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Classification and Structural Analysis of Live and Dead *Salmonella* Cells Using Fourier Transform Infrared Spectroscopy and Principal Component Analysis

Jaya Sundaram,* Bosoon Park, Arthur Hinton, Jr., Seung Chul Yoon, William R. Windham, and Kurt C. Lawrence

ARS, Russell Research Center, U.S. Department of Agriculture, 950 College Station Road, Athens, Georgia 30605, United States

ABSTRACT: Fourier transform infrared spectroscopy (FT-IR) was used to detect *Salmonella* Typhimurium and *Salmonella* Enteritidis food-borne bacteria and to distinguish between live and dead cells of both serotypes. Bacteria cells were prepared in 10^8 cfu/mL concentration, and 1 mL of each bacterium was loaded individually on the ZnSe attenuated total reflection (ATR) crystal surface (45° ZnSe, 10 bounces, and 48 mm × 5 mm effective area of analysis on the crystal) and scanned for spectral data collection from 4000 to 650 cm^{-1} wavenumber. Analysis of spectral signatures of *Salmonella* isolates was conducted using principal component analysis (PCA). Spectral data were divided into three regions such as 900−1300, 1300−1800, and 3000−2200 cm^{-1} based on their spectral signatures. PCA models were developed to differentiate the serotypes and live and dead cells of each serotype. Maximum classification accuracy of 100% was obtained for serotype differentiation as well as for live and dead cells differentiation. Soft independent modeling of class analogy (SIMCA) analysis was carried out on the PCA model and applied to validation sample sets. It gave a predicted classification accuracy of 100% for both the serotypes and its live and dead cells differentiation. The Mahalanobis distance calculated in three different spectral regions showed maximum distance for the 1800−1300 cm^{-1} region, followed by the 3000−2200 cm^{-1} region, and then by the 1300−900 cm^{-1} region. It showed that both of the serotypes have maximum differences in their nucleic acids, DNA/RNA backbone structures, protein, and amide I and amide II bands.

KEYWORDS: *FT-IR spectroscopy, Salmonella Typhimurium, Salmonella Enteritidis, principal component analysis, foodborne pathogen, food safety, Mahalanobis distance, SIMCA classification*

INTRODUCTION

Various technologies have been developed for pathogen detection using optical, electrochemical, biochemical, and physical properties of microorganisms. Conventional microbiological identification methods require days to week for completion. Furthermore, because food is a complex matrix, it is difficult to directly detect the presence of pathogens, and traditional methods are time-consuming and labor intensive.[1] Therefore, there is a need for the development of rapid, accurate detection and identification methods. Recently, more research has been conducted on the use of physicochemical methods for identifying and characterizing pathogens. Fourier transform infrared spectroscopy (FT-IR) has been used by many researchers for rapid detection, identification, and characterization of pathogens.[2−8]

The FT-IR technique requires little or no sample preparation. This technique has been used successfully in the classification and differentiation of microorganisms at the species and subspecies level. It measures molecular vibrations of chemical composition and structure of pathogens. FT-IR produces characteristics fingerprints of biochemical composition and structural features that help identify and differentiate various pathogens.[9−15]

Principal component analysis (PCA) is a widely used technique in infrared spectral data analysis. PCA minimizes the multidimensional data sets into the most dominant features obtained during analysis. Furthermore, excessive noise in analysis is eliminated, and principal components (PC) that contain related sample spectral variation are retained.[16] PCA produces natural clusters in the data sets and describes similarities and differences in the data set.[17] PC1 contains most of the analytical information, then PC2, and followed by PC3, PC4, etc.[18] Scores obtained for each PCA classification are weighed and are used to create the best fit eigenvector for each sample. The eigenvector is a nonzero vector in a square matrix, whose values would not be changed even after multiplied by matrix, and remains parallel to the original vector. Soft independent modeling of class analogies (SIMCA) solves the problem in chemical pattern recognition. The principle of SIMCA is based on PCA classification models.[19]

Salmonella are Gram-negative, facultative bacteria, and they belong to the family of Enterobacteriaceae.[20] *Salmonella* Typhimurium is responsible for most cases of salmonellosis in United States.[21] In 2007, a *Salmonella* outbreak linked to peanut butter contamination caused 329 illnesses. In 2008, more than 700 cases of *Salmonella* were reported in United States, and in 2010, *Salmonella*-tainted eggs caused 1200 illnesses. Improper cooking, reheating, and handling of food may lead to *Salmonella* outbreaks.[22] Poultry and poultry

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products are the major sources of *Salmonella* contamination and causes human illness. Because *Salmonella* can grow and survive even in adverse environmental conditions (e.g., low nutrient concentrations and extreme temperatures as low as 5.9 °C and as high as 54 °C), control of the pathogen in agricultural and food-processing industries is a major problem in food safety.[23] Thus, the development of methods for rapid detection of *Salmonella* on food continues to be high-priority research. Furthermore, rapid pathogen detection is very useful in the quality control of food while processing in a large scale. Also, rapid detection and differentiation of live and dead cells after sterilization would be useful in predicting the effect of lethality of food-processing treatments.

The main objective of this study was to determine if FT-IR could detect and differentiate between *Salmonella* Typhimurium and *Salmonella* Enteritidis live serotypes and between live and dead *Salmonella* cells. Additionally, PCA was performed to classify the *Salmonella* serotypes and to differentiate live and dead *Salmonella* cells. PCA results were used to establish a classification model for detection and differentiation of *Salmonella* serotypes using SIMCA. Mahalanobis distances were then calculated to determine spectral signatures of bacterial structural components that contribute more in the classification.

MATERIALS AND METHODS

Live Bacteria Samples Preparation. Bacterial strains used in this study were obtained from the Poultry Processing and Swine Physiology Unit, of the Russell Research Center in Athens, GA. Isolates of *Salmonella* Typhimurium, strain MH 68123 (isolated from chicken rinse), and *Salmonella* Enteritidis, strain MH 42841 (isolated from ground chicken), were used. Cultures of the isolates were grown in tryptic soy broth (TSB) extract (Becton, Dickinson, and Co., Sparks, MD) incubated at 37 °C for 24 h. Stock cultures were stored at 4 °C. Fresh *Salmonella* Typhimurium and *Salmonella* Enteritidis cultures were prepared by transferring aliquots of stock cultures to fresh TSB medium and incubating for 24 h at 37 °C. After incubation, 30 mL of broth of each culture was transferred to 50 mL sterile centrifuge tubes and centrifuged at room temperature of 20−22 °C at 2800 rcf (5000 rpm) for 10 min (Labnet, model Hermle Z 300, Hermle LaborTechnik, Germany). Bacterial pellets were suspended in 10 mL of sterile deionized water and centrifuged as previously described for a total of three times. Live cell membrane damage occurs if the samples are in water more than 12 h. Therefore, FT-IR measurements were taken as soon as the cell suspensions were made. The viability of the resuspended cells was confirmed by streaking cells on a tryptic soy agar (Becton Dickinson) plate just after FT-IR measurement. It was incubated for 24 h at 37 °C, and the colony formation was observed.

Dead Bacteria Samples Preparation. Fresh cultures of *Salmonella* in TSB were immersed in water heated to 100 °C for 30 min. Heated cultures were immediately transferred to an ice−water bath for cooling. The death of cultures was confirmed by streaking cultures on tryptic soy agar (Becton Dickinson), incubating for 24 h at 37 °C, and observing for bacterial growth on the agar. Also, microscopic observation of cultures was done to confirm the death by observing any bacterial motion. Cells in heated bacterial cultures were harvested, washed, and resuspended in sterile deionized water as described above.

FT-IR Spectral Measurements. FT-IR analysis of *Salmonella* serotypes was performed using Thermo Nicolet 380 FT-IR spectrometer (Thermo Electron Corp., San Jose, CA). Bacteria of live and dead cells from each serotype were placed directly in contact with an zinc selenide (ZnSe ATR crystal surface with 45° angle, 10 bounces, and 48 mm × 5 mm effective surface area on crystal) attenuated total reflection (ATR) crystal trough plate. Spectra were recorded from 4000 to 650 cm^{-1} wavenumber with a resolution of 4 cm^{-1} and data spacing of 1.928 cm^{-1}. Thirty spectra were acquired at room temperature (20−22 °C) for each sample, that is, *Salmonella* Typhimurium live and dead cells and *Salmonella* Enteritidis live and dead cells to yield 120 spectra. The number of scans done on each spectrum was 32. Omnic 8.1 software (Thermo Electron Corp.) was used to collect the spectra that were automatically baseline corrected and normalized. Each spectrum was also subtracted from the background spectra of the empty ZnSe crystal surface using the same software.

Data Analysis. Data analysis was performed using multivariate data analysis software (Unscrambler, version 9.8, CAMO Software, Inc., Woodbridge, NJ). FT-IR spectral features were overlapped as shown in Figure 1. To eliminate overlapping of spectral features, second derivative transformation and smoothing pretreatments were given to separate overlapping absorption bands and remove the baseline offset. The Norris Gap second derivative[16] was applied to the collected spectra with gap size of 5. Derivatively transformed spectra were smoothed using a Savitzky−Golay algorithm[24] with a gap segment of 11. Multivariate statistical analysis technique of PCA was performed on pretreated spectra. PCA was used to show the natural clusters in the data set and to describe the difference between the sample clusters.[16,25] The score value obtained for each PC provided the best fit value for each sample. A cross-validation procedure was performed in PCA using the SIMCA procedure.

