Identification and Quantification of Foodborne Pathogens in Different Food Matrices Using FTIR Spectroscopy and Artificial Neural Networks

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Abstract. *FTIR absorbance spectra of four foodborne pathogens suspended in four common food matrices at three different concentrations were used with artificial neural networks (ANNs) for identification and quantification. The classification accuracy of the ANNs was 93.4% for identification and 95.1% for quantification when validated using a subset of the data set. The accuracy of the ANNs when validated for identification of the pathogens studied at four different concentrations using an independent data set had an accuracy range from 60% to 100% and was strongly influenced by background noise. The pathogens could be identified irrespective of the food matrix in which they were suspended, although the classification accuracy was reduced at lower concentrations. More sophisticated background noise filtration techniques are needed to further improve the predictions.*

Keywords. ANNs, Differentiation, Food matrices, Food pathogens, FTIR spectroscopy, Quantification.

As concerns in food safety and security increase, it has become imperative to develop simple but effective methods for rapid identification of foodborne pathogens even at the single cell level (Tauxe et al., 1997; Mead et al., 1999; Crutchfield et al., 2000). Fourier transform infrared spectroscopy (FTIR) has been widely used for the identification and characterization of bacteria (Naumann et al., 1989; Yang and Irudayaraj, 2003; Naumann et al., 1991a, 1991b; Curk et al., 1994; Helm et al., 1991a, 1991b) as it permits discrimination of intact microbial cells based on molecular fingerprints that are distinct and reproducible without extensive sample preparation. Real-time applications of these spectral signatures in previous research have used unsupervised classification methods such as analysis maps and cluster analysis, where perceived closeness of similar objects is used to form respective clusters (Helm et al., 1991a). Over the past few years, "supervised" methods such as chemometrics have been applied to analyze FTIR data. Artificial neural networks (ANNs), which belong to the class of "supervised" learning methods, have excellent potential for processing FTIR data because they possess the ability to learn based on training and predictions (Argov et al., 2002; Beksac et al., 1997; Chun et al., 1993; Freeman et al., 1994; Goodacre et al., 1994, 1998; Kirschner et al., 1999; Udelhoven et al., 2000).

Direct detection of pathogens in food matrices presents a major obstacle to most detection schemes because of the complexity and time involved in sampling (Hanes, 1999). Traditional methods involve isolation and enrichment, but for rapid online assessment, identification, and quantification should occur directly in a food matrix. To our knowledge, no study has been reported on the identification and quantification of foodborne pathogens in food matrices. Therefore, the focus of this study was to obtain absorbance spectra of food pathogens suspended directly in selected real food matrices such as orange juice (pulp free), chicken broth, fat-free skim milk, and milk with 1% fat, and to use ANNs for qualitative and quantitative assessment. The proposed study is an extension of the previous work by Gupta and Irudayaraj (2004) on quantification and differentiation of foodborne pathogens in phosphate-buffered saline solution based on their FTIR spectral signatures using ANNs.

The objectives of this study were: (1) to obtain FTIR fingerprints of four selected food pathogens at three concentration levels in four different food matrices and to construct a database of their absorbance spectra in the key fingerprint region, (2) to develop ANN models to differentiate and quantify the various food pathogens in different food matrices, and (3) to validate the differentiation and quantification models using independent data sets.

Material and Methods

Sample Preparation for FTIR

Four food pathogens (*E. coli* O26, *Salmonella typhimurium*, *Yersinia enterocolitis*, and *Shigella boydii*) were obtained from the Gasteroenteric Disease Center (GDC) at Pennsylvania State University (University Park, Pa.). The samples were cultured in 100 mL of broth media at 37°C and

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Table 1. Concentrations of microorganisms after incubation for 24 h by plate counting.

