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

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Abstract. *Routine identification of pathogenic microorganisms predominantly based on nutritional and biochemical tests is a time-consuming process, but 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 mixtures of different* E. coli *strains could be accurately identified using our method, at a concentration level of 103 CFU mL^{-1}. The procedure was also applied to determine the presence or absence of pathogenic microorganisms in a simple but real food matrix (apple juice). Results indicated that microorganisms in a cocktail of up to eight different species suspended in an apple juice matrix could be accurately identified for its presence or absence.*

Keywords. *IR absorption, Microorganism identification and differentiation, Nanoparticles, Surface enhanced.*

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 microorganism 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, and then spread them onto an agar plate and separate them into single colonies. Subsequently, the single colonies are cultured for another 16 to 24 h on solid or broth culture medium to obtain enough biomass, 10^6 to 10^9 colony forming units (CFU) mL^{-1}, for further analysis by biochemical methods, which are labor-intensive and time-consuming (about 2 to 3 days or more are needed). Methods that allow detection of microorganisms at the earliest time are highly desirable.

Over the last two decades, 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; Jean et al., 2004; Batt, 2007). 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; Fredricks and Relman, 1998; Nolte and Caliendo, 2003). In addition, 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, 2003).

An alternative approach to microbial characterization is the use of spectroscopic methods. Pyrolysis mass spectrometry has been evaluated as a method for bacterial characterization (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 (Naumann, 2000; Mouwen et al., 2005; Rebuffo-Scheer et al., 2006, 2007; Bosch et al., 2008; Amiali et al., 2008). 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 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 Maquelin 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 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).

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To further increase the discriminating power of the method to analyze microbial mixtures at strain level, or samples with complicated backgrounds such as food matrices, 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) fields around the particles, it also causes enhancement of the infrared absorption of molecules (on the order of 10 to 100) that are in close proximity to the particles, just as 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 the microbial composition of mixtures of different *E. coli* strains, and (2) 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 Pennsylvania State University (University Park, Pa.). Each strain was cultured in a 100 mL broth medium (5 g yeast extract, 8 g tryptone, and 5 g NaCl in 500 mL distilled water) at 35°C and shaken at 100 rpm for 24 h. Suspensions of microbial cells of each strain in saline buffer solution were prepared by adding 1 mL of the culture to 9 mL phosphorus buffer saline (PBS) solution (pH 7.2 to 7.6). Four possible combinations (table 1) of mixtures of three strains (5 mL of each strain) were prepared for analysis. To prepare the samples for NPEIRA measurement, 1 mL of nanoparticle solution was added into the 10 mL saline microbial cell suspension, the sample was then vortexed at 100 rpm for 2 min to mix the cells and nanoparticles, and then centrifuged at 2000 rpm for 5 min to obtain a mixture of cells and nanoparticles. After disposal of the supernatant, the sample was ready for NPEIRA 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*, and *Hafnia alvei*) were obtained from the GDC. Each species was cultured in a 100 mL broth medium (5 g yeast extract, 8 g tryptone, and 5 g NaCl in 500 mL distilled water) at 35°C and shaken at 100 rpm for 24 h. Suspensions of microbial cells of each species in autoclaved apple juice (White House brand, National Fruit Product Co., Winchester, Va.) were made by adding 1 mL of each culture to 9 mL of apple juice. Three different mixtures with 6, 7, or 8 microorganism species were prepared by mixing 5 mL each of the suspensions containing the respective microorganisms. Subsequently, 1 mL of nanoparticle solution was added to the mixture sample and stirred for 1 min, and cells were collected along with nanoparticles by centrifugation at 2000 rpm for 5 min.

Table 1. Composition of the *E. coli* mixtures in PBS buffer solution.

Mixture	Present	Absent
em1	O103, O55, O121	O30, O26
em2	O103, O55, O26	O121, O26
em3	O103, O121, O26	O55, O30
em4	O55, O121, O26	O103, O30

All water used in this study was purified (18.2 mΩ) using a Nanopure water system (Direct-Q 3, Millipore Co., Billerica, Mass.).

