Detection and Identification of Microorganisms in Mixed Cultures by Nanoparticle-Induced Nanospray Enhanced FTIR Spectroscopy and Chemometrics

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Keywords
Nanoparticles, Surface enhanced IR absorption, Microorganism Identification and differentiation

Disciplines
Agriculture | Bioresource and Agricultural Engineering

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Abstract
Routine identification of pathogenic microorganisms predominantly based on nutritional and biochemical tests is a time-consuming process; the delay may lead to fatal consequences at times. In this work, nanoparticle-induced nanoSPR enhanced IR spectroscopy was used in conjunction with a background elimination data processing algorithm to directly identify microorganisms in mixed cultures. It was demonstrated that the microbial composition of
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**INTRODUCTION**

Conventional microbiological analysis of food, environmental, and clinical samples is largely based on nutritional and biochemical characteristics of microorganisms. Generally, more than one species of microorganisms could be present in the samples; hence the first step in an identification process is almost always the isolation step. Routine practice is to incubate microorganisms extracted from a sample to reach a workable number, then spread them onto an agar plate and separate them into single colonies. Subsequently, the single colonies were cultured for another 16~24 hours on solid or broth culture medium to obtain enough biomass ($10^6$-$10^9$ cells/mL) for further analysis by biochemical methods, which are labor-intensive and time-consuming (about 2~3 days or more are needed). Methods that allow detection of microorganisms at the earliest time are highly desirable.

Over the last decade, molecular biological techniques have been applied to the identification of microorganisms and the detection of specific antibiotic resistance genes (Tang et al., 1997; Nikkari and Relman 1999; Tenover et al., 1994; Clark et al., 1999). Although these techniques are potentially rapid, they are expensive, elaborate, and need skilled personnel. False positive reactions due to DNA contamination and false negative reactions due to inhibitors introduced while preparing or collecting a sample may result in incorrect results in the DNA amplification-based molecular diagnostics (Vaneechoutte and Eldere, 1997; Noordhoek et al., 1996; Ieven and Goossens, 1997; Fredricks and Relman, 1998). Also as the first step, isolation of a single strain is still necessary. Currently molecular diagnostics are usually second lines of investigation and are seldom the sole basis for microbial identification (Maquelin et al., 2000).

An alternative approach in microbial characterization is the use of spectroscopic methods. Pyrolysis mass spectrometry has been evaluated as a method for bacterial characterization (Goodacre et al., 1996; Timmins et al., 1998; Barshick et al., 1999; Demirev et al., 1999); however, high instrument costs have prevented the widespread use of this method (Busse et al., 1996). The use of Fourier Transform Infrared (FTIR) spectroscopy for microbial identification and characterization has been gaining acceptance since the pioneering work by Naumann and co-workers (Naumann et al., 1991; Helm et al., 1991). Most of the past work has dealt with characterizing single colonies and to a large extent the suspensions were in buffer systems (Timmins et al., 1998; Zeroual et al., 1995; Sockalingum et al., 1997; Bouhedja et al., 1997; Naumann, 2000). A major impediment in the analysis of mixed cultures or single colonies in real food matrices is the inability of the spectral procedure (experimental and/or analysis) to account for competing similar background contributions. In our previous work (Yu and Irudayaraj, 2006), we have demonstrated that this difficulty could be overcome if appropriate mathematical
procedures could be used to extract the true fingerprint before chemometric analysis of the spectral data. Built upon the vector algebra procedure developed by Maqueulin et al. (2000) to extract the ‘real’ microbial Raman fingerprint by removing the contributions from the background medium and water, and the Hybrid Linear Analysis (HLA) multivariate calibration algorithm developed by Berger et al. (1998), we developed a background elimination algorithm to acquire the “true” fingerprints of each individual microbial species in a microbial cocktail. Using this method, the presence or absence of specific pathogens in microbial mixtures of up to three in PBS buffer was successfully predicted (Yu and Irudayaraj, 2006).