	Plate Count Values ($\times 10^9$ CFU/mL)	
Microorganism	TSA Broth	Nutrient Broth
E. coli O26	8.09	3.29
Salmonella typhimurium	9.26	8.66
Yersinia enterocolitis	9.98	5.44
Shigella boydii	9.99	3.98

shaken at 100 rpm for 24 h to attain a concentration of about 10^9 colony-forming units per mL (CFU/mL). Broth media used were tryptic soy agar (TSA) broth (5 g yeast extract, 8 g tryptone, and 5g NaCl in 500 mL distilled water; referred to as media I) and nutrient broth (referred to as media II). The concentrations of pathogens after incubation are listed in table 1.

Sterile samples of orange juice, chicken broth, pasteurized skim milk, and milk with 1% fat were inoculated with the different organisms and diluted to six levels ($\times 10^6$, 10^5, 10^4, 10^3, 10^2, and 10^1) to result in seven different concentrations (10^9, 10^8, 10^7, 10^6, 10^5, 10^4, and 10^3 CFU/mL). The dilutions of 10^9, 10^8, 10^6, and 10^3 CFU/mL were then used for FTIR spectroscopic measurements.

FTIR MEASUREMENTS

Different bacteria suspended in the respective food matrices were evenly applied onto a ZnSe (zinc selenide) ATR crystal using a pipette and analyzed using a Nexus 870 FTIR spectrometer (Thermo Electron, Madison, Wisc.). FTIR signals were collected in the spectral region between 600 and 4000 cm^{-1} (wave numbers) at a resolution of 2 cm^{-1}. For each spectrum, 256 interferograms were co-added, averaged, and Fourier transformed to give a resolution of 2 cm^{-1}. Mixtures of plain broth and the respective food matrices in corresponding dilutions were first measured and used as the background spectra and subtracted from the respective sample spectra. For each sample, the spectra were recorded in replicates of ten. The spectra were then smoothed using an automatic smoothing function to reduce the noise and differentiated to the first order as a spectra enhancement operation using the difference equation to reduce the baseline variability with OMNIC E.S.P. software (v5.2, Madison, Wisc.).

DATA ANALYSIS
Principal Component Analysis (PCA)

In our previous study (Gupta and Irudayaraj, 2004), absorbance data in the entire 600 to 4000 cm^{-1} range was used as input to the ANN. The training time required for this network configuration was 30 to 90 min, and the classification accuracy from the validation set was between 0% and 30%, possibly due to the presence of a high level of noise in the spectra due to the interference from water and carbon dioxide. Hence, the absorbance spectra in the 600 to 1750 cm^{-1} region were used because they contained typical fingerprints (600 to 900 cm^{-1}) characterized by a combination of weak absorptions due to aromatic ring vibrations of phenylalanine, tyrosine, tryptophan, and several nucleotides; the polysaccharide region (900 to 1200 cm^{-1}) was used due to symmetric stretching vibrations and peaks due to C−O−C and C−O−P; the mixed region (1200 to 1500 cm^{-1}) was used due to the bending modes of lipids and proteins; and the

intense amide I and amide II region was used due to the presence of α and β and structures of the cellular proteins (Naumann, 2000). The prediction accuracy of this network was 90% to 100%.

To reduce the network development and training time, the 20 principal components were extracted from the first derivative smoothed spectra (ten replications each for the four food pathogens) in the spectral region between 600 and 4000 cm^{-1} wave numbers and used as input to the ANNs.

Development of the Artificial Neural Network

The neural network analysis was carried out using the Neuroshell 2 Release 4.0 software (Ward Systems Group, Inc., Frederick, Md.), and the probabilistic neural network (PNN) based algorithm was used for training. The network consists of an input layer with 20 elements corresponding to the PCs of the spectra. It has a pattern layer, which organizes the training set in such a manner that each input vector is represented by an individual processing element (thus, it is 40-120 for the bacteria differentiation network and 30 for the quantification network). The final network consists of an output layer, called the summation layer, with as many processing elements as the number of classes to be recognized (three classes for the quantification network, i.e., one element corresponding to each concentration class for each bacterium, and four classes for the bacteria differentiation network, corresponding to each bacterium for each concentration considered).