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 IR spectral measurement.

Fabrication of Gold Nanorods

Gold nanorods were made through seed-mediated growth. Gold nanoparticle seeds with diameters around 4 nm were made as follows: 0.6 mL of 0.01 M freshly prepared, ice-cold $NaBH_4$ solution was added to a mixture solution composed of 5 mL of 0.2 M cetyltrimethylammonium bromide (CTAB), 0.25 mL of 0.01 M $HAuCl_4$, and 4.75 mL of water. The solution was vigorously mixed for 2 min. The seeds were kept at 27°C for 3 h to allow complete degradation of the remaining $NaBH_4$ and were then used in subsequent nanorod growth: 5 mL of 0.2 M CTAB solution and 5 mL of 0.2 M benzyldimethylhexadecylammonium chloride (BDAC) solution were mixed to create a bisurfactant solution, and 80 μL of 0.01 M $AgNO_3$ and 0.5 mL of 0.01 M $HAuCl_4$ were added to the bisurfactant solution. After gentle mixing, 65 μL of 0.1 M ascorbic acid was added. The solution was mixed until the color 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 were made with an aspect ratio (length / width) of approximately 3.

Fabrication of Silver-Tipped Gold Nanorods

The as-made gold nanorod (GNR) solution was centrifuged twice and washed twice with purified water from a Nanopure system (Direct-Q 3, Millipore, Billerica, Mass.). The washed GNR pellets were resuspended in bisurfactant solution. The concentration of GNR template solution was adjusted to around 4.2×10^{-10} M. An additional 0.1 mL of 10 mM $HAuCl_4$, 0.3 mL of 10 mM $AgNO_3$, and 20 μL of 0.1 M ascorbic acid were added to 5 mL of purified GNR template solution, and 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.

Fabrication of Gold-Silver Alloy Nanocages

Silver nitrate (0.12 g, Aldrich) was dissolved in anhydrous ethylene glycol (2.5 mL, 99.8% Aldrich) 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; 100 μL of 0.3 mM Na_2S was then pipetted into the vial; 8 min later, 1.5 mL of the PVP solution

and 0.5 mL of the $AgNO_3$ solution were added to the reaction vial; and 15 min later, the reaction vial was removed from the heated oil bath and 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* for 30 min. After being washed three times with deionized water, the nanocubes were redispersed in 1.5 mL of deionized water by sonication. The silver nanocubes were 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 20 mL vial and heated to a mild boil for approximately 10 min; 0.1 mM $HAuCl_4$ solution was then added to the reaction vial at a rate of 0.75 mL min^{-1}. A series of color changes were observed, which indicated the wavelength of the SPR peak for the Au-Ag nanocages. Addition of $HAuCl_4$ solution was discontinued 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 1380*g* for 30 min and redispersed in purified 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 FTIR spectrometer (Alpha, Bruker Optics Inc., Billerica, Mass.) and 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. To obtain the spectra of each sample, 256 scans were averaged. Each experiment was repeated ten times.

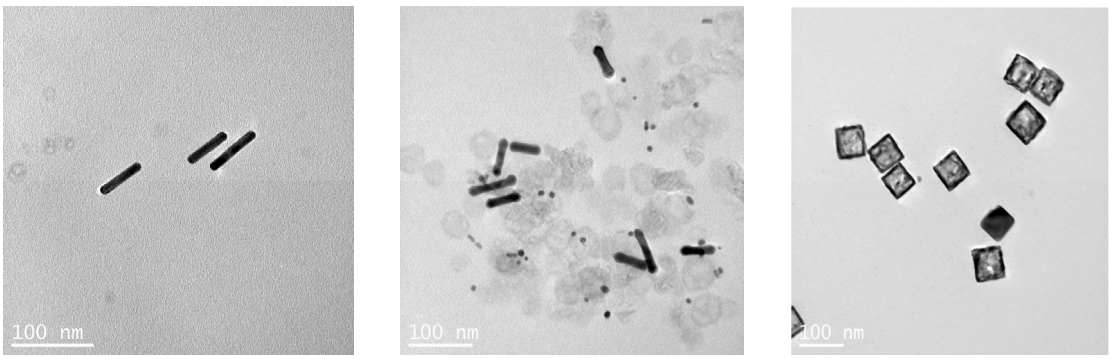
Mathematical Procedure to Extract Fingerprint Features of Bacterial Spectra

