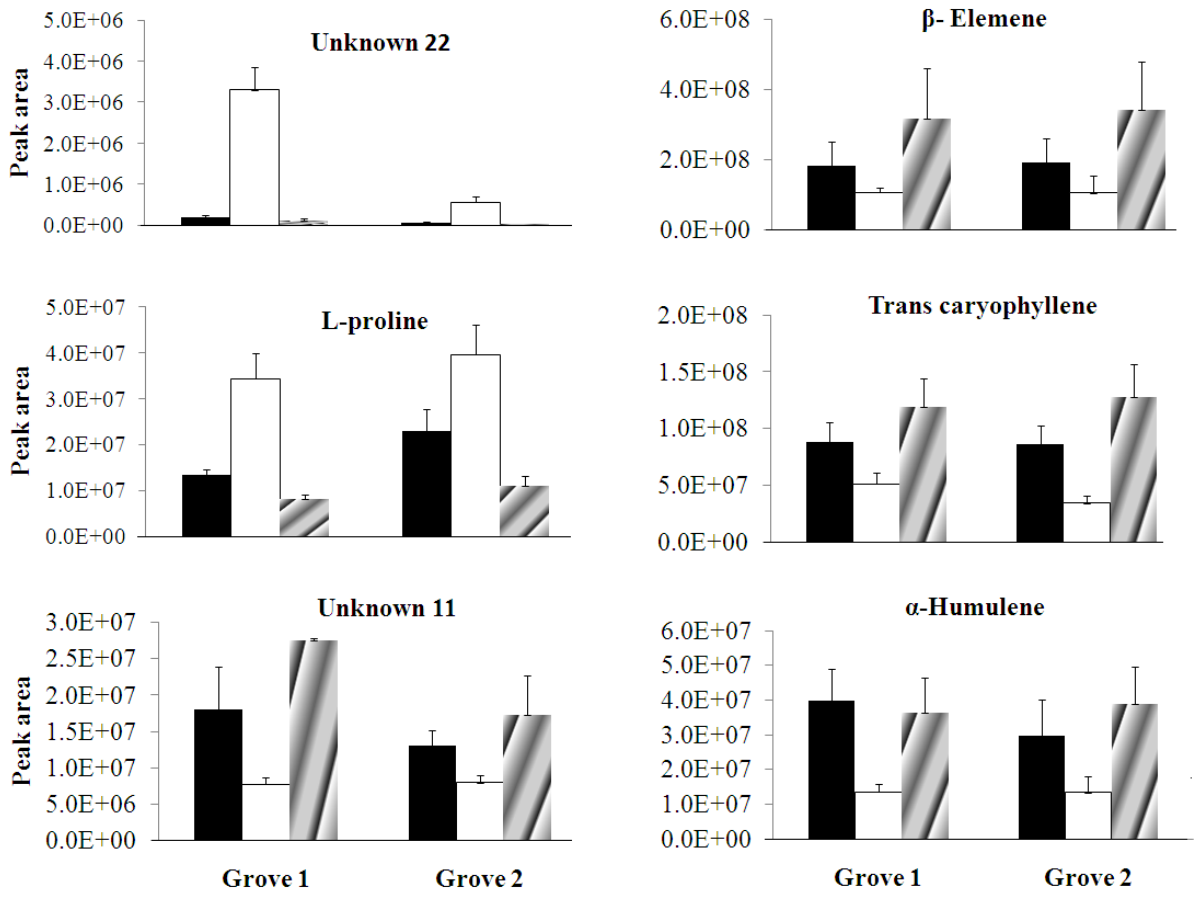

Figure 4-11. Significantly different metabolites of HLB-infected (□) when compared to zinc deficient (■) and healthy (◪) leaves from grove 1 (Plant City, FL) and grove 2 (Lake Alfred, FL).

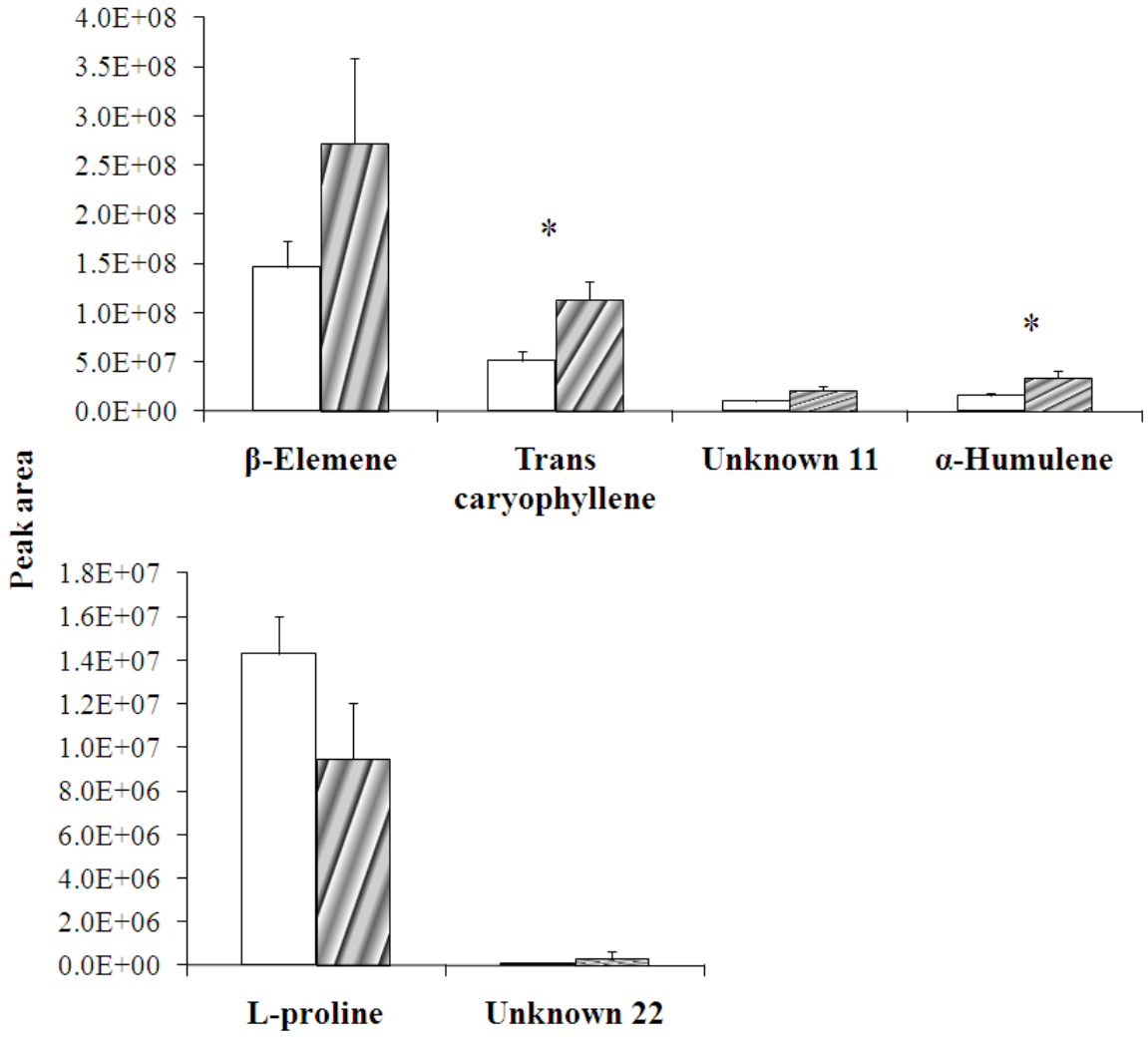

Figure 4-12. Behavior of potential biomarkers in mild or asymptomatic HLB-infected (☐) and healthy (◪) leaves. Compounds marked with * showed significant differences between the two types of samples.

CHAPTER 5
METABOLOMICS OF FODBORNE PATHOGES

Overview

Escherichia coli O157:H7 and *Salmonella* spp. have been related to several outbreaks in United States during the past years. In particular, *Salmonella* spp. have been regarded as the leading cause of foodborne illnesses in the US (Jarquin and others 2009) and one of the most predominant pathogens in Europe (Malorny and others 2009). Several foods such as peanut butter in 2008-2009, peppers in 2008, and poultry in 2007 have been the vehicle of numerous *Salmonella* outbreaks in the US as reported by the Center for Disease Control (CDC). In addition, several *E. coli* O157:H7 outbreaks such as those linked to romaine lettuce in 2010, ground beef and cookie dough in 2009, and spinach in 2006 were reported by CDC. Rapid methods for detection of these microorganisms are needed as an aid to reduce the number of outbreaks. Table 5-1 summarizes recent progress on the development of rapid methods for detection of *E. coli* and *Salmonella*. Pathogen detection by polymerase chain reaction (PCR) is very specific but requires pre-enrichment that may last up to 18 h (Malorny and others 2009) making the total analysis time, in many cases, longer than 24 h. Other methods such as surface plasmon resonance (SPR) are very rapid (~1 h) but have higher limit of detection (LOD) (Table 5-1). Most novel methods such as those based on immunosensors have yielded best results when used in uncontaminated food samples or after enrichment in selective media (Tokarskyy and Marshall 2008). Furthermore, most methods are specific to one single pathogen, making it difficult to test several pathogens at the same time. Therefore, simultaneous detection of pathogens in real food samples is desirable. Recent studies on multiplex detection of *E. coli* and *Salmonella* along with other pathogens have been carried out using immunoassays (Magliulo and

others 2007) and PCR (Seidavi and others 2008; Wang 2008; Suo and others 2010). However, the required LOD of 1 CFU/25g of food along with the analysis time of 24 h or less were not met. Therefore, methods that allow testing of food that meet current regulations that require the absence of pathogenic *E. coli* and *Salmonella* per 25 g of food are still needed (Table 5-1).

