analytical chemistry

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pubs.acs.org/ac

Detection of Huanglongbing Disease Using Differential Mobility Spectrometry

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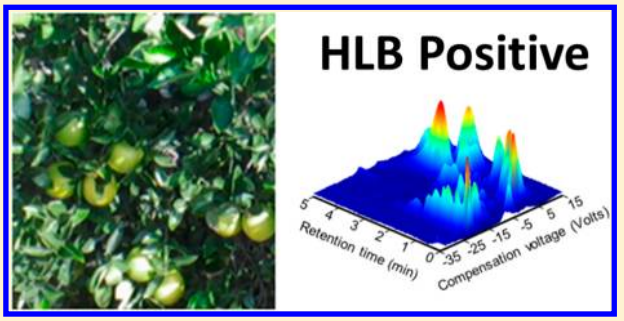
Supporting Information

ABSTRACT: The viability of the multibillion dollar global citrus industry is threatened by the "green menace", citrus greening disease (Huanglongbing, HLB), caused by the bacterial pathogen *Candidatus Liberibacter*. The long asymptomatic stage of HLB makes it challenging to detect emerging regional infections early to limit disease spread. We have established a novel method of disease detection based on chemical analysis of released volatile organic compounds (VOCs) that emanate from infected trees. We found that the biomarkers "fingerprint" is specific to the causal pathogen and could be interpreted using analytical methods such as gas chromatography/mass spectrometry (GC/MS) and gas chromatography/differential mobility spectrometry (GC/DMS). This VOC-based disease detection method has a high accuracy of ~90% throughout the year, approaching 100% under optimal testing conditions, even at very early stages of infection where other methods are not adequate. Detecting early infection based on VOCs precedes visual symptoms and DNA-based detection techniques (real-time polymerase chain reaction, RT-PCR) and can be performed at a substantially lower cost and with rapid field deployment.



Citrus greening disease, or Huanglongbing (HLB), is caused by the bacterium *Candidatus Liberibacter* and is the most destructive and devastating citrus plant disease currently identified by the agriculture community.[1,2] HLB has destroyed citrus fruit production throughout the world, including Southeast Asia, North America, and South America. An example of devastation left by the disease can be seen in Florida, where there was a relatively recent introduction of the disease several years ago. Spread of HLB has led to the lowest acreage levels in recent history of about half of its peak value. Without an adequate response, the citrus industry faces a rapid decline in fruit production and subsequent large economic impacts. In 2012, the *C. Liberibacter* pathogen was identified in California[3] and Texas,[4] the two other major citrus-producing states in the U.S., and timely detection for HLB has become a critical issue.

If detected at an early stage, transmission of the disease from infected trees to their healthy noninfected neighbors can be diminished or even halted through selective tree removal. However, early HLB detection is challenging because infected citrus plants remain asymptomatic, sometimes for years. The infection is especially hard to detect in ornamental residential plantings, where trees are often kept in poor conditions. These plants can act as reservoirs of infection, leading to disease propagation into nearby commercial orchards. A prolonged incubation period and regional dispersal make eradication nonviable.[5] In Florida, the greatest threat is infections that spread from abandoned groves.

Currently, the only widely accepted method used for the identification of the *C. Liberibacter* is polymerase chain reaction (PCR) based assays,[6,7] based on amplification of the 16S rRNA gene of the *liberibacters* with specific primers.[8] However, PCR is an expensive and time-consuming process that is particularly challenging because *C. Liberibacter* pathogen loads are distributed unevenly in plant tissues, may be present at low titer, and can fluctuate with time. Because of the possibility of false negatives from sampling, many trees that are determined to be uninfected continue to be a source of infection for months to years after initial testing.

VOCs are naturally produced by all living organisms, including plants. Released VOCs are closely associated with plant metabolism and can serve as an indicator of the status of a plant's health.[9,10] Disease detection via scent would be advantageous for in-field use, primarily due to its ease and

Received: October 21, 2013
Accepted: February 2, 2014

   dx.doi.org/10.1021/ac403469y | *Anal. Chem.* XXXX, XXX, XXX−XXX

noninvasive nature compared to other methods. However, determining the nature of volatile metabolite variations for complex living organisms poses a formidable challenge from the analytical chemistry standpoint.

Changes in VOCs can be influenced by a variety of conditions, including changes in environment such as water and nutrient stress, plant-to-plant communication, and host-to-insect communication.[11−18] It has been reported that the presence of a plant pathogen can increase the attractiveness of infected plants to specific insect vectors due to elevated emissions and changes in the profile of induced plant volatiles.[19,20] Many genes that encode specific steps in the biosynthesis of terpenoids, green-leaf volatiles, and other types of volatiles that are induced by herbivory have also been identified.[21] Furthermore, it was shown that a bacterial pathogen, such as citrus canker could be identified using volatiles.[22,23] The potential of a portable VOC monitoring device such as the E-nose has been demonstrated for real-time pest and disease monitoring in agricultural and horticultural settings,[24] and the diagnostic capabilities of VOC monitoring are poised to move from concept studies to real-world applications. In the present work, we show how the analytical chemistry approaches could be applied for plant disease detection.

