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ARTICLE

Rapid Detection and Differentiation of *Alicyclobacillus* Species in Fruit Juice Using Hydrophobic Grid Membranes and Attenuated Total Reflectance Infrared Microspectroscopy

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Pasteurized juices may undergo spoilage during normal shelf life due to *Alicyclobacillus* spp. Metabolic byproducts during germination of these thermoacidiophilic, endospore-forming bacteria impart off-flavors. The objective was to develop a simple, rapid, and sensitive approach for differentiation of *Alicyclobacillus* spp. by attenuated total reflectance infrared (ATR-IR) microspectroscopy after isolation onto hydrophobic grid membrane (HGM) filters. Dilutions of four different species of *Alicyclobacillus* were filtered onto HGM, incubated on orange serum agar (50 °C, 36−48 h), and dried under vacuum. Spectra were collected using ATR-IR microspectroscopy and analyzed by multivariate analysis. Results indicated that soft independent modeling of class analogy models exhibited clusters that permitted classification at species and strain levels. The methodology was validated by correctly predicting *Alicyclobacillus* (100%) in blind tests. The proposed procedure permits chemically based classification of intact microbial cells. Implementation provides the juice industry with a rapid screening procedure to detect and monitor *Alicyclobacillus* that threatens the quality of pasteurized juices.

KEYWORDS: *Alicyclobacillus* spp.; Fourier transform infrared microspectroscopy; hydrophobic grid membrane filters

INTRODUCTION

The United States is a largely agricultural nation, with economic crop values of over 69.5 billion dollars in 2005−2006 for apples, pears, oranges, peaches, grapes, and tomatoes (*1*). Much of these fruit products are used in the production of pasteurized, noncarbonated juices. An emerging concern for the food industry is the spoilage of fruit juices with *Alicyclobacillus* spp. (mainly *Alicyclobacillus acidoterrestris* and *Alicyclobacillus acidocaldarius*) (*2*−*4*). *Alicyclobacillus* spp. are thermoacidophilic spoilage endospore-formers found in soil environments (*2*, *3*, *5*) and are resistant to pasteurization and hot-filling techniques used by the juice industry to eliminate pathogenic and spoilage microbiota (*6*). Growth of these bacteria in acid products, such as fruit juices, results in flat and sour spoilage (*7*, *8*). Metabolic products of *Alicyclobacillus* spp. include guaiacol and halophenols, which cause undesirable "medicinal" or "antiseptic" taint in fruit juice (*2*, *7*). Spoilage by these bacteria has been observed in apple, pear, orange, peach, and white grape juices (*2*, *4*, *7*, *9*, *10*). *Alicyclobacillus* spp. also have caused problems with juice blends, fruit juice-containing drinks, tomato juice, and canned tomatoes (*5*). In apple juice, *Alicyclobacillus*-associated spoilage is manifested as an off-flavor and off-odor in shelf-stable products (*9*, *10*).

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Currently, batches of juice are not routinely monitored for the presence of *Alicyclobacillus* spp. Conventional testing for *Alicyclobacillus* spp. is a time-consuming culture-based procedure, and it take days to weeks to positively identify the targeted bacterium (*4*, *5*).

Rapid techniques for identification of *Alicyclobacillus* spp. have relied on genetic-based methods (*5*). A polymerase chain reaction-based identification relies on the amplification of a 16S rRNA region of suspect bacterium, and the method can detect as little as 100 cells (*5*). However, the procedure requires skilled personnel and may not be easily adapted to routine testing. Lin et al. (*11*, *12*) have shown the potential for the detection and identification of *Alicyclobacillus* isolates in apple juices by collecting the bacterial biomass through filtration using an aluminum oxide membrane filter and direct infrared measurements by attenuated total reflection equipped with a diamond crystal.