Metabolomics, the study of small metabolites present in a system, has been regarded as an alternative for biomarker identification. Metabolomics, has been proposed for discovery of specific biomarkers of foodborne pathogens growing in culture media which in turn can be used to predict the presence of the pathogen (Cevallos-Cevallos and others 2009b). Metabolomic strategies have been used to identify biomarkers formed during plant-pathogen infections by capillary electrophoresis (CE) (Cevallos-Cevallos and others 2009c). Foodborne pathogens have also been analyzed by metabolomic techniques. Maddula and others (2009) used a multi capillary column coupled to ion mobility spectrometry to analyze volatiles produced by generic *E. coli* and confirmed with GC-MS that ethanol, propanone (acetone), heptan-2-one, and nonan-2-one correlated with bacterial growth. Maharjan and Ferenci (2005) were able to differentiate *E. coli* strains from different origins by two-dimensional high-performance thin layer chromatography. Other studies on cellular metabolomics have been carried out on *E. coli* (Rabinowitz 2007) and recently, modified strains of *E. coli* were classified from their wild type counterparts by using GC-FID and GC-MS finding that succinic acid, proline, and asparctic acid were the main metabolites responsible of the classification (Tian and others 2008). Metabolite-based efforts for rapid detection of *E. coli* and *Salmonella* have been carried out using an electronic nose (Siripatrawan and others 2006) and neutral desorption sampling coupled to extractive electrospray-ionization MS (Chen and others 2007). However, no specific biomarkers for each target pathogen were reported.

Even though these techniques are better suited when bacteria levels in food are above 10^5 CFU/g, they show the potential of metabolomics for rapid detection of foodborne pathogens. Therefore, there is still a need of metabolomics-based methods capable of detecting pathogens at levels as low as 1 CFU/25 g of food in less than 24 h. The objective of this study was to determine the suitability of CE, HPLC-MS, and GC-MS based metabolomic techniques for rapid and simultaneous detection of *E. coli* O157:H7, *Salmonella* Typhimurium, *Salmonella* Muenchen, and *Salmonella* Hartford in beef and chicken.

Materials and Methods

Reagents and Bacterial Strains

HPLC grade methanol, putrescine, L-threonine, L-alanine, butanedioic acid, methoxyamine hydrochloride (20mg mL^{-1}) in pyridine (MOX), and *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) were purchased from Fisher Scientific Inc. (Pittsburg, PA). The Difco tryptic soy broth (TSB) and nutrient broth (NB), as well as Sorbitol Mac Conkey were from Becton, Dickinson and Company (Sparks, MD). Chormagar ECC and Chromagar *Salmonella* Plus were from CHROMagar Microbiology (Paris, France).

The bacteria cultures used in this experiment were from various sources: *Escherichia coli* O157:H7 associated with leafy greens (PVTS88) , *Salmonella* Hartford associated with an orange juice outbreak (HO778), *Salmonella* Typhimurium LT2 (ATCC 15277), *Salmonella* Muenchen associated from an orange juice outbreak (IJH592), *E. coli* K12 (LJH506), *Pseudomonas aeruginosa, Staphylococcus* from wound (ATCC 29213), *Saccharomyces cerevisiae* was from molasses distillery yeast ATCC4132 and ATCC 26785, and *Aspergillus Oryzae* from soy sauce ATCC 14895. ATCC refers to American Type Culture Collection (Manassas, VA).

Equipment and Software

The GC model HP 5890 coupled to an HP 5971 series mass spectrometer with ChemStation B.02.02 data acquisition software and the Wiley 138K mass spectral database were from Hewlett Packard (Palo Alto, CA). The chromatographic column used for both headspace and liquid extract analyses was a DB5-MS 60 m x 0.25 mm (length x I.D.) from J & W Scientific (Folsom, CA). The water bath (model Isotemp 3016s) and sonicator (model FS20H) were from Fisher Scientific (Pittsburg, PA). The Class II Type A Biological safety cabinet model NU-425-600 was from Nuaire Inc. (Plymouth, MN). The HPLC system was composed of a Surveyor HPLC, autosampler, and PDA detector; the MS was a LCQ Advantage Ion Trap with ESI (electrospray ionization) as ion source, and the data was processed by using the Xcalibur 2.0 software. The whole HPLC-MS-software system was from Thermo Scientific Inc. (Waltham, Ma). The CE system model P/ACE MDQ with photodiode array detector (PDA), the data acquisition and analysis software Karat 32 version 5.0 was from Beckman Coulter (Fullerton, CA). The capillary was bare fused silica from Polymicro Technologies (Phoenix, AZ) 50 µm I.D. 56 cm total length (48 cm to the detector). Data was aligned to correct deviations in retention time by using an in-house alignment program and compounds were tentatively identified prior to principal components analysis (PCA) and partial least square regression (PLS) as described in the compound identification section. PCA and PLS were carried out to compare the overall metabolite profile of each bacteria by using MATLAB R2008a from The MathWorks (Natick, MA). PLS models were built by using the metabolite profile obtained after growth of microorganisms in TSB as the training data set. Pure cultures growth in TSB as well as inoculated raw chicken and ground beef samples were used as the test data set.

Experimental Design

All microorganisms were individually grown in TSB and NB at 37 °C for 18 h. Then, one hundred microliters of each microorganism were added to 10 mL of TSB to prepare bacteria cocktails. Three cocktails containing all the bacteria (cocktail A), all except *E. coli* O157:H7 (cocktail A-O), or all except *Salmonella* spp. (cocktail A-S) were prepared and grown in both TSB and NB as described before. Samples were taken at 2-h intervals during 24 h and processed as in sample preparation section.

Ground beef and chicken samples obtained from two different supermarkets located in Winter Haven and Haines City, FL. were surface inoculated with *E. coli* O157:H7, *Salmonella* Hartford, *Salmonella* Typhimurium, and *Salmonella* Muenchen at levels of approximately 1 CFU/25 g of food. Inoculation levels were achieved by serially diluting in phosphate buffer the microorganisms grown in TSB. Inoculated meats were allowed to dry for 20 min in the biological safety cabinet. Twenty-five-gram samples were suspended in 225 mL of phosphate Butterfield water and hand-shaken for 2 min to extract bacteria. One milliliter of the suspension was inoculated in 10 mL of TSB and incubated for 18 h at 37 °C.

HPLC-MS Analysis

Two-milliliter aliquots of each sample taken after incubation were analyzed by HPLC-MS. The HPLC-MS system operated with a stationary phase C-18 column and a mobile phase consisting of 80% of an acetic acid solution (0.05% in water) and a 20% of acetonitrile (with 0.05% acetic acid also) during the first 12 minutes, then a gradient phase was applied during the following 47 min to reach a final concentration of 90% acetonitrile and 10% acetic acid solution. The final concentration was held for 11 min. The MS worked with an electrospray ionization source and was operated in the 80 – 1000 m/z range.

CE Analysis

Two-milliliter aliquots of each sample were analyzed by CE. The CE analysis was done using a background electrolyte solution consisting of 76% 11.2mM sodium borate solution at pH 9.3 and 5% ACN. Separation was done at 20 kV and detection was done by scanning the total range of 190 to 600 nm. These conditions were selected because they yielded no peak coelution.

Sample Preparation for GC-MS Analysis

After incubation, samples were spiked with approximately 50 mg of internal standard (IS1) malic acid and with 20 µL of (E,E,)-2,4 nonadienal as second internal standard (IS2). IS1 and IS2 were added as quality control of the derivatization and headspace extraction respectively as well as to assure adequate GC-MS analysis and library matching. Endogenous IS1 and IS2 were not detected in either sample category under tested conditions, and did not interfere with any peaks in the chromatograms.