In addition to the laboratory-based analytical method of choice, gas chromatography/mass spectrometry (GC/MS), we employed a portable sensing platform based on the differential mobility spectrometry (DMS) that can be deployed to directly "sniff" the VOCs produced by plants *in situ*. DMS is a variation of a method called high field asymmetric waveform ion mobility spectrometry (FAIMS)[25−27] with planar geometry of electrodes, and it is a very suitable technology for the challenges associated with VOC detection in agriculture. The miniature DMS is a small, portable device with great sensitivity and specificity and relatively low power consumption. It can also operate at ambient temperatures and pressures. The FAIMS technology exploits differences in ion behavior under alternating low and high electric fields. Each chemical species has a unique dependence of its mobility on the electric field strength; therefore, the differences in ion mobilities can be used to identify specific chemicals. An additional direct current (DC) voltage, called a "compensation voltage" (CV), is applied to compensate for ion drift under differential field conditions and to allow a particular chemical species to pass through the device for detection. The CV value is related to the ion structure[28] and mass[29] and is, therefore, particular to a specific ion.[28] Although limited resolution of FAIMS (typical resolution ranges from ∼10 up to ∼500)[30] does not immediately allow for unambiguous identification of species, in combination with portable gas chromatography (GC), DMS can be used to profile very complex VOC distributions such as biological mixes of metabolites at trace concentrations.[31,32] Similarly to the E-nose, the GC/DMS provides a "fingerprint" of the measured VOCs. However, unlike E-nose, GC/DMS also allows conjecturing the identity of biomarker chemicals measured by GC/DMS via the use of chemical standards to build libraries. In the present work, we demonstrate how a field portable VOC detection technology coupled with advanced algorithm development can be applied to tackle the greatest current challenge facing the citrus industry.

EXPERIMENTAL SECTION

GC/DMS Experiment. The healthy and infected citrus trees with varying degrees of symptoms were sampled in the green house and in the orchard as described in the Supporting Information (Citrus Plants (Greenhouse Study), Citrus Plants (Field Study), Independent Confirmatory Study, and GC/DMS Sampling sections). The GC/DMS analysis was carried out using a custom-made device which included a Carbopack X/Carbopack B sorbent trap preconcentrator connected to a DB-XLB 10 m × 0.25 mm × 1.0 μm GC column interfaced to a commercial DMS sensor (Sionex Corporation).[33] The detailed description of the device is given elsewhere.[33] Briefly, the sample is introduced into the device via inlet equipped with a check valve and is sorbed on a trap for a predetermined amount of time. Following the sample intake, the trap is heated and desorbed chemicals are introduced into GC column. The outlet of the GC column is interfaced with the DMS unit. The GC eluent at the outlet of the column is mixed with DMS carrier gas (scrubbed air), passed through a ^{63}Ni ionization source, and introduced into DMS for orthogonal separation and detection. The following optimized experimental settings were used for all of the GC/DMS experiments: sorbent trap preconcentration time 120 s, desorption temperature 300 °C. The GC carrier gas flow was ∼1 mL/min. The GC column temperature was ramped from 40 to 180 °C over 250 s and then held at 180 °C for 230 s, followed by a cool down to 40 °C. The total length of the GC separation protocol was 10 min. The DMS sensor has 500 μm gap size and was operated under the following conditions: carrier gas flow ∼500 mL/min; asymmetric waveform amplitude, 1450 V; RF frequency, 1.196 MHz with the asymmetric waveform 34% high field and 66% low field; CV scan range, −40 to +10 V; 200 steps across the CV scan; 2 s per complete CV scan. The total length of a single GC/DMS measurement was 12 min, and the sampling was repeated continuously for the duration of the experiment.

GC/DMS Data Analysis. A total of 427 independent VOC recordings (samples) were collected. Prior to classification, a visual inspection and principal component analysis (PCA)[34] were applied in order to detect outliers that might occur due to various factors such as chemical background changes (e.g., pesticide spray), if a branch was accidentally damaged, an instrument malfunction, or other occurrences that could invalidate a sample. In addition, overnight or long-term storage of the GC/DMS instrument resulted in accumulation of extraneous chemicals on the cold sorbent trap, leading to background interference in our analysis. Consequently, the first several analyses from each day of sampling where the background chemicals were being flushed out of the system were identified as outliers through PCA and discarded. The outlier detection was done by analyzing the first two principal components. The sample was considered an outlier if it fell out of 95% confidence interval. A total of 394 samples out of the initial 427 were selected for data analysis after outlier removal. All data were autoscaled before application of NPLS: $x_{scaled} = (x - \bar{x})/\sigma$, where σ is the standard deviation, x are all of the values in the sample, and $\bar{x}$ is the mean value. Such scaling is intended to correct for possible bias between volatile abundances in different samples due to factors such as foliage thickness differences among trees and branches and wind impact. A smoothing procedure using a Savitzky−Golay noise-removal filter and a baseline correction were performed on all samples over constant compensation voltage.[35]