A summary of all the models is given in table 2. Each element in this layer combines via processing elements within the pattern layer that relate to the same class and prepares that category for output. Sometimes a fourth layer is added to normalize the input vector. Based on previous results (Gupta and Irudayaraj, 2004), the network was trained using 70% of the data, of which 50% was used for training and 20% was used as a test set for calibration of the smoothing parameter. The remaining 30% was used for validation of the trained network. A binary coding system was used for differentiation and quantification of the input classes.

Differentiation of Foodborne Pathogens Irrespective of Food Matrix

Although the ANNs developed for differentiation of bacteria in a given food matrix are more accurate, an exhaustive set of ANNs for all possible food matrices is needed. Hence, an attempt was made to assess whether the spectra of the foodborne pathogens from the four different food matrices studied could be considered as a single group for pathogen detection independent of the food matrix. Thus, the PCs were extracted with 40 spectra (10 per food matrix) for each of the four foodborne pathogens as a single group. These PCs were then used as a training set in the ANN analysis.

Time-Course Validation of ANNs for Differentiation Using an Independent Data Set

Since calibration models are not stable over time due to drift in instrumental and measurement parameters (e.g., source intensity, temperature and humidity of the surroundings, cleanliness of the ZnSe crystal, laser intensity), a preliminary evaluation was made to demonstrate the effectiveness of the model using a time-course study. The ANNs developed using the data collected in March and April

Table 2. Summary of models developed and used for analysis.

Models	Input Data Used	Remarks
1-3	Principal components of spectral data of the four bacteria grown in media I at each of the concentration (10^8, 10^6, 10^3) in orange juice	Done separately for each concentration
4-6	Principal components of spectral data of the four bacteria grown in media II at each of the concentration (10^8, 10^6, 10^3) in orange juice	Done separately for each concentration
7-9	Principal components of spectral data of the four bacteria grown in media I at each of the concentration (10^8, 10^6, 10^3) in chicken broth	Done separately for each concentration
10-12	Principal components of spectral data of the four bacteria grown in media II at each of the concentration (10^8, 10^6, 10^3) in chicken broth	Done separately for each concentration
13-15	Principal components of spectral data of the four bacteria grown in media I at each of the concentration (10^8, 10^6, 10^3) in skim milk	Done separately for each concentration
16-18	Principal components of spectral data of the four bacteria grown in media II at each of the concentration (10^8, 10^6, 10^3) in skim milk	Done separately for each concentration
19-21	Principal components of spectral data of the four bacteria grown in media I at each of the concentration (10^8, 10^6, 10^3) in milk with 1% fat	Done separately for each concentration
22-24	Principal components of spectral data of the four bacteria grown in media II at each of the concentration (10^8, 10^6, 10^3) in milk with 1% fat	Done separately for each concentration
25-28	Principal components of spectral data of *E. coli* O26 at all concentrations (10^9, 10^8, 10^6, 10^3) in each food matrix	Done separately for each food matrix
29-32	Principal components of spectral data of *Salmonella typhimurium* at all concentrations (10^9, 10^8, 10^6, 10^3) in each food matrix	Done separately for each food matrix
33-36	Principal components of spectral data of *Yersinia enterocolitis* at all concentrations (10^9, 10^8, 10^6, 10^3) in each food matrix	Done separately for each food matrix
37-40	Principal components of spectral data of *Shigella boydii* at all concentrations (10^9, 10^8, 10^6, 10^3) in each food matrix	Done separately for each food matrix
41-44	Principal components of spectral data of the four bacteria in all food matrices done at each concentration	Done separately for each concentration

2004 were further validated using the data collected in July 2004 for one pathogen. *Yersinia enterocolitica* was grown in TSA broth for 24 h at 37°C to a concentration of 10^9 CFU/mL and suspended in sterile chicken broth at concentrations of 10^8, 10^6, and 10^3 CFU/mL. The absorbance spectra at these three concentrations were then measured in replications of ten using the Nicolet Nexus 870 FTIR spectrometer (Thermo Electron Corp., Madison, Wisc.).