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 details of this procedure were reported elsewhere (Yu and Irudayaraj, 2006). Briefly, it is a three-step procedure. In step 1, a reference spectrum (b_a) is 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* is 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 are retained in a reference set of spectra (*BF*). In step 3, the same operations are conducted on each microbial species that needs to be tested, and a discriminant model is established for the group of *BF* values. Once the discriminant model was in place, then we could test whether or not a microbial species was present in the microbial mixture. A testing set was created for the microbial species by projecting *M* onto b_a. If a particular microorganism was present in the mixture, then 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 (Chichester, U.K.: Wiley and Sons, Ltd.) To further confirm the prediction, a linear discriminant analysis (LDA) model using Mahalanobis distance as the differentiating criteria was also implemented against the test set using Matlab (Math Works, Inc., Natick, Mass.). The details of the mathematical modeling were reported elsewhere (Yu and Irudayaraj, 2006).

Results and Discussion

Nanoparticle Enhanced FTIR Spectra of Microorganisms

Figure 1 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 FTIR spectroscopic measurements of microbial samples. Figure 2 shows the typical spectra of *E. coli* O103 with no nanoparticles, with gold nanorods, with silver-tipped gold nanorods, and with gold-silver alloy nanocages with one-bounce attenuated total reflectance (ATR). The concentration of *E. coli* O103 was 10^3 CFU mL^{-1}. Without the nanoparticle-induced nanoSPR enhancement, one droplet of microbial sample did not yield a meaningful FTIR spectrum, as shown in figure 2. However, when



(a) (b) (c)

Figure 1. TEM images of (a) gold nanorods, (b) silver-tipped gold nanorods, and (c) Au-Ag alloy nanocages.

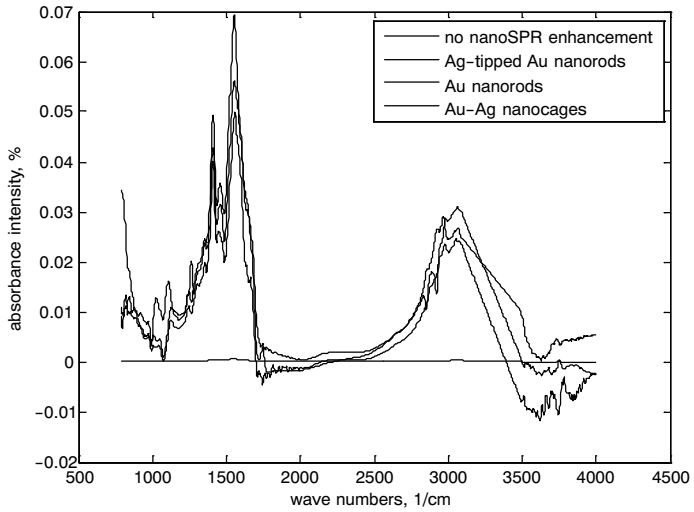


Figure 2. nanoSPR enhanced IR spectrum of *E. coli* O103 at 10^3 CFU mL^{-1}.

nanoparticles were present, the nanoSPR enhancement yielded spectra with identifiable peaks that could be used for identification and differentiation between microbial species. This enhancement was mainly caused by the strong local electromagnetic field (EF) surrounding the nanoparticles, which resulted from the interaction between incident light (IR) and the plasmons in the nanoparticles. The anisotropic shapes of the nanoparticles further enhanced the local EF; the geometrical singularity of these anisotropic particles created very intensive EF around them. The overall enhancement effect to the FTIR spectra is by a factor of 100 to 1000.

