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Rapid Detection and Identification of *Pseudomonas aeruginosa* and *Escherichia coli* as Pure and Mixed Cultures in Bottled Drinking Water Using Fourier Transform Infrared Spectroscopy and Multivariate Analysis

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Fourier transform infrared (FT-IR) spectroscopy and multivariate analysis were used to identify *Pseudomonas aeruginosa* and *Escherichia coli* ATCC 25922 inoculated into bottled drinking water. Three inoculation treatments were examined: (i) *E. coli* ATCC 25922 ($N = 3$), (ii) *P. aeruginosa* ($N = 3$), and (iii) a 1:1 (v:v) mixed culture of both *P. aeruginosa* and *E. coli* ATCC 25922 ($N = 3$). The control treatment was noninoculated drinking water ($N = 3$). Second derivative transformation and loadings plots over the range of 1800−900 cm^{-1} indicate variations in the following bacterial constituents: amide I band ca. 1650 cm^{-1}, amide II band ca. 1540 cm^{-1}, phosphodiester backbone of nucleic acids ca. 1242 and 1080 cm^{-1}, and polysaccharide compounds ca. 1050−950 cm^{-1}. Cells with the different treatments were clearly segregated from a mean centered principal component analysis. By using soft independent modeling of class analogy analysis, spectra from a given treatment could be correctly classified 83−88% of the time. These results suggest that FT-IR spectroscopy can determine whether a pure culture is present, in addition to confirming that this method can discriminate between closely related bacteria based on differences in biochemical and phenotypic characteristics that can be detected in this spectral region.

KEYWORDS: FT-IR; spectroscopy; drinking water contamination; *P. aeruginosa*; *E. coli*; PCA; SIMCA; bacterial differentiation; mixed bacterial cultures

INTRODUCTION

The public health standards for safe drinking water for human consumption have the following guidelines: a maximum of 500 colony-forming unit (CFU) per mL of heterotrophic plate count (HPC), free from coliforms, and a nephelometric turbidity of less than 2 (*1*). Drinking water should not contain any bacteria indicative of fecal pollution such as *Pseudomonas* spp. (*2*). Microbial contamination is the most common and widespread health risk associated with drinking water, either directly or indirectly, by animal or human excreta (*3*). In developing nations, more than 250 million new cases of waterborne diseases are reported annually. This has resulted in high morbidity and mortality rates, especially in young children (*4*).

Oligotrophic bacteria such as pseudomonads are capable of reproducing and forming biofilms under conditions that are usually considered nutrient restricted. Such organisms are found in low nutrient environments such as drinking water, groundwater, and surface water (*4*), attaining population densities of 10^6-10^7 cells per mL in distilled water (*5*). *Pseudomonas aeruginosa* is a Gram-negative, rod-shaped, strictly aerobic, oxidase-positive, catalase-positive bacterium that produces diffusible and/or insoluble pigments (*6*). This bacterium does not require organic growth factors. It has traditionally served as a surrogate or indicator for the presence of other opportunistic pathogens.

Now, *P. aeruginosa* is considered to be an opportunistic pathogen in patients with low general or local resistance against infections (*6*) and its presence is unacceptable because it has been implicated in waterborne and foodborne diseases; it is now considered to be a primary infectious agent (*7*). The presence of *P. aeruginosa* as a means of assessing the hygienic quality of drinking water has been advocated, and levels of this

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organism provide an indication of the general cleanliness of the water distribution system.

The coliform bacteria are members of the family Enterobacteriaceae and consist of genera *Escherichia*, *Enterobacter*, *Citrobacter*, and *Klebsiella*. The presence of coliforms in drinking water suggests inadequate treatment, post-treatment contamination, or excessive nutrients (*3*), and levels for acceptable drinking water should be nondetectable in a 100 mL sample. Traditionally, detection of coliform bacteria in drinking water has been viewed as an indicator of fecal contamination through a cross-connection, an inability to maintain a disinfectant residual in distributed water, or inadequate treatment (*8*). *Escherichia coli* specifies the presence of human or mammalian feces and is rarely found in water or soil that has not been subjected to fecal pollution.