dx.doi.org/10.1021/ac403469y | *Anal. Chem.* XXXX, XXX, XXX−XXX

The chemometric data processing was performed using MATLAB (The Mathworks Inc., Natick, MA, 2007) and PLS Toolbox 6.5 (Eigenvector Research, Inc., Manson, WA). To investigate the classification of healthy and infected trees, multiway partial least-squares (NPLS)[36] was employed to quantitatively examine the classification results. NPLS regression is a method that identifies the largest variance in a set of independent variables (X) that maximize correlation with the variance of a dependent variable (Y). In our experiments, the dependent variable is the categorization of each sample: healthy or infected (asymptomatic, mild, or severe symptoms). A validation process was applied to provide the accuracy of the model through a standard confusion matrix.[37] The samples were randomized and split into training (70%) and testing sets (30%), with similar proportions of the samples within each category (healthy or infected) assigned to each set. Before creating the model, the correct number of latent variables was identified through k-fold validation of the samples within the training set (70%). Using the determined number of latent variables and only the training set (70%), NPLS was used to build a model. The model was validated using the testing set (30%). This validation identified the accuracy of the model for each iteration by calculating the percentage of true positive, true negative, false positive, and false negative in a standard confusion matrix. This entire model creation and testing process, from separation into a training and a testing set through the generation of the confusion matrix, was repeated 300 times. The number of iterations was chosen because it appeared to exceed the threshold required to produce consistent results. The accuracies collected from these iterations were combined to represent the expected behavior of the model.

Once the model is generated, the values are returned for each unknown sample tested. These values relate to the categorizations assigned during the generation of the model and correspond to a probability of accuracy for each category. When the probability value that corresponds to healthy exceeds 0.8 and the values within the other categories are below 0.4, there is a strong indication that the sample was taken from a healthy tree. However, since there were a greater number of samples from healthy trees to generate the model, the constraints are relaxed for the other classification groupings. If the value obtained for a specification of infected (e.g., asymptomatically infected) is greater than 0.5 and is the largest value compared to any other class, that sample is considered infected.

GC/MS Analysis. The VOCs were collected *in situ* using the polydimethylsiloxane (PDMS)-based Twister (Gerstel, Inc.) sorbent beads as described in the Sample Collection for GC/MS Analysis section in the Supporting Information. Volatile compounds captured by the Twister were thermally desorbed for analysis and analyzed by GC/MS.[38] The employed instrument was a 6890 GC (Agilent Technologies, Santa Clara, CA) equipped with a thermal desorption unit (TDU, Gerstel, Inc., Muehlheim, Germany), a cryo-cooled injection system inlet (CIS4, Gerstel, Inc.), and a robotic sampler (MPS2, Gerstel, Inc.) interfaced to the Pegasus IV time-of-flight mass spectrometer (LECO, St. Joseph, MI). The volatiles trapped by Twisters were thermally desorbed in the TDU in splitless mode (50 mL/min flow rate, solvent vent mode) at an initial temperature of 30 °C, ramped to 250 °C at a rate of 12 °C/s, and then held at the final temperature for 3 min. The desorbed analytes are cryofocused in the CIS4 inlet with liquid nitrogen (−120 °C). After desorption the inlet is heated from −120 to 260 °C at a rate of 12 °C/s and held at 260 °C for 3 min. Chromatographic separation was performed on a Rtx-5SilMS column with a 10 m integrated guard column (95% dimethyl/5% diphenyl polysiloxane film; 30 m × 0.25 mm (inside diameter) × 0.25 μm d_f (Restek, Bellefonte, PA)). The GC oven temperature program was set as follows: initial temperature of 45 °C with a 2 min hold, followed by a 20 °C/min ramp up to 300 °C with a 2 min hold, followed by a 20 °C/min ramp up to 330 °C with a 0.5 min hold. The carrier gas (99.9999% He) flow was held constant at 1 mL/min. The transfer line temperature between the gas chromatograph and mass spectrometer was held at 280 °C. Mass spectra were acquired at 25 spectra/s with a mass range of 35−500 m/z. The detector voltage was set at 1800 V and the ionization energy at 70 eV. The ion source temperature was 250 °C.

GC/MS Data Analysis. Raw GC/MS data were preprocessed using the LECO ChromaTOF software (v. 2.32) parameters,[38] including a baseline setting just above noise (value = 1), no smoothing, and a signal-to-noise ratio minimum of 20. The compounds are then identified based on mass spectral similarity to prior database entries and retention index. If a compound does not meet the similarity score threshold, it is presumed unidentified and assigned a database entry number. The data files contain the information on Fiehn retention indices[39] base peak m/z and 70 eV EI fragments listed for the identified and unidentified compounds are stored at the UC Davis Metabolomics Core Lab database.[40] The full list of compounds (both identified and unidentified) with their corresponding abundances was generated for each Twister sample. The compounds for all Twisters were then compounded into raw data tables. In these compound tables, each peak was then normalized against the sum of the peak intensities. A Student's t-test was applied within each study to the December 2010, March 2011, and September 2011 samples, in order to identify distinguishable peaks for healthy and HLB samples. To directly compare the sample sets from the three time periods, the HLB subgroups (asymptomatic, mild, and severe) were combined into a single HLB group for March 2011 and September 2011. The p-value for the t-test was set to 0.1 to provide the widest possible inclusion of putative biomarker chemicals. A total of 245 discriminating compounds were found in the December 2010 samples that were statistically different ($p < 0.1$) between healthy and infected trees, 82 discriminating compounds were found in the March 2011 samples, and 38 discriminating compounds were found in the September 2011 samples.