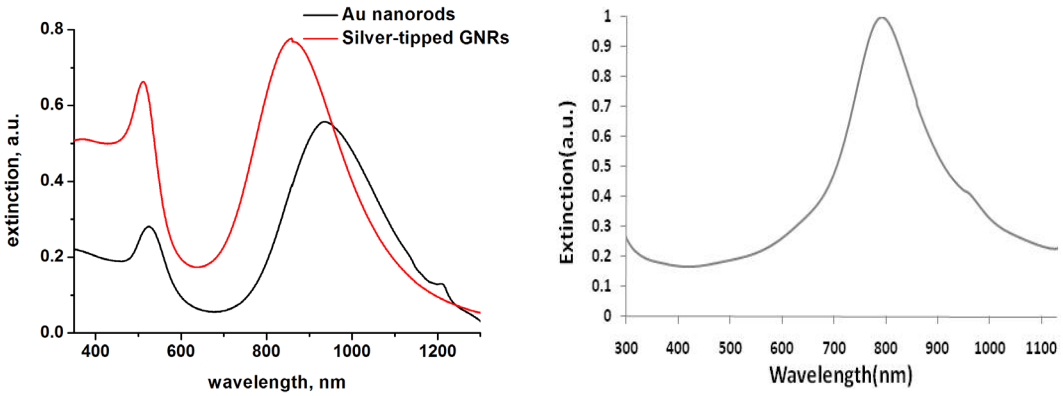
All three types of nanoparticles studied exhibited strong enhancement effects, which was consistent with their surface plasmon characteristics. As shown in figure 3, the surface plasmon resonance bands of the silver-tipped gold nanorods (λ_{pl} = 850 nm), gold nanorods (λ_{pl} = 920 nm), and Au-Ag alloy nanocages (λ_{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.

Strain Level Analysis: Mixtures of *E. coli* Strains

The Au-Ag alloy nanocage-induced nanoSPR enhanced mid-IR spectra of the five *E. coli* strains studied are shown in figure 4. To simplify the description, we will use numbers 1, 2, 3, 4, and 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 figure 5.

In order to test the bacterial composition of the mixtures, we first generated the real fingerprint of each species with respect to the mixture. These spectral fingerprints were used to develop the discriminant models, as shown in figure 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.

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 dominated 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. Figure 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



(a) (b)

Figure 3. Surface plasmon characteristics of (a) gold nanorods and silver-tipped gold nanorods and (b) Au-Ag alloy nanocages.

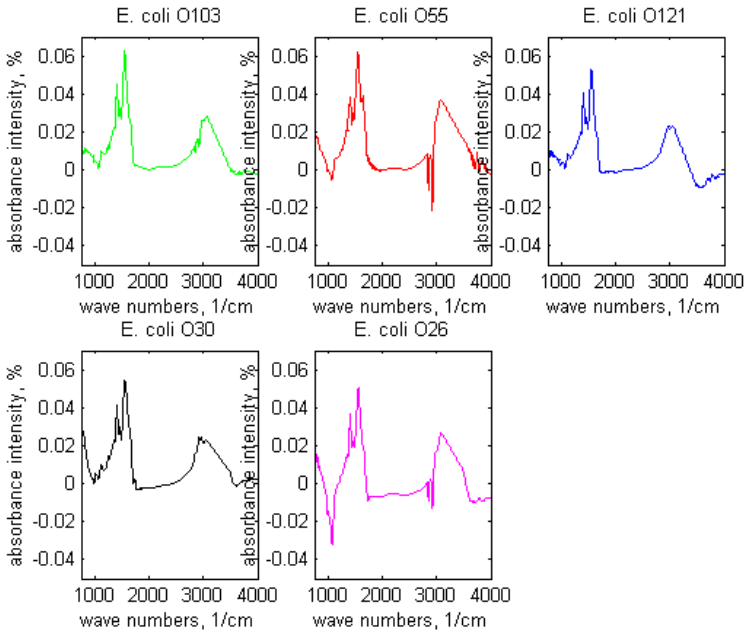


Figure 4. Spectra of the five *E. coli* strains under nanoSPR enhancement of Au-Ag alloy nanocages.