One milliliter of each spiked sample was mixed with 3 mL of methanol and metabolites were further extracted by sonication on ice for 10 min and stored at 20 °C for 24 h. Quenching and metabolite extraction by cold methanol have been shown to yield the best results in *E. coli* studies (Prasad Maharjan and Ferenci 2003; Winder and others 2008). Extracts were derivatized prior to GC-MS analysis as described in liquid extracts analysis section. The remaining of each sample (10 mL) was submitted to headspace analysis as described in the headspace analysis section. Levels of *E. coli* O157:H7 and *Salmonella* spp were determined by serial dilution in Butterfield's phosphate buffer followed by inoculation on Sorbitol Mac Conkey agar and Chromoagar ECC (for *E. coli* strains) as well as Chromoagar Salmonella Plus (for *Salmonella* strains).

Headspace Analysis

A solid phase micro-extraction (SPME) fiber 50/30 µm DVB/Carboxen ™/ PDMS StableFlex ™ for manual holder 57328-U from Supelco (Bellefonte, PA) was conditioned at 270 ºC for 1 h prior to its first use, and daily at 240 ºC for 5 min. Ten milliliters of the incubated samples were transferred to a 50-mL vial and equilibrated at 47 ºC for 30 min while stirring. The pre-conditioned SPME fiber was exposed to the headspace of the equilibrated samples for 40 min at 47 ºC and then splitlessly injected into the GC-MS. The injector temperature was 250 ºC, the oven was initially held at 55 ºC for 1 min, the temperature rate was 7 ºC min^{-1}, and the final temperature of 300 ºC was held for 5 min. Ultrapure hydrogen was used as the carrier gas at 0.8 mL min^{-1}. The MS was tuned to maximum sensitivity in electron impact mode, positive polarity, and the total ion current was recorded for a mass range of 25-650 amu. The GC-MS interface was set to 318 ºC.

Liquid Extract Analyses

For liquid extracts, 540 µL were transferred into a 2-mL GC vial and dried under a nitrogen flow. Thirty microliters of MOX were added to the dried extract and allowed to react for 17 h at room temperature as recommended in previous reports (Gullberg and others 2004). After methoximation with MOX, silylation reactions were induced by adding 80 µL of MSTFA for 70 min at room temperature. Other amounts of MSTFA and reaction times yielded lower number of detected peaks and poorer reproducibility. Volumes of 0.3 µL of derivatized sample were splitlessly injected into the GC-MS. The injector was at 250 ºC, the initial oven temperature was 70 ºC held for 1 min, the temperature rate was 10 ºC min^{-1}, and the final temperature was 315 ºC held for 10 min. After 8 min of solvent delay the total ion current of mass fragments in

the range of 50 – 650 amu was recorded. Other MS conditions were identical to that used for headspace analysis.

Compound Identification

Visual examination of mass spectra obtained at the beginning, middle, and end width of each peak revealed no coelution in any of the chromatogram peaks. Compound identification was done by library matching of mass spectra using the Wiley library and our internal databases. Compound identity was obtained and reported in Tables 5-3 and 5-4 when the matching value of the mass spectra comparison was 70 or higher and retention time matched those of the pure compound run under identical conditions.

Results and Discussion

Detection and Culture Media Analysis

Individual and cocktail samples were run by HPLC-MS and CE-PDA to determine potential biomarkers. Only one peak with potential difference was detected by using any of these two technologies by comparing any set of pathogens. Figure 5-1 A and 5-1 B show the results for *E. coli* O157:H7 and cocktail A-O. However, GC-MS yielded the highest number of detected peaks and greatest significant differences (Figure 5-1C). These results were expected, because GC-MS is a better alternative for detecting compounds with low polarity and molecular weight (Fancy and Rumpel 2008) which are the ones involved in bacterial metabolism in this study. Therefore GC-MS was used for the rest of the study.

Metabolites were measured and compared during and after growth in both TSB and NB by GC-MS. A higher number of metabolites were detected in TSB (Table 5-2) and all the compounds detected in NB were also present in TSB but in higher concentrations. This may be

due to a greater nutrient availability in TSB. Therefore, TSB was selected for the rest of the study.

GC-MS Analyses of Derivatized Samples

Analysis of derivatized samples yielded 62 compounds. Table 5-3 shows the metabolites with potential ID detected in each type of sample. Metabolites not detected (ND) in cocktails A, A-S, and A-O as well as in the pure culture of *Salmonella* Typhimurium and *E. coli* O157:H7 and reported in Table 5-3 were detected in control samples (TSB with no inoculation). All Salmonellae produced metabolite profile with no significant differences; hence only T was reported in Table 5-3. Among all metabolites detected, only putrescine was identified as a potential biomarker for *Salmonella* spp. because of its presence in all the samples containing any of the *Salmonella* strains tested and its absence in any cocktail containing non-*Salmonella* microorganisms. However, putrescine is known to be produce by other bacteria such as *Citrobacter freundii*, *Enterobacter* spp. strains, *Serratia grimesii*, *Proteus alcalifaciens*, *Morganella morganii*, and *Proteus mirabilis* (Durlu-Ozkaya and others 2001) among others that were not included in this study. Therefore, putrescine and all of the detected compounds were not regarded as biomarkers.

Multivariate techniques such as PCA were tested to compare overall metabolite profile of each pathogen and cocktail. Data were aligned to the total area prior to PCA. Figure 5-2A shows that PCA of derivatized samples was not able to fully classify samples, suggesting similarities between the metabolite profiles of sample groups. Principal components (PC) 1 and 2 accounted for only 35% of the variation. A weak discrimination of control, staph, *Saccharomyces cereviseae*, and cocktails A-S and AO can be seen in PC1, suggesting small differences in the compounds with the highest PC1 loading values. Out of the nine identified compounds with the

highest absolute loading values in PC1 and 2 (Figure 5-2B), only cadaverine, glucose, and the amino acids glycine, histidine, and tyrosine showed significant differences among sample groups. Figure 5-3 shows the levels of these differences. Further research is needed to determine the ID of the unidentified compounds with the highest loading values.

Cadaverine was produced by all microorganisms tested but in much lower amounts in staph and *Saccharomyces cereviseae*. Cadaverine formation by *Saccharomyces cereviseae* has been linked to ornithine decarboxylase activity (Walters and Cowley 1996). Therefore, the lower levels of cadaverine in these microorganisms may be due to enzyme activity lower than in other micros. Cadaverine formation has often been related to the presence of members of the *Enterobacteriaceae* family (Halasz and others 1994) such as *E. coli* and some *Salmonella* and mostly due to decarboxylation of lysine by lysine decarboxylase. Dextrose was significantly higher in C due to its consumption by all the microorganisms tested. Changes in the amino acids glycine, histidine, and tyrosine may be due to the different metabolic pathways of each microorganism tested. Further research is needed to determine the reasons of these differences. Glycine was significantly higher in *E. coli* K12, *E. coli* O157:H7, and cocktails. Glycine synthesis by *E. coli* has been suggested as a result of the metabolism of L-serine as precursor (Pizer 1965). This is in agreement with L-serine levels reported in Table 5-3 because L-serine was initially present in TSB and not detectable in any sample containing any *E. coli* or *Salmonella*, supporting the observation of L-serine as glycine precursor in *E. coli* and suggesting the same for *Salmonella* metabolism as well. L-Histidine was significantly higher in staph and *Saccharomyces cereviseae*, but consumed by *Aspergillus oryzae*, all *E. coli* and *Salmonella* suggesting possible synthesis of this amino acid by staph and *Saccharomyces cereviseae*. Pathways for biosynthesis of histidine has been previously discussed for staph (Burke and Pattee

1972) and *Saccharomyces cereviseae* (Fink 1966) reporting clusters of genes involved in the biosynthesis process. L-tyrosine was significantly lower in control samples, suggesting biosynthesis or release of the amino acid by specific proteases possibly present in each of the microorganisms tested. Further research is needed to test this hypothesis and to better understand the reasons of the differences in metabolite production of the microorganisms tested.

SPME Analyses

Headspace metabolomic analysis has been shown to provide additional information that may be lost during derivatization of extracts (Cevallos-Cevallos and others 2010). Analysis based on headspace-SPME yielded 39 compounds. None of the compounds detected by SPME were detected after derivatization of liquid extracts. Therefore, SPME analysis provided an additional list of metabolites. Tentative identity was obtained for the compounds listed in Table 5-4. All *Salmonella* strains used in this study produced similar metabolite profile; hence only *Salmonella* Typhimurium was reported in Table 5-4. None of the compounds detected were exclusively related to any single pathogen. Therefore, no potential biomarker was reported for *E. coli* O157:H7 and all Salmonellae. As with liquid extracts, statistical analysis was carried out after data alignment and normalization to the total area. Analysis by PCA (Figure 5-4A) achieved a full classification of the samples tested. Only E. coli O157:H7 samples were not fully classified from *Salmonella* samples, suggesting similarities between these two types of pathogens. Figure 5-4B shows the loading plot as well as the 8 compounds with known identity with the highest absolute loading values in PC1 and 2. Only 5 compounds showed significant differences among samples analyzed (Figure 5-5). The alcohol 1-octanol was significantly higher in samples containing *Aspergillus oryzae*. Significant production of higher alcohols including 1-octanol by *Aspergillus oryzae* has been previously reported (Kaminski and others 1974).