To further increase the discriminating power of the method to analyze microbial mixtures at strain level, or samples with complicated background like food matrixes, the accuracy and sensitivity of the IR spectral measurement would need to be improved. One way to achieve this is to utilize surface enhanced infrared absorption (SEIRA) (Jensen et al., 2000). In SEIRA, the infrared-active vibrational modes of molecules are intensified when they are in close proximity to nanometer-thick metal films. It is believed that the enhancement of the spectral signal mainly comes from the highly intensified local electromagnetic field caused by surface plasmon resonance (SPR) of the nanometer-thick metal film. Since the interaction between nanosized metal particles and incidental electromagnetic radiation creates strong localized SPR (LSPR) field around the particles, they also cause enhancement to the infrared absorption of molecules (on the order of 10~100) that are in close proximity to the particles, just like in the case of surface enhanced Raman scattering (Kosower et al., 2007).

In this study we used nanoparticle-induced nanoSPR enhanced infrared absorption (NPEIRA) in conjunction with the previously developed background elimination algorithm to 1) identify microbial composition of mixtures of different E. coli strains, and 2) to identify multiple pathogens present in apple juice.

MATERIALS AND METHODS

Sample preparations

Mixtures of three were made from the five E. coli strains (O103, O55, O121, O30 and O26) obtained from the Gastroenteric Disease Center (GDC) at the Pennsylvania State University (University Park, PA). Each strain was cultured in a 100 ml broth medium (5g yeast extract, 8g tryptone, and 5g NaCl in 500 ml distilled water) at 35 ºC and shaken at 100 RPM for 24 hours. Suspensions of microbial cells of each strain in saline buffer solution were prepared by adding 1 ml of the culture in 9 ml saline buffer solution (PBS, pH 7.2~7.6). Four possible combinations (Table 1) of mixtures of the three strains (each strain 5 ml) were prepared for analysis. To prepare the samples for BPEIRA measurement, 1 ml of nanoparticle solution was added into the 10 ml saline microbial cell suspension, the sample was then vertexed at 100 rpm for 2 minutes to mix the cells and the nanoparticles, then centrifuged at 2000 rpm for 5 minutes to obtain a mixture of cells and nanoparticles. After disposal of the supernatant, the sample was ready for BPEIRA measurement.

To address the second objective, nine bacteria (Enterobacter cloacae, Salmonella typhimurium, Enterobacter aerogenes, Salmonella choleraesuis, Serratia marcescens, Pseudomonas vulgaris, E. coli O26, Vibrio cholerae, Hafnia alvei) were obtained from the Gastroenteric Disease Center (GDC) and each species was cultured in a 100 ml broth medium (5g yeast extract, 8g tryptone, and 5g NaCl in 500 ml distilled water) at 35ºC and shaken at 100 RPM for 24 hours. Suspensions of microbial cells of each species in autoclaved apple juice
(Whitehouse®) were made by adding 1 ml of each culture to 9 ml of apple juice. Three different mixtures with 6, 7, or 8 microorganisms were prepared by mixing 5 ml of each of the apple juice sample with the respective microorganism for analysis. Subsequently, 1 ml of nanoparticle solution was added into the mixture sample and stirred for 1 minute, and cells were collected along with nanoparticles by centrifugation at 2000 rpm for 5 minutes.

**Nanoparticle fabrication**

Three different types of nanoparticles (Gold nanorods, silver-tipped gold nanorods and gold-silver alloy nanocages) were used in this work to achieve the optimal enhancement effect for the IR spectral measurement.

**a. Fabrication of gold nanorods**

Gold nanorods were made through the seed-mediated growth. Gold nanoparticles with diameter around 4 nm were made as follows as seeds: 0.6mL, 0.01M freshly prepared, ice-cold NaBH4 solution was added to a mixture solution composed of 5mL, 0.2M cetyltrimethylammonium bromide (CTAB), 0.25mL, 0.01M HAuCl4 and 4.75mL water. The solution was vigorously mixed for 2 min. The seeds were then kept at 27 °C for 3 hours to allow complete degradation of the remaining NaBH4, and were then used in subsequent nanorod growth: 5mL of 0.2 M CTAB solution and 5mL of 0.2 M benzylhexadecylammonium chloride (BDAC) solution were mixed to get bisurfactant solution. 80 µL of 0.01 M AgNO3 and 0.5 mL of 0.01 M HAuCl4 were added to the bisurfactant solution. After gentle mixing, 65 µL of 0.1 M ascorbic acid was added. The mixture solution was mixed until color of the solution changed from yellow to colorless. After addition of 12 µL of seed solution, the growth solution was incubated at 27 °C overnight. Using this procedure gold nanorods with aspect ratio (width/length) ~3 were made.

