(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 September 2020 (17.09.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/183437 A1

(51) International Patent Classification:
C12Q 1/18 (2006.01) *G01N 21/00* (2006.01)

(21) International Application Number:
PCT/IB2020/052339

(22) International Filing Date:
13 March 2020 (13.03.2020)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/818,039 13 March 2019 (13.03.2019) US

(71) Applicant: **MONASH UNIVERSITY** [AU/AU]; Wellington Road, Clayton, Australian Capital Territory 3800 (AU).

(72) Inventors: **HERAUD, Philip Robert**; Melbourne, Victoria (AU). **KOCHAN, Kamila**; Krakow (PL). **WOOD, Bayden Robert**; Melbourne, Victoria (AU). **PELEG, Anton**; Melbourne, Victoria (AU).

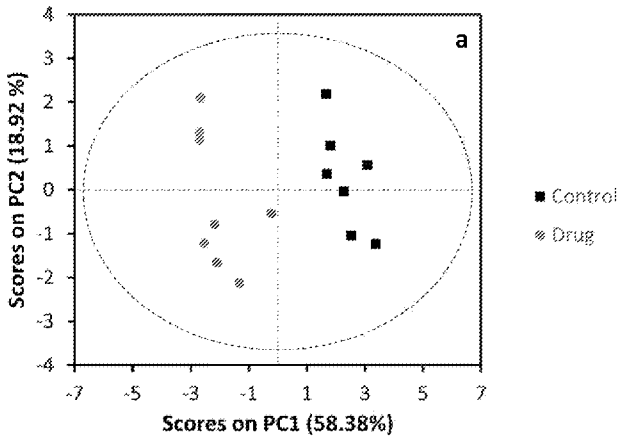
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

(54) **Title:** SYSTEMS AND METHODS FOR SPECTRAL DETECTION OF DRUG-RESISTANT PATHOGENS

FIG. 22



(57) **Abstract:** Methods of determining the treatment susceptibility or resistance of a pathogen include collecting spectra from a pathogen cultured with a treatment and from the same pathogen cultured without the treatment. The spectra are compared to determine if the pathogen is susceptible or resistant to the treatment. Systems for determining the treatment susceptibility or resistance of a pathogen are also disclosed and may include a remote computer network, which may be cloud-based.

WO 2020/183437 A1

SYSTEMS AND METHODS FOR SPECTRAL DETECTION OF DRUG-RESISTANT PATHOGENS

TECHNICAL FIELD

The present disclosure relates generally to systems and methods of determining the susceptibility or resistance of a pathogen to a treatment using spectroscopy.

BACKGROUND

Antimicrobial and antifungal resistance ("AMR") are major global health threats as resistant infections are presently claiming 700,000 lives each year globally, and morbidity and mortality from resistant infections is predicted to rise. The proportion and absolute number of resistant strains has continuously increased over the last decade. Apart from the burden on patients, AMR is associated with a significant economic cost: the cumulative loss to the global economy associated with such resistance is estimated to reach $100 trillion between 2016 and 2050.

In the absence of AMR detection, patients are treated with broad spectrum drugs, which are not as effective and which increases the chance that new resistant strains will develop.

Conventional methods of AMR detection, such as antibiotic susceptibility testing, generally include culturing a pathogen-containing sample, often on an agar plate, for at least one day and up to one week for slow-growing species. AMR-specific therapy is delayed during this time, which can impact a patient's chance of survival.

Discrimination between drug-susceptible and resistant pathogens via spectroscopy has involved the direct detection of phenotypic differences between the pathogens. Direct phenotype-based detection is unreliable due to the variability of drug resistance-related phenotypes and errors resulting from even minor variations in culture conditions.

SUMMARY

Embodiments disclosed herein relate to systems and methods for determining the drug susceptibility or resistance of a pathogen using spectroscopy.