A simple IR microspectroscopy technique, combined with multivariate analysis, could provide the juice industry with a rapid and reagent-free screening procedure to detect and monitor *Alicyclobacillus* spoilage microorganisms that threaten the quality of pasteurized fruit juices. Attenuated total reflection−infrared (ATR-IR) spectroscopy provides spectral signature profiles or "fingerprints" that would permit the classification of intact microbial cells based on their chemical structure. ATR-IR microspectroscopy in conjunction with multivariate analysis

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could allow for a simple, rapid, and reagent-free microbial differentiation method (*13*). The objective of this research was to develop a rapid method to detect *Alicyclobacillus* spp. in fruit juices, relying on hydrophobic grid membrane (HGM) filtration for isolation and ATR-IR microspectroscopy for identification of species and differentiation among strains.

MATERIALS AND METHODS

Organisms and Growth Conditions. Cultures of six *Alicyclobacillus* strains, including *A. acidocaldarius* (ATCC 43030 and ATCC 43032), *A. acidoterrestris* (ATCC 49025 and ATCC 49026), *Alicyclobacillus sendaiensis* (ATCC BAA-609), and *Alicyclobacillus vulcanalis* (ATCC BAA-609), were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The stock cultures were streaked onto orange serum agar (OSA; Becton, Dickinson and Co., Sparks, MD) selective plates, pH 5.4, and incubated at 50 °C for approximately 48 h. Stock cultures were also prepared into 10 mL of OSA broth at the same conditions.

Overnight cultures [$\sim 10^8$ colony-forming unit (CFU)/mL] of the six strains of *Alicyclobacillus* were diluted to $\sim 10^3$ CFU/mL in a 0.85% saline solution (NaCl; Fisher Scientific, Fair Lawn, NJ). Aliquots of 10 mL were vacuum filtered through 0.45 μm HGMs (ISO-GRID, Neogen Corp., Lansing, MI). The HGM filters were placed on OSA plates and incubated at 50 °C for 48 h. Following incubation, the HGM filters were peeled off the agar and dried under vacuum in a desiccator containing calcium sulfate mineral absorbent (W.A. Hammond Drierite Co. Ltd., Xenia, OH). Complete drying of the HGM filters and associated colonies was aided by the nitrogen stream flowing through the sample stage of the IR microspectroscope.

Fatty Acid Analysis. Cultures for fatty acid analysis were grown on OSA (50 °C, 48 h). Following incubation, a loopful (10 μL) biomass of each *Alicyclobacillus* strain was aseptically harvested and suspended in 1 mL of ddH_2O. Fatty acid methyl esters were obtained via the two-step Bligh and Dyer method (*14*). Following centrifugation, both the lipid and the nonlipid (pellet) fractions were collected and analyzed by ATR-IR microspectroscopy.

Infrared Microspectroscopy. Spectra of individual *Alicyclobacillus* colonies on HGM filters were measured using an infinity-corrected Fourier transform (FT) IR microscope (UMA 600 series IR microscope interfaced with a FTS Excalibur 3100GX FTIR spectrometer; Varian, Walnut Creek, CA). The microscope was equipped with a motorized $x-y$ stage, a 4× and 16× objective, a broadband mercury cadmium telluride detector, and slide-on ATR germanium objective (Varian 600 UMA, Palo Alto, CA). The spectrometer was controlled using Win-IR Pro control software programmed to record spectra over the frequency range 700−4000 cm^{-1}. The spectral resolution was 4 cm^{-1}, and 128 spectra were coadded and averaged to improve the signal-to-noise ratio. Spectra were displayed in terms of absorbance as calculated from the transmittance spectra using the Win IR Pro software. The absorbance spectrum was obtained by rationing the single beam spectrum against that of the air background.

Six individual colonies were harvested from each cultured *Alicyclobacillus* strain, and their spectra were collected. The reproducibility of cultures grown on four different days was also examined, resulting in ~24 spectra per strain to construct the training model.