**b. Fabrication of silver-tipped gold nanorods**

The as-made GNRs solution was centrifuged twice and washed with nanopure water twice. The washed GNR pallet was resuspended to bisurfactant solution. The concentration of GNRs template solution was adjusted to around 4.2 × 10^{-10} M. Additional 0.1mL of 10mM HAuCl4, 0.3mL of 10mM AgNO3 and 20 µL of 0.1 M ascorbic acid were added to 5 mL of purified GNRs template solution. 0.1 M NaOH solution was used to adjust the solution’s pH to 11. Finally the solution was kept at 27 °C overnight and silver-tipped gold nanorods were acquired.

**c. Fabrication of gold-silver alloy nanocages**

Silver nitrate (0.12 g, Aldrich) was dissolved in anhydrous ethylene glycol (2.5 mL, Aldrich, 99.8%) in a glass vial. In a separate vial, PVP (MW = 55000, 0.14 g, Aldrich) was dissolved in ethylene glycol (7 mL). In another reaction vial, 6 ml of ethylene glycol was heated at 150°C for 60 min under constant stirring. Then 100 µl of 0.3 mM Na2S was pipetted into the vial; 8 minutes later, 1.5 ml of the PVP solution and 0.5 ml of the AgNO3 solution was also added into the reaction vial. 15 minutes later, the reaction vial was removed from the heated oil bath and was placed in a water bath at room temperature to quench the reaction. The product was predominately silver nanocubes. The nanocubes were collected by centrifugation at 1380 g force for 30 min. After washed three times with deionized water, the nanocubes were re-dispersed in 1.5 ml of deionized water by sonication. The silver nanocubes are the starting material for Au-Ag alloy nanocage fabrication. 100 µl of the Ag nanocubes were pipetted into 5 ml of 9 mM PVP solution in a 20ml vial, and heated to a mild boil for approximately 10 min. 0.1 mM HAuCl4 solution was then added to the reaction vial at a rate of 0.75 ml/min. A series of color changes were observed which indicated the wavelength of the SPR peak for the Au-Ag nanocages. Stop.
adding HAuCl4 solution when the appropriate blue color was observed. NaCl was added into the mix until saturation was reached. The Au-Ag nanocages were collected by centrifugation at 1,380g for 30 mins and re-dispersed in nanopure water.

**FTIR measurements**

Mixtures of the bacterial cells and nanoparticles, together with the residual background matrixes (PBS and apple juice) after centrifugation were loaded onto a platinum-ATR module of an ALPHA FT-IR spectrometer (Bruker Optics Inc., Billerica, MA) and were subjected to FTIR measurements. FTIR signals were collected in the spectral region between 800 and 4000 cm⁻¹, at a resolution of 2 cm⁻¹. Plain buffer and apple juice were measured first as the background, and subtracted from all of the sample spectra. 256 scans were averaged to obtain the spectra of each sample. Each experiment was repeated ten times.

**Mathematical procedure to extract fingerprint features of bacterial spectrum**

We hypothesize that the FTIR spectrum of each bacterial species is a superposition of contributions from several biomolecules that absorb in the mid-IR range. Variations among different replications in the biomaterial analyzed are to be expected and it is assumed that the spectra of different species share many common features, hence the difference in spectral fingerprints between the different types of bacteria could be minor or hardly visible. To extract the unique features of each spectrum embedded within the common features, a mathematical multivariate calibration procedure was developed, the detail of the procedure was reported elsewhere.²¹ Briefly, it was a three steps procedure. In step 1, a reference spectrum (bₐ) was generated for a microbial species by calculating the average over a set of replicated measurements on the same microbial species (B); in step 2, each spectrum in B was processed with respect to a set of spectra measured for a sample of microbial mixture (M), such that only the “true” signatures that are unique to the microbial species were retained in a reference set of spectra (BF); in step 3, same operations were conducted on each microbial species that need to be tested, and a discriminant model was established for the group of BFs.