RESULTS AND DISCUSSION

DIFFERENTIATION OF THE FOODBORNE PATHOGENS

Figure 1 shows the FTIR absorbance spectra of four matrices in which the bacteria were suspended. Similarly, figure 2 shows the FTIR absorbance spectra of the four microorganisms suspended in chicken broth, and table 3 lists the major chemical groups in these matrices and microorganisms. Figures 1 and 2 show that the spectra of the microorganisms are different from that of the matrix (chicken broth in this case). Slight variations among the four bacterial spectra are visible in figure 2. These variations are too minute to be visually differentiated; hence, sophisticated statistical methods were used for the analysis of the spectral data.

ANNs were developed to differentiate four microorganisms at various concentration levels (10^9 to 10^3 CFU/mL). Data compression of the absorption spectra in the 600-4000 cm^{-1} range was done using principal component analysis (PCA), and 20 principal components (figs. 3 and 4) were obtained and used as input to the ANNs. The network could successfully differentiate all four microorganisms grown in the two different media at an accuracy ranging between 87.5% and 100%. The classification accuracy of the training set was 100%. As reported in earlier studies (Gupta

and Irudayaraj, 2004), there were no correlations between the pathogen concentration, growth media used, food matrix analyzed, and classification accuracy. Errors could be attributed to outlier patterns in the validation set. The classification was 100% correct for data obtained from suspensions in chicken broth at a concentration of 10^6 CFU/mL (using bacteria grown in nutrient broth) and in skim milk at the same concentration (using bacteria grown in TSA broth). The overall classification accuracy for the validation set using the data from the bacteria grown in the two different media was 93.4%.

QUANTIFICATION OF THE FOODBORNE PATHOGENS

The concentration of bacteria in a suspension is directly reflected by the change in intensity of the peaks in various fingerprint regions, and this change in peak intensity linearly correlates with the concentration of bacteria (fig. 5). ANNs were developed separately for each pathogen to differentiate and quantify the organisms at the three different concentrations (10^8, 10^6, 10^3). The purpose here is to explore the potential of FTIR spectroscopy as a counting tool. Results from the analysis of the absorbance spectra using the 20 principal components in the ANN model is summarized in figures 6 and 7 for bacteria grown in media I and media II, respectively, in different food systems. Samples at all three concentrations could be classified correctly with an accuracy of about 100% using the data in the training set for bacteria grown in both media. The validation accuracy range was 93.3% to 100% for bacteria grown in media I and 77.8% to 100% for bacteria grown in media II. There was no correlation between the concentration of the pathogens and the accuracy of quantification. The errors could only be attributed to outlier patterns in the validation set. The
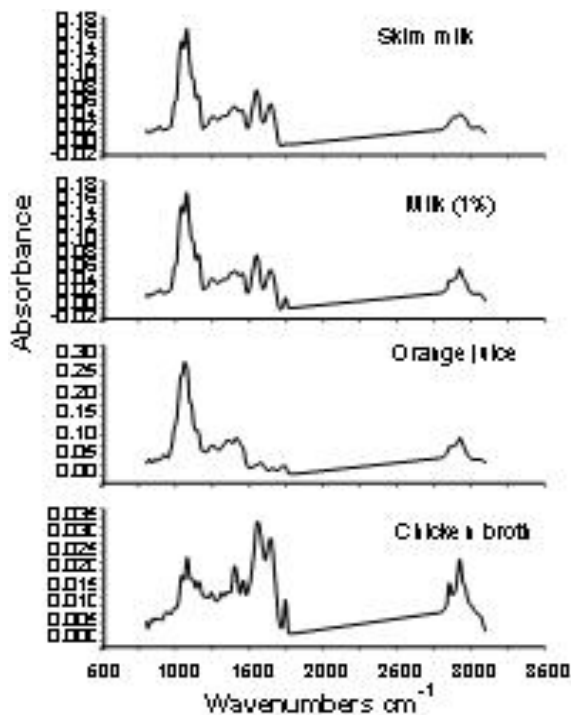
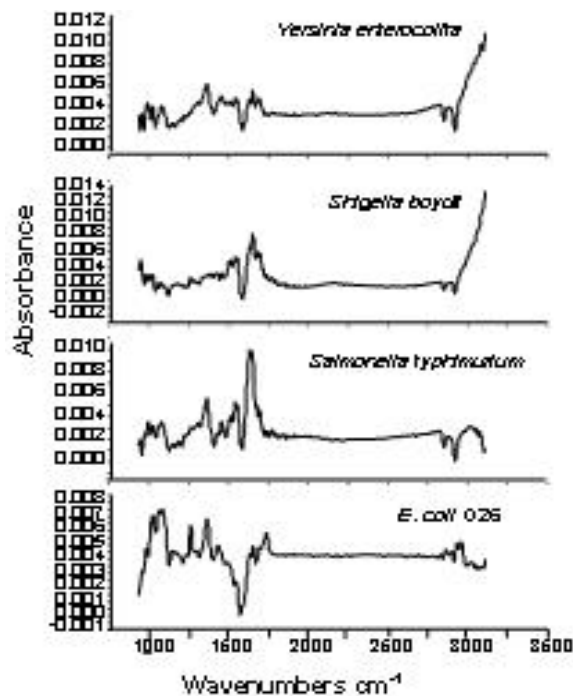Figure 1. FTIR absorbance spectra of the various food matrices.