PLS was then applied to quantitatively examine the separability of the selected peaks, using a standard 5-fold cross-validation strategy. The systematic classification accuracies based on the discriminating compounds were as follows: 95.0% for the December 2010 samples (53/57 correct for HLB and 62/64 correct for healthy); 83.5% for the March 2011 samples (54/59 correct for HLB and 12/20 correct for healthy); 83.3% for the September 2011 samples (22/24 correct for HLB and 8/12 correct for healthy). These discriminating compounds are considered to be putative HLB biomarkers, given that differences in their abundances are associated with the presence of the *C. Liberibacter* pathogen and enable determining the health status of citrus trees with high accuracy. An example of the top 5 of the most discriminating analytes selected by a t test for spring season is given in the Table 3. A comprehensive list of discriminating compounds,

dx.doi.org/10.1021/ac403469y | *Anal. Chem.* XXXX, XXX, XXX−XXX

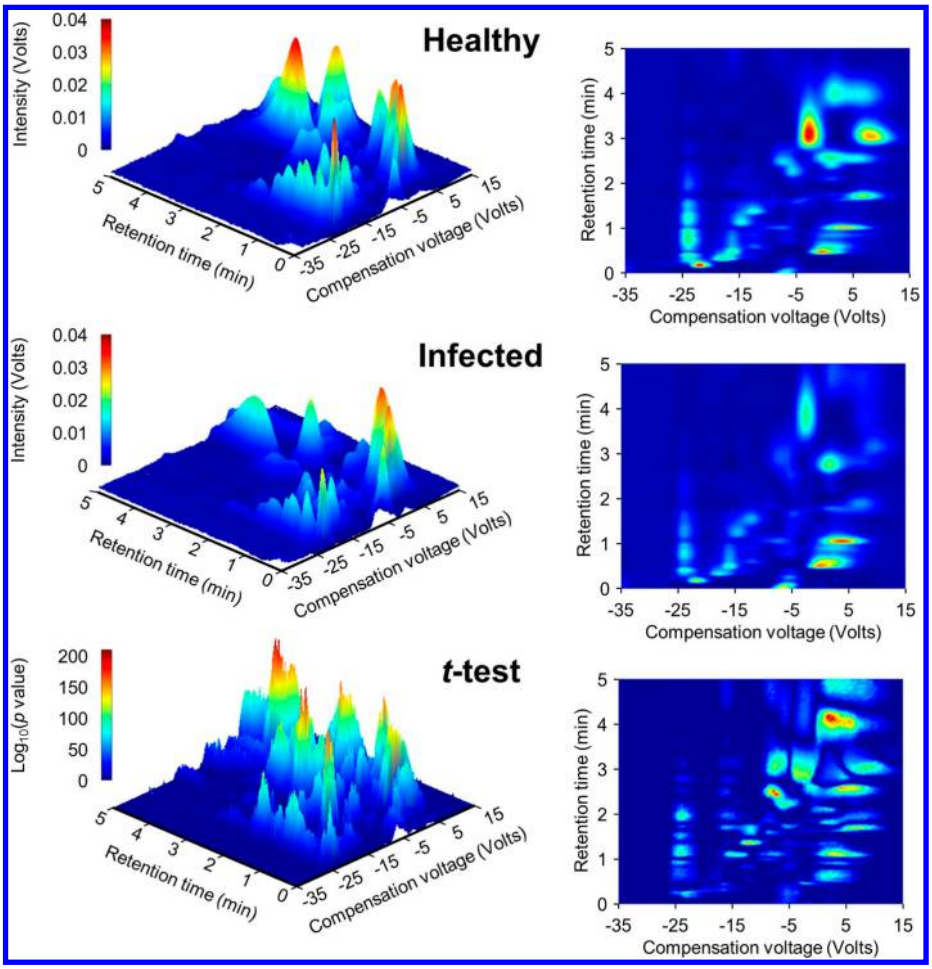


Figure 1. Averaged GC/DMS plots that reflect the VOC signatures for healthy (noninoculated) (top) and inoculated (both PCR+ and PCR−) (middle) trees. All trees are asymptomatic; all of the samples were collected in a single experiment within an 8 h time span. The differences in abundances of various compounds produced by citrus plants foliage recorded at corresponding retention times (GC parameter) and compensation voltages (DMS parameter) are very apparent and easily discernible. The differences are plotted on the Student's *t*-test plot ($p < 0.05$) (bottom).

sorted by PLS coefficient, is given in Table S1 in the Supporting Information.