Once the discriminant model was in place, whether or not a microbial species was present in the microbial mixture could be tested. A testing set was created for the microbial species by projecting M onto bₐ. If a particular microorganism was present in the mixture, the projection would contain enough common features with their corresponding spectral fingerprints (BF) and further processing by discriminant analysis would show that the testing set and BF of the microbial species are the same group. The discriminant analysis in this study was performed using canonical variate analysis in WIN-DAS software (Wiley and Sons, Ltd., Chichester, U.K.) To further confirm the prediction, an LDA model using Mahalanobis distance as the differentiating criteria was also implemented against the test set using Matlab (Math Works, Inc., Natick, MA). The details of the mathematical modeling was reported elsewhere.²¹

**RESULTS AND DISCUSSION**

**Nanoparticle enhanced FT-IR spectra of microorganisms**
Fig. 1 TEM images of (a) gold nanorods; (b) silver-tipped gold nanorods and (c) Au-Ag alloy nanocages

Fig 1a-c shows the typical gold nanorods, silver-tipped gold nanorods and gold-silver alloy nanocages made in this study. All of them were used in nanoparticle-induced nanoSPR enhanced FT-IR spectroscopic measurements of microbial samples. Fig 2 shows the typical spectra of *E. coli* O103 with a) no nanoparticles, b) gold nanorods, c) silver-tipped gold nanorods and d) gold-silver nanocages with one-bounce ATR setting. Without the nanoparticle-induced nanoSPR enhancement, one droplet of microbial sample did not yield a meaningful FT-IR spectrum, as shown in fig.2. However, when nanoparticles were present, the nanoSPR enhancement effects yield spectra with identifiable peaks that could be used for identification and differentiation between microbial species. All three types of nanoparticles studied exhibited strong enhancement effects, which was consistent with their surface plasmon characteristics. As shown in Fig3a-b, the surface plasmon resonance bands of the silver-tipped gold nanorods ($\lambda_{pl}$= 850 nm), gold nanorods ($\lambda_{pl}$= 920 nm) and Au-Ag alloy nanocages ($\lambda_{pl}$=725 nm) were all strongest at the near infrared range, and hence led to enhancement of IR absorption of microbial cells that are in close vicinity to these particles. Similar trends were observed for all the microbial species investigated in this study.
The Au-Ag alloy nanocage-induced nanoSPR enhanced mid-IR spectra of the five *E. coli* strains studied are shown in Fig.4. To simplify the description we will use numerals 1, 2, 3, 4, 5 to represent O103, O55, O121, O30 and O26, respectively. The ability of the spectroscopic procedure to differentiate between the chosen strains using principal component analysis (PCA)
followed by canonical variate analysis (CVA) on the ten samples studied is demonstrated in fig. 5.

Fig. 4 Spectra of the five *E. coli* strains under nanoSPR enhancement of Au-Ag alloy nanocages

In order to test the bacterial composition of the mixtures, first we generated the real ‘fingerprint’ of each species with respect to the mixture. These spectral fingerprints were first used to develop the discriminant models, as shown in fig 5. Then for each mixture in Table 1, Discriminant analysis (DA) was performed using CVA to test for the presence of a specific species in the mixture.

Fig. 5 Differentiation of the five *E. coli* strains in buffer

(Group1: *E. coli* O103; Group2: *E. coli* O55; Group3: *E. coli* O121; Group4: *E. coli* O30; Group5: *E. coli* O26)
Table 1. Composition of the E. coli mixtures in PBS buffer solution

<table>
<thead>
<tr>
<th>Mixture name</th>
<th>Present</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>em1</td>
<td>O103, O55, O121</td>
<td>O30, O26</td>
</tr>
<tr>
<td>em2</td>
<td>O103, O55, O26</td>
<td>O121, O26</td>
</tr>
<tr>
<td>em3</td>
<td>O103, O121, O26</td>
<td>O55, O30</td>
</tr>
<tr>
<td>em4</td>
<td>O55, O121, O26</td>
<td>O103, O30</td>
</tr>
</tbody>
</table>