Classical microbiological examinations performed in drinking water to detect waterborne pathogens include most probable number, membrane filtration techniques, and, most recently, enzyme substrate tests. However, these techniques are time consuming, require further identification and verification tests, and are labor intensive. There is a need for rapid and accurate screening methods to detect and verify waterborne pathogens.

Fourier transform infrared (FT-IR) absorbance spectroscopy (in the mid-IR range, usually defined as 4000−400 cm^{-1} or 2500−25000 nm) has sufficient resolution to distinguish intact microbial cells at the strain level (*9*). When FT-IR is applied in combination with multivariate statistical techniques, quantitative analysis of the cellular contents for bacterial cells is possible. The resulting spectra for microbial cells represent the "total" biochemical composition of those cells (*10*). FT-IR spectroscopy measures vibrations of functional groups and highly polar bonds. These spectral features of many important biochemical constituents create a "fingerprint" for a bacterial strain. For microbial samples, these spectral are most commonly DNA/RNA, proteins, and membrane and cell wall amine- and fatty acid-containing components (*11*).

FT-IR has been successfully applied on pure microbial cultures to identify strains of bacteria, yeast, and fungi (*12*−*15*) and, more recently, specific foodborne pathogens (*13*, *16*) including *Listeria* spp., *Staphylococcus* spp., *Clostridium* spp., *Streptococcus* spp., *Legionella* spp., *P. aeruginosa*, *Bacillus cereus*, and *E. coli* (*13*, *17*, *18*). Chemometric methods that include principal component analysis (PCA) and soft independent modeling by class analogy (SIMCA) are widely used in the analysis of infrared spectra within the fields of biology and agricultural sciences (*19*). PCA is a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components (PCs). PCA analysis is a helpful technique to show if there are natural clusterings in the data and if there are outlier samples. The first PC is the combination of variables that explains the greatest amount of variation. The second PC defines the next largest amount of variation and is independent (orthogonal) to the first PC. The end result of PCA is to get graphical representations of similarities and differences from infrared multivariate spectral data (*19*).

The main objective of this study was to examine the feasibility of FT-IR spectroscopy combined with multivariate statistical methods to identify *P. aeruginosa* and *E. coli* in pure and mixed cultures in bottled drinking water.

MATERIALS AND METHODS

Microorganisms and Growth Conditions. Bacterial cells that used in this study were obtained from the culture collection in the Department of Food Science and Human Nutrition, Washington State University. Bacterial strains including *E. coli* ATCC 25922 and *P. aeruginosa* were activated by transferring bacterial cells from refrigerated slant to tryptic soy agar (TSA) (Difco, MD), and cultured plates were incubated at 37 °C for 24 h. A representative colony was then inoculated into 50 mL of brain heart infusion broth (Bacto, MD), and strains were grown aerobically at 37 °C for 24 h to reach the concentration of ($\sim 1 \times 10^8$−10^9 CFU per mL).

Microbial Testing of Drinking Water Samples. Bottled drinking water (treated by reverse osmosis and ozone sterilization) was purchased from a local grocery store the day that the experiment was conducted. To examine the microbial quality of purchased bottled water, microbiological tests including total HPC, fecal coliform (i.e., *E. coli*), and *P. aeroginosa* were carried out for each sample in a duplicate manner using a membrane filtration technique (*20*). Under aseptic conditions, 100 mL of drinking water sample was filtered through a gridded sterile cellulose-nitrate membrane filter (0.45 μm pore size, 47 mm diameter, Sartorius type filters) under partial vacuum. The membrane filters were immediately removed with sterile forceps and placed on the following media with rolling motion to avoid entrapment of air: TSA (for detection of HPC), m-Endo agar LES (Difco) for selective detection and enumeration of *E. coli* from water, and M-PA-C agar (BBL, MD) for selective detection and enumeration of *P. aeruginosa* from water (*20*). Petri dishes were incubated at 37 °C for 48 h.