RESULTS AND DISCUSSION

In our study, we carried out *in situ* and *in vitro* profiling of the VOCs produced by citrus plants (*Citrus sinensis* L. Osbeck) using a portable GC/DMS.[25] We tracked both the physiological changes and the phenotypic changes that occurred in the studied plant systems (as described in the Experimental Section).

A simple proof-of-concept experiment conducted under controlled laboratory conditions demonstrated the power of VOC analysis for disease detection (refer to the Experimental Section). In this experiment, potted greenhouse trees graft-inoculated with *C. Liberibacter* were studied during early asymptomatic infection stages. The infected trees appeared visually indistinguishable from healthy trees, as none had symptoms of disease. Some of the graft inoculated trees had not yet tested positive with PCR, despite definitive infection. In an orchard, such trees would not be properly identified and would remain a source of infection. The VOC testing shows all infected trees (regardless of their PCR status) are vastly different compared to uninfected controls (Figures 1 and 2). These VOC signature differences are specific to the presence of the *C. Liberibacter* pathogen and did not result from other

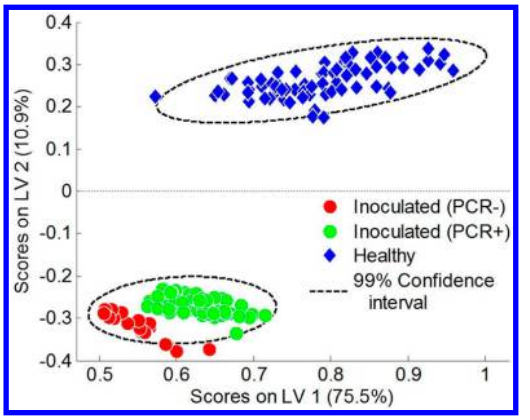


Figure 2. N-PLS score plot of VOC signatures. The Hamlin sweet orange potted seedlings were tested in a greenhouse several months after inoculation. The PCR test is positive (Ct < 40) for some of the studied inoculated trees and negative (Ct > 40) for others. All trees are asymptomatic; all of the samples were collected in a single experiment within an 8 h time span. PCR positive, PCR negative, and noninoculated trees (healthy controls) were tested using GC/DMS in laboratory (constant temperature and humidity). The 99% confidence intervals were calculated for healthy scores and infected (PCR−, PCR+) scores.

dx.doi.org/10.1021/ac403469y | *Anal. Chem.* XXXX, XXX, XXX−XXX

differences in plant health status or experimental conditions, as all of the plants were potted and cultivated identically under well-controlled greenhouse conditions. These obvious VOC differences likely reflect a major disruption in tree metabolism due to the host response to infection.[41]

We further explored this VOC detection paradigm under field conditions. We conducted a longitudinal study of citrus trees naturally infected with *C. Liberibacter* in an outdoor orchard (experimental design described in the Experimental Section). The study encompassed all four seasons across 16 months, and this included large weather variations and the yearly cycles of blooming and fruit production. In actual field experiments, ambient conditions are not controlled and nonspecific VOC alterations can reflect changes in plant metabolism due to light, temperature, water, or nutrients.[42] Also, as noted above, changes in volatiles may occur due to a variety of other factors, such as herbivore feeding and physical damage to the plants, pathogen coinfections, the presence of insects, and other biological sources of volatiles. Nonbiogenic factors such as chemical backgrounds due to insecticide sprays may also be present and could confound pathogen identification in the trees. To discern the features of the VOC signature that indicated the presence of the *C. Liberibacter* pathogen, it was essential to evaluate the contribution of various confounding factors to the VOC profile and establish the sources of variability. These variations occurred partially due to baseline humidity and temperature differences throughout the year as well as temperature and humidity variances between consecutive days in different seasons (shown in the weather log and Figures S1 and S2 in the Supporting Information). In order to reliably discern differences in volatiles specific to the health of each tree as opposed to other causes of variation, hundreds of independent VOC measurements were collected and analyzed through chemometric approaches.

A multiway partial least-squares (NPLS)-based mathematical model was built for health status classification based on collected VOC profiles. Application of this model on seasonal test data subsets resulted in high classification accuracy throughout the year (Table 1). This accuracy was especially high during the summer, where close to 100% accuracy was observed, likely due to the diminished impact of day-to-day weather variations. An example of PLS-DA score plot is shown in Figure S3 in the Supporting Information. This underscores the importance of testing under the optimal conditions in order to achieve the best classification outcomes. The classification accuracies for infected and healthy trees were found to be approximately equal. The aberration of the relatively low rate of true positives in the spring (~63% classification accuracy for healthy trees) may be attributed to the interference of background VOCs produced by the spring blossoming of trees in the test orchard. The total abundance of produced VOCs was noticeably greater for infected than for healthy trees (all the data were autoscaled as described in the Experimental Section). The large output of volatiles from blossom may have masked lower abundances of emitted VOCs for healthy trees. The rate of false negatives remained low throughout all four seasons. A low rate of false negatives is very important for a detection method as it is critical to identify all infected trees and take precautionary measures to curb propagation of the infection. An important factor that may have affected classification accuracy in our trial is initial incorrect health status assignment. While infected trees were visually selected by scouts and their health status confirmed by PCR, it is possible that some trees classified as healthy were in fact infected. This can occur at very early stages of infection, since PCR may produce false negative results. This would have resulted in decreased classification accuracy in our trials.