Similarly, significantly higher concentrations of 1-propanol and 1-butanol were observed in samples containing *Saccharomyces cereviseae*. Reports describing a significant production of 1-propanol (Carrau and others 2008) and 1-butanol (Valero and others 2002) by *Saccharomyces cereviseae* are common in the literature. Higher content of 2,5-dimethylpyrazine was found in *E. coli* K12 samples. Production of 2,5-dimethylpyrazine by pathogenic and non-pathogenic *E. coli* (Yu and others 2000) has been previously reported. Additionally, the significantly higher production of 2-ethyl-1-hexanol was also related to *E. coli* K12 samples. Further research is needed to elucidate the pathways required for the production of the reported compounds and determine the reason of differences in production levels by each microorganism.

Prediction Model and Validation in Food Samples

Although the change in concentration of no single compound may be exclusively attributed to *Salmonella* or *E. coli* O157:H7, the use of the overall metabolite profile may increase specificity. To test this hypothesis, PLS prediction models were built based on the overall profile of volatile metabolites in the headspace for each sample group (training set). The microorganisms volatile profile was chosen as the training set because of its better classification by PCA (Figure 5-4A). Two PLS models were built, one for prediction of *E. coli* O157:H7 and another for *Salmonella*. Three test sets were selected for testing the model by inoculating each pathogen at the level of ~1 CFU/mL of sample into approximately 10 independent samples of TSB (set 1), raw ground beef (set 2), and raw chicken (set 3). Data from training sets was aligned and normalized prior to PLS regression. Poor prediction models were obtained when test sets were not normalized (data not shown).

Models for prediction of *E. coli* O157:H7 and *Salmonella* were tested in the same samples. Figures 5-6 and 5-7 show the results of the models built for *E. coli* O157:H7 and

Salmonella respectively. The model for *E. coli* O157:H7 consisted on 13 PLS components which accounted for the 86% of the mean square error (MSE) of the response variables and 99% MSE of the predictor variables with a coefficient R^2 of 0.86. The model was able to predict 100% (Figure 5-6A) of the blind samples tested in TSB at levels of 1 CFU/mL after 15-18 h incubation. However, when testing the model in ground beef and chicken samples less than 80% was predicted (with 10% of false negatives). This may be due to the high number of bacteria present in these raw samples that were not included in the training set. To correct for these differences, two samples of raw ground beef and two samples of chicken inoculated with *E. coli* O157:H7 were added to the training set to create additional PLS models that will work in these two matrices. The model for ground beef consisted in 11 components whereas the model for chicken contained 8. Each model accounted for more than 70% of the response MSE and 97% of the predictors MSE with R^2 greater than 0.8. Prediction of ~ 1CFU/mL in 15 – 18 h was 100% for both ground beef (Figure 5-6B) and chicken (Figure 5-6C) models.

Similarly, PLS model for detection of *Salmonella* in TSB was accurate in all the samples tested (Figure 5-7A) but poor prediction was achieved in ground beef and chicken samples. To account for this matrix effect two additional models were created by adding two ground beef and two chicken samples to the training set respectively. The resulting models consisted of 34 and 33 components for ground beef and chicken samples respectively, accounting for 98% of the response MSE and 99% of the predictors MSE with a R^2 value greater than 0.98 each. As reported for *E. coli* O157:H7 models, ground beef (Figure 5-7B) and chicken (Figure 5-7C) models were able to predict 100% of the samples inoculated with *Salmonella* at levels of ~ 1 CFU/mL after 15 – 18 h after adjustment of the models. Other studies reporting low LOD values have been based in expensive techniques such as immunomagnetic separation and PCR (Notzon

and others 2006) or take ~ 24 h to complete (Techathuvanan and others 2010). The LOD of 1 CFU/mL achieved in this study in less than 18 h shows the potential of the use of metabolomics for rapid bacterial detection.

This research shows evidence of the potential use of GC-MS based metabolomics for rapid detection of *Salmonella* and *E. coli* O157:H7 at levels as low as 1 CFU/25 g of food. The potential use of this technique in food processing environments will allow reducing holding time of product before shipment while waiting for microbial results carried out by traditional methods. Additionally, the use of independent models for *E. coli* O157:H7 and *Salmonella* in the same culture media, reduces labor and saves the cost of buying additional microbiology reagents.

Table 5-1. Recent studies on rapid methods for detection of *E. coli* and *Salmonella*

Target bacteria	Method	Sample	Time	LOD	Ref.
E. coli O157:H7	Gold nanoparticle-Inductively coupled plasma MS	PBS	< 1 h	500 CFU/mL	(Li and others 2010)
E. coli O157:H7	Optical biosensing	PBS	2 h	10-100 CFU/mL	(Li and Su 2006)
Salmonella spp.	Magnetic bead-electrochemical detection	Milk	8 h	2.7 CFU/25g	(Liebana and others 2009)
E. coli	Signal enhanced-SPR	Spinach	<5 h	10^4 CFU/mL	(Linman and others 2010)
E. coli O157:H7 and *Salmonella* Typhimurium	Multiplex chemiluminescent immunoassay	PBS	~10 h	10^4-10^5 CFU/mL for each	(Magliulo and others 2007)
Salmonella Typhimurium	SPR	Milk	1 h	10^5 cells/mL	(Mazumdar and others 2007)
Salmonella Typhimurium and Heidelberg	Multiplex PCR	Cheddar cheese and turkey (raw and cooked)	~45 h	7, 10^3 and < 1 (raw and cooked turkey) CFU/mL	(McCarthy and others 2009)
Salmonella spp.	Real time PCR	Meat carcass	26 h	1-10 CFU/100 cm^2	(McGuinness and others 2009)
E. coli	Nanoparticles – surface enhanced Raman scattering	Milk and apple juice	<1 h	10^3 cells/mL (both)	(Naja and others 2010)
Salmonella Typhimurium	Label free electrochemical impedance spectroscopy	Lennox broth	6 min	500 CFU/mL	(Nandakumar and others 2008)
Salmonella spp.	Immunomagnetic separation/RT PCR	Meat	13 h	10 CFU/25 g	(Notzon and others 2006)
E. coli O157:H7	Immunomagnetic separation and real time PCR	Fresh produce	7 h	0.04-0.4 CFU/g	(Prentice and others 2006)
Salmonella spp.	Nested PCR	Fresh produce and poultry	~8 h	4 CFU/25g	(Saroj and others 2008)

Table 5-1. Continued

Target bacteria	Method	Sample	Time	LOD	Ref.
E. coli O157:H7	Array biosensor	Ground beef, turkey sausage, apple juice	~30 min	10^4 CFU/mL	(Shriver-Lake and others 2007)
Salmonella Enteritidis	Immunomagnetic separation and PCR	Milk	16 h	1-10 CFU/mL	(Taban and others 2009)
Salmonella Typhimurium	Loop-mediated isothermal amplification	Pork	24 h	10^2 CFU/25g	(Techathuvanan and others 2010)
Salmonella Typhimurium	Reverse transcriptasa PCR	Pork	24 h	10^1 CFU/25g	(Techatruvanan and others 2010)
E. coli K12	Nanoimmunoseparation and surface enhanced Raman	Water	30 min	4-5 CFU/mL	(Temur and others 2010)

Table 5-2. Differences in peaks detected in samples run in NB and TSB

	Total area	Number of peaks
Nutrient broth	523453543	62
Tryptic soy broth	23456781	25

Table 5-3. Average peak areas +/- standard deviation of the metabolites identified in derivatized samples