In embodiments, a method of determining if a pathogen is susceptible or resistant to a treatment is provided. The method includes providing a sample including the pathogen; identifying the pathogen in the sample; culturing the sample in each of the presence of the treatment and the absence of the treatment; preparing the cultured samples for spectral analysis; measuring spectral data of the sample cultured in each of the presence of the treatment and the absence of the treatment; analyzing the spectral data by comparing the

spectral data of the sample cultured in the presence of the treatment to the spectral data of the sample cultured in the absence of the treatment to determine if at least one difference in the spectral data is present; and supplying a result that the pathogen is susceptible to the treatment or resistant to the treatment.

In embodiments, a method of determining if a pathogen is susceptible or resistant to a treatment is provided. The method includes culturing the pathogen in the presence of the treatment and in the absence of the treatment; collecting a plurality of spectral data from the cultured pathogens; and transmitting the plurality of spectral data to a processor. The processor is configured to process the plurality of spectral data from the pathogen cultured in the absence of the treatment to produce a control average spectra, process the plurality of spectral data from the pathogen cultured in the presence of the treatment to produce a test average spectra, compare the test average spectra to the control average spectra to determine if at least one difference between the test average spectra and the control average spectra is present, and generate a result that the pathogen is susceptible to the treatment or resistant to the treatment.

In embodiments, a method of determining if a pathogen is susceptible to a treatment is provided. The method includes receiving each of a test sample exposed to a treatment and a control sample not exposed to the same treatment; acquiring at least one spectrum from the control sample, the spectrum having a plurality of absorbance and wavenumber values; calculating a threshold value from a wavenumber region of the spectrum of the control sample that would display absorbance values indicative of biochemical composition changes to the control sample; acquiring at least one spectrum from the control sample, the spectrum having a plurality of absorbance values and wavenumbers; calculating a test value from a wavenumber region of the spectrum of the test sample that would display absorbance values indicative of biochemical composition changes to the test sample; and comparing the test value to the threshold value. A difference between the test value and the threshold value indicates the treatment susceptibility of the pathogen.

In embodiments, a system for determining if a pathogen is susceptible or resistant to a treatment is provided. The system includes a remote processing network. The network is configured to receive spectral data from a control system, the spectral data including a plurality of test spectra gathered from the pathogen cultured in the presence of the treatment and a plurality of control spectra gathered from the pathogen cultured in the absence of the treatment, each of the spectra having a plurality of absorbance values and wavenumbers; process the spectral data by calculating a test average spectra from a wavenumber region of

the test spectra that would include absorbance values indicative of the biochemical composition of the pathogen, calculating a control average spectra from the control spectra in the wavenumber region, and comparing the control average spectra to the test average spectra to produce a result describing the susceptibility or resistance of the pathogen to the treatment; and return the result to the control system.

Features from any of the disclosed embodiments may be used in combination with one another, without limitation. In addition, other features and advantages of the present disclosure will become apparent to those of ordinary skill in the art through consideration of the following detailed description and the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The drawings illustrate several embodiments of the invention, wherein identical reference numerals refer to identical or similar elements or features in different views or embodiments shown in the drawings.

Figure 1A is a schematic illustration of a method of determining the drug susceptibility or resistance of a pathogen according to an embodiment.

Figure 1B is a flow diagram of a method of determining the drug susceptibility or resistance of a pathogen according to an embodiment.

Figure 2A is a flow diagram of a method of providing a sample of bodily fluid according to an embodiment.

Figure 2B is a flow diagram of a method of providing a sample from a culture plate according to an embodiment.

Figure 3 is a flow diagram of a method of culturing a sample according to an embodiment.

Figure 4A is a flow diagram of a method of preparing a sample for spectral analysis according to an embodiment.

Figure 4B is a flow diagram of a method of collecting infrared spectral data from a sample according to an embodiment.

Figure 4C is a flow diagram of a method of collecting Raman spectral data from a sample according to an embodiment.

Figure 5 is a schematic of a spectroscopy processing system according to an embodiment.