PCA was conducted at different spectral regions to differentiate the different serotypes of *Salmonella* and live and dead cells of each stereotype individually on the basis of difference in their spectral features of cell structure components. PCA was carried out at three different spectral regions. One spectral region was between 1300 and 900 cm^{-1}, the second one was between 1800 and 1300 cm^{-1}, and the third one was between 3000 and 2200 cm^{-1}. PCA was not done at the spectral region where the water O−H band stretch appears, around 3400 cm^{-1}. In general, it could be useful to divide the spectral regions based on the major biological composition of bacteria such as fatty acids in the spectral regions 3000−2800 cm^{-1}; proteins and amide I and amide II at 1700−1500 cm^{-1}; phospholipids, DNA, and RNA at 1500−1200 cm^{-1}; and polysaccharides at 1200−900 cm^{-1} regions.[26,27] The second derivative curve in Figure 2 showed a very clear difference between live and dead cells of *Salmonella* in the selected spectral regions.

The PCA model obtained from the calibration set of spectral data was applied to the validation set to examine its classification performance using SIMCA analysis. X-Loading plots of spectral data were used to identify the frequencies that are responsible for the classification to show how the X variables (spectral regions) contributed in meaningful variation in the data and to interpret the classification. Score plots of classification models were used to identify the PC that had higher score in the model development. The Mahalanobis distance, which separates the live and dead cell groups, was calculated at various spectral regions to quantify the separation between live and dead cells. The spectral region, which gives a higher distance, has more variation between the groups and is responsible for classification. The Mahalanobis distance between two groups is given by their mean and is calculated as

$$M_{ij}^{\ 2} = (x_i - x_j)' S^{-1} (x_i - x_j)$$

where x_i and x_j are the means of two groups i and j and S is the covariance matrix. The minimum distance of 4 and above between the groups shows that the two groups are compositionally different from each other.[28]

RESULTS AND DISCUSSION

Average FT-IR absorbance spectra of live and dead cells of *Salmonella* Enteritidis and *Salmonella* Typhimurium are shown in Figures 1 and 2. The *Y*-axis shows the difference in absorbance value [absorbance = log (100/transmission %)] obtained by subtracting background spectra from the sample spectra using a software Omnic 8.1 (Thermo Electron Corp.).

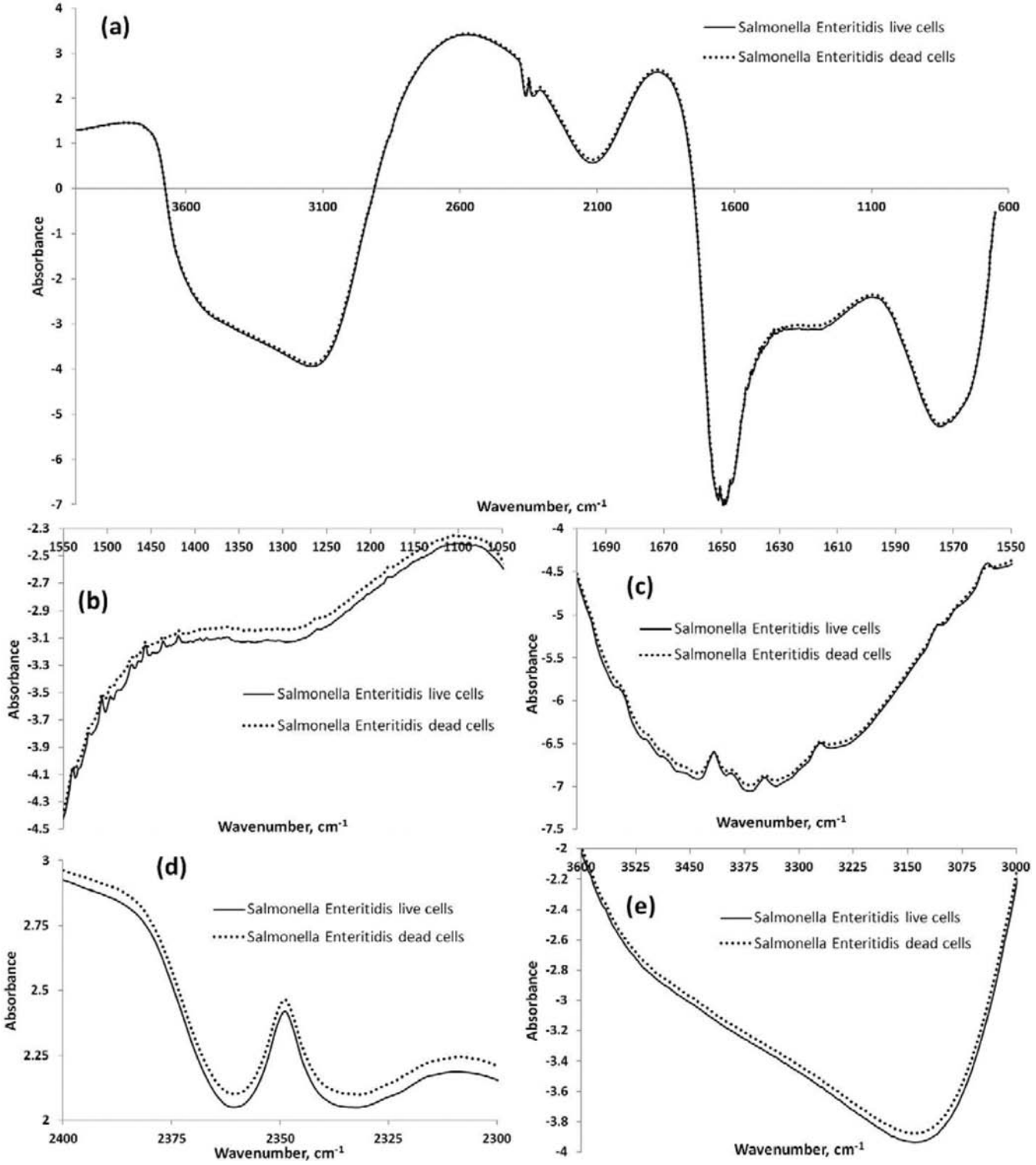

Figure 1. Average absorption spectra of *Salmonella* Enteritidis live and dead cells.

The two spectra showed similar trends but demonstrated differences in absorbance values. Each strain exhibited characteristic absorbance at the frequencies between 4000 and 650 cm^{-1} as shown in Figures 1a and 2a. These figures are not showing more spectral information except the short and sharp peak around 2360−2340 and 1655−1635 cm^{-1}. Peaks at these regions are responsible for the amide structure of protein and sulfenic and sulfonic acids of cystein in bacteria protein.[29] To show other characteristic peaks with more clarity, entire spectral regions were divided into four as shown in panels b−e of Figures 1 and 2. Figures 1d and 2d show the broad peak of the cystein components around 2345 cm^{-1} and the difference in the absorbance values of live and dead cells. Figures 1e and 2e show the difference in absorbance of live and dead cells very clearly in the spectral region 3600−3100 cm^{-1}, of which region has N−H stretching (3200 cm^{-1}) of amide A protein and O−H water bands (3400 cm^{-1}). To analyze further the spectral signature of bacteria structural components, spectra in Figures 1a and 2a

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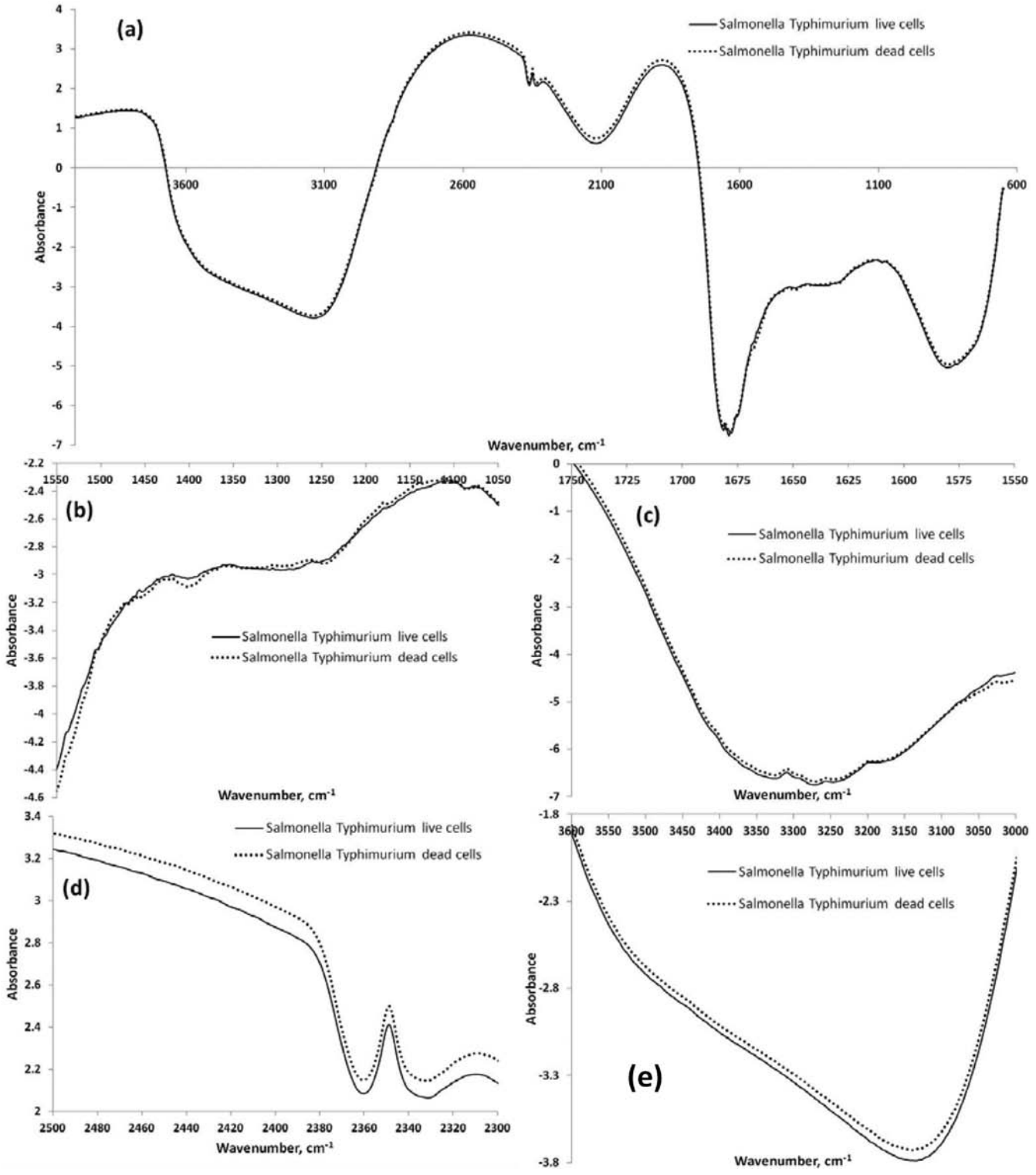

Figure 2. Average absorption spectra of *Salmonella* Typhimurium live and dead cells.

were pretreated to its second derivative analysis as mentioned in data analysis.