Figure 2. Absorbance spectra of the four bacteria.

Table 3. Major chemical groups in the food matrices and bacteria.

Food Matrix	Wavenumbers (cm^{-1})	Assignment
Skim milk and milk with 1% fat	1200-900	C–O–C, C–O dominated by ring vibrations of carbohydrates C–O–P, P–O–P
	1076	Lactose
	1200-1450	Acetone
	1402	C=O str (sym) of COO$^-$
	1548	Amide-II band of protein
	1745	>C=O str vibration of esters
	2924	C–H str (asym) of >CH$_2$ in fatty acids
Orange juice	1200-900	C–O–C, C–O dominated by ring vibrations of carbohydrates C–O–P, P–O–P
	~1400	C=O str (sym) of COO$^-$
	2926	C–H str (asym) of >CH$_2$ in fatty acids
Chicken broth	1200-900	C–O–C, C–O dominated by ring vibrations of carbohydrates C–O–P, P–O–P
	1220-1250	P=O str (asym) of >PO$_2^-$ phosphodiesters
	1240-1310	Amide III band
	1400	C=O str (sym) of COO$^-$
	1460	C–H def of >CH$_2$
	1558	Amide II
	1645	Amide I of α-helical structures
	1716	>C=O str vibration of esters
	1743	>C=O str vibration of esters
	2854	C–H str (sym) of >CH$_2$ in fatty acids
	2873	C–H str (sym) of –CH$_3$
	2926	C–H str (asym) of >CH$_2$ in fatty acids
Bacteria	900-600	"Fingerprint region"
	1200-900	C–O–C, C–O dominated by ring vibrations of carbohydrates C–O–P, P–O–P
	1310-1240	Amide III
	~1400	C=O str (sym) of COO$^-$
	1540-70	Amide II
	~1630	Amide I of β-turns of proteins
	~1655	Amide I of α-helical structures
	1740	>C=O str vibration of esters
	2919-21	C–H str (asym) of >CH$_2$ in fatty acids
	2960	C–H str (sym) of –CH$_3$
	~3100	N–H str (amide A) of proteins
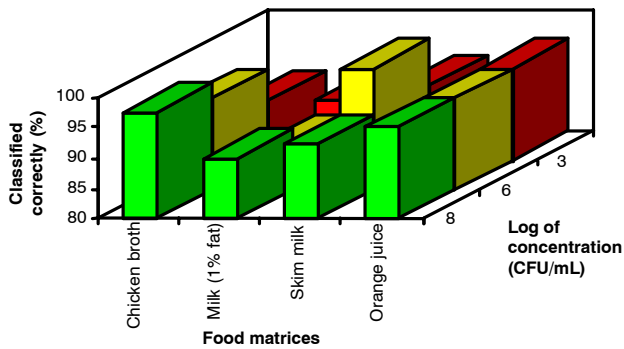
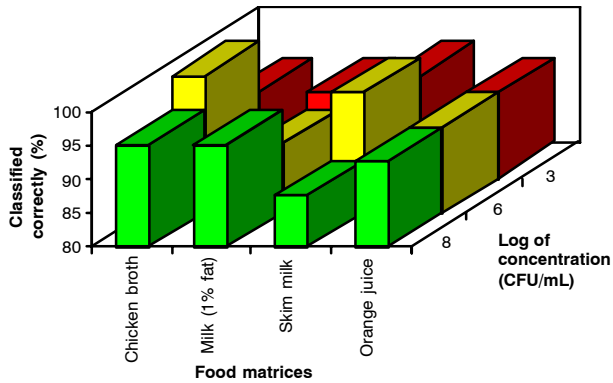**Figure 3. Food pathogens grown in media I and suspended in various food systems.**