Table 1. VOC "Fingerprinting" Validation Accuracies for Different Seasons

	classification	true positive (average ± σ)	number of samples
winter	healthy	89.60 ± 7.63	49
	HLB	94.67 ± 7.21	75
summer	healthy	99.90 ± 0.5	46
	HLB	98.19 ± 0.82	27
fall	healthy	95.49 ± 4.11	48
	HLB	88.48 ± 11.62	36
spring	healthy	63.31 ± 20.86	39
	HLB	90.94 ± 7.06	74
		total	394

The abundance of produced VOCs for each sample was autoscaled. This can account for an array of different reasons that the intensity of VOCs may vary between samples such as foliage thickness or time of day of the collection but does not target any single confounding factor. The total abundance of VOCs was noticeably greater for HLB-infected trees than for healthy trees, which corresponds with evidence in a study of transcriptome profiling of citrus fruit that *C. Liberibacter* infection up-regulates production of multiple volatile compounds in citrus plants.[43]

After successfully developing NPLS classification models from seasonal subsets of the 16 month sample set, we applied the model to predict the health status of the trees in a previously unseen orchard with an independently confirmed emerging HLB infection. This challenging scenario included asymptomatic trees from an orchard in a different state with a different geographic location and included a different citrus varietal (Valencia sweet orange instead of Hamlin). In order to validate the developed methodology, the study was double blinded. The PCR-based health status was unknown to us during the VOC sampling and analysis. The classification outputs of the model for the VOC signatures are provided in Table 2. As can be seen, the health status was correctly identified in all cases through the VOC identification method. Two trees (trees 3 and 4) were not outright classified by the VOC monitoring method as infected but were flagged as "suspicious". Our prediction was later confirmed. One of the trees was asymptomatically infected at the time (October 2012) but not identified as infected by PCR until April 2013. The other tree continued to test negative by PCR but was removed in April 2013 due to apparent yellow mottling symptoms. We believe this successful prediction in the double-blinded study underscores the robustness of our proposed detection approach. While PCR detects the pathogen itself, VOC analysis measures the host response to infection. The cells of a host plant overwhelmingly outnumber the cells of the (Liberibacter) pathogen in the system and changes in the host metabolism are potentially detectable at earlier stages of infection.

Many biochemical pathways are up- or down-regulated in infected plants and likely cause the VOC signature alteration (Figure 1). We sought to determine the chemical identity of gas phase biomarkers of HLB infection using *in vivo* VOC collection by Twister followed by GC/MS analysis. The longitudinal GC/MS study was performed in parallel with the

dx.doi.org/10.1021/ac403469y | *Anal. Chem.* XXXX, XXX, XXX−XXX

Table 2. VOC GC/DMS Testing Compared to PCR in an Independent Confirmatory Study in an Orchard with Emerging Infection[a]

	VOC analysis				PCR analysis	
	model probabilities					
tree index	healthy	HLB+ but asymptomatic	HLB+ with mild symptoms	final prediction	HLB titer lever (PCR)	PCR status
1	−0.12	0.86	0.10	HLB+	33.91	HLB+ (control, PCR status known prior)
	VOC analysis shows clear indication of infected but asymptomatic					
2	0.91	0.31	−0.31	healthy	>37	healthy (control, PCR status known prior)
	VOC analysis indicates healthy tree					
3	0.61	0.45	−0.15	likely very early stage of infection; likely case of PCR negative but infected	>37[b]	healthy[b]
	suspicious tree; did not fall strongly in either direction; should not classify as healthy					
4	0.68	0.42	−0.20	likely very early stage of infection; likely case of PCR negative but infected	>37[c]	healthy[c]
	suspicious tree; did not fall strongly in either direction; should not classify as healthy					
5	0.83	0.27	−0.23	healthy	>37	healthy
6	0.82	0.34	−0.22	healthy	>37[d]	healthy[d]
7	0.80	0.32	−0.21	healthy	>37	healthy
8	0.80	0.36	−0.24	healthy	>37	healthy
9	0.91	0.29	−0.29	healthy	>37	healthy
10	0.22	0.54	0.10	HLB+	21.33	HLB+
	not healthy; suspect transition from healthy to HLB+ but asymptomatic					
11	0.07	0.41	0.29	HLB+	26.58	HLB+
	not healthy; suspect transition from HLB+ but asymptomatic to HLB+ with mild symptoms					

[a]No trees have exhibited discernible HLB symptoms at the time of sampling. [b]The tree tested PCR positive in April 2013 with a Ct value of 23.65. [c]The tree has developed apparent HLB symptoms (yellow mottling) and was removed in April 2013. Up until removal, PCR testing continued to produce negative results. [d]The tree tested PCR positive in April 2013 with Ct value of 22.7; the date of infection is not known.