***Salmonella* Serotypes Spectral Characteristics.** Figure 3 shows the representative second derivative spectra in their fingerprint region between 1300 and 900 cm^{-1} for *Salmonella* Enteritidis (Figure 3a) and *Salmonella* Typhimurium (Figure 3b) live and dead cells. The spectral region between 1300 and 900 cm^{-1} is a characteristic region for nucleic acids, cell membranes, and cell wall components.[9−11]

It gives vibrational features of these components bands such as P═O (1080 cm^{-1}) of the phosphodiester, which is the backbone of the nucleic acid, C−O−C stretching vibrations of cell wall peptidoglycan layer, and lipopolysaccharide outer layer,[9−15] which is a unique cell wall component of Gram-negative bacteria like *Salmonella*. The spectral region between

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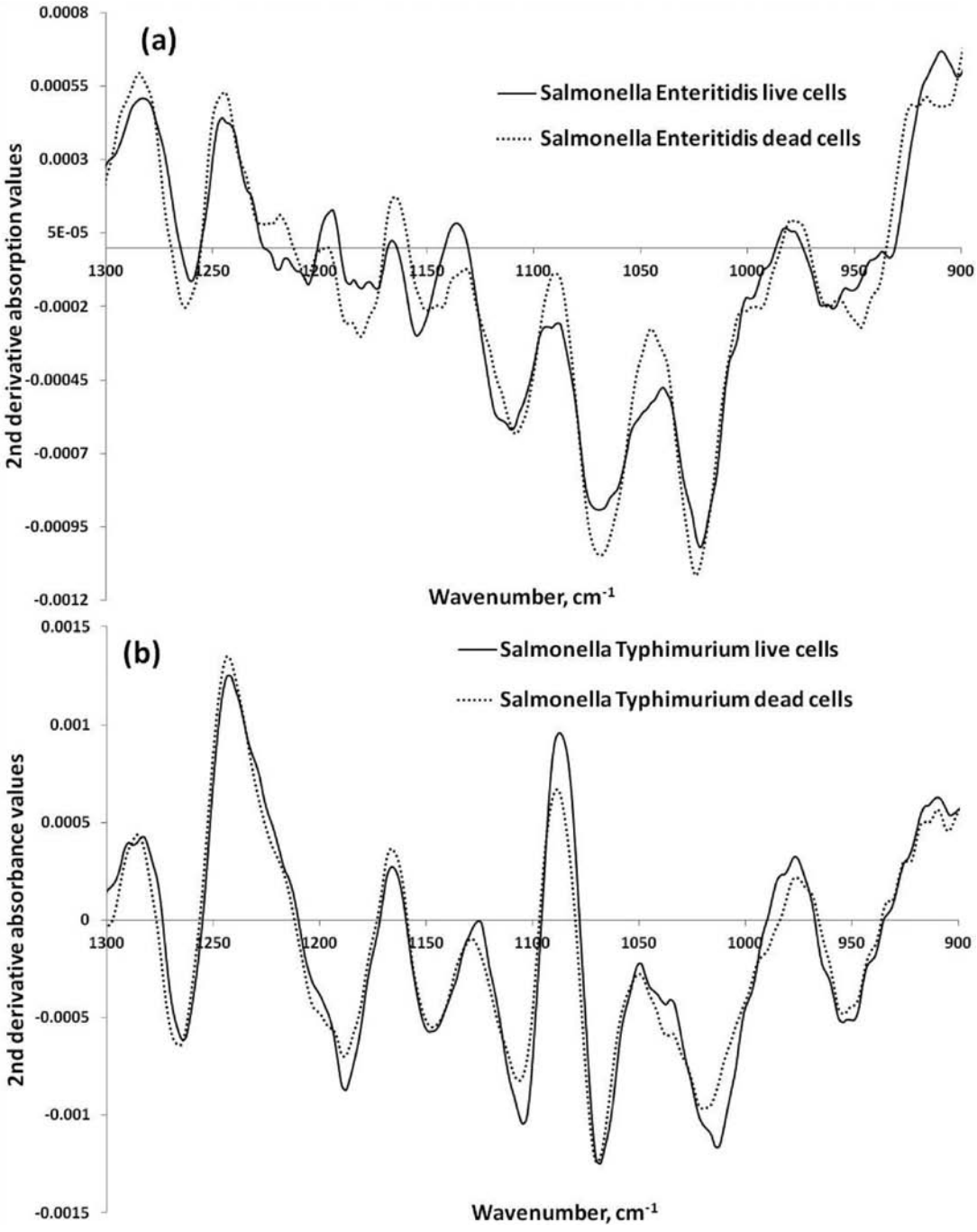

Figure 3. Representative second derivative spectra in the fingerprint region 1300−900 cm^{-1} for (a) *Salmonella* Enteritidis and (b) *Salmonella* Typhimurium live and dead cells.

1300 and 900 cm^{-1} for dead cells of *Salmonella* Enteritidis in Figure 3a and *Salmonella* Typhimurium in Figure 3b indicates nucleic acid denaturation, which could be observed approximately at 1242 cm^{-1} .It is due to P═O antisymmetric stretching mode of phosphodiester. A symmetric stretch mode of P═O of nucleic acid ribosome or deoxyribosome at 1080 cm^{-1} was also observed for dead *Salmonella* cells. Stretching vibrations of C−O−C band of bacterial cell wall polysaccharides happened in the spectral region from 1100 to 950 cm^{-1}.

Figure 4 shows the second derivative spectra of live and dead cells of *Salmonella* serotypes in their fingerprint region from 1800 to 1300 cm^{-1}. The spectral region between 1800 and 1300 cm^{-1} is denoted for the amide I band. Figure 4a,b shows a C═O stretching vibration band of the amide group at 1650 cm^{-1}.[9−11] N−H deformation and a C−N mixture of amide II occur at 1540 cm^{-1}. A band at 1455 cm^{-1} is for CH_2 and CH_3 groups symmetric and antisymmetric deformation, respectively. The symmetric deformation of the CH_3 band was assigned approximately at 1398 cm^{-1}, which is close to its antisymmetric deformation. Heat applied to kill the live cells changed the properties of protein, enzymes, and nucleic acids in dead cells, which could be observed through the spectral changes in the region between 1800 and 1300 cm^{-1}. By comparing Figures 3 and 4, it can be found that more variation in absorbance value occurs between the live and the dead cells in the spectral region of 1500 to 900 cm^{-1} (P═O stretching mode

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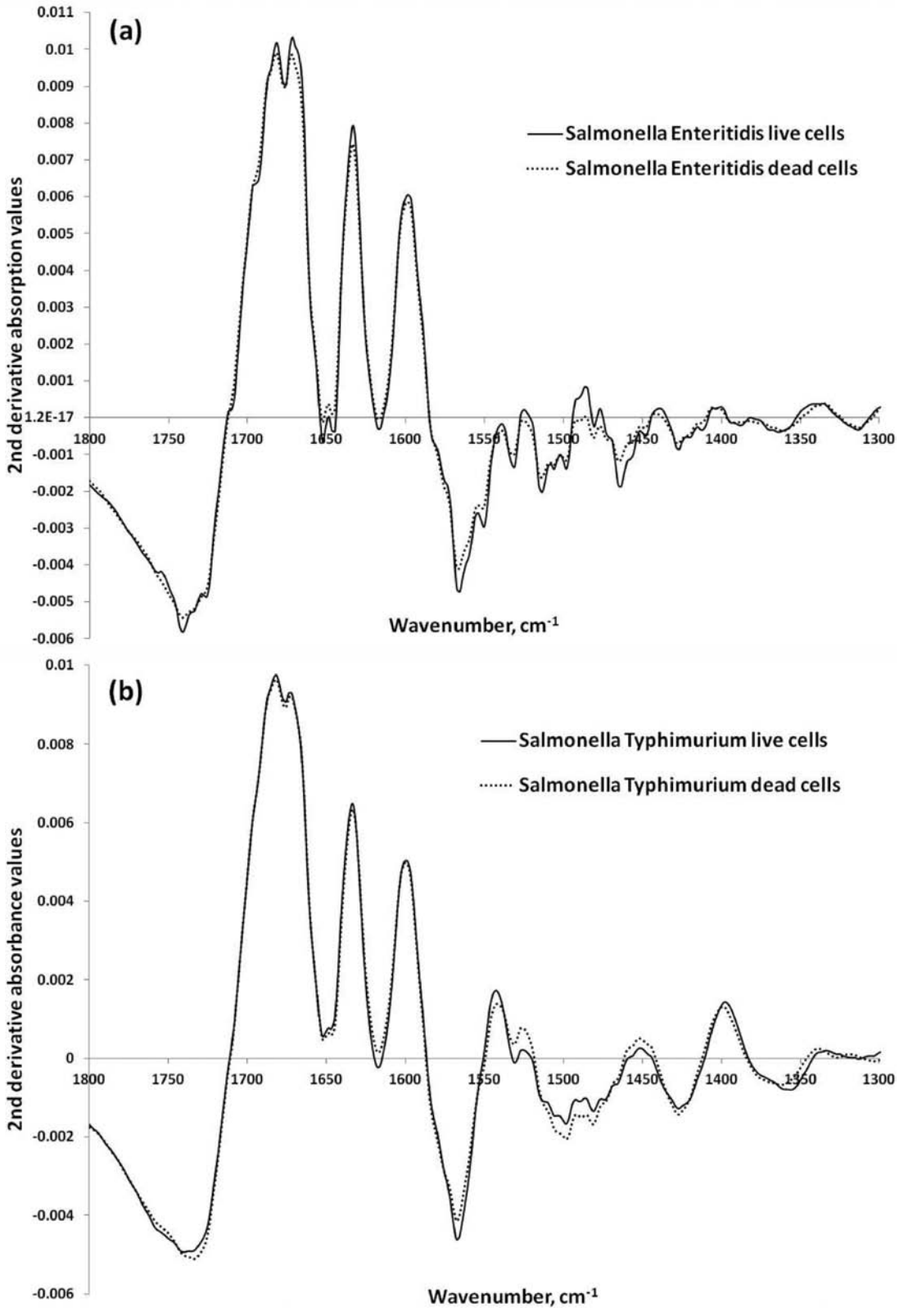