Inoculation of Drinking Water. Samples ($N = 3$) of 45 mL of drinking water were inoculated with 5 mL of *E. coli* ATCC 25922 broth (stationery phase culture), 5 mL of *P. aeruginosa* broth (stationery phase culture), or 2.5 mL of *E. coli* ATCC 25922 broth and 2.5 mL of *P. aeruginosa* broth. The control treatment was prepared by adding 5 mL of noninoculated broth to the drinking water samples. Samples were then incubated at 37 °C for 48 h. High cell concentrations were used because researchers (*14*, *21*) have found that bacterial concentrations of less than 10^4 CFU per mL may not provide an adequate FT-IR signal. The inoculated and control drinking water samples were examined for *E. coli*, *P. aeruginosa*, and mixed cultures at the end of the incubation period. Appropriate serial dilutions (using sterile 0.1% peptone) were recovered for membrane filtration techniques as previously described (*20*).

After incubation at 37 °C for 48 h, 10 mL of each inoculated and control drinking water sample was filtered through an aluminum oxide membrane filter (0.2 μm pore size, 25 mm o.d.) (Anodisc, Whatman Inc., Clifton, NJ) to harvest bacterial cells; this membrane filter did not contribute significantly to the bacterial spectra and gave a smooth and flat surface to form a bacterial film (*19*, *21*). At the end of each sample filtration and with the filter still in place, the interior surface of the funnel was rinsed with 20 mL of sterile 0.9% saline solution to remove bacterial metabolites from the surface of the filter (*21*). The Anodisc filters were then air-dried under laminar flow at room temperature for 5 min to allow a homogeneous dried film of bacterial cells. This filtration technique was found to give more homogeneous distribution of cells and more reliable results than the centrifugation one.

Using this filtration method to harvest bacterial cells on an Anodisc membrane gave a more homogeneous distribution of cells and more reliable spectral results than recovering cells by centrifugation and then applying them to the membrane (*19*), thereby increasing the reproducibility of the spectroscopic method.

FT-IR Spectroscopy and Measurement. FT-IR spectra were recorded using a Nicolet 380 FT-IR spectrometer (Thermo Electron Inc., San Jose, CA). The Anodisc membrane filters coated with a very thin layer of bacterial cells were placed in direct contact with an infrared attenuated total reflection (ATR) diamond crystal. This technique was used to study the chemical composition of a smooth surface (i.e., biofilms) (*22*). FT-IR spectra were recorded from 4000 to 500 cm^{-1} at a resolution of 2 cm^{-1}. Sixty spectra ($N = 60$) were acquired at room temperature, and each spectrum was composed of an average of 36 separate scans. FT-IR spectra were mean-centered and baseline corrected.

Multivariate Analysis. OMNIC (Thermo Electron Inc.) and Delight version 3.2.1 (Textron Systems, Wilmington, MA) software were used to conduct data analysis. Data preprocessing algorithms were performed to reduce overlapping spectral features (*23*). Spectral data were first

Table 1. Average Microbial Concentration after Inoculation of Drinking Water

sample treatment	count (CFU/mL)	no. of cells per mm^{2a}
control	0	0
P. aeruginosa	8.1×10^9	1.65×10^8
E. coli ATCC 25922	6.2×10^9	1.26×10^8
mixed culture[b]	9.2×10^9	1.87×10^8

[a] Volume of filtered sample = 10 mL, and surface area of anodisc membrane = 490.9 mm^2. [b] Microbial count for *P. aeruginosa* = 5.1×10^9; and microbial count for *E. coli* ATCC 25922 = 4.1×10^9.

binned by 2 cm^{-1} and then smoothed with a Gaussian function of 4 cm^{-1} and followed by a second derivative transformation of 15 cm^{-1} gap (*24*).

The multivariate statistical analysis techniques of PCA and SIMCA were used for spectral data analysis. PCA was used to capture the related variations among FT-IR spectra, remove random noise (*26*), and determine whether there was natural clustering within the data (*10*, *19*, *25*). SIMCA was extensively used to classify samples according to their analogy to the training set samples (*21*).

RESULTS AND DISCUSSION

The drinking water before inoculation showed that the water samples were free from heterotrophic bacterial cells, coliform, and *P. aeruginosa*. **Table 1** shows the average microbial loads after inoculation of drinking water samples at 48 h and a number of bacterial cells per mm^2 of Anodisc membrane filter. Both *P. aeruginosa* and *E. coli* cells could survive and grow in drinking water despite a lack of essential nutrients, with *P. aeruginosa* growing to a slightly higher density than *E. coli* under the conditions tested here.