Multivariate Analysis. Spectra were exported to the Pirouette multivariate analysis software (version 3.1, InfoMetrix, Inc., Woodville, WA) as ".spc" files. Second derivative transformations of the spectra removed the baseline shifts and resolved the overlapping peaks, helping to reduce the variability between replicates (*13*). Principal component analysis (PCA), specifically soft independent modeling of class analogy (SIMCA), was used to build a predictive model based on the construction of separate PCA models for each class to describe and model the variation (*13*). SIMCA class models were interpreted based on class projections, misclassifications, discriminating power, and interclass distances. Class projections were visible through a three-dimensional graph of clustered bacteria. Probability clouds (95%) were built around the clusters based on PCA scores, allowing SIMCA to be used as a predictive modeling system. Total misclassifications were analyzed and interpreted for the input data and also validation unknowns to assess the power of the model.

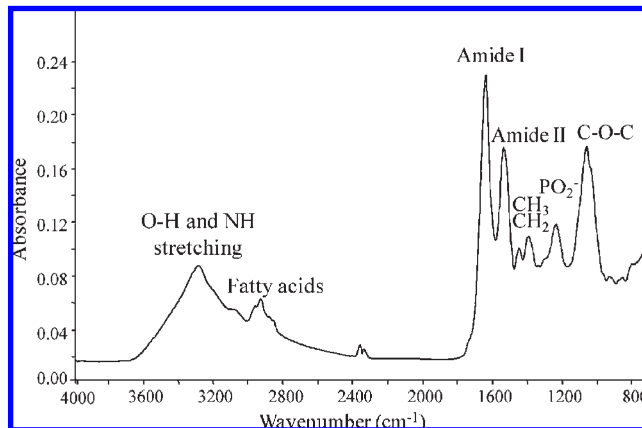

Figure 1. ATR-IR microspectroscopy spectrum of an intact single cell of *A. acidocaldarius* ATCC 43030 grown on OSA (50 °C, 48 h) using a germanium crystal accessory in reflectance mode.

The discriminating power was used to strengthen the model, through removal of spectral bands, which do not aid in the separation and differentiation of the *Alicyclobacillus* strains. The discriminating power allowed wavenumbers (cm^{-1}) of bands of interest to be determined, aiding in the identification of functional groups present in the *Alicyclobacillus* strains, which allowed differentiation. Interclass distances, values that represent Mahalanobis distances between the bacterial classes, allowed for determination of separations based on factor loadings. The identity of unknown samples can be predicted using the training models with three possible outcomes: (i) The unknown is part of one class, (ii) the unknown is part of more than one class, or (iii) the unknown does not belong to one class (*15*).

Model Validation. Individual cultures of five different *Alicyclobacillus* strains, used in completion of the SIMCA model, were prepared as stated earlier and labeled using an "unknown" coding system. The spectra of the "unknown" *Alicyclobacillus* strains were evaluated, and class predictions were performed by SIMCA models using Pirouette software. Validation of the model was conducted separately using apple and white grape juices (Dole, Thousand Oaks, CA), inoculated with "test" *Alicyclobacillus* strains. Strains were plated onto OSA as previously described, and an isolated colony ($\sim 10^8$ CFU) was collected and resuspended into 1 mL of saline solution (0.9% NaCl). An aliquot (100 μL) of the suspension ($\sim 10^7$ CFU/mL) was inoculated into 9.9 mL of juice. Following incubation (~25 °C, 60 min) in the juice, serial dilutions were made in to $\sim 10^3$ CFU/mL. The "spiked" juice samples were filtered using HGM filters and grown at 50 °C for 48 h on OSA. Following growth, HGM filters were removed from contact with agar, dried, and analyzed as previously described. The predictive ability of the SIMCA model was tested by collecting the spectra of bacterial biomass in three randomly chosen squares on the HGM filters per juice sample to develop the validations set.