Figure 6. Quantification of four foodborne grown in media I based on their FTIR absorbance spectra using (PNN and PCA).

Figure 4. Food pathogens grown in media II and suspended in various food systems.

Figure 7. Quantification of four foodborne grown in media II based on their FTIR absorbance spectra using (PNN and PCA).

maximum errors in classification occurred in the quantification of *Yersinia enterocolitis* in chicken broth using the bacteria grown in nutrient broth and in the quantification of *Shigella boydii* in skim milk using the bacteria grown in media II. The overall accuracy of classification was 95.1%.

DIFFERENTIATION OF FOODBORNE PATHOGENS IRRESPECTIVE OF FOOD MATRIX

ANNs were developed for the differentiation of bacteria irrespective of the food matrix at four different concentrations (10^9, 10^8, 10^6, 10^3). Thus, for a given concentration, the absorbance spectra of each bacteria suspended in the four
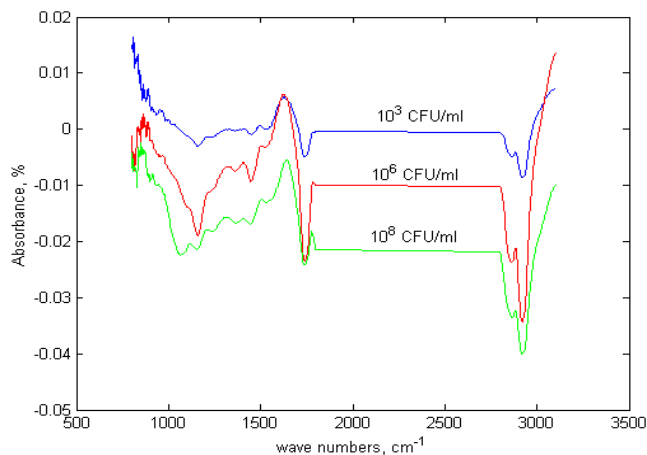

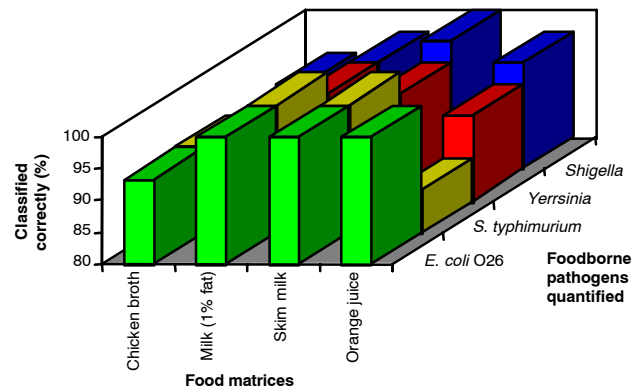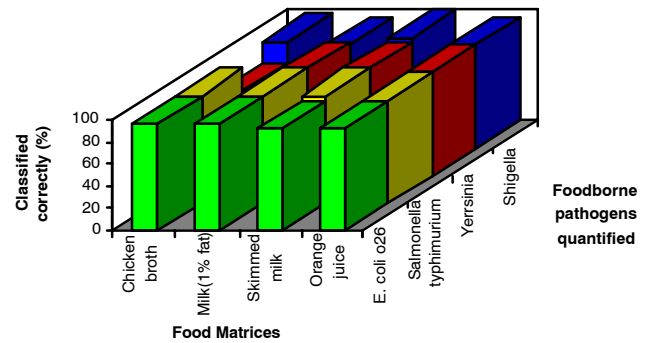**Figure 5. FTIR absorbance spectra of *Yersinia enterocolitis* in chicken broth at three concentrations.**