Ret. Time (min)	Possible ID	Cocktail A	*Salmonella* Typhimurium	*E. coli* O157:H7
8.46	Butanoic acid, trimethylsilyl ester 12	ND	ND	801 +/- 33
9.40	Propanoic acid, 2-[(trimethylsilyl)ox	8565 +/- 1255	8205 +/- 1078	6663 +/- 1615
10.06	L-Alanine, N-(trimethylsilyl)-, trime	2247 +/- 584	1741 +/- 1032	2446 +/- 782
11.22	Butanoic acid, 2-[(trimethylsilyl)ami	1917 +/- 273	1334 +/- 1140	577 +/- 475
11.88	L- valine	3992 +/- 729	3350 +/- 1794	4170 +/- 985
12.80	Silanol, trimethyl-, phosphate (3:1)	36993 +/- 5986	29992 +/- 22757	35874 +/- 10077
13.11	L-Isoleucine, N-(trimethylsilyl)-, tr	3064 +/- 560	2564 +/- 1410	3295 +/- 725
13.34	Glycine, N,N-bis(trimethylsilyl)-, tr	8451 +/- 445	9481 +/- 1186	8806 +/- 2086
14.14	L-Serine, N,O-bis(trimethylsilyl)-	ND	ND	ND
14.58	L-Threonine, N,O-bis(trimethylsilyl)-	ND	150 +/- 300	1556 +/- 567
16.58	L-Methionine, N-(trimethylsilyl)-, tr	1527 +/- 363	1374 +/- 839	1627 +/- 398
16.67	L-Proline, 5-oxo-1-(trimethylsilyl)-,	6784 +/- 209	7124 +/- 2188	7017 +/- 1848
16.99	Phenylalanine 1tms	511 +/- 472	1397 +/- 1346	435 +/- 240
17.83	Unknown	1986 +/- 449	1776 +/- 1433	2158 +/- 726
18.02	Phenylalanine 2tms	5639 +/- 996	5438 +/- 2375	5748 +/- 1520
18.50	L-Asparagine, N,N2-bis(trimethylsilyl	ND	148 +/- 297	ND
19.38	Trimethylsilyl of putrescine	3253 +/- 2163	2746 +/- 754	ND
20.68	Cadaverine tetratms	14894 +/- 3184	14137 +/- 6682	14807 +/- 3903
20.84	Xylitol 5tms	1069 +/- 121	350 +/- 701	840 +/- 471
21.06	1H-Indole-3-carboxaldehyde, 1-(trimet	209 +/- 362	500 +/- 650	188 +/- 293
21.39	Glucose oxime 6TMS	ND	ND	ND
21.47	L-Histidine, N,1-bis(trimethylsilyl)-	ND	ND	ND
21.71	L-Tyrosine, N,O-bis(trimethylsilyl)-,	1306 +/- 186	1338 +/- 244	1094 +/- 268
21.90	Glucose 5tms	162 +/- 281	134 +/- 269	82 +/- 200

Table 5-3. Continued

Ret. Time (min)	Possible ID	Cocktail A	*Salmonella* Typhimurium	*E. coli* O157:H7
22.67	Butanoic acid, 4-[bis(trimethylsilyl)	230 +/- 398	264 +/- 304	83 +/- 203
24.74	Tryptophan 2tms	983 +/- 418	1287 +/- 703	1116 +/- 351
29.12	Ribitol-1,2,3,4,5-pentatms	682 +/- 303	4680 +/- 536	3652 +/- 944
30.13	.Alpha.-d-glucopyranoside, methyl 2,3	ND	456 +/- 537	ND

Table 5-4. Average peak areas +/- standard deviation of the metabolites identified in headspace-SPME samples

Ret. Time (min)	Compound Id	Cocktail A			*Salmonella* Typhimurium			*E. coli* O157:H7		
3.35	Ethanol	73054	+/-	10364	23617	+/-	20430	27900	+/-	19986
3.71	1-Propanol	24351	+/-	3455	7872	+/-	6810	9300	+/-	6662
3.93	2-Butanone	26360	+/-	8848	14477	+/-	6687	16203	+/-	5761
4.35	1-Butanol	4255	+/-	3162	ND			ND		
5.34	1-Butanol, 2-methyl-	53889	+/-	4522	32229	+/-	10845	36975	+/-	8748
5.4	1-Butanol, 3-methyl-	192492	+/-	35750	127879	+/-	9782	147583	+/-	22789
8.92	Pyrazine, 2,5-dimethyl-	14118	+/-	8110	33338	+/-	10343	17619	+/-	2759
11.68	1-Hexanol, 2-ethyl-	43998	+/-	20844	103627	+/-	18299	58698	+/-	3524
12.71	1-Octanol	80446	+/-	71878	10125	+/-	2927	12215	+/-	3174
13.58	Nonanal	227	+/-	393	ND			807	+/-	746
15.99	2-Nonen-1-ol, (E)-	26580	+/-	14228	6972	+/-	1217	6726	+/-	2378
22.51	Phenol, 2,6-bis(1,1-dimethyl	10032	+/-	5296	2436	+/-	1303	1791	+/-	306
24.01	Propanoic acid, 2-methyl-, 1-(1,1	1023	+/-	1771	ND			528	+/-	1182

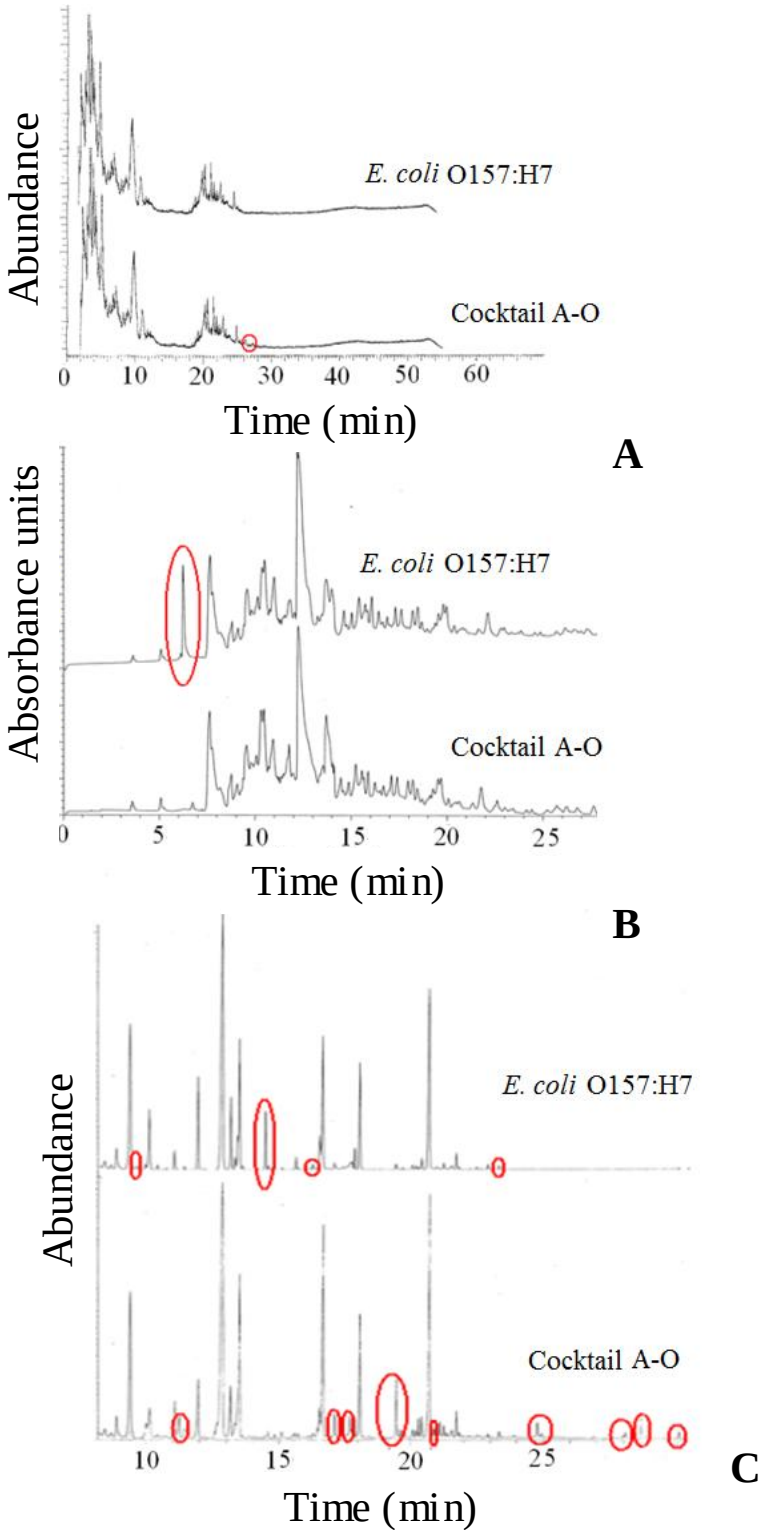

Figure 5-1. Metabolite differences between E. coli O157:H7 and cocktail A-O. Analysis done by HPLC-MS (A), CE-PDA (B), and GC-MS (C). Circled compounds show potential differences.