Table 3. List of Top Five Most Discriminating Compounds for a Particular Health Status As Determined by *t*-Test for Spring Season (March 11, 2011)[a]

Healthy		Asymptomatic		Mild		Severe	
Analyte	$-\text{LOG}_{10}(p)$	Analyte	$-\text{LOG}_{10}(p)$	Analyte	$-\text{LOG}_{10}(p)$	Analyte	$-\text{LOG}_{10}(p)$
geranyl acetone	5.095	(1-) tetradecene	4.016	(trans-2-tert-butyl) cyclohexanol acetate	2.494	methyl salicylate	4.137
hexacosane	4.869	linalool	2.640	(4-OH-4-Me-2-) pentanone	1.969	hexacosane	2.040
pentadecane	3.985	nonadecane	2.453	octanol acetate	1.683	(1-) tetradecene	1.795
linalool	3.089	phenylacetaldehyde	2.308	(n-) pentadecanol	1.668	docosane	1.762
(n-) tetradecanol	2.796	ethylhexanol	2.216	benzaldehyde	1.465	ethylhexanol	1.561

[a]A two group *t*-test was run on each identified analyte, separating the samples into each category and the combination of all other categories. Outlier samples were removed and chemicals that are potential environmental contaminants were disregarded. The $-\text{LOG}_{10}(p)$ column gives the value for the corresponding category (i.e. healthy, asymptomatic, etc...) compared with the combination of samples for all other categories for that analyte. A 95% confidence limit is defined as a value of 1.3010 on this table ($-\text{LOG}_{10}(0.05)$). The analyte background color of blue indicates that the analyte is downregulated in comparison to the other categories, while red indicates that the analyte is upregulated.

in situ GC/DMS studies (details provided in the Experimental Section). An example of chromatograms for healthy and infected plants is shown in Figure S4 in the Supporting Information. An example of the top five compounds that are most important for health status discrimination based on Student's *t* test for the spring sampling is given in Table 3 for each category (note that the symptoms severity status assignment is subjective; also, the symptoms change throughout the year, so no direct comparison among seasons is possible). The entire list of discriminating compounds for each season sorted by the PLS coefficient is given in the Supporting Information, Table S1. An example of a principal component analysis based on selected biomarkers is shown in Figure 3. The VOC biomarkers identified by GC/MS differentiated the infected and healthy plants throughout the year, with accuracies ranging from ∼83% to 95%, as described in the Experimental Section. These classification accuracies are similar to the classification based on VOC analysis using the field portable GC/DMS method. From our data it is clear that VOC variations observed by GC/DMS were also very evident in the GC/MS analysis method. Both the overall VOC distributions and the discriminating compounds used as putative HLB biomarkers were found to change significantly season-to-season. Several biomarker compounds were found to be important in more than one season, while some biomarkers are season-specific. Also, some biomarkers are differently expressed at different stages of infection. For example, methyl salicylate is noticeably upregulated in trees with pronounced (severe) symptoms at later stages of infection (Figure S6 in the Supporting Information). This biomarker compound was previously reported as upregulated in infected plants.[20] However, biomarkers that may be able to distinguish asymptomatic or mildly symptomatic trees would be of more interest. For example, geranyl acetone appears to be

dx.doi.org/10.1021/ac403469y | Anal. Chem. XXXX, XXX, XXX−XXX

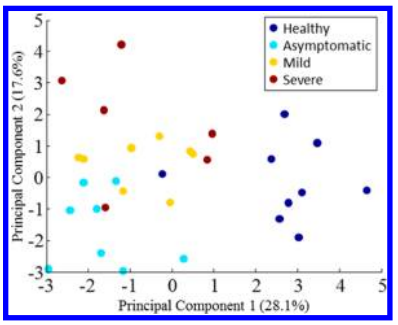


Figure 3. Principal component analysis for the 16 analytes given in Table 3 (4 compounds overlap between different classes: hexacosane, linalool, 1-tetradecene, and ethylhexanol). The "healthy" category has a clear differentiation along the first principal component with the largest coefficients corresponding to geranyl acetone, hexacosane, and (*n*-) tetradecanol. The different levels of infection are harder to differentiate and primarily vary along the second principal component, with the largest coefficients correlating to ethylhexanol, methyl salicylate, and benzaldehyde.

upregulated at all of the infection stages, including the early, asymptomatic stage compared to noninfected plants. This suggests that compounds such as geranyl acetone would be more suitable VOC biomarkers for HLB detection. In general, our results suggest that VOC-based HLB disease detection needs to be guided by season-specific VOC distribution "maps", rather than rely on identification and measurement of a single (or even several) biomarker(s). This may be attributed to seasonal alterations in plant metabolism, fluctuating titer of *C. Liberibacter*[44−47] and differences in the pathogen's life cycle, such as differential expression of *C. Liberibacter* genes in the plant host and insect vector.[48] We see evidence of these complex and season-dependent VOC distributions in both our GC/DMS and GC/MS studies. Because of differences of employed sorbents and ionization sources, the GC/DMS and GC/MS data might not be immediately comparable. Future studies are needed to unambiguously determine which specific chemicals are responsible for producing signal in discriminating regions of GC/DMS chromatograms.