Figure 4. Representative second derivative spectra in the fingerprint region 1800−1300 cm^{-1} for (a) *Salmonella* Enteritidis and (b) *Salmonella* Typhimurium live and dead cells.

and C−O−C stretching mode of polysaccharides and lipopolysaccharide) as compared to the region 1800 to 1500 cm^{-1} (amide I and amide II). In dead cells, because of heat treatment, lipid A has been released from cell wall lipopolysaccharide.[30] Thus, it gives more spectral signature difference between live and dead cells of *Salmonella* in the region from 1300 to 900 cm^{-1}.

Figure 5 shows the second derivative spectra of live and dead cells of *Salmonella* serotypes in their functional group region from 3000 to 2200 cm^{-1}. This region shows the major bands of

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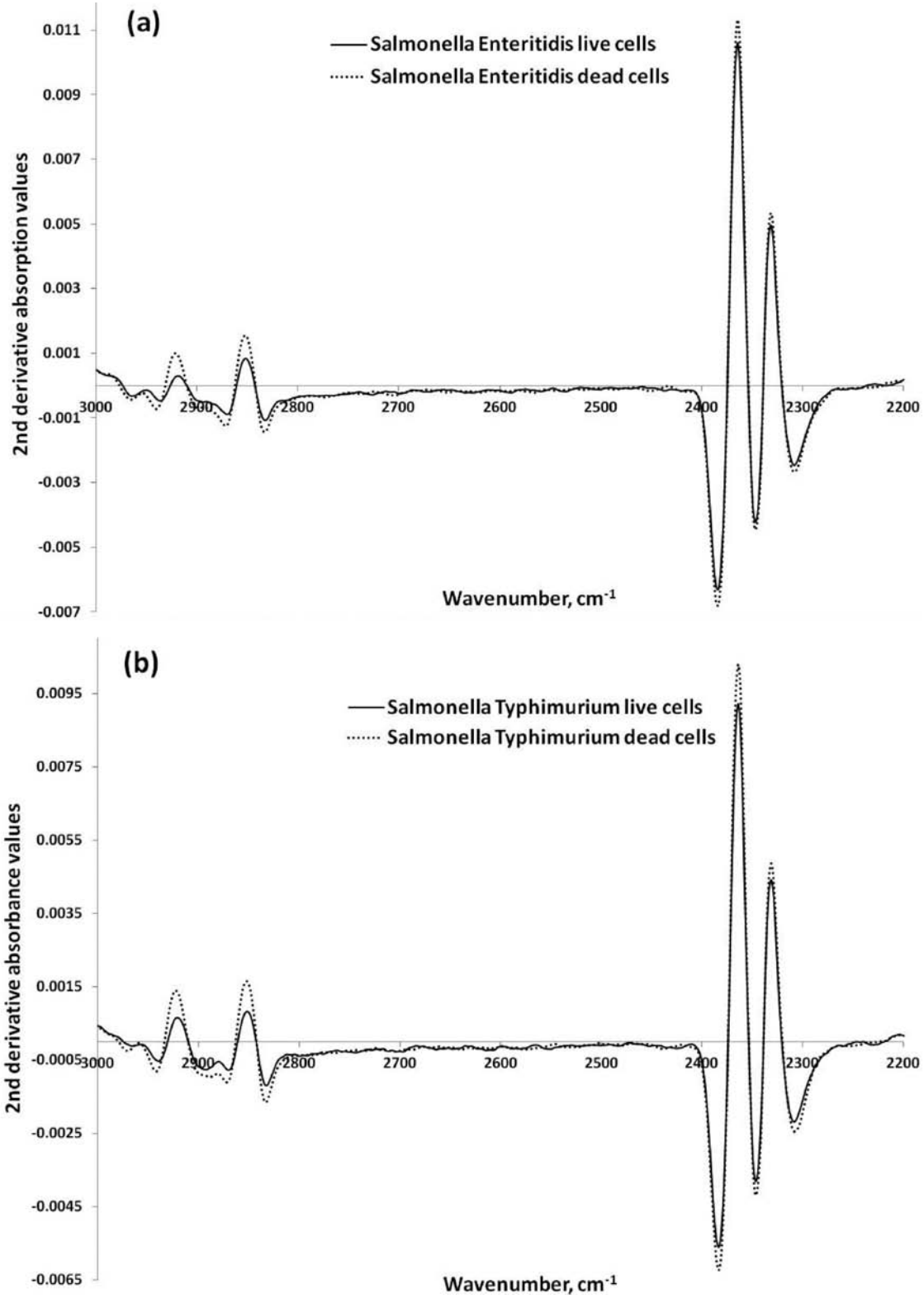

Figure 5. Representative second derivative spectra in the functional group region for (a) *Salmonella* Enteritidis and (b) *Salmonella* Typhimurium live and dead cells.

fatty acids such as $\nu(CH_2)$ asymmetric band at 2919 cm^{-1}, >C−H symmetric stretching of $-CH_2$ in fatty acids at 2850 cm^{-1}, and C−H symmetric stretching of CH_3 in fatty acids at 2870 cm^{-1}.[9−11] These are the fatty acids from phopolipids matrix of cell wall membrane in which the membrane protein is embedded. In the process of making dead cells, a change in protein structure happened, and it indirectly affected the phospholipids structures also. That reflects the change in spectral absorbance of dead cells as compared to live cells in this region.[30]

Signature bands around at 2300, 2330, 2345, 2360, and 2385 cm^{-1} are the characteristic bands of $RSO_2{\cdot}OH$ (2300 cm^{-1}) of cystein

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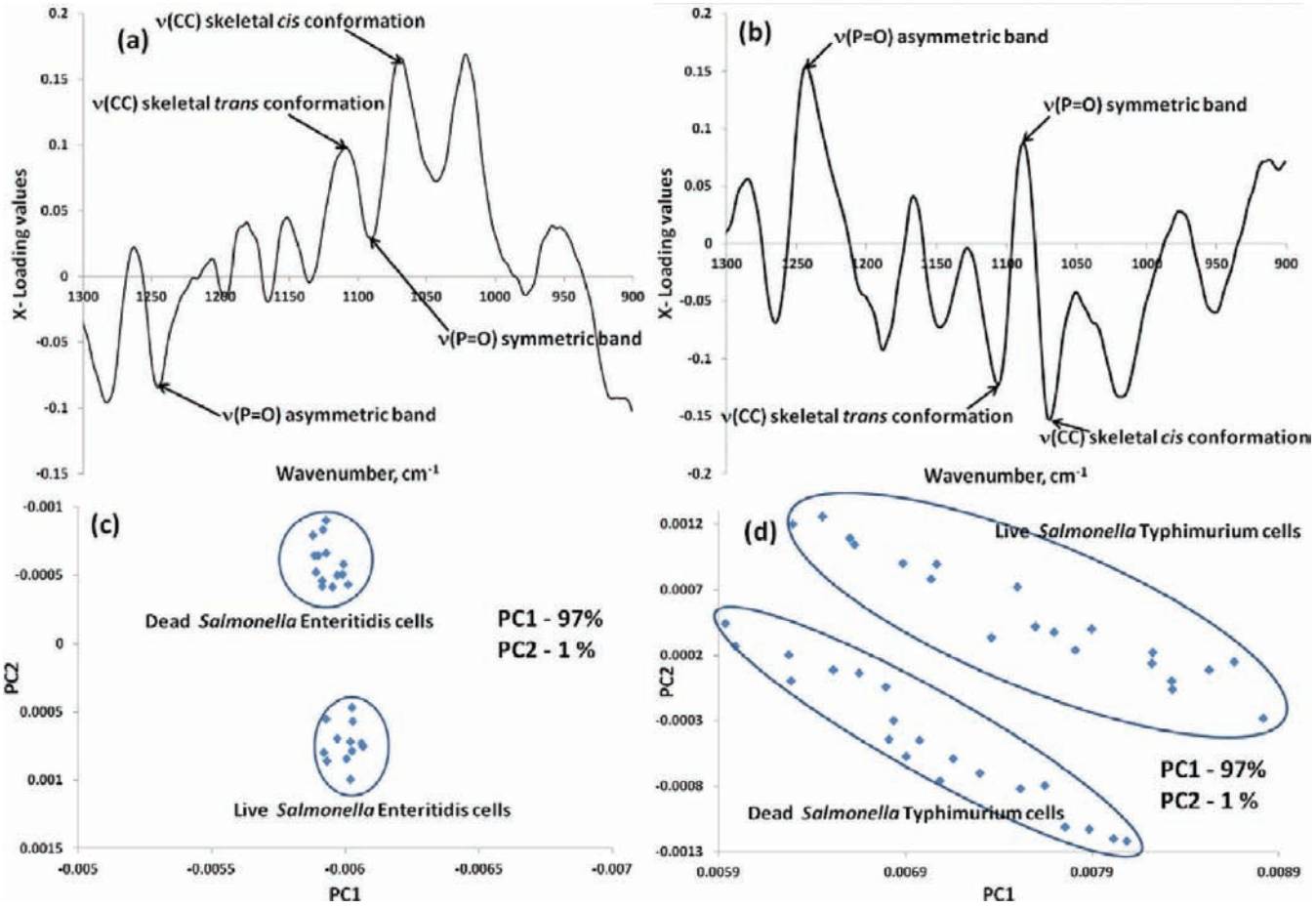