be absent, but the differences shown by present or absent strains are not conspicuous. Using the LDA model, the average Mahalanobis distance from the ten entries in the 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 a 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 through 5 list the calculated Mahalanobis distances using the LDA model for the four 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* strains correctly with good accuracy at relatively low levels of *E. coli* concentration (10^3 CFU mL^{-1}).

The results demonstrate that the mathematical procedure developed could successfully determine the species present in a cocktail even at the strain level. Combined with the high spectral sensitivity of nanoparticle-induced nanoSPR enhanced IR spectroscopy, the 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 the presence of pathogenic microbial contaminations in food and water.

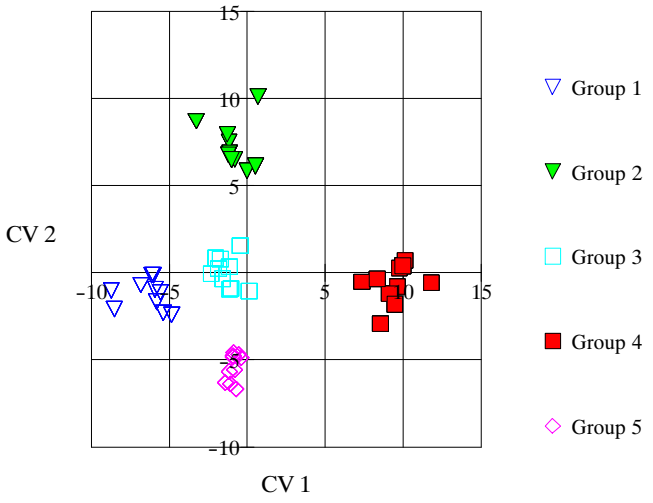


Figure 5. Differentiation of the five *E. coli* strains in buffer (group 1 = *E. coli* O103, group 2 = *E. coli* O55, group 3 = *E. coli* O121, group 4 = *E. coli* O30, and group 5 = *E. coli* O26).

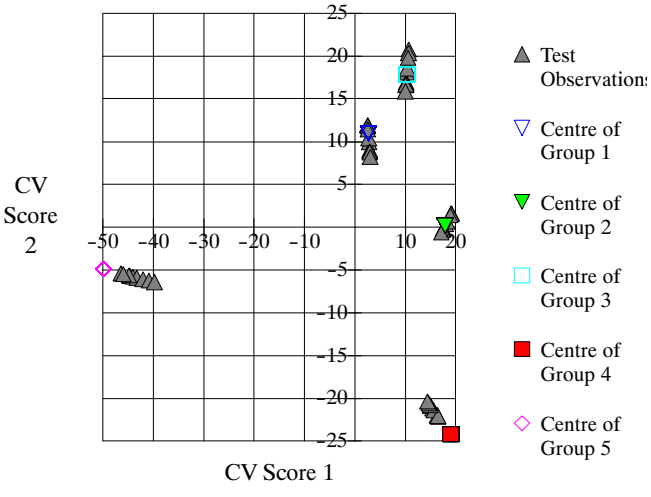


Figure 6. Testing of mixture em1 to determine the presence or absence of bacterial targets.

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 and 5 are absent; 1, 2, and 3 are present).

Test Data Set	Distance from Group 1	Distance from Group 2	Distance from Group 3	Distance from Group 4	Distance from Group 5	Prediction
Test for 1	1.3968	14.9835	21.8059	43.0641	78.2243	Present
Test for 2	15.3623	0.8769	28.998	28.6162	84.2112	Present
Test for 3	21.9929	27.8125	1.6917	57.954	90.6371	Present
Test for 4	38.3623	22.4619	47.206	9.5335	93.8745	Absent
Test for 5	70.0277	74.213	90.641	89.8754	12.634	Absent

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 and 4 are absent; 1, 2, and 5 are present).