At the strain level, the spectral signals due to features unique to each individual strains could be much weaker than what could be expected for features representing different microbial species, and the spectral signatures are dominant by features that are common to the same microbial species. Hence the overall signal difference due to the presence or absence of different strains of the same species could be very weak. Fig. 6 shows the typical results for one mixture, em1, which contained three different E. coli strains. Visually inspecting the CVA plotting, it might be deduced that microorganisms 1, 2, 3 are present, and 4, 5 are likely to be absent, but the differences shown by present or absent strains are not conspicuous. Using LDA model, the average Mahalanobis distance from the ten entries in test set to the center of each cluster representing the strain in question was calculated. As reported previously (Yu and Irudayaraj, 2006), for species level differentiation, a minimum Mahalanobis distance calculated for an absent species was normally 100 times larger than that of the present species. For strain level differentiation, the differences in Mahalanobis distances were not as large. Nevertheless, the minimum Mahalanobis distance calculated for an absent strain was still at least 10 times larger than that of a present strain. A definite prediction could still be made based on this criterion: a decrease in Mahalanobis distance by a factor of 10 indicates the presence of a strain in the mixture that is being tested.

Tables 2~5 listed the calculated Mahalanobis distances using the LDA model for the 4 mixtures of different E. coli strains (table 1). It is clearly shown that the developed technique can identify specific strains in a cocktail of E. coli strain mixture with 100% accuracy.

Table 2. Prediction of identity based on average Mahalanobis distances of entries in test set to each cluster for mixture em1 using LDA model; 4, 5 are absent and 1, 2, 3 are present

<table>
<thead>
<tr>
<th>Test Data set</th>
<th>Distance from group 1</th>
<th>Distance from group 2</th>
<th>Distance from group 3</th>
<th>Distance from group 4</th>
<th>Distance from group 5</th>
<th>prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test for 1</td>
<td>1.3968</td>
<td>14.9835</td>
<td>21.8059</td>
<td>43.0641</td>
<td>78.2243</td>
<td>Present</td>
</tr>
<tr>
<td>Test for 2</td>
<td>15.3623</td>
<td>0.8769</td>
<td>28.998</td>
<td>28.6162</td>
<td>84.2112</td>
<td>Present</td>
</tr>
<tr>
<td>Test for 3</td>
<td>21.9929</td>
<td>27.8125</td>
<td>1.6917</td>
<td>57.954</td>
<td>90.6371</td>
<td>Present</td>
</tr>
<tr>
<td>Test for 4</td>
<td>38.3623</td>
<td>22.4619</td>
<td>47.206</td>
<td>9.5335</td>
<td>93.8745</td>
<td>Absent</td>
</tr>
<tr>
<td>Test for 5</td>
<td>70.0277</td>
<td>74.213</td>
<td>90.641</td>
<td>89.8754</td>
<td>12.634</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Table 3. Distances Prediction of identity based on average Mahalanobis distances of entries in test set to each cluster for mixture em2 using LDA model; 3, 4 are absent and 1, 2, 5 are present

<table>
<thead>
<tr>
<th>Test Data set</th>
<th>Distance from group 1</th>
<th>Distance from group 2</th>
<th>Distance from group 3</th>
<th>Distance from group 4</th>
<th>Distance from group 5</th>
<th>prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test for 1</td>
<td>1.1646</td>
<td>37.3557</td>
<td>21.9885</td>
<td>14.7469</td>
<td>70.0231</td>
<td>Present</td>
</tr>
<tr>
<td>Test for 2</td>
<td>37.3539</td>
<td>1.1385</td>
<td>47.8068</td>
<td>22.6162</td>
<td>84.2112</td>
<td>Present</td>
</tr>
<tr>
<td>Test for 3</td>
<td>21.9929</td>
<td>47.8125</td>
<td>15.8525</td>
<td>29.2060</td>
<td>90.6371</td>
<td>Absent</td>
</tr>
<tr>
<td>Test for 4</td>
<td>44.7463</td>
<td>22.619</td>
<td>29.206</td>
<td>8.635</td>
<td>73.8745</td>
<td>Absent</td>
</tr>
<tr>
<td>Test for 5</td>
<td>70.0277</td>
<td>84.213</td>
<td>90.641</td>
<td>73.8754</td>
<td>2.634</td>
<td>Present</td>
</tr>
</tbody>
</table>
Table 4. Prediction of identity based on average Mahalanobis distances of entries in test set to each cluster for mixture em3 using LDA model; 2, 4 are absent and 1, 3, 5 are present