Both *P. aeruginosa* and *E. coli* are Gram-negative rods and have many similarities in cell wall and cell membrane structure, specifically outer membrane components including: lipopolysaccharides (LPS), phospholipids, and peptidoglycans (*27*, *28*).

Figure 1 shows representative FT-IR spectra (4000−800 cm^{-1}) of control (**A**), *E. coli* ATCC 25922 (**B**), *P. aeruginosa* (**C**), and mixed culture of both microbes (**D**) isolated from drinking water. The absorption peaks around 3000 cm^{-1} (∼2960 and ∼2929 cm^{-1}) are mainly due to the asymmetric stretch of CH_2 and CH_3 of bacterial cell wall fatty acids and other components (*10*, *22*). To exmine the FT-IR spectral patterns of bacterial cells, we concentrated on two different regions: (I) 1800−1300 cm^{-1} is mainly due to (i) C═O stretching vibrations of ester functional groups basically from lipids and fatty acids (∼1740 cm^{-1}) (*15*), (ii) C═O stretching vibrations of amides associated with proteins (amide I band at ∼1650 cm^{-1}) (*10*, *16*, *18*), (iii) N−H deformation of amides associated with proteins (amide II band at ∼1540 cm^{-1}), and (iv) CH_3 and CH_2 asymmetric and symmetric deformation of proteins (∼1455 and ∼1398 cm^{-1}) (*10*, *18*). The second region (II) located between 1300 and 900 cm^{-1} consists of the vibrational features of DNA/RNA, proteins, and membrane and cell wall components (*11*). The most significant differences between the bacteria spectral features in the 1300−900 cm^{-1} region are mainly due to (i) P═O asymmetric and symmetric stretches of the phosphodiester backbone of nucleic acids DNA and RNA at ∼1242 and ∼1080 cm^{-1}, respectively, and (ii) C−O−C stretching vibrations of polysaccharides content of bacterial cells (1200−900 cm^{-1}) (*10*, *16*, *18*).

As shown in **Figure 1**, there are variations among *E. coli* and *P. aeruginosa* in regions associated with amide I band (1650 cm^{-1}), amide II band (1550 cm^{-1}), CH_3 and CH_2 asymmetric and symmetric deformation (∼1455 and ∼1398 cm^{-1}), and the stretching vibrations of polysaccharides (1200−900 cm^{-1}). To make differences in the spectral features more distinctive, data preprocessing algorithms were used to amplify spectral variations (*10*, *29*).