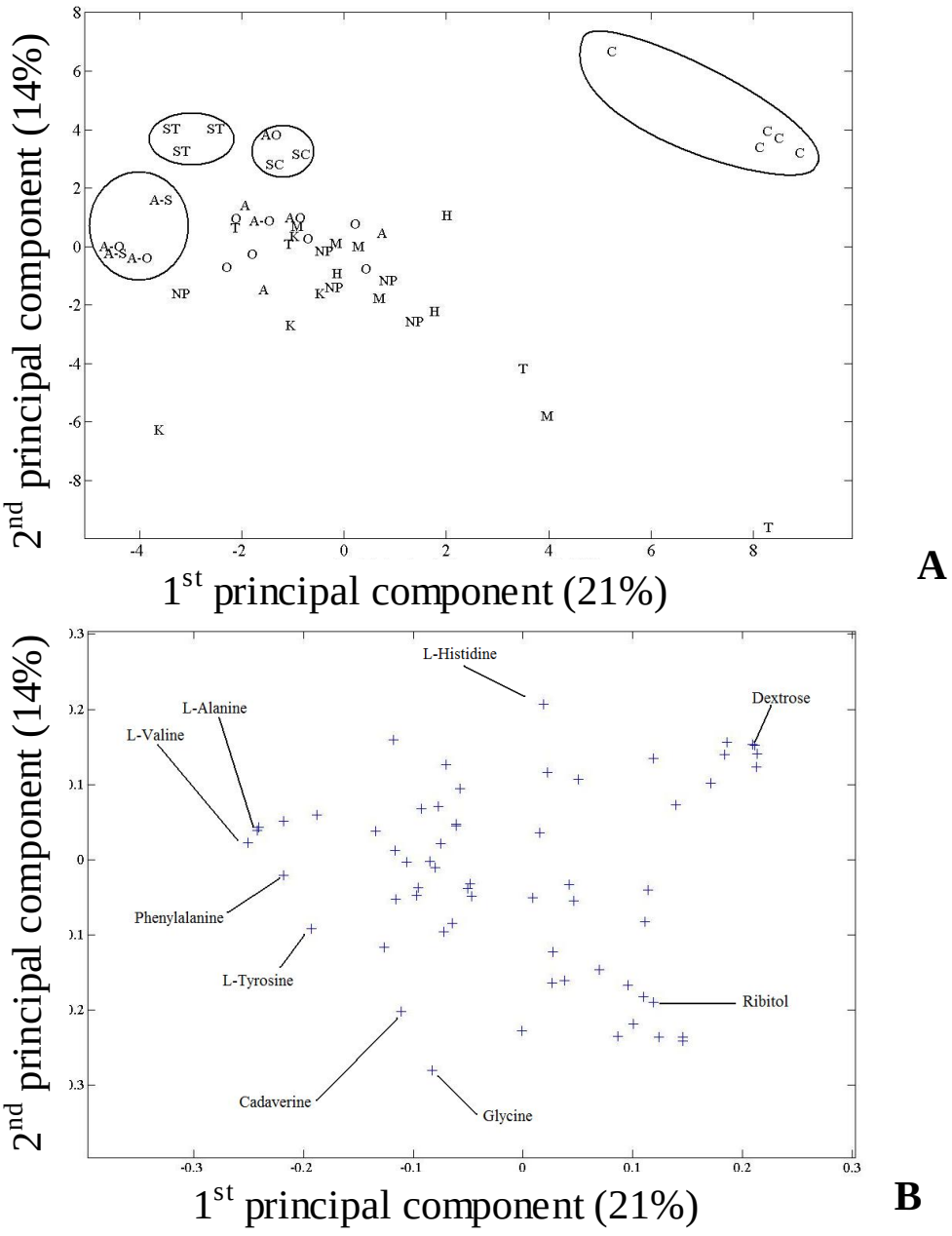

Figure 5-2. Scores plot (A) and loadings plot (B) of the principal components analysis of liquid extracts of *Escherichia coli* O157:H7 (O), *Salmonella* Hartford (H), *Salmonella* Typhimurium (T), *Salmonella* Muenchen (M), *E. coli* K12 (K), *Staphylococcus aureus* (ST), *Saccharomyces cerevisiae* (SC), and *Aspergillus Oryzae* (AO). Significant differences are in Figure 5-5.

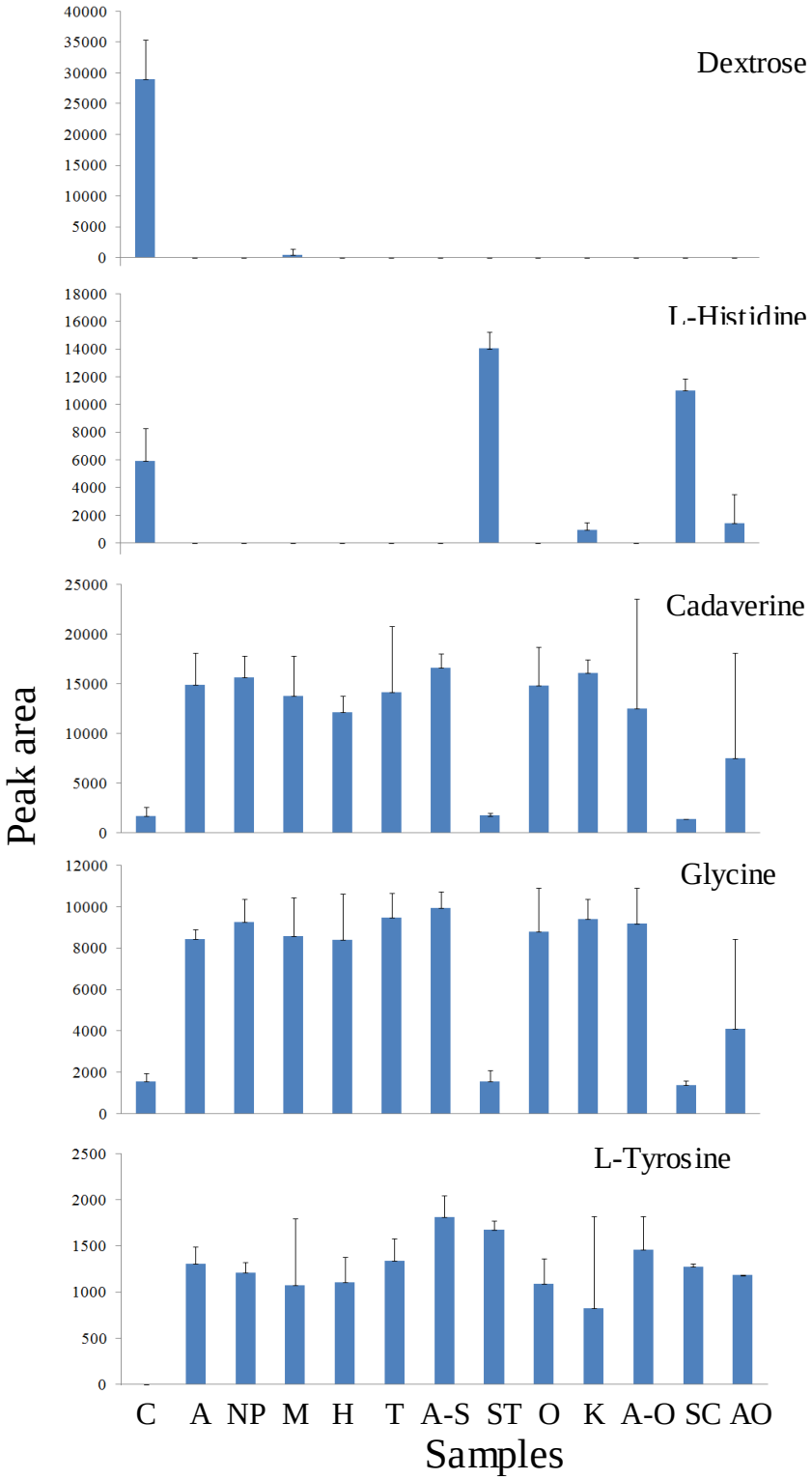

Figure 5-3. Metabolites responsible for PCA classification in derivatized samples. Codes are as in Figure 5-2.