RESULTS AND DISCUSSION

Four species of *Alicyclobacillus*, each represented by one or two strains for a total of six strains, were tested in this study. The application of HGM filters allowed for single CFUs to be isolated in a hydrophobic square, which limited horizontal spread and overlap of microcolonies. Isolated colonies within the confines of the hydrophobic grids and subsequent vacuum drying of the membrane limited the IR interference from water absorption bands. This protocol allowed for the collection of reproducible infrared absorption spectra directly from biomass of individual colonies isolated with HGM filters. A typical spectrum for *Alicyclobacillus* spp. (*A. acidocaldarius* ATCC 43030) is shown in **Figure 1**. HGM filters have previously been used for the detection and quantification of microorganisms in food (*16*) and are thought to aid in the automation and high-throughput analysis (*17*).

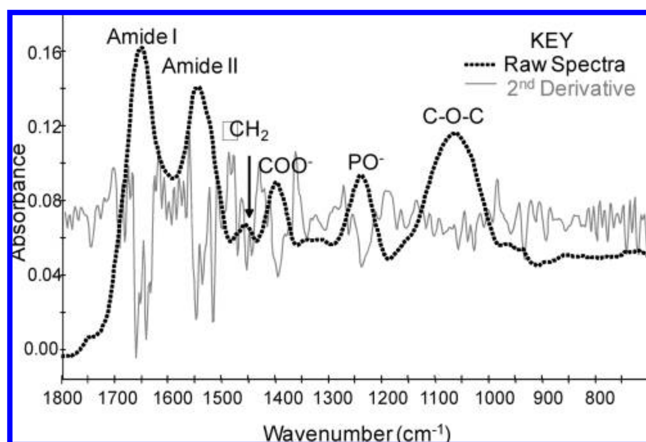

Figure 2. Typical ATR-IR microspectroscopy spectrum and its respective second derivative transformation of *A. acidocaldarius* ATCC 43030 grown on OSA (50 °C, 48 h) using a germanium crystal accessory in reflectance mode.

The slide-on ATR germanium accessory provided a fixed absorbance path length with band intensities for the largest amide I band (~1650 cm^{-1}) from 0.20 to 0.24 absorbency units. In addition, the highly specific spectral patterns related to fundamental vibrational transitions were associated with functional groups of major cellular constituents reflecting the total biochemical composition of the microorganism (*18−20*). All strains had very similar raw spectral bands that required mathematical transformations (second derivative, **Figure 2**) of the spectra to removed baseline shifts and resolved overlapping peaks, helping to reduce the variability between replicates (*13*). The bands of highest proportion in the raw spectrum at approximately 1650 and 1520 cm^{-1} were associated with amide I and amide II group vibrations, respectively (*20*). The fingerprint region of the spectra (900−1200 cm^{-1}) is a very reproducible and robust region that has been associated with polysaccharide and phosphate stretching vibrations (*18*, *20*).

The complex cellular composition of the six *Alicyclobacillus* strains evaluated yielded infrared vibrational transitions that permitted microbial discrimination by using multivariate analysis, SIMCA. The SIMCA class projection plot (**Figure 3**) of transformed spectra (900−1225 cm^{-1} region) aided in the visualization of clustering patterns and exhibited nonoverlapping and well-separated grouping in a multidimensional space permitting accurate strain level classification. SIMCA's misclassification algorithm for *Alicyclobacillus* strains showed zero misclassifications, indicating that the training set was reasonably homogeneous, and all samples were correctly classified into their corresponding categories.

A 95% confidence interval probability cloud around each class is assigned based on the standard deviations in each principle component direction (*15*) allowing for reliable classification and prediction of new samples (unknowns). Five of the six *Alicyclobacillus* strains were comprised of repeated observations on cells collected from stationary phase cultures (six measurements) from cultures grown on four different days (replications) to build the SIMCA training model. Difficulties arose with the culturing of *A. acidoterrestris* ATCC 49026, resulting in the collection of ~5 individual spectra from colonies grown on two different days, resulting in a total of 10 spectra.