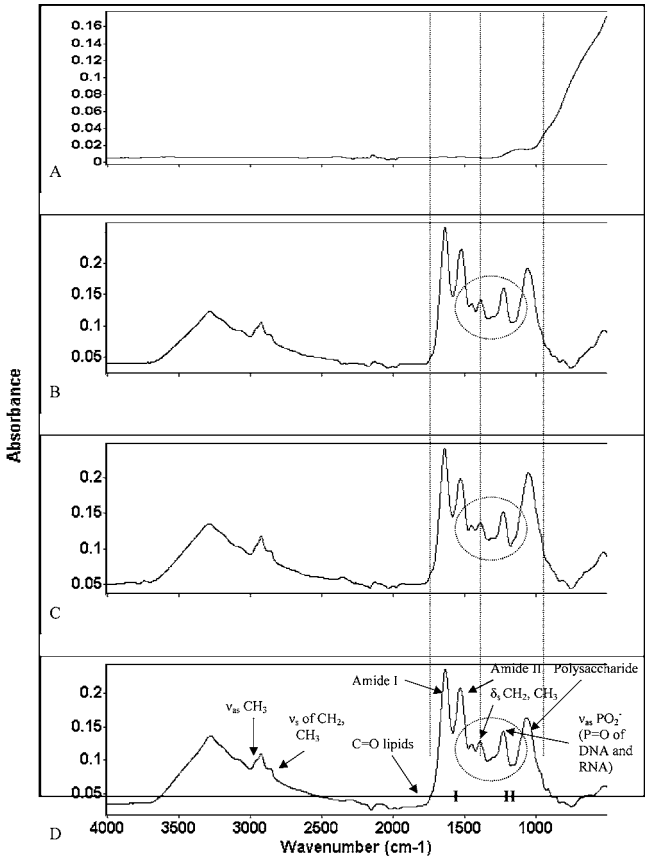

Figure 1. Representative FT-IR spectra (4000–800 cm^{-1}) of control (**A**), *E. coli* ATCC 25922 (**B**), *P. aeruginosa* (**C**), and mixed culture (**D**) isolated from drinking water. ν_{as} = asymmetric stretch, ν_s = symmetric stretch, and δ_s = symmetric deformation.

The major preprocessing algorithms involved binning and smoothing to reduce overlapping spectral features and to eliminate high-frequency instrumental noise and normalization of bacterial spectra that result from different concentrations of the same bacterial cells to balance the variations in path length strength and to allow for comparison (*30*). However, the most important steps in evaluating spectral features may be the second derivative transformation, which separates overlapping bands (*29*), reducing the variability between replicate spectra due to baseline offsets (*10*), generating an estimate of the number of overlapped bands within the region (*29*). The net result of spectral derivatives is to enhance the clarity of bacterial spectral features and to improve spectral resolution.

Figure 2 shows the same spectral region used in **Figure 1**, after a 15-point second-order derivatization (1800−1300 cm^{-1}). Differences between the spectra of bacterial strains are more distinctive particularly in the amide I and amide II regions due to C═O stretching vibration and N−H deformation of amides associated with proteins in the range of ∼1650 and ∼1540 cm^{-1}, respectively. Other variations due to C═O stretching vibration of ester functional groups from lipids and fatty acids (∼1740 cm^{-1}) and CH_3 and CH_2 asymmetric and symmetric deformation of proteins (∼1455 and ∼1398 cm^{-1}) were apparent. **Figure 3** illustrates the second derivative transformation (15-point) of bacterial spectra in region II, 1300−900 cm^{-1}. Differences

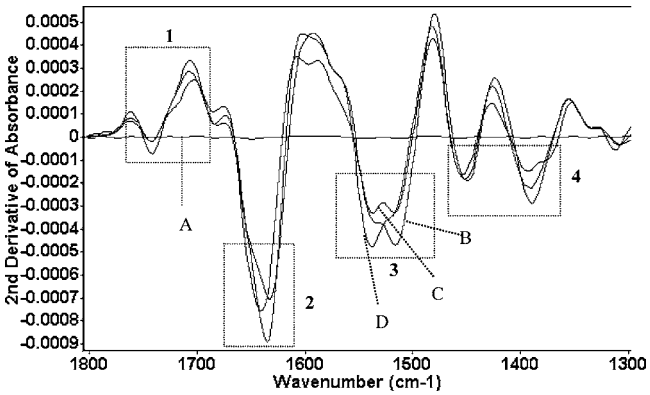

Figure 2. Representative second derivative transformation FT-IR spectra (1800–1300 cm^{-1}) of control (**A**), *E. coli* ATCC 25922 (**B**), *P. aeruginosa* (**C**), and mixed culture (**D**). Boxes: 1, C═O stretching vibration of ester functional groups from lipids and fatty acids; 2, C═O stretching vibration of amides associated with proteins (amide I); 3, N–H deformation of amides associated with proteins (amide II); and 4, CH_3 and CH_2 asymmetric and symmetric deformation of proteins.