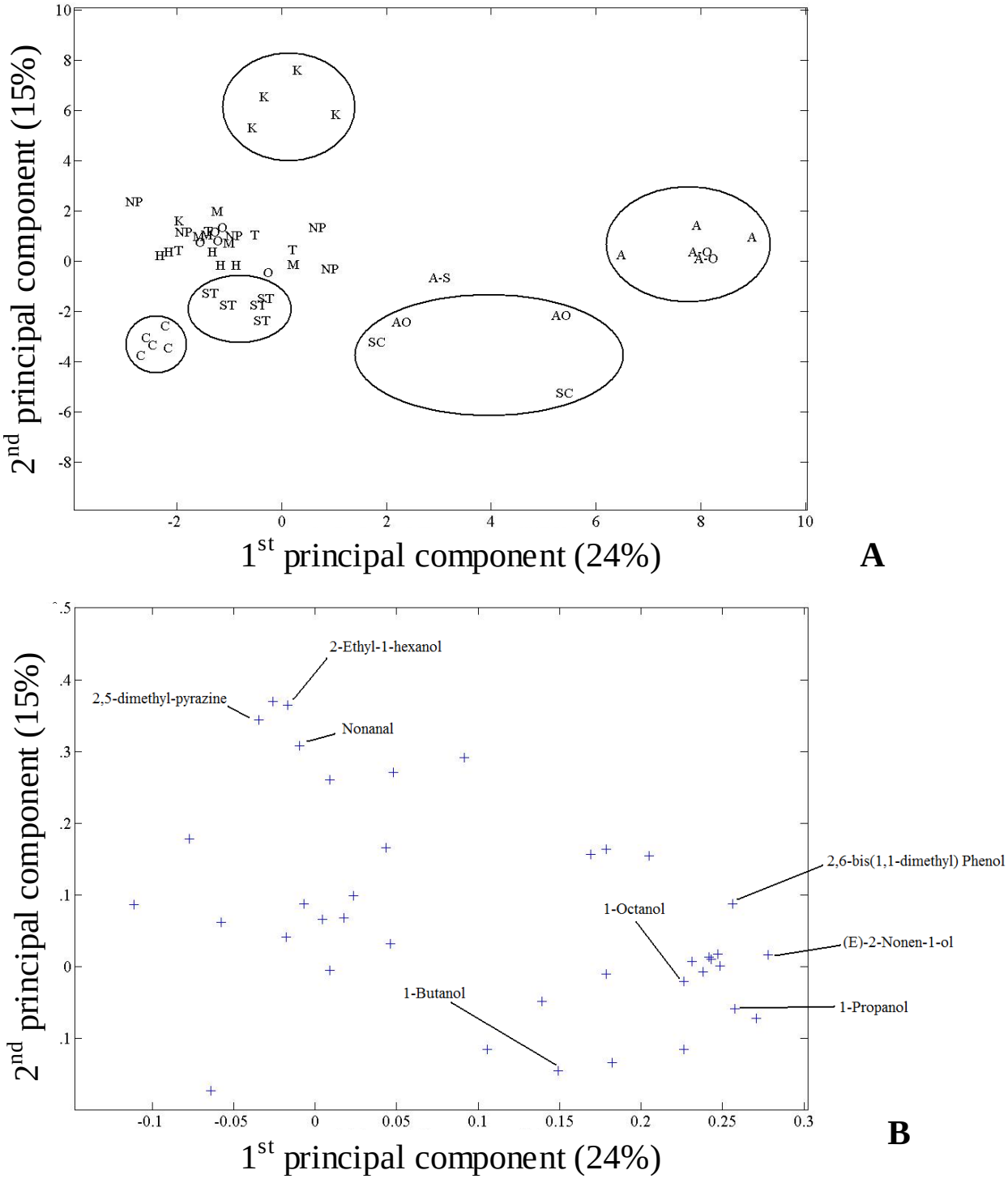

Figure 5-4. Scores plot (A) and loadings plot (B) of the principal components analysis of headspace-SPME samples. Codes are as in Figure 5-2. Significant differences are in 5-5.

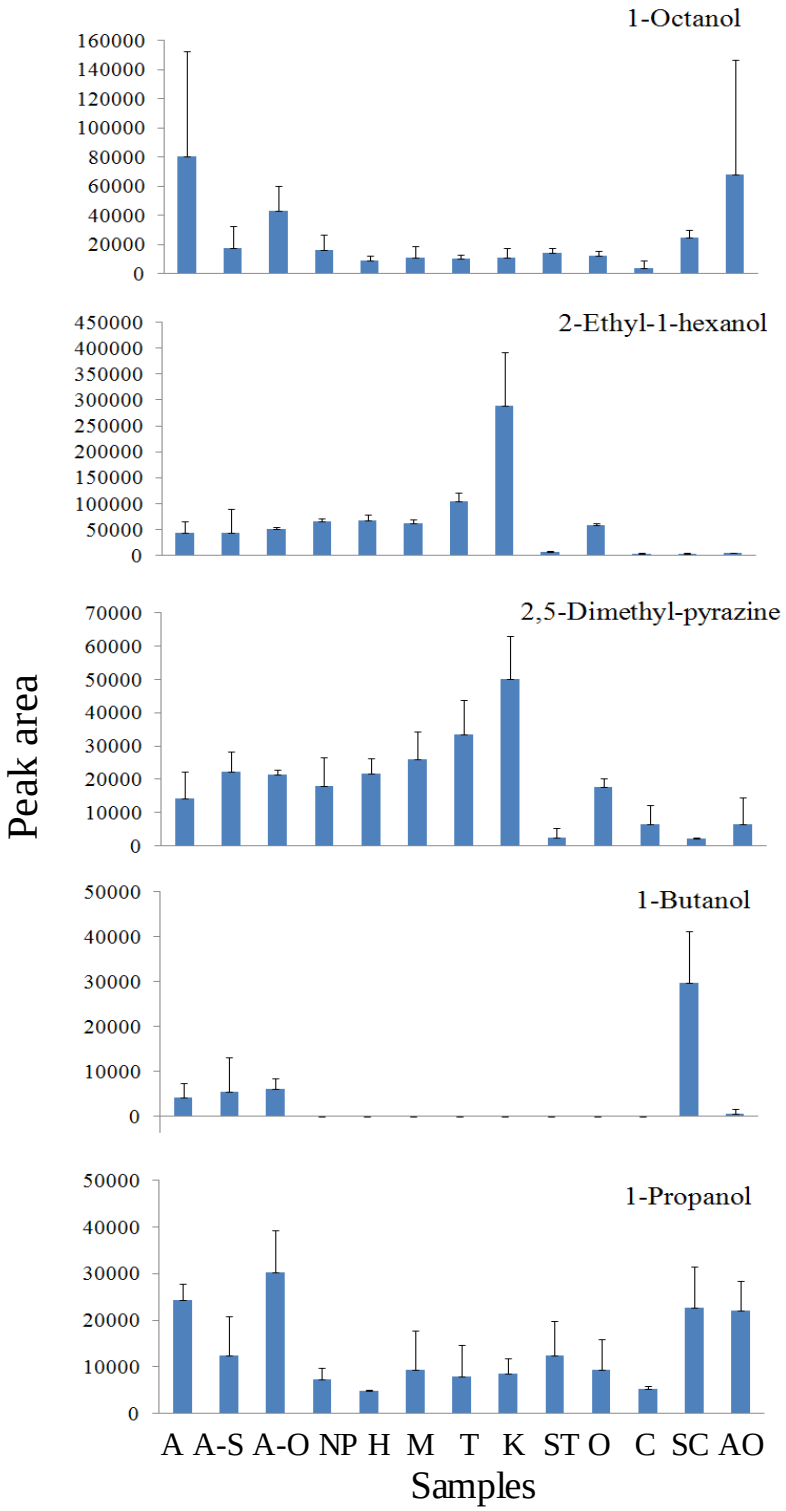

Figure 5-5. Metabolites responsible for PCA classification in headspace SPME samples. Codes are as in Figure 5-2.

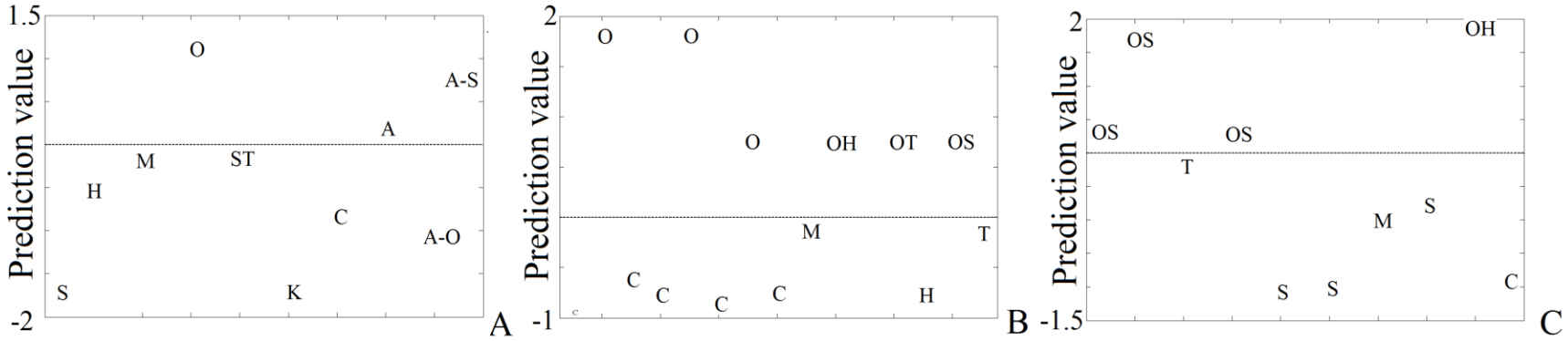

Figure 5-6. Prediction models for *E. coli* O157:H7 tested in pure culture (A), and adjusted models for raw ground beef (B) and raw chicken (C). Codes are as in Figure 5-2