food matrices (table 3) were combined and used for developing the network. Bacteria grown in media I and media II were used at the 10^9 CFU/mL concentration. For the other three concentrations (10^8, 10^6, 10^3), the bacteria grown in media II were considered. The data were randomly split into a training set (50%) and a validation set (50%). Thus, four ANNs (one for each concentration) were developed and validated. The results are summarized in table 4. The correct assignment numbers in the table indicate the number of spectra of bacteria that were correctly classified. The concentration of pathogens had a strong effect on the accuracy of network prediction. When the accuracy of the validation sets was considered, at a concentration of 10^9 CFU/mL, with samples comprised of pathogens grown in both types of growth media (media I and media II), the ANNs could classify all four pathogens correctly with an average accuracy of 87.5 %. At 10^8 CFU/mL, when pathogens suspended in all four food matrices were considered, the

Table 4. Performance of the differentiation network for pathogens suspended in all food matrices.

Concentration Class (CFU/mL)	Suspension Matrix	Correct Assignment, (number) %	
		Training Set	Validation Set
10^9	TSA broth and nutrient broth	(40) 100	(35) 87.5
10^8	Food matrices[a]	(80) 100	(76) 95
10^6	Food matrices	(80) 100	(62) 77.5
10^3	Food matrices	(70) 87.5	(38) 47.5
Overall results		96.88	73.33

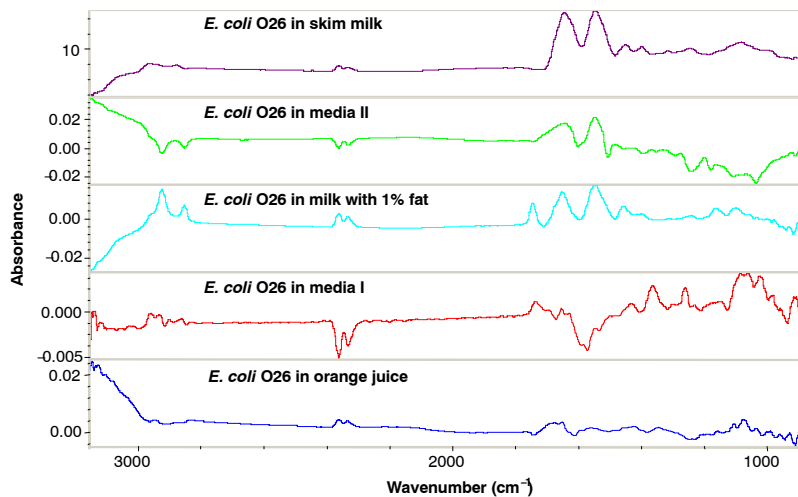[a] Chicken broth, milk (1% fat), orange juice, and skim milk

Figure 8. FTIR absorbance spectra of *E. coli* O26 in different matrices.

ANN developed had an accuracy of 95%. For the pathogens suspended in the same matrices at 10^6 and 10^3 CFU/mL, the accuracy dropped to 77.5% and 47.5%, respectively.

The error at 10^9 CFU/mL is greater than that at 10^8 CFU/mL, as indicated by the accuracy of classification in table 4, and this can be justified by the fact that the combined spectral data are from bacteria grown on two different growth media, whereas the spectral data at the other three concentrations are for bacteria cultured in TSA and grown in TSA broth. The spectral characteristics of cells depend on a number of factors, such as cell cycle, growth conditions, and sample preparation (Maquelin et al., 2002). Although the growing conditions were similar, the difference in the growth media must have contributed significantly to the difference in the spectral signatures, and hence more errors in classification were noted. The absorbance spectra of *E. coli* O26 grown in different matrices (fig. 8) clearly show the difference in the respective spectral signatures.