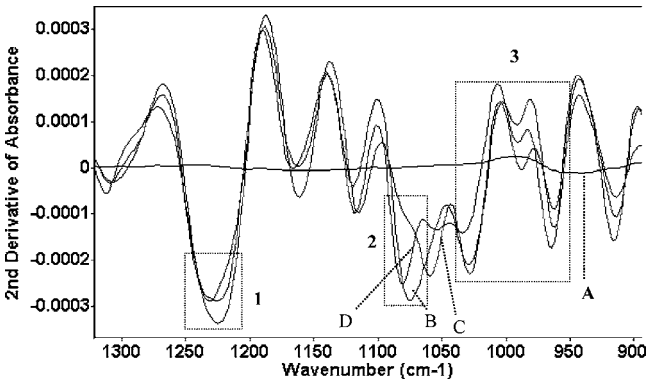

Figure 3. Representative second derivative transformation for FT-IR spectra (1300–900 cm^{-1}) of control (**A**), *E. coli* ATCC 25922 (**B**), *P. aeruginosa* (**C**), and mixed culture (**D**). Boxes: 1, ν_{as} P═O of the phosphodiester backbone of nucleic acids; 2, ν_s P═O of the phosphodiester backbone of nucleic acids; and 3, C–O–C stretching vibration of polysaccharides.

between *P. aeruginosa* and *E. coli* and for the mixed culture can be seen in the P═O asymmetric and symmetric stretches of the phosphodiester backbone of DNA (~1242 cm^{-1}) and RNA (~1080 cm^{-1}). Features attributable to bacterial cell wall peptidoglycan due to C−O−C stretching vibration of the polysaccharides content of bacterial cells can be seen around 1050−950 cm^{-1}.

To differentiate between bacterial cells, multivariate statistical analysis techniques (PCA and SIMCA) were performed. PCA is widely used to explain infrared spectral data variances and to capture related variations (*29*) and cluster samples depending upon differences in the FT-IR spectral patterns that result from variations in the major cellular constituents of microorganisms.

A mean-centered PCA was carried out on the second derivative FT-IR spectra over the range of 1800−900 cm^{-1}. **Figure 4** shows two-dimensional PCA clustering results from FT-IR spectra and shows clear segregation between pure and mixed cultures of *E. coli* ATCC 25922 and *P. aeruginosa* resulting from variations in quantity and distribution of major bacterial cellular constituents such as nucleic acids, proteins, phospholipids, peptidoglycan, and LPSs of these closely related bacteria (*22*). The distinctive clustering for the mixed culture could also be due to factors associated with the growth of these

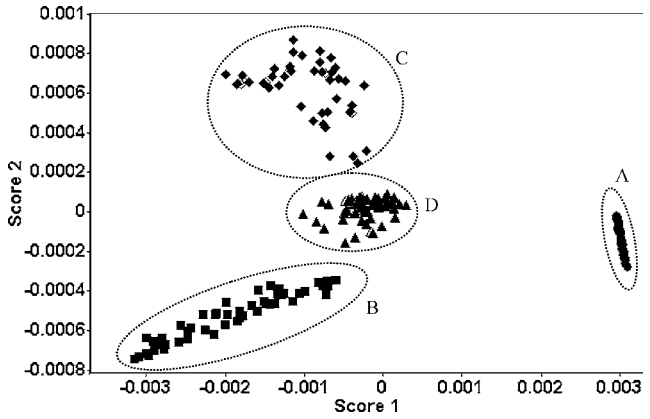

Figure 4. Principle components analysis (PCA) for control (**A**), *E. coli* ATCC 25922 (**B**), *P. aeruginosa* (**C**), and mixed culture (**D**).

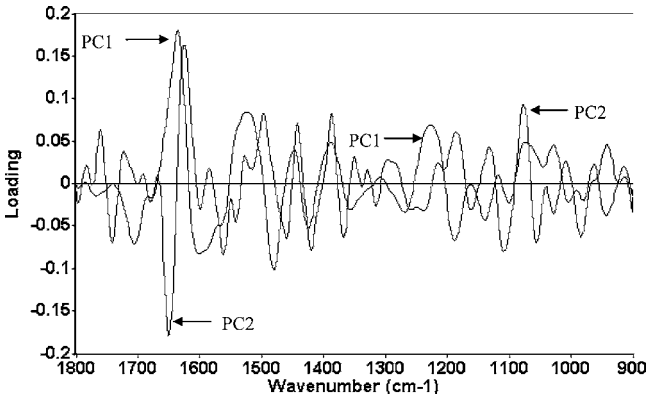

Figure 5. Loadings plot of the first and the second principle components (PCs) obtained from PCA analysis of FT-IR spectra of the four treatments.

microbes in mixed culture. *E. coli* ATCC 25922 and *P. aeruginosa* shared minimal available nutrients from drinking water, and this may have affected metabolic process for both and their resultant spectral features.