SIMCA classification models showed limited intraclass variation (**Figure 3**), which permitted tight clustering of the *Alicyclobacillus* strains. Interclass distances, a measure of the distance between samples based on factor loadings, provided further information regarding separation of classes. Interclass distances, a measure of the separation between the bacterial classes, ranged from 1.15 to 7.53 (**Table 1**), showing differences between the biochemical patters in all classes. SIMCA's class distance values between groups of objects above 3.0 are regarded as significant to identify two groups of samples as different classes (*21*).

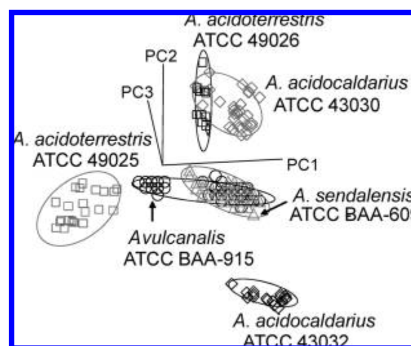

Figure 3. SIMCA class projections of transformed (second derivative) ATR-IR microspectroscopy spectra (900−1225 cm^{-1}) of *Alicyclobacillus* strains grown on OSA (50 °C, 48 h) using a germanium crystal accessory in reflectance mode. Data were obtained by measurements of six different bacterial cells per day per strain for 4 consecutive days.

Table 1. SIMCA Interclass Distances of *Alicyclobacillus* Strains Using Transformed (Second Derivative) ATR-IR Microspectroscopy Spectra (900−1225 cm^{-1}) Using a Germanium Crystal Accessory in Reflectance Mode

	ATCC 43030[a]	ATCC 43032[a]	ATCC 49025[b]	ATCC 49026[b]	ATCC BAA-609[c]	ATCC BAA-915[d]
ATCC 43030	0					
ATCC 43032	5.55	0				
ATCC 49025	5.28	7.53	0			
ATCC 49026	2.06	6.82	6.30	0		
ATCC BAA-609	2.78	4.41	4.42	3.42	0	
ATCC BAA-915	3.25	4.68	4.11	3.94	1.15	0

[a] *A. acidocaldarius.* [b] *A. acidoterrestris.* [c] *A. vulcanalis.* [d] *A. sendaiensis.*

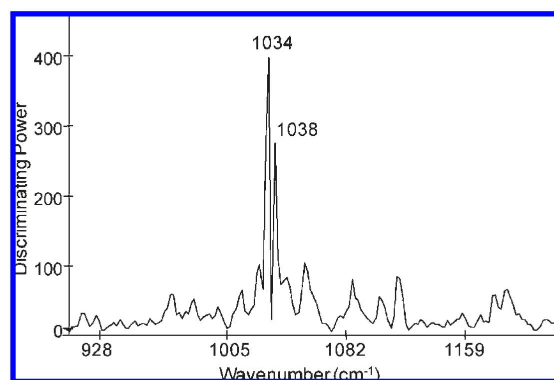

Figure 4. Discriminating power of *Alicyclobacillus* strains grown on OSA (50 °C, 48 h) by transformed (second derivative) ATR-IR microspectroscopy spectra (900−1225 cm^{-1}) using SIMCA multivariate analysis software and a germanium crystal accessory in reflectance mode.