Test Data Set	Distance from Group 1	Distance from Group 2	Distance from Group 3	Distance from Group 4	Distance from Group 5	Prediction
Test for 1	1.1646	37.3557	21.9885	14.7469	70.0231	Present
Test for 2	37.3539	1.1385	47.8068	22.6162	84.2112	Present
Test for 3	21.9929	47.8125	15.8525	29.2060	90.6371	Absent
Test for 4	44.7463	22.619	29.206	8.635	73.8745	Absent
Test for 5	70.0277	84.213	90.641	73.8754	2.634	Present

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 and 4 are absent; 1, 3, and 5 are present).

Test Data Set	Distance from Group 1	Distance from Group 2	Distance from Group 3	Distance from Group 4	Distance from Group 5	Prediction
Test for 1	0.6679	26.527	41.2185	34.6219	90.4879	Present
Test for 2	24.4817	6.2324	68.5925	24.3558	89.5554	Absent
Test for 3	24.1356	36.894	1.5225	58.7985	73.8063	Present
Test for 4	40.1235	19.8564	26.5412	9.521	82.4331	Absent
Test for 5	68.7322	64.5431	70.641	69.8745	2.7367	Present

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 and 4 are absent; 2, 3, and 5 are present).

Test Data Set	Distance from Group 1	Distance from Group 2	Distance from Group 3	Distance from Group 4	Distance from Group 5	Prediction
Test for 1	10.9005	24.7181	26.043	64.9126	58.8112	Absent
Test for 2	25.006	1.2332	60.5578	26.726	57.161	Present
Test for 3	26.074	42.257	0.9118	52.457	87.658	Present
Test for 4	55.2145	24.5652	32.2145	12.2540	77.5289	Absent
Test for 5	89.251	28.7136	65.1	58.2134	1.0219	Present

MICROORGANISM MIXTURE IN A FOOD MATRIX (APPLE JUICE)

To identify microorganisms embedded in complex matrices like foods, two things are important. First, the predominant contributions from the background need to be removed. Second, when numerous components are present, the differences between the microorganisms become complex. If one species is very different from all of the rest, then its contributions in CVA will dominate and minor differences between the other microorganisms will be masked and become less apparent. This can be seen by the close clustering of the groups that represent these microorganisms. Consequently, a multi-step differentiation needs to be adopted. First, those microorganisms that could be well differentiated were identified during the first round of differentiation, and 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^{-1} were investigated and compared to the fingerprint database of the nine microorganisms. To simplify the description, we will use letters A, B, C, D, E, F, G, H, and I to represent *Enterobacter cloacae*, *Salmonella typhimurium*, *Enterobacter aerogenes*, *Salmonella choleraesuis*, *Serratia marcescens*, *Pseudomonas vulgaris*, *E. coli* O26, *Vibrio cholerae*, and *Hafnia alvei*, respectively.

Table 6. Composition of mixtures in apple juice.

Mixture	Composition
sm1	*Enterobacter cloacae*, *Salmonella typhimurium*, *Enterobacter aerogenes*, *Salmonella choleraesuis*, *Serratia marcescens*, and *Pseudomonas vulgaris*
sm2	*Enterobacter cloacae*, *Salmonella typhimurium*, *Enterobacter aerogenes*, *Salmonella choleraesuis*, *Serratia marcescens*, *Pseudomonas vulgaris*, and *E. coli* O26
sm3	*Enterobacter cloacae*, *Salmonella typhimurium*, *Enterobacter aerogenes*, *Salmonella choleraesuis*, *Serratia marcescens*, *Pseudomonas vulgaris*, *E. coli* O26, and *Vibrio cholerae*