<table>
<thead>
<tr>
<th>Test Data set</th>
<th>Distance from group 1</th>
<th>Distance from group 2</th>
<th>Distance from group 3</th>
<th>Distance from group 4</th>
<th>Distance from group 5</th>
<th>prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test for 1</td>
<td>0.6679</td>
<td>26.527</td>
<td>41.2185</td>
<td>34.6219</td>
<td>90.4879</td>
<td>Present</td>
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<tr>
<td>Test for 2</td>
<td>24.4817</td>
<td>6.2324</td>
<td>68.5925</td>
<td>24.3558</td>
<td>89.5554</td>
<td>Absent</td>
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<tr>
<td>Test for 3</td>
<td>24.1356</td>
<td>36.894</td>
<td>1.5225</td>
<td>58.7985</td>
<td>73.8063</td>
<td>Present</td>
</tr>
<tr>
<td>Test for 4</td>
<td>40.1235</td>
<td>19.8564</td>
<td>26.5412</td>
<td>9.521</td>
<td>82.4331</td>
<td>Absent</td>
</tr>
<tr>
<td>Test for 5</td>
<td>68.7322</td>
<td>64.5431</td>
<td>70.641</td>
<td>69.8745</td>
<td>2.7367</td>
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</tr>
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</table>

Table 5. Prediction of identity based on average Mahalanobis distances of entries in test set to each cluster for mixture em4 using LDA model; 1, 4 are absent and 2, 3, 5 are present

<table>
<thead>
<tr>
<th>Test Data set</th>
<th>Distance from group 1</th>
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<th>Distance from group 3</th>
<th>Distance from group 4</th>
<th>Distance from group 5</th>
<th>prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test for 1</td>
<td>10.9005</td>
<td>24.7181</td>
<td>26.043</td>
<td>64.9126</td>
<td>58.8112</td>
<td>Absent</td>
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<tr>
<td>Test for 2</td>
<td>25.006</td>
<td>1.2332</td>
<td>60.5578</td>
<td>26.726</td>
<td>57.161</td>
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<td>Test for 3</td>
<td>26.074</td>
<td>42.257</td>
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<td>52.457</td>
<td>87.658</td>
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<tr>
<td>Test for 4</td>
<td>55.2145</td>
<td>24.5652</td>
<td>32.2145</td>
<td>12.2540</td>
<td>77.5289</td>
<td>Absent</td>
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<tr>
<td>Test for 5</td>
<td>89.251</td>
<td>28.7136</td>
<td>65.1</td>
<td>58.2134</td>
<td>1.0219</td>
<td>Present</td>
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</table>

Results demonstrate that the mathematical procedure developed could successfully determine the species present in a cocktail even at the strain level. Combining with the high spectral sensitivity of the nanoparticle-induced nanoSPR enhanced IR spectroscopy, microbial composition of samples that contain multiple strains of the same species of microorganisms can be determined with high accuracy. Since little sample preparation is needed in this approach, it is very rapid. Potentially it can lead to fast detection schemes for determination of presence of pathogenic microbial contaminations in food and water.

**Microorganism mixture in a food matrix (apple juice)**

To identify microorganisms embedded in complex matrixes like foods, two things are important. First, the predominant contributions from the background need to be removed. Second, when numerous components are present, the difference between the microorganisms becomes complex. If one species is very different from all of the rest, its contributions in CVA would dominate and minor differences between the other microorganisms will be masked and become less apparent. It can be seen by the close clustering of the groups that represent these microorganisms. Consequently, a multi-step differentiation needs to be adopted. Firstly, those microorganisms that could be well differentiated were identified during the first round of differentiation; then a second round of CVA was conducted to differentiate species that were left out in the first round.

Three mixtures (Table 6) that contained a cocktail of 6, 7, or 8 species at a concentration of $10^4$ CFU/ml were investigated and compared to the fingerprint database of the nine microorganisms. To simplify the description we will use numerals 1, 2, 3, 4, 5, 6, 7, 8, 9 to represent *Enterobacter cloacae, Salmonella typhimurium, Enterobacter aerogenes, Salmonella choleraesuis, Serratia marcescens, Pseudomonas vulgaris, E. coli O26, Vibrio cholerae, Hafnia alvei*, respectively.