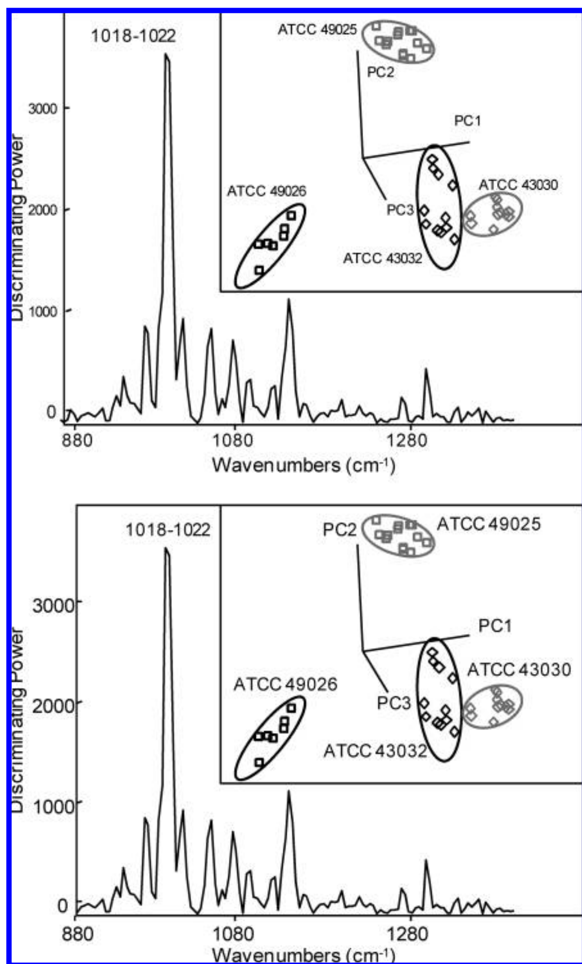

Figure 5. Discriminating power and SIMCA class projections of transformed (second derivative) ATR-IR microspectroscopy spectra (900−1225 cm^{-1}) of the pellet fraction from *Alicyclobacillus* strains grown on OSA (50 °C, 48 h).

The discriminating power was used to define the variables, infrared frequencies, which had a predominant effect on sample classification (*22*). Major discrimination (**Figure 4**) occurred at 1034 and 1038 cm^{-1}, presumably due to stretching C−O vibrations of sugar chains. Although *Alicyclobacillus* are structurally comprised of unique *ω*-alicylic fatty acids (*23*, *24*), we feel that carbohydrate-containing compounds may be causing our differentiation (*25*). Prior research has reported that *Alicyclobacillus* differentiation was due to the *ω*-alicyclic fatty acids in the cell membrane (*12*), so we used the Bligh and Dyer method to extract the lipid- and nonlipid (pellet)-soluble components of the *Alicyclobacillus* cultures to determine which portion was responsible for our results (**Figure 4**). **Figures 5** and **6** represent the SIMCA analysis of the pellet- and lipid-soluble fractions of *Alicyclobacillus*, respectively. The nonlipid microbial pellet had a higher discriminating power (**Figure 5**) than the lipid-soluble portion (**Figure 6**) and also ranged closer to the bands (1034 and 1038 cm^{-1}) used for intact cell direct ATR-IR analysis (**Figure 4**), indicating that in whole cell analysis, sugar-containing moieties, that is, glycoproteins and/or extrapolysaccharide cellular components, are responsible for the differentiation of *Alicyclobacillus* spp.

Model validation proved successful for saline and fruit juice matrices inoculated with blind-coded strains of *Alicyclobacillus*. For the saline matrix, a total of five *Alicyclobacillus* strains were tested in triplicate, and the SIMCA model correctly identified 15 out of 15 of the blind-coded samples for a 100% correct predictions at the *Alicyclobacillus* strain level (**Table 2**). The apple juice