Figure 5 shows the first and the second loadings plot from PCA analysis over the range of 1800−900 cm^{-1}. Loadings plot emphasizes the contribution of each variable (wavenumber) to each PC providing an indication of which spectral region makes the most significant contributions to data variation (*10*, *29*). Large loadings (positive or negative) are linked to the regions within the original spectra that are responsible for bacterial discrimination (*31*). Over the range of 1800−900 cm^{-1}, loadings 1 and 2 were accounted for 81% of the total variability (71 and 10%, respectively) As shown in **Figure 5**, important loadings were observed at the following regions: amide I at ~1650 cm^{-1}, amide II at ~1540 cm^{-1}, CH_3 and CH_2 of protein components around 1455 and 1398 cm^{-1}, P═O of the phosphodiester backbone of nucleic acids around 1242 and 1080 cm^{-1}, and polysaccharide compounds around 1050−950 cm^{-1}.

SIMCA analysis was used to classify samples using models developed from spectra from *E. coli* and *P aeruginosa*. **Figure 6** shows SIMCA classification results of *E. coli* ATCC 25922 as compared to the other test treatments over the range of 1800−900 cm^{-1}. The SIMCA procedure was used to classify bacterial spectra. It aims to assign a new treatment to the class where it shows the largest similarity. Accordingly, it allows for treatments to have individualities and only the common properties of the classes are modeled. Here, 53 out of 60 spectra (88.3%) of *E. coli* ATCC 25922 were correctly classified. **Table 2** shows SIMCA classification results for each sample treatment.

Previous results indicate that FT-IR spectroscopy could be

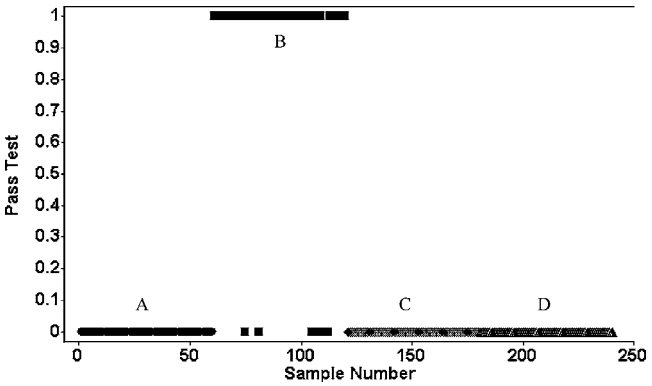

Figure 6. SIMCA classification of *E. coli* ATCC 25922 (**B**) as compared to control (**A**), *P. aeruginosa* (**C**), and mixed culture (**D**).

Table 2. SIMCA Classification Results of Each Sample Treatment as Compared to the Other Test Treatments

	correctly classified spectra	
sample treatment	no.	%
control	56	93.3
E. coli ATCC 25922	53	88.3
P. aeruginosa	50	83.3
mixed culture	50	83.3

used to discriminate between closely related bacterial cells and could be a powerful tool to subtype these cells based upon differences in biochemical and phenotypic characteristics of microbial cells using multivariate analysis techniques and to differentiate pure from mixed cultures of closely related bacteria (*9*, *11*, *21*).

In conclusion, by using FT-IR spectroscopy and multivariate analysis (PCA and SIMCA), it is possible to detect and identify the primary bacterial contaminants *P. aeruginosa* and *E. coli*. in drinking water. Furthermore, FT-IR spectra of the mixed cultures are distinct from those of pure cultures. The greatest spectral variation between these microbes was observed between 1800 and 900 cm^{-1}: for the amide I band at ~1650 cm^{-1}, amide II band at ~1540 cm^{-1}, CH_3 and CH_2 of protein components around 1455 and 1398 cm^{-1}, P═O of the phosphodiester backbone of nucleic acids around 1242 and 1080 cm^{-1}, and polysaccharide compounds around 1050−950 cm^{-1}. Further development of this technique will be to establish a spectral library of major food- and waterborne pathogens and to evaluate other statistical analysis methods such as artificial neural networks for data analyses.

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