Table 6. Composition of mixtures in apple juice
<table>
<thead>
<tr>
<th>Mixture name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>sm1</td>
<td><em>Enterobacter cloacae, Salmonella typhimurium, Enterobacter aerogenes, Salmonella choleraesuis, Serratia marcescens, Pseudomonas vulgaris</em></td>
</tr>
<tr>
<td>sm2</td>
<td><em>Enterobacter cloacae, Salmonella typhimurium, Enterobacter aerogenes, Salmonella choleraesuis, Serratia marcescens, Pseudomonas vulgaris, E. coli O26</em></td>
</tr>
<tr>
<td>sm3</td>
<td><em>Enterobacter cloacae, Salmonella typhimurium, Enterobacter aerogenes, Salmonella choleraesuis, Serratia marcescens, Pseudomonas vulgaris, E. coli O26, Vibrio cholerae</em></td>
</tr>
</tbody>
</table>

Fig. 7a shows the typical results for the mixture sm2 given in Table 6. The assignments of groups (Table 8) were based on whether or not 75% or more of the data in the test set were within the 95% confidence interval of the respective groups. After the first round of analysis, it was clear that organisms 8 and 9 were reported as not present and 1, 5, and 7 were identified as present. A clear presence/absence decision on the microorganisms 2, 3, 4, and 6 could not be made at this time, hence a second round of CVA was performed using the remainder of the data and the results are shown in Fig. 8b. According to the criteria (75% of the data should lie within the 95% confidence interval of each group), all these four groups were identified as positively present. Results of the final analysis on the mixture sm1 shown in Table 8 indicated a 100% correct classification.

Similar results were obtained for the microorganism cocktail sm1 and sm3 as shown in Table 8 and 9 respectively. After two rounds of analysis using the canonical variate procedure, the microorganisms present in the cocktail sm1 and sm3 could be correctly identified.
Table 7. Group assignments after 1st and 2nd round CV A analysis for mixture sm2

<table>
<thead>
<tr>
<th>Test set</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group, 1st round</td>
<td>1</td>
<td>2, 4</td>
<td>3, 6</td>
<td>2, 4</td>
<td>5</td>
<td>6, 3</td>
<td>7</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Group, 2nd round</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

Table 8. Group assignments after 1st and 2nd round CV A analysis for mixture sm1

<table>
<thead>
<tr>
<th>Test set</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group, 1st round</td>
<td>1</td>
<td>2, 4</td>
<td>3</td>
<td>2, 4, 5</td>
<td>5</td>
<td>6</td>
<td>none</td>
<td>3</td>
<td>none</td>
</tr>
<tr>
<td>Group, 2nd round</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>none</td>
<td>3</td>
<td>none</td>
</tr>
</tbody>
</table>

Table 9. Group assignments after 1st and 2nd round CV A analysis for mixture sm3

<table>
<thead>
<tr>
<th>Test set</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group, 1st round</td>
<td>1</td>
<td>2</td>
<td>3, 5, 8</td>
<td>4</td>
<td>5, 3, 2, 8</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>none</td>
</tr>
<tr>
<td>Group, 2nd round</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>none</td>
</tr>
</tbody>
</table>

In this process, only one incubation operation was needed to prepare the samples for investigation and the whole process could be finished within 24 hours from the first collection of bacteria samples. This is a significant improvement compared to the traditional approaches. To make the most use of this method, accurate FTIR fingerprints of the different bacteria are required. Thus, more work need to be done to establish the database for most of the important pathogenic microorganisms.

CONCLUSION

A nanoparticle induced nanoSPR-enhanced FTIR based procedure was successfully used to detect the presence or absence of microorganisms in microbial mixtures at the strain level. Four mixtures each containing three *E. coli* strains were analyzed with the developed procedure with 100% accuracy. A two-step procedure was used to extend the methods developed to identify specific microorganisms in microbial mixtures of up to eight organisms in apple juice. Experiments conducted with various combinations of the microorganism mixtures using the proposed spectroscopic approach gave 100% prediction accuracy. The easy-to-operate, user-friendly, and rapid analysis capability could reduce the analysis time needed to perform the identification of microbiological samples from a week to a day. With an appropriate database of key microorganisms the procedure can be extended to identifying clinically relevant microorganisms for rapid diagnosis of infectious pathogens down to the strain level.

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References