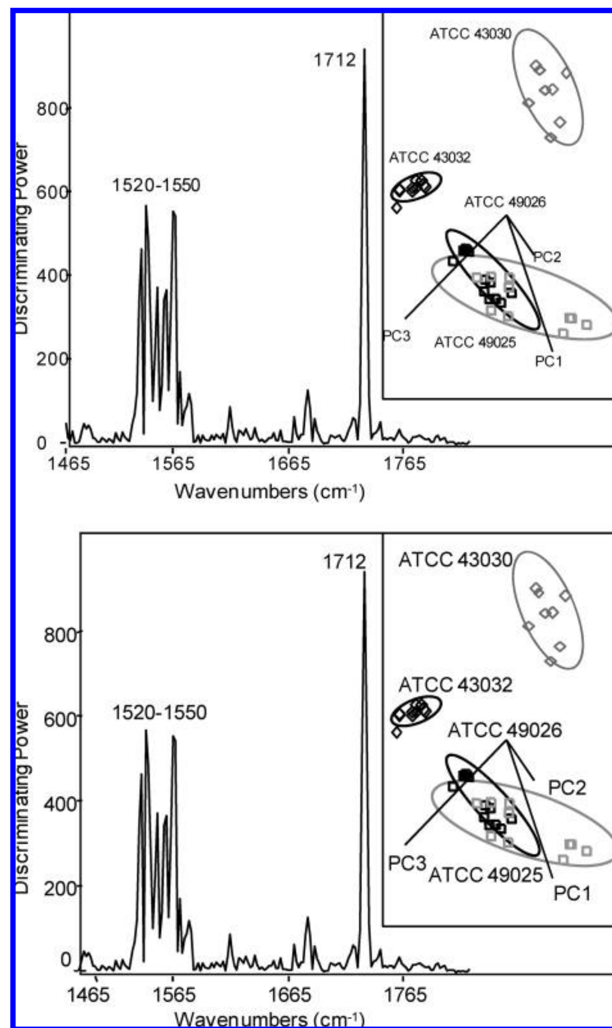

Figure 6. Discriminating power and SIMCA class projections of transformed (second derivative) ATR-IR microspectroscopy spectra (900−1225 cm^{-1}) of the lipid-soluble fraction from *Alicyclobacillus* strains grown on OSA (50 °C, 48 h).

Table 2. SIMCA Model Validations for *Alicyclobacillus* Strains Using Transformed (Second Derivative) ATR-IR Microspectroscopy Spectra (900−1225 cm^{-1}) for Saline, Apple Juice, and White Grape Juice Matrices Using a Germanium Crystal Accessory in Reflectance Mode[a]

product	organism	correct validation[b]
saline	ATCC 43030[c]	3
	ATCC 49025[d]	3
	ATCC 49026[d]	3
	ATCC BAA-609[e]	3
	ATCC BAA-915[f]	3
apple juice	ATCC 43030[c]	3
	ATCC 49025[d]	3
	ATCC 49026[d]	3
	ATCC BAA-609[e]	3
	ATCC BAA-915[f]	3
white grape juice	ATCC 43030[c]	3
	ATCC 49025[d]	3
	ATCC 49026[d]	3
	ATCC BAA-915[f]	3

[a] Model validation was completed with *Alicyclobacillus* strain "unknowns" grown on OSA (50 °C, 48 h) and tested in the previously constructed SIMCA training model. [b] Out of three trials for each matrix. [c] *A. acidocaldarius.* [d] *A. acidoterrestris.* [e] *A. vulcanalis.* [f] *A. sendaiensis.*

validation tested five blind-coded samples in triplicate, and 15 out of 15 spectra (100%) were correctly identified (**Table 2**). Similarly, white grape juice (**Table 2**) gave 12 out of 12 correct spectra for four *Alicyclobacillus* strains tested in triplicate (100%).

Combining HGM isolation of bacteria with ATR-IR microspectroscopy and supervised multivariate analysis has shown promise as an easy, rapid, reagent-free, high-throughput, and robust procedure to complement the elaborate molecular identification methods for *Alicyclobacillus* spp. This HGM-IR microspectroscopic approach could help streamline the analytical procedure so that it is more applicable to higher sample throughput and automation and provide juice manufacturers the ability to prescreen incoming raw materials and finished products for the presence of spoilage *Alicyclobacillus*. This technique could allow for the analysis of *Alicyclobacillus* isolates from mixed culture juice samples, which overcomes limitations inherent to aluminum oxide filtration used in recent ATR-IR studies of *Alicyclobacillus* isolates (*12*). Generation of a library of major spoilage organisms is needed for this approach to become a standard typing tool.

ABBREVIATIONS USED

ATCC, American Type Culture Collection; ATR-IR, attenuated total reflectance−infrared; CFU, colony-forming units; FT, Fourier transform; HGM, hydrophobic grid membrane; OSA, orange serum agar; PCA, principal component analysis; SIMCA, soft independent modeling of class analogy.

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