Figure 6 is a schematic of an architecture of a spectroscopy computing system according to an embodiment.

Figures 7A and 7B are growth curves of methicillin-sensitive *Staphylococcus aureus* (7A) and methicillin-resistant *S. aureus* (7B) cultured with or without oxacillin. The legend applies to both Figures 7A and 7B.

Figure 8A is an overlay of the individual ATR-FTIR spectra of Figures 8B-8H, collected after culturing methicillin-sensitive *S. aureus* for up to 360 minutes.

Figure 9A is an overlay of the individual Raman spectra of Figures 9B-9H, collected after culturing methicillin-resistant *S. aureus* for up to 360 minutes.

Figures 10A and 10B are graphs of relative changes in intensity of selected ATR-FTIR (Figure 10A) and Raman (Figure 10B) spectra from methicillin-sensitive *S. aureus* cultured for up to 360 minutes.

Figure 11A is a scores plot of multimodal Principal Component Analysis (PCA) of methicillin-sensitive *S. aureus* cultured with or without oxacillin. Portions of the ATR-FTIR (Figures 11B, 11D) and Raman (Figures 11C, 11E) spectra obtained from loading 1 (Figures 11B, 11C) and loading 2 (11D, 11E) are also shown, with prominent bands marked.

Figure 12A is a scores plot of multimodal PCA of methicillin-resistant *S. aureus* cultured with or without oxacillin. Portions of the ATR-FTIR (Figure 12B) and Raman (Figure 12C) spectra obtained from loading 1 are also shown, with prominent bands marked.

Figures 13A and 13B are scores plots of multimodal PCA of five strains of methicillin-sensitive (Figure 13A) and five strains of methicillin-resistant (Figure 13B) *S. aureus* cultured with or without oxacillin. The legend applies to both Figures 13A and 13B.

Figures 14A and 14B are spectral analyses of peptidoglycan standards showing an ATR-FTIR spectrum (Figure 14A) and a 2nd derivative of the ATR-FTIR spectrum (Figure 14B) with labeled major bands at about 1622 cm^{-1} and about 1515 cm^{-1}.

Figure 15A is a scores plot of PCA of methicillin-susceptible *S. aureus* cultured with (0.5 µg/mL or 4 µg/mL) or without oxacillin for 120 minutes. Portions of the ATR-FTIR (Figure 15B) spectra are also shown, with prominent bands marked.

Figures 16A and 16B are scores plots of PCA of methicillin susceptible *S. aureus* cultured with (0.5 µg/mL or 4 µg/mL) or without oxacillin for 90 minutes (16A) or 60 minutes (16B). Portions of the ATR-FTIR (Figure 16C, 90 minutes; Figure 16D, 60 minutes) spectra are also shown, with prominent bands marked.

Figures 17A-C are scores plots of PCA of methicillin-resistant *S. aureus* cultured with (0.5 µg/mL or 4 µg/mL) or without oxacillin for 120 minutes (17A), 90 minutes (17B), or 60 minutes (17C).

Figures 18A and 18B are a scores plot (18A) and IR spectra (18B) of MSSA cells exposed to oxacillin (MIC) for 120 minutes and then subjected to a delicate ("three washes") or intense ("six washes") external stress factor.

Figures 19A-F show an SVM-C model and its validation for prediction of susceptibility towards β-lactam antibiotics for susceptible and resistant *S. aureus*.

Figures 19A-F show a PLS-DA model and its validation for prediction of susceptibility towards β-lactam antibiotics for susceptible and resistant *S. aureus*.

Figure 21 shows SVM-C and PLS-DA models and their use in predicting the results of double-blind testing.

Figure 22 shows the results of PCA (scores plot, 22A; IR spectra, 22B) of vancomycin susceptible *S. aureus* exposed to vancomycin for 120 minutes compared to an unexposed control group.

Figure 23 shows the results of PCA (scores plot, 23A; IR spectra, 23B) of vancomycin intermediate-resistant *S. aureus* exposed to vancomycin for 120 minutes compared to an unexposed control group.