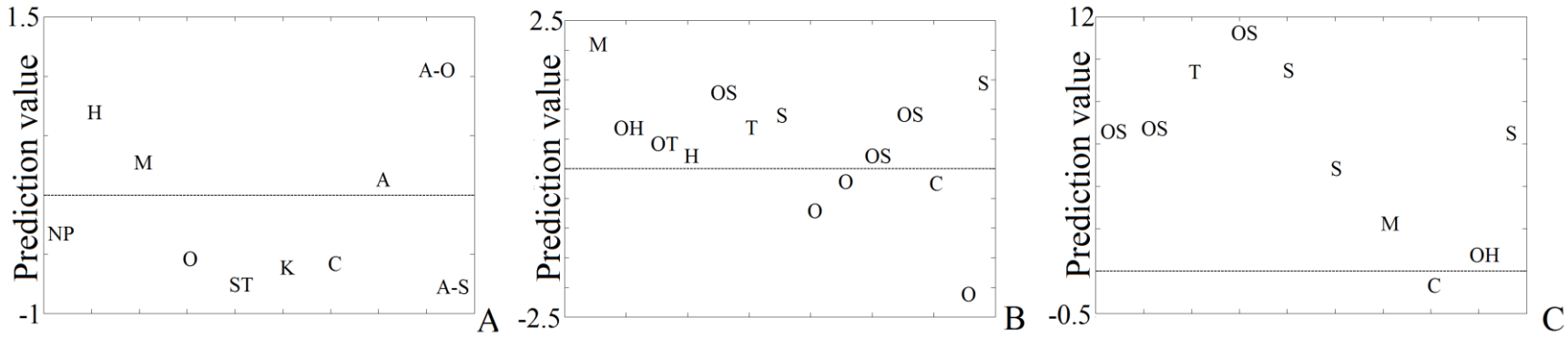

Figure 5-7. Prediction models for *Salmonella* tested in pure culture (A), raw ground beef (B) and raw chicken (C). Codes are as in figure 5-2

CHAPTER 6
SUMMARY AND FUTURE WORK

Metabolomics has shown to be an important tool for the progress of the main food science areas such as compliance of regulations, processing, quality, safety, and microbiology. This research suggests that the potential of metabolomics can also be expanded to the rapid diagnosis of plant diseases and foodborne pathogens. Two forms of rapid detection can be achieved by metabolomics: by using biomarkers or multivariate analysis. This dissertation can be divided into three major areas: Metabolomics of Huanglongbing (HLB), metabolomics of foodborne pathogens, and general metabolomics in food science.

Metabolomics of HLB

Our research on metabolomics for rapid detection of HLB showed several metabolites with a strong correlation between their peak area and the infection scale. These metabolites were detected by HPLC-MS but no individual identification was reported due to lack of databases for this technique. Further research will involve a selective extracts of compounds in the area of interest number two (flavonoids derivatives) for better identification, other techniques such as capillary electrophoresis (CE) were also tested in this study. Capillary electrophoresis coupled with photo-diode array detection (PDA) was able to detect significant differences in metabolite profile between healthy and HLB infected citrus leaves. Results show CE-PDA potential for untargeted metabolomic analysis for plant diseases as well as its use for monitoring HLB infection based on the concentration of hesperidin, naringenin, and quercetin in leaf extracts. A limitation of PDA is the difficulty in identifying compounds using their UV-Vis spectrum. Identification of unknown compounds from this research will require further use of alternative methods, such as mass spectrometry (MS). Additionally, further research will involve application

of new detection methods such as cyclic voltammetry to aid identification by UV-Vis spectra. Testing the specificity of potential biomarkers reported by CE needs to follow.

Metabolomics by GC-MS was also performed in zinc deficient trees and compared to HLB by GC-MS. Metabolite profiling by combined headspace and liquid extracts GC-MS analysis increased the number of candidate biomarkers for HLB detection. Compounds identified via headspace analysis were not detected in the derivatized liquid extracts, strengthening our hypothesis that headspace volatiles were lost during sample drying or they were present in a very low amount in the liquid phase needing to be concentrated in the headspace through solid-phase micro-extraction (SPME). The clear principal components analysis (PCA) classification of the derivatized liquid extracts showed greater metabolite differences among sample groups when comparing with the headspace analysis. Even though PCA was not able to discriminate among SPME samples, ANOVA reported significant differences in the levels of sesquiterpenes of each treatment, sowing the importance of the use of ANOVA in metabolic differentiation studies. The significant changes in content of L-proline and the sesquiterpenes β-elemene, trans-caryophyllene, and α-humulene in HLB-infected samples were comparable to those reported with drought stress in citrus. Therefore, water transport in HLB-infected plants may be compromised. Future research is needed to determine physiological similarities between HLB and drought stress-affected plants. The low amounts of the sesquiterpenes known to have antimicrobial properties in HLB-infected samples propose a post-infection inhibitory mechanism of the synthesis of these compounds, increasing the susceptibility of 'Valencia' sweet orange to HLB. Further research comparing the sesquiterpene profile of tolerant and susceptible orange varieties after HLB-infection is recommended. Additional research in greenhouse plants is needed to determine if pre-symptomatic and pre-PCR-positive changes in the metabolite profile

occur in citrus. Untargeted GC-MS metabolite analyses were also able to find possible biomarkers for zinc deficiency, showing the potential of liquid and headspace-based GC-MS analysis for biomarker identification in both citrus disease and stress conditions. Proposed biomarkers have the potential of being targeted by traditional chemical assays or biosensors, reducing the analysis costs and improving portability as well as rapidity. Even though an initial validation involving two groves was done in this research, additional studies involving different groves, seasons, levels of infection, and stresses are needed. More work is also needed to establish the sugars and proteins profile of diseased trees and to compare these profiles with the ones obtained under other stress conditions. Finally, metabolite profiling of the tree's phloem is needed because HLB is caused by phloem-limited bacteria. Therefore, possible biomarkers may be present in the phloem in concentrated amounts.

Metabolomics of Food Pathogens

This study shows the potential of the use of metabolomics along with multivariate data analysis such as PLS for rapid and simultaneous detection of *E. coli* O157:H7 and *Salmonella* in food samples. This study showed that metabolomic research of microorganisms can be done by GC-MS as opposed to CE and HPLC-MS because of the nature of the bacteria metabolites which are usually small in size. GC-MS based metabolomics was able to detect *E. coli* O157:H7 and *Salmonella* at levels of ~1CFU/25g of food. The rapid nature of this method (less than 18 h) makes it possible to predict presence of *E. coli* O157:H7 and *Salmonella* in food samples while waiting for confirmation by conventional methods. Due to the differences in microbial flora of each sample type, models built by partial least squares (PLS) were matrix specific. Therefore, models were calibrated for each type of food. Even thought food samples analyzed in this study were from different supermarkets, further research is needed to validate models in samples

coming from different sources and seasons. Further research is needed to find potential biomarkers produced during pathogen growth in selective culture media. Moreover, more work is needed to establish metabolic pathways for production of metabolites in bacteria.

Future Directions of Metabolomics in Food Science

Metabolomics has been mostly based on CE, HPLC, and GC. However, the development of rapid technologies such as direct infusion MS (DIMS), ion mobility spectrometry (IMS), and extractive electrospray ionization (EESI) has helped the growth of metabolomics in food science. Further improvement on these techniques is necessary to overcome sensitivity and compound identification issues. Even though metabolomics analyses in food have been much diversified, most studies can be considered as discriminative (Table 1-1) with very few compounds identified. Therefore, the development of a food metabolome database, as suggested by Wishtar (2008b) is needed in order to facilitate compound identification and the development of informative metabolomics. In addition, most reports have focused on fruit and vegetables (Table 1-1) leaving the meat, seafood, and related areas still underexplored. Because of some metabolic similarities, identification of many compounds in red meat can be carried out by using available human metabolome databases Wishtar (2008b).

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BIOGRAPHICAL SKETCH

Juan Manuel Cevallos was born and raised in Manta, Ecuador. He is the sixth of seven children born to Eddie Cevallos and Esperanza Cevallos. He attended Julio Pierregrosse elementary and high school and graduated with honors being the best student graduating in 1999. He was admitted to do an undergrad program in food engineering and received a full tuition waiver at the Escuela Superior Politecnica del Litoral in Guayaquil, Ecuador. He graduated with honors being the best student graduating with a food engineering degree in 2004.

In early 2004, he started working in the quality assurance department for one of the biggest tuna processing companies in Ecuador: Sociedad Ecuatoriana de Alimentos y Frigorificos Manta C.A. (SEAFMAN C.A.). In 2005, he was awarded a Fulbright fellowship to attend University of Florida to pursue a master's degree in food science. At the same time, he pursued a master's degree in agribusiness. He was awarded both degrees in 2007 and started his Ph.D. program in food science at the University of Florida immediately. He obtained his degree in summer 2010 and will continue his research as a postdoctoral associate at the University of Florida's Emerging Pathogens Institute.