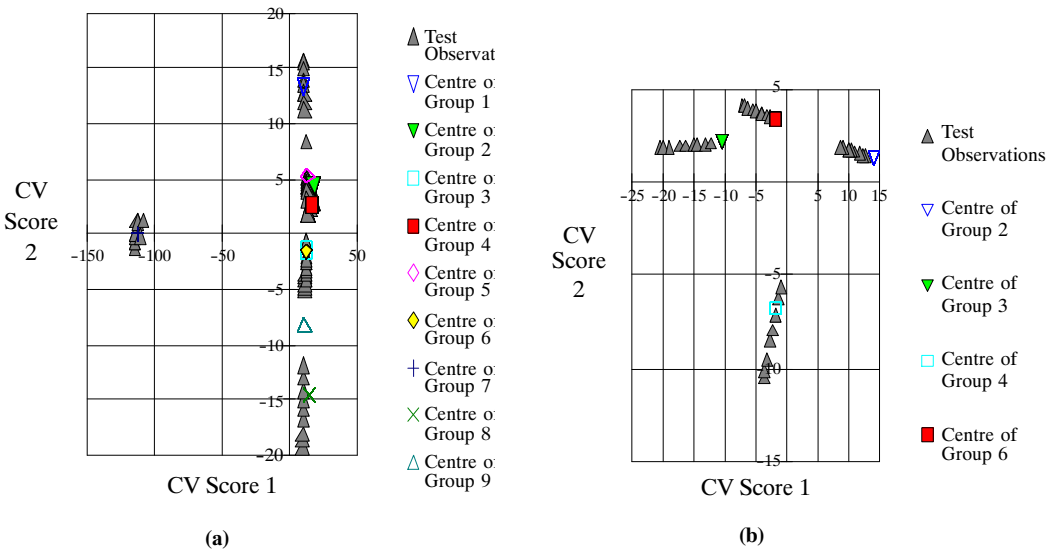


Figure 7. CVA analysis of sm2 (table 6): (a) first round and (b) second round.

Figure 7a shows the typical results for mixture sm2 given in table 6. The assignments of groups (table 7) 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 H and I were reported as not present and A, E, and G were identified as present. A clear presence or absence decision for microorganisms B, C, D, and F 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 figure 7b. According to the criteria (75% of the data should lie within the 95% confidence interval of each group), these four groups were all identified as positively present. Results of the final analysis of mixture sm2, shown in table 7, indicated a 100% correct classification.

Similar results were obtained for the microorganism cocktails sm1 and sm3, as shown in tables 8 and 9, respectively. After two rounds of analysis using the canonical variate procedure, the microorganisms present in mixtures sm1 and sm3 could be correctly identified.

In this process, only one incubation operation was needed to prepare the samples for investigation, and the whole process could be finished within 24 h 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 needs to be done to establish a database of the most important pathogenic microorganisms.

Table 7. Group assignments after first and second round CVA analysis for mixture sm2.

	Test Set								
	A	B	C	D	E	F	G	H	I
Group, first round	A	B, D	C, F	B, D	E	C, F	G	None	None
Group, second round	A	B	C	D	E	F	G	None	None

Table 8. Group assignments after first and second round CVA analysis for mixture sm1.

	Test Set								
	A	B	C	D	E	F	G	H	I
Group, first round	A	B, D	C	B, D, E	E	F	None	H	None
Group, second round	A	B	C	D	E	F	None	H	None

Table 9. Group assignments after first and second round CVA analysis for mixture sm3.

	Test Set								
	A	B	C	D	E	F	G	H	I
Group, first round	A	B	C, E, H	D	E, C, B, H	F	G	H	None
Group, second round	A	B	C	D	E	F	G	H	None

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 at the relatively low concentration of 10^3 CFU mL^{-1}. Four mixtures, each containing three *E. coli* strains, were analyzed with the developed procedure with good 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 microorganisms using the proposed spectroscopic approach gave correct prediction about the composition of the mixtures. This easy-to-operate, user-friendly, and rapid analysis method could reduce the time needed to identify 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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