Figure 6. X-loading and PCA score plot for (a, c) *Salmonella* Enteritidis and (b, d) *Salmonella* Typhimurium live and dead cells in the spectral region of 1300−900 cm^{-1}.

sulfonic acid, OH stretch of −SO−OH, that is, cystein sulfinic acid (2345 cm^{-1}), OH stretch in the

bond at 2360 and 2385 cm^{-1} for phosphine oxide, respectively.[29] These are oxidized compounds of cystein sulfenic acid (cys-SOH) present in protein. This Cys-SOH is novel class of protein-derived redox cofactors in specific antioxidant enzymes such as the bacterial NADH peroxidase and peroxiredoxins that have been characterized in bacteria cell systems. These compounds are relating various biochemical, biological, and genetic information of bacteria cells. *Salmonella* cells have an alkyl hydroperoxide reductase system that protects the cells from toxic effects of reactive oxygen species generated during its aerobic metabolism. This system has an AhpC protein in which two redox-active cysteins connected with disulfide bonds.[31−33] This can be seen in Figure 5a,b by the appearance of a peak at 2330 cm^{-1}. It is possible that the onset of heat shock protein during the preparation of dead cells.[34] Those proteins might be led into cystein oxidation and left more oxidized compounds of sulfonic and sulfinic acid.[31,32] This shows the remarkable change in spectral bands of dead cells at their signature spectral region as compared to live cells.

The cellular composition of dead cells may be distorted by heating and contribute to change characteristics of spectral bands to make them different from the live cells. Heat could damage the bacterial cell wall, protein, and nucleic acid moieties and trigger physiological response in the production of some compounds such as heat shock proteins.[34] These proteins have unique absorption in the infrared region and gives specific signatures for dead *Salmonella* cells. Also, denatured protein and damaged cell walls and cell membranes produced by heating could also be responsible for the spectral changes in dead bacterial cells. Clear separation with distinct sample clusters was observed between all live and dead cells of *Salmonella* Typhimurium and *Salmonella* Enteritidis at the spectral regions of 1300−900, 1800−1300, and 3000−2200 cm^{-1}.

PCA Classification. Figure 6 shows that PC1 explained a maximum of 97% of the variation between live and dead *Salmonella* Enteritidis (Figure 6c) as well as live and dead *Salmonella* Typhimurium cells (Figure 6d). PC2 explained 1% of the variation and in total 98% of the variation between live and dead cells of both serotypes at the spectral regions 1300−900 cm^{-1}. Figure 6c also illustrates that samples in each group of *Salmonella* Enteritidis are close to each other on a score plot, which indicates that the cell characteristics are similar in their composition. Samples of *Salmonella* Typhimurium in each group were not as closely related as *Salmonella* Enteritidis; however, the difference between the groups is 98% in total. *Salmonella* Typhimurium cells have more positive score values than *Salmonella* Enteritidis cells. It means that *Salmonella* Typhimurium has more absorbance values over the selected spectral region than *Salmonella* Enteritidis cells, which have lesser values than average absorbance values. Negative score values are a result of mean centering data; that is, it is the geographic center of the *Salmonella* Enteritidis cells based on

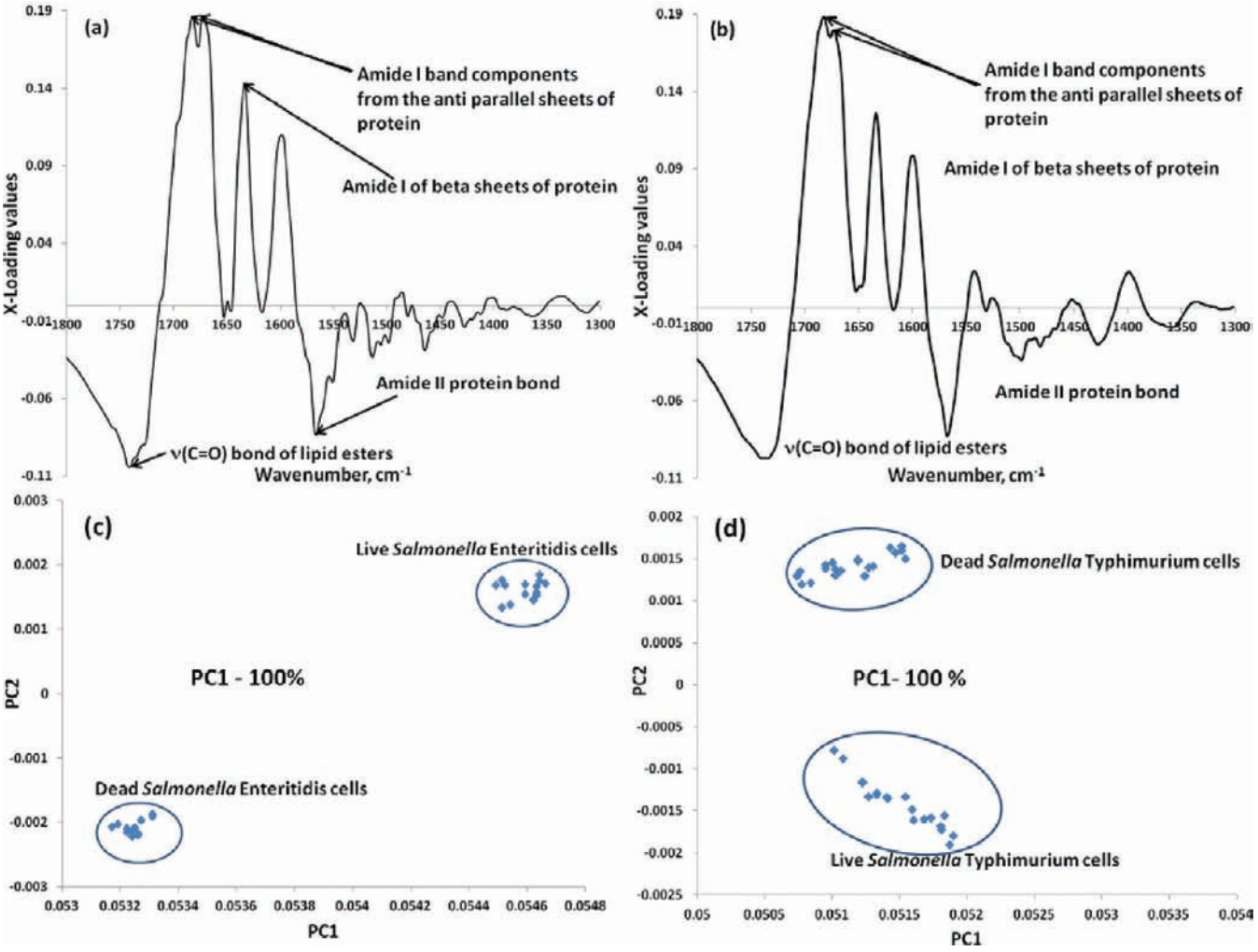

Figure 7. X-loading and PCA score plot for (a, c) *Salmonella* Enteritidis and (b, d) *Salmonella* Typhimurium live and dead cells in the spectral region of 1800−1300 cm^{-1}.

the absorbance values obtained in the given spectral region. It gives the shape of cell distribution in the score plot as a circle as compared to the scattered shape of *Salmonella* Typhimurium cells. This also helps to reduce the standard error of calibration in the PCA model developed to classify *Salmonella* Enteritidis live and dead cells. The score plot also indicates the PC that distinguishes variation among the samples. For example, in *Salmonella* Enteritidis cells, PC1 is able to distinguish the samples of live cells from dead cells. A similar separation is obtained for *Salmonella* Typhimurium live and dead cells.

Loading plots (Figure 6a,b) show the frequencies that have significant contribution to the variation described by the PC1. This can be identified by their higher loading values (absolute values). Frequencies with a higher loading value carried most of the information about the difference between live and dead cells of *Salmonella*. For both of the *Salmonella* serotypes, the spectral region between 1150 and 1000 cm^{-1} and 1280 −1170 cm^{-1} has higher loading values and shows their significant contribution in the classification of live and dead cells. These regions are responsible for ν(CC) skeletal *cis* conformation of DNA and RNA backbones (1076 cm^{-1}), ν(P═O) symmetric band of DNA and RNA phospholipids (1085 cm^{-1}), ν(CC) skeletal *trans* conformation of DNA and RNA backbones (1105 cm^{-1}), and ν(P═O) asymmetric band of DNA and RNA phospholipids (1240 cm^{-1}).[9−11]

In Figure 7, PC1 shows 100% of the variation between live and dead *Salmonella* Enteritidis as well as live and dead *Salmonella* Typhimurium cells. Total classification of live and dead cells is given by PC1 for both serotypes at the spectral region 1800−1300 cm^{-1}. It can be seen in Figure 7c that samples in each group of *Salmonella* Enteritidis are closely grouped on the score plot, which indicates that the cell characteristics are more similar in their composition. In the case of *Salmonella* Typhimurium, the samples in each group are not as close as *Salmonella* Enteritidis; however, variation between the groups is given as 100% in total. The score plot in Figure 7d indicates that PC1 distinguished live and dead *Salmonella* Typhimurium cells. The spectral region between 1800 and 1550 cm^{-1} gives major contribution to the classification of live and dead cells of both *Salmonella* serotypes. It contains the information about the bacteria cell protein and their amide bands, DNA and RNA bases.[9−15] The amide II protein bond appears at 1548 cm^{-1}, and the amide I of β-sheets of protein appears at 1637 cm^{-1}. Figure 7a,b shows the maximum loading value (absolute values) at these frequencies. Similarly, the amide I band that results from antiparallel sheets of protein structure appeared at frequencies 1675 and 1685 cm^{-1} as shown in Figure 7a,b, and they have higher loading values. Also, the ν(C═O) bond of lipid esters is responsible for live and dead cell classification of *Salmonella*.