The increase in errors with a decrease in the concentration could be attributed to the basic principle of attenuated total reflectance (ATR), the technique used for obtaining the mid-infrared spectra of the microbial cells on the internal reflection element (IRE), i.e., zinc selenide (ZnSe). IR radiation is focused onto the end of the IRE, and the incident light is reflected down the length of the crystal. At each internal reflection, the IR radiation actually penetrates a short distance (1 to 4 microns) from the surface of the IRE into the sample on top of the crystal (Compton and Compton, 1993; Smith, 1996). It is this unique physical phenomenon that enables one to obtain infrared spectra of samples placed in contact with the IRE.

Since the samples in our experiment were retained only for a short time on the crystal, the depth of penetration of the evanescent wave might not be sufficient to uniformly cover all the samples in the 1 to 4 micron range of penetration of the ATR infrared radiation. However, the software assumes that the spectra of the matrix or media with bacteria constitute the spectra of the background and the microorganisms. Hence, it subtracts the spectra of the background from the spectra of the matrix with microorganisms to give the specific fingerprint of the spectra with microorganisms. This leads to an increased level of noise in the signal. Settling time of microorganisms might also be a factor; however, since all

experiments were conducted in static conditions, settling time was not a factor in this work. However, if a continuous flow-through analysis is to be devised, then settling time should be considered as a parameter in sensing protocol development. Nevertheless, at lower concentrations, a lesser area of the crystal surface is covered by the microorganisms. Hence, weaker signals are possible, contributing to a lower signal-to-noise ratio and possibly increased errors in classification. Thus, more sophisticated analytical methods for subtraction of the background, which take into consideration settling time of the suspended bacteria and settling rate, are needed for more accurate identification of pathogens, irrespective of the food matrices.

VALIDATION OF ANNs FOR DIFFERENTIATION WITH INDEPENDENT DATA SETS

The ANNs developed using spectral data collected in March and April 2004 were validated using another independent spectral data set for *Yersinia enterocolitis* suspended in chicken broth, collected in July 2004 at the three concentrations. The validation results are summarized in table 5.

As observed earlier when the classification was done with smoothed and differentiated FTIR absorbance spectra, the ANN predictions were poor. This could be due to instrumental and environmental factors noted earlier (e.g., stability of the source, intensity of the laser, variations in environmental conditions such as temperature and relative humidity, and even the accuracy of the background spectra). As a first step in this analysis, the smoothed spectral data were normalized using Origin software (v7.7, SR2, OriginLab Corp., Northampton, Mass.). The normalized data were then compressed into 20 principal components and used as input to the ANNs.

Table 5. Validation of the differentiation for an independent data set (*Yersinia enterocolitis* in chicken broth).

Concentration Class (CFU/mL)	Correct Assignment, (number) %	
	Training Set	Validation Set
10^9	(40) 100	(8) 80
10^8	(40) 100	(7) 70
10^6	(40) 100	(10) 100
10^3	(26) 65	(6) 60
	(36.5) 91.25	(7.75) 77.5

The network was influenced strongly by the poor signal-to-noise ratio and performed best with data obtained from the 10^6 CFU/mL sample, where a classification accuracy of 100% was achieved. However, the average classification accuracy for the validation set was 77.5%. Hence, better post-processing procedures to remove the background noise from the spectral data are needed.

CONCLUSIONS

ANNs combined with PCs have been successfully used for the identification and quantification of four foodborne pathogens (*E. coli* O26, *Salmonella typhimurium*, *Yersinia enterocolitis*, and *Shigella boydii*) in four different food matrices. The classification accuracy of the ANNs for identification and quantification was 93.4% and 95.1%, respectively. The networks were also validated using an independent data set obtained from a pathogen cultured and grown separately. The network had an accuracy of 64% to 100% for such a validation in detecting *Yersinia enterocolitis*. In this work, we have thus successfully demonstrated that pathogens could be identified irrespective of the food matrices. More sophisticated background noise filtration techniques are needed to further refine the ANNs to improve predictions, especially at lower concentrations (<10^3 CFU/mL).

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