From our data, it is evident that the observed VOC signatures, at least in part, are specific to the citrus host response to the *C. Liberibacter* pathogen rather than a generic indication of a "plant in distress". For example, a ubiquitous coinfecting pathogen citrus tristeza virus (CTV) was also present in all of our originally tested orchard trees but was absent in the greenhouse and at the secondary field test site. Yet the VOC signatures observed did not confound identification of *C. Liberibacter* infection in the geographically removed and CTV-free field test. In addition to CTV, other diseases such as citrus canker (bacterial infection), phytophthora (a member species of Oomycetes (water molds)), as well as black spot (fungal infection) were sporadically present in many of the tested trees but did not appear to produce VOC signatures which would overlap and prevent successful classification of *C. Liberibacter* infection.

Our reported methodology for disease detection provides a novel and powerful tool to combat emerging crop infections. This approach has many other advantages over currently used methods, such as speed, noninvasiveness, and low cost.

The potential value of this early detection solution to the citrus industry is tremendous. By "flagging" infected trees early at the asymptomatic stage, eradication would be both more effective and minimized, since it would take place well before other trees become infected and enter the latent period. This would interrupt the deadly infestation cycle at the source, significantly reducing the heavy costs of losing trees and citrus produce.

However, further advances beyond our study will still be needed. The device employed in the present study is a prototype that can be significantly improved to achieve rapid in-field evaluation, reduced energy consumption, and enhanced portability. Fast responses are critical, especially for surveying citrus groves. The GC analysis time can be significantly reduced while maintaining or even increasing resolution by using more advanced GC techniques such as fast GC or application of a microcapillary array column. By keeping sampling times limited to 1 to 5 min, the VOC monitoring approach can be practical for monitoring large commercial groves with thousands of trees. The method can be initially employed as a global screening tool with further confirmation of suspicious trees by validated molecular methods such as PCR. Low cost analysis is essential for longitudinal monitoring which, in turn, will aid in reducing false negative results through constant surveillance of acreage. Development of instrumentation for use by the citrus industry is already underway.

Finally, an important advantage of this method is flexibility, and our approach may be further extended to other citrus pathogens or other plant systems. This would require only the collection of a VOC database corresponding to a specific plant-pathogen combination, through which an appropriate analysis model can be generated. Because the GC/DMS is not a reagent-based detection method (like PCR), simple database upgrades of the new gas phase biomarkers can be implemented to encompass any number of crops or pathogens of interest. Multiple infections could also be screened in parallel for the same crop. Future advances in chemical analysis and analytical instrumentation, such as introduction of portable GC/MS systems,[49] would allow for further enhancements in detection accuracy. We envision that the "scent-based" detection of a host-pathogen response demonstrated in this study will spur further advances which will aid global efforts to ensure reliable and sustainable food supplies.

CONCLUSIONS

We have shown that volatiles released by plants are closely associated with plant metabolism and can serve as an indicator of the status of plant health. We demonstrate for the first time how this knowledge can be practically utilized. We have shown how application of analytical methods to analyze VOC profiles emitted by trees enables detection of citrus greening disease. The volatiles-based detection method described here has a high accuracy of ∼90% throughout the year, approaching 100% under optimal testing conditions and precedes visible symptoms and DNA-based detection techniques.

ASSOCIATED CONTENT

Supporting Information

Experimental: Citrus Plants (Greenhouse Study), Citrus Plants (Field Study), Independent Confirmatory Study, GC/DMS Sampling, and Sample Collection for GC/MS Analysis; principal component analysis (PCA) plot for all GC/DMS VOC samples collected on October, 2011; PCA plot for the GC/DMS VOC samples collected in July 2011; PLS-DA score of samples from healthy trees and samples from trees infected with *C. Liberibacter*; example of chromatograms for the healthy

dx.doi.org/10.1021/ac403469y | *Anal. Chem.* XXXX, XXX, XXX−XXX

and tree infected with *C. Liberibacter* (severe symptoms); gas chromatography/differential mobility spectrometry (GC/DMS) sampling in-field; examples of biomarker compounds for HLB infection for the spring season (March 2012 study) for different health statuses; table with a list of compounds differentially expressed for healthy and infected trees for different seasons; and weather log. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): US patent application No. 61/465,649, March 21, 2011 has been submitted.

ACKNOWLEDGMENTS

The authors are grateful to Prof. Tim Spann (University of Florida), Applied Nanotech, Inc., and Prof. Mamoudou Setamou (Texas A&M) for assistance. This study was supported by the California Citrus Research Board (CRB), the Industry-University Cooperative Research Program (UC Discovery), and the Florida Citrus Production Advisory Council (FCPRAC).

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