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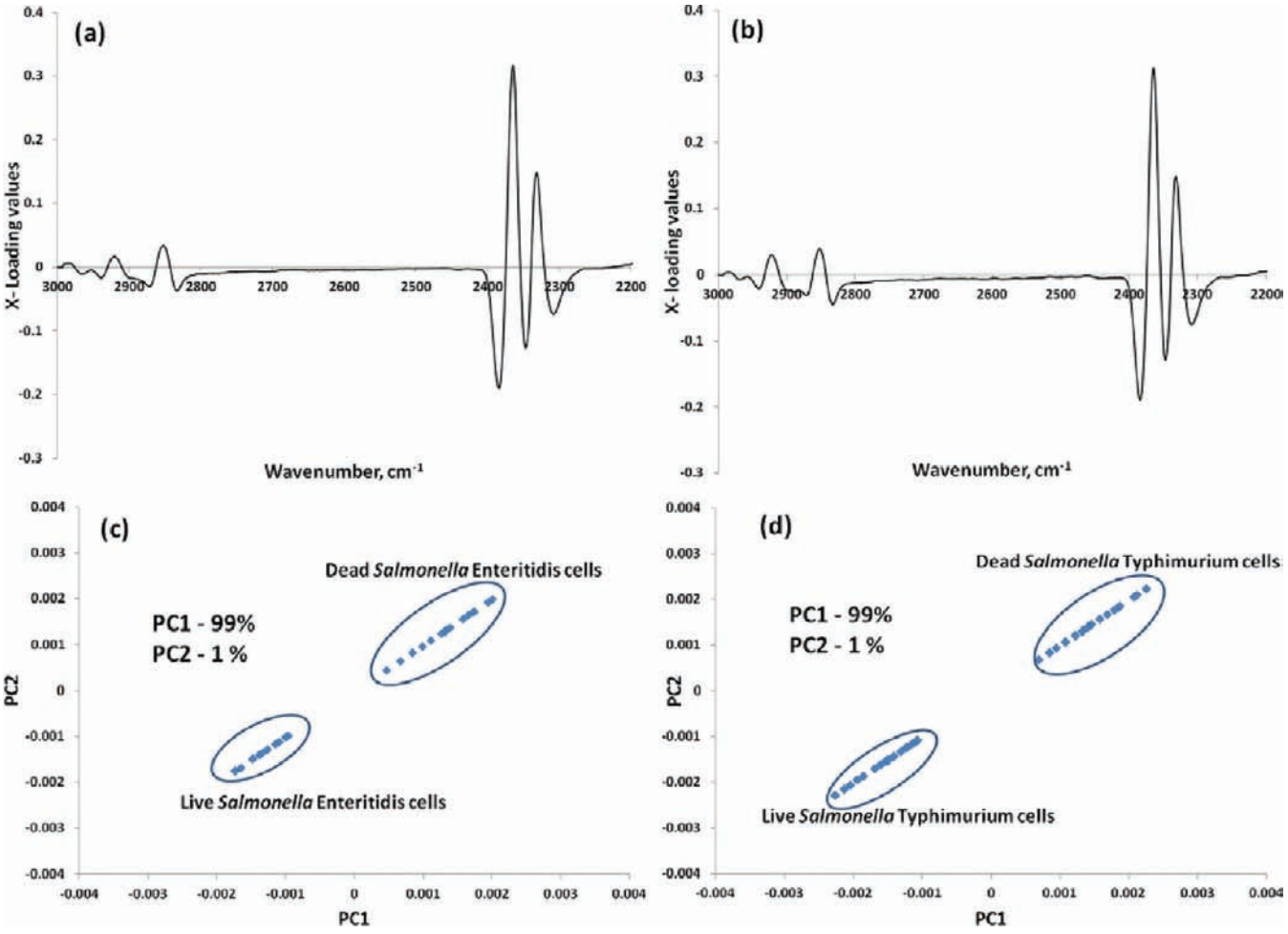

Figure 8. X-loading and PCA score plot for (a, c) *Salmonella* Enteritidis and (b, d) *Salmonella* Typhimurium live and dead cells in the spectral region of 3000−2200 cm^{-1}.

In Figure 8, PC1 indicates the maximum 99% of the variation obtained between live and dead *Salmonella* Enteritidis as well as between live and dead *Salmonella* Typhimurium cells. PC2 explains 1% of variation and in total 100% of variation obtained between live and dead cells of both serotypes at the spectral region 3000−2200 cm^{-1}. Figure 8 also illustrates that samples in live cells of both the *Salmonella* serotypes are in close proximity in the score plot, which indicates that cellular compositions are similar at this spectral region. Dead cells of both serotypes have positive scores, and the live cells have negative scores. This indicates that live cells have less IR absorbance than the dead cells at certain frequencies, which are responsible for the classification. It also tells that live cells were geographically centered in the score plot space along the axis of PC1. Loading plots show that the region between 2400 and 2300 cm^{-1} has higher loading values (absolute); therefore, it has a significant contribution in the classification between live and dead cells of both serotypes. As mentioned before, it is due to the onset of heat shock protein during the preparation of dead cells, which led into cystein oxidation and left more oxidized compounds of sulfonic and sulfinic acid.[31−34] This shows the remarkable change in spectral bands of dead cells at their signature region as compared to live cells.

By comparing the percentage classification obtained at different spectral regions of 1300−900, 1800−1300, and 3000−2200 cm^{-1}, except the first region other two regions gave 100% classification, whereas the first region gave 98%. However, the classification difference among the spectral regions is not very significant; therefore, all three spectral regions showed a difference in the structural composition between live and dead cells of both *Salmonella* Enteritidis and *Salmonella* Typhimurium cells.

SIMCA Analysis. Table 1 shows the PCA classification accuracy obtained for *Salmonella* live and dead cells from the calibration sample set at three different spectral regions. The PCA model shows the maximum of 100% classification obtained in the spectral region that are responsible for amide I and amide II of protein present in the cell wall membrane and nucleic acid, fatty acids of the cell wall structure, and oxidative cystein compounds of DNA protein. This model was applied

Table 1. PCA Classification Result of Live and Dead *Salmonella* Cells at Different Spectral Regions for the Calibration Set of Data

spectral region	*Salmonella* Enteritidis		*Salmonella* Typhimurium	
wavenumber (cm^{-1})	no. of samples	PC classification (%)	no. of samples	PC classification (%)
1300−900	15	98	20	98
1800−1300	15	100	20	100
3000−2200	15	100	20	100

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to the validation sample set of spectral data using SIMCA classification.

Table 2 shows the classification obtained through SIMCA for the validation set samples of live and dead *Salmonella* cells of both serotypes. SIMCA was applied to the different spectral regions that were used for PCA calibration model. These spectral regions are significant for biological composition of live and dead cells. For both serotypes, maximum classification accuracy of 100% was obtained from the regions 3000−2200 and 1800−1300 cm^{-1}, which correspond to the composition of cell wall and cell membrane. The classification accuracy of 98% was obtained in the spectral region 1300−900 cm^{-1}, which has the majority in phospholipids, polysaccharide, and lipopolysaccharide bands.

Table 2. SIMCA Classification Result of Live and Dead *Salmonella* Cells at Different Spectral Regions for the Validation Set of Data

spectral region	*Salmonella* Enteritidis		*Salmonella* Typhimurium	
wavenumber (cm^{-1})	no. of samples	correctly classified spectra (%)	no. of samples	correctly classified spectra (%)
1300−900	15	98	20	98
1800−1300	15	100	20	100
3000−2200	15	100	20	100

PCA Classification of *Salmonella* Typhimurium and *Salmonella* Enteritidis Live Cells. Figures 9 and 10 show the PCA classification of *Salmonella* Typhimurium and *Salmonella* Enteritidis live cells obtained at three different FT-IR spectral regions, 1300−900, 1800−1300, and 3000−2200 cm^{-1}. The PCA score plot gives 84% of PC1, 15% of PC2, and in total 99% of correct classification between *Salmonella* Typhimurium and *Salmonella* Enteritidis at 1300−900 cm^{-1}. At the spectral region 1800−1300 cm^{-1}, the total accuracy of classification obtained is 100 (PC1, 98%; and PC2, 2%), and at the spectral region 3000−2200 cm^{-1}, it is 100% by PC1. Figure 10c,d illustrates that the samples in *Salmonella* Enteritidis are close to each other on a score plot, which indicates that the cell characteristics are similar in their composition. In the case of *Salmonella* Typhimurium; however, cells are not as close as the *Salmonella* Enteritidis. The variation between the stereotypes is given as 100% in total. *Salmonella* Typhimurium cells have positive scores at all three spectral regions, and the *Salmonella* Enteritidis cells have negative scores, which means that *Salmonella* Enteritidis cells have more IR absorption than *Salmonella* Typhimurium cells. Also, the scattered arrangement of *Salmonella* Typhimurium cells obtained in the score plot is due to its positive scores. SIMCA classification of two serotypes

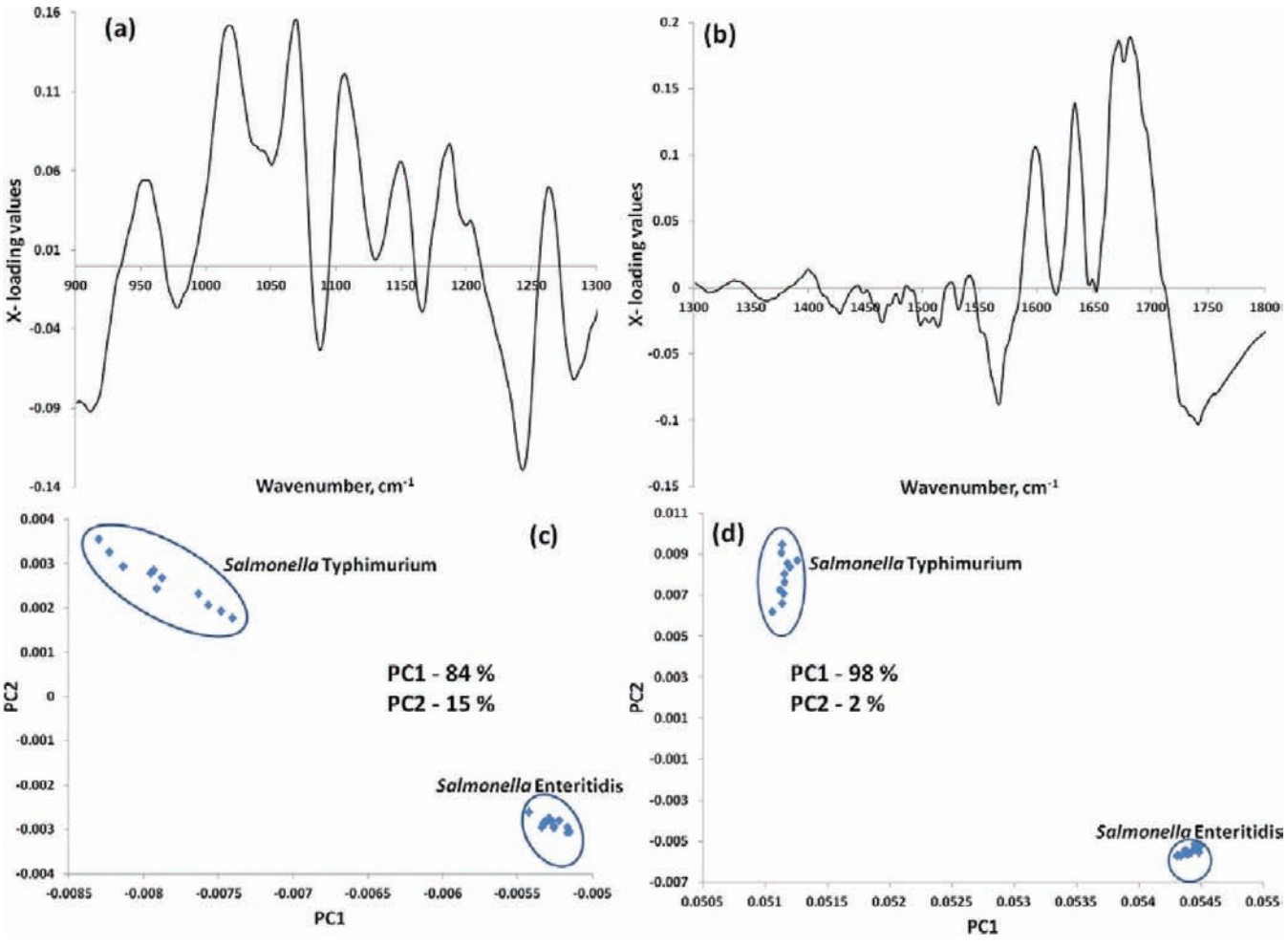

Figure 9. X-loading and PCA score plot for *Salmonella* Enteritidis and *Salmonella* Typhimurium live and dead cells in the spectral region (a, c) 900−1300 and (b, d) 1300−1800 cm^{-1}.

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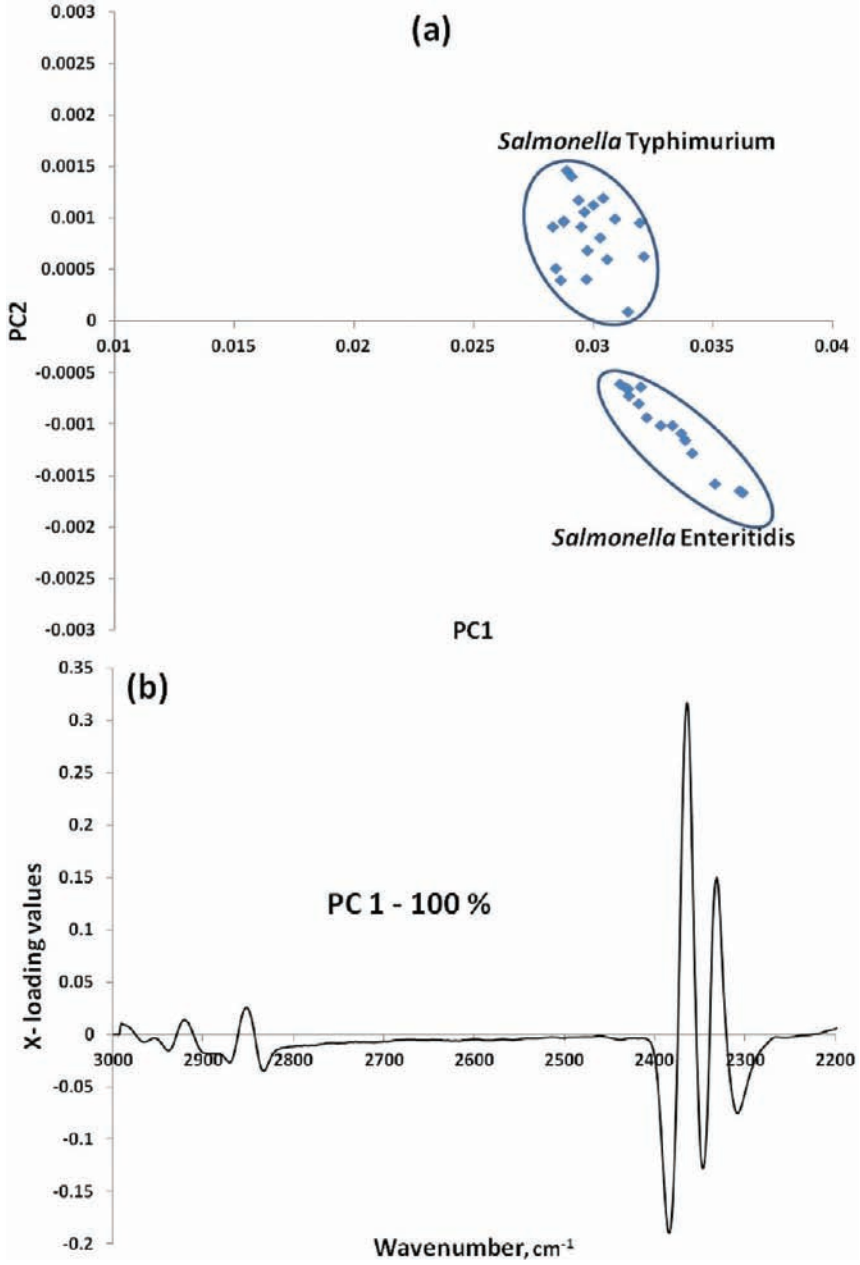

Figure 10. (a) X-loading and (b) PCA score plot for *Salmonella* Enteritidis and *Salmonella* Typhimurium live and dead cells in the spectral region of 3000−2200 cm^{-1}.

of *Salmonella* samples in validation set gave a correct classification of 98% (PC1 96% and PC2 2%) at the 1300−900 cm^{-1} region and 100% for other two spectral regions.

Mahalanobis Distance. Table 3 shows the Mahalanobis distance calculated between live and dead cells at three different spectral regions for both the *Salmonella* serotypes. These regions are characteristic spectral signatures of polysaccharides, phospholipids, proteins, amides I and II, and lipids of bacteria cells. A high distance value gives more contribution in classification between the samples. In comparison of *Salmonella* Typhimurium and *Salmonella* Enteritidis live and dead cells, the Mahalanobis distance was maximum at the spectral region 1800−1300 cm^{-1} followed by 3000−2200 cm^{-1} and 1300−900 cm^{1}, respectively. In both of the serotypes, the spectral region responsible for cell wall polysaccharide (1300−900 cm^{-1}) has lesser contribution in classification as compared to other regions. However, the Mahalanobis distance obtained for this region is more than 4, which indicates that this region also has a significant difference between live and dead cells.[35−37] Table 3 also shows that live *Salmonella* Enteritidis and *Salmonella* Typhimurium cells have a higher Mahalanobis distance at the spectral region 1800−1300 cm^{-1}, followed by 3000−2200 and

Table 3. Mahalanobis Distance between Live and Dead Cells Calculated at Various Spectral Regions

spectral region	1300−900 cm^{-1}		1800−1300 cm^{-1}		3000−2200 cm^{-1}	
	SE dead	ST dead	SE dead	ST dead	SE dead	ST dead
SE live	7.10		19.32		13.19	
ST live		13.59		22.52		15.19
SE and ST live	19.63		49.33		11.65	

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1300−900 cm^{-1}, respectively. This shows that larger differences between *Salmonella* Enteritidis and *Salmonella* Typhimurium are in their nucleic acids, DNA/RNA backbone structures and its protein, amide I, and amide II bands. There is less structural difference in their phospholipids, polysaccharides, and lipopolysaccharides.

This study demonstrates that FT-IR can differentiate between live and dead cells of *Salmonella* Typhimurium and *Salmonella* Enteritidis. FT-IR was effective to discriminate between live and dead cells based on their spectral difference in the cell wall, cell membrane, cytoplasm, polysaccharide, protein and peptides, amide bands, and nucleic acid moieties. This method is also able to distinguish between live cells of *Salmonella* Typhimurium and *Salmonella* Enteritidis. The Mahalanobis distance calculated at different spectral regions showed that differentiation of cells is due to differences detected in the spectral region that has the structural information and signature peaks of fatty acids, cystein oxidative compounds, RNA, DNA, protein, and amide compositions. SIMCA analysis classified live and dead cells of *Salmonella* serotypes with a maximum of 100% for the validation set of samples. Similar classification accuracy was obtained to classify the two different serotypes of *Salmonella*. Overall, the PCA models developed for calibration set of live and dead cells gave maximum accuracy to classify the validation set of samples through SIMCA analysis. Further work on this needs to be continued to develop a spectral library for various *Salmonella* serotypes and verify sensitivity as well as selectivity through common microbiological validation procedures. In the future, this method could be applied to detect and differentiate *Salmonella* cells from food samples.

AUTHOR INFORMATION

Corresponding Author

*Tel: 706-546-3346. Fax: 706-546-3607. E-mail: jaya.sundaram@ars.usda.gov.

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REFERENCES

(1) Hanes, D. E. FDA-CFSAN research activities: Virulence assessment and molecular pathogenesis of multi-drug resistant *Salmonella* Typhimurium. In *Proc. Food Safety Symposium on Antimicrobial Resistance*; FDA: Rockville, MD, 1999; pp 201−204.

(2) Naumann, D.; Figala, V.; Labischinski, H.; Giesbrecht, P. The rapid differentiation and identification of pathogenic bacteria using Fourier transform infrared spectroscopic and multivariate statistical analysis. *J. Mol. Struct.* **1989**, *174*, 165−170.

(3) Naumann, D.; Labischinski, H.; Giesbrecht, P. The characterization of microorganisms by Fourier-Transform infrared spectroscopy (FT-IR). In *Modern Techniques for Rapid Microbiological Analysis*; Nelson, W. H., Ed.; VCH: New York, 1990.

(4) Yang, H.; Irudayaraj, J. Rapid detection of food borne microorganisms on food surface using Fourier transform Raman spectroscopy. *J. Mol. Struct.* **2003**, *646*, 35−43.

(5) Naumann, D.; Helm, D.; Labischinski, H. Microbiological characterizations by FT-IR spectroscopy. *Nature* **1991a**, *351* (6321), 81−82.

(6) Naumann, D.; Helm, D.; Labischinski, H.; Giesbrecht., P. The characterization of microorganisms by Fourier transform infrared spectroscopy (FT-IR). In *Modern Techniques for Rapid Microbiological Analysis*; Nelson, W. H., Ed.; VCH: New York, 1991b; pp 67−85.

(7) Curk, M. C.; Peladan, F.; Hubert, J. C. Fourier transform infrared (FT-IR) spectroscopy for identifying *Lactobacillus* species. *FEMS Microbiol. Lett.* **1994**, *123* (3), 241−248.

(8) Helm, D.; Labischinski, H.; Schallehn, G.; Naumann, D. Classification and identification of bacteria by Fourier transform infrared spectroscopy. *J. Gen. Microbiol.* **1991**, *137*, 69−79.

(9) Choo-Smith, L. P.; Maquelin, K.; Vreeswijk, T. V.; Bruining, H. A.; Puppels, G. J.; Ngo Thi, N. A.; Krischner, C.; Naumann, D.; Ami, D.; Villa, A. M.; Orsini, F.; Doglia, S. M.; Lamfarraj, H.; Sockalingum, G. D.; Manfait, M.; Allouch, P.; Endtz, H. P. Investigating microbial (micro) colony heterogeneity by vibrational spectroscopy. *Appl. EnViron. Microbiol.* **2001**, 1461−1469.

(10) Ellis, D. I.; Broadhurst, D.; Kell, D. B.; Rolwland, J. J.; Goodacre, R. Rapid and quantitative detection of the microbial spoilage of meat by Fourier transform infrared spectroscopy and machine learning. *Appl. Environ. Microbiol.* **2002**, *68*, 2822−2828.

(11) Schmitt, J.; Flemming, H.-C. FT-IR-spectroscopy in microbial and material analysis. *Int. Biodeterior. Biodegrad.* **1998**, *41*, 1−11.

(12) Kim, S.; Reuhs, B. L.; Mauer, L. J. Use of Fourier transform infrared spectra of crude bacterial lipopolysaccharides and chemometrics for differentiation of *Salmonella enterica* serotypes. *J. Appl. Microbiol.* **2005**, *99*, 411−417.

(13) Kim, S.; Kim, H.; Reuhs, B. L.; Mauer, L. J. Differentiation of outer membrane proteins from *Salmonella enterica* serotypes using Fourier transform infrared spectroscopy and chemometrics. *Lett. Appl. Microbiol.* **2006**, *42*, 229−234.

(14) Baldauf, N. A.; Rodriguez-Romo, L. A.; Yousef, A. E.; Rodriguez-Saona, L. E. Differentiation of selected *Salmonella enterica* serovars by Fourier transform mid-infrared spectroscopy. *Appl. Spectrosc.* **2006**, *60*, 592−598.

(15) Baldauf, N. A.; Rodriguez-Romo, L. A.; Männig, A.; Yousef, A. E.; Rodriguez-Saona, L. E. Effect of selective growth media on the differentiation of *Salmonella enterica* serovars by Fourier-transform mid-infrared spectroscopy. *J. Microbiol. Methods* **2007**, *68*, 106−114.

(16) Goodacre, R.; Timmins, E. M.; Burton, R.; Kaderbhai, R.; Woodward, A. M.; Kell, D. B. Rapid identification of urinary tract infection bacteria using hyperspectral whole-organism fingerprinting and artificial neural networks. *Microbiology* **1998**, *144*, 1157−1170.

(17) Nilsen, H.; Esaiassen, M.; Heia, K.; Sigernes, F. Visible/near infrared spectroscopy: a new tool for the evaluation of fish freshness. *J. Food Sci.* **2002**, *67*, 1821−1826.

(18) Gremlich, H.-U.; Yan, B. Infrared and Raman spectroscopy of biological materials. *Practical Spectroscopy Series*; Marcel Dekker Inc.: New York, 2001; Vol. *24*.

(19) Krzanowski, W. J. *Principles of Multivariate Analysis: A User's Perspective*; Oxford University Press, Oxford, United Kingdom, 1988.

(20) Garbutt, J. *Essentials of Food Microbiology*, 1st ed.; Arnold: London, United Kingdom, 1997.

(21) Zhang, G.; Ma, L.; Patel, N.; Swaminathan, B.; Wedel, S.; Doyle, M. P. Isolation of *Salmonella* Typhimurium from outbreak-associated cake mix. *J. Food Prot.* **2007**, *70*, 997−1001.

(22) Gorman, R.; Adley, C. C. Characterization of *Salmonella enterica* serotype Typhimurium isolates from human, food, and animal sources in the Republic of Ireland. *J. Clin. Microbiol.* **2004**, *42*, 2314−2316.

(23) Tietjen, M.; Fung, D. Y. *Salmonella* and food safety. *Crit. Rev. Microbiol.* **1995**, *21*, 53−83.

(24) Savitzky, A.; Golay, M. J. E. Smoothing and differentiation of data by simplified least squares procedures. *Anal. Chem.* **1964**, *36*, 1627−1633.

(25) Martens, H.; Naes, T. *MultiVariate Calibration*; Wiley: New York, 1989.

(26) Naumann, D. Infrared spectroscopy in microbiology. In *Encyclopedia of Analytical Chemistry*; Meyers, R. A., Ed.; John Wiley & Sons Ltd.: Chichester, United Kingdom, 2000; pp 102−131.

(27) Davis, R.; Burgula, Y.; Deering, A.; Irudayaraj, J.; Reuhs, B. L.; Mauer, L. J. detection and differentiation of live and heat-treated *Salmonella enteric* serovars inoculated onto chicken breast using Fourier transform infrared (FT-IR) spectroscopy. *J. Appl. Microbiol.* **2010**, *109*, 2019−2031.

(28) Kemsley, E. K. *Discriminant Analysis and Class Modeling of Spectroscopic Data*; John Wiley and Sons: Chichester, United Kingdom, 1998.
(29) Socrates, G. *Infrared and Raman Characteristics Group Frequencies; Tables and Charts*; John Wiley and Sons: Chichester, United Kingdom, 2001.
(30) Yong-Mei Zhang, C. O. Rock. Membrane lipid homeostasis in bacteria. *Nature* **2008**, *6*, 222−232.
(31) Giles, N. M.; Giles, G. I.; Jacob, C. Multiple roles of cystein in biocatalysis. *Biochem. Biophys. Res. Commun.* **2003**, *300*, 1−4.
(32) Barford, D. The role of cystein residues as redox-sensitive regulatory switches. *Curr. Opin. Struct. Biol.* **2004**, *14*, 679−686.
(33) Poole, L. B.; Karplus, P. A.; Claiborne, A. Protein sulfenic acids in redox signaling. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 325−347.
(34) Pagan, R.; Manas, P.; Palop, A.; Sala, F. J. Resistance of heat shocked cells of *Listeria monocytogenes* to mano-sonication and mano-thermo-sonication. *Lett. Appl. Microbiol.* **1999**, *28*, 71−75.
(35) Kansiz, M.; Heraud, P.; Wood, B.; Burden, F.; Beardall, J.; McNaughton, D. Fourier transforminfrared microspectroscopy and chemometrics as a tool for the discrimination of cyanobacterial strains. *Phytochemistry* **1999**, *52*, 407−417.
(36) Filip, Z.; Hermann, S. An attempt to differentiate *Pseudomonas* spp. and other soil bacteria by FT-IR spectroscopy. *Eur. J. Soil Biol.* **2001**, *37*, 137−143.
(37) Lin, M.; Al-Holy, M.; Mousavi-Hesary, M.; Al-Qaridi, H.; Cavinato, A. G.; Rasco, B. A. Rapid and quantitative detection of the microbial spoilage of chicken breasts by diffused reflectance spectroscopy (600−1100 nm). *Lett. Appl. Microbiol.* **2004**, *39*, 148−155.