Figure 24 shows the results of applying a PLS-DA and SVM model to the vancomycin-treated *S. aureus* of Figures 22A-23B. "S" – susceptible; "R" – resistant.

Figure 25 shows the results of PCA (scores plot, 25A; IR spectra, 25B) of clindamycin susceptible *S. aureus* exposed to clindamycin for 120 minutes compared to an unexposed control group.

Figure 26 shows the results of PCA (scores plot, 26A; IR spectra, 26B) of clindamycin resistant *S. aureus* exposed to clindamycin for 120 minutes compared to an unexposed control group.

Figure 27 shows the results of PCA (scores plot, 27A; IR spectra, 27B) of vancomycin susceptible *E. faecium* exposed to vancomycin for 60 minutes compared to an unexposed control group.

Figure 28 shows the results of PCA (scores plot, 28A; IR spectra, 28B) of vancomycin resistant *E. faecium* exposed to vancomycin for 60 minutes compared to an unexposed control group.

Figure 29 shows the results of PCA (scores plot, 29A; IR spectra, 29B) of amoxicillin susceptible *E. faecium* exposed to vancomycin for 120 minutes compared to an unexposed control group.

Figure 30 shows the results of PCA (scores plot, 30A; IR spectra, 30B) of amoxicillin resistant *E. faecium* exposed to vancomycin for 120 minutes compared to an unexposed control group.

Figure 31 shows the results of PCA (scores plot, 31A; IR spectra, 31B) of ciprofloxacin susceptible *E. coli* exposed to ciprofloxacin for 120 minutes compared to an unexposed control group.

Figure 32 shows the results of PCA (scores plot, 32A; IR spectra, 32B) of ciprofloxacin resistant *E. coli* exposed to ciprofloxacin for 120 minutes compared to an unexposed control group.

Figure 33 shows the results of PCA (scores plot, 33A; IR spectra, 33B) of gentamicin susceptible *E. coli* exposed to gentamicin for 120 minutes compared to an unexposed control group.

Figure 34 shows the results of PCA (scores plot, 34A; IR spectra, 34B) of gentamicin resistant *E. coli* exposed to gentamicin for 120 minutes compared to an unexposed control group.

Figure 35 shows the results of PCA (scores plot, 35A; IR spectra, 35B) of ceftriaxone susceptible *E. coli* exposed to ceftriaxone for 120 minutes compared to an unexposed control group.

Figure 36 shows the results of PCA (scores plot, 36A; IR spectra, 36B) of ceftriaxone resistant *E. coli* exposed to ceftriaxone for 120 minutes compared to an unexposed control group.

Figure 37 shows the results of PCA (scores plot, 37A; IR spectra, 37B) of ceftriaxone susceptible *P. aeruginosa* exposed to ceftriaxone for 120 minutes compared to an unexposed control group.

Figure 38 shows the results of PCA (scores plot, 38A; IR spectra, 38B) of ceftriaxone resistant *P. aeruginosa* exposed to ceftriaxone for 120 minutes compared to an unexposed control group.

Figure 39 shows the results of PCA (scores plot, 39A; IR spectra, 39B) of ciprofloxacin susceptible *P. aeruginosa* exposed to ciprofloxacin for 120 minutes compared to an unexposed control group.

Figure 40 shows the results of PCA (scores plot, 40A; IR spectra, 40B) of ciprofloxacin resistant *P. aeruginosa* exposed to ciprofloxacin for 120 minutes compared to an unexposed control group.

Figure 41 shows the results of PCA (scores plot, 41A; IR spectra, 41B) of gentamicin susceptible *P. aeruginosa* exposed to gentamicin for 120 minutes compared to an unexposed control group.

Figure 42 shows the results of PCA (scores plot, 42A; IR spectra, 42B) of gentamicin resistant *P. aeruginosa* exposed to gentamicin for 120 minutes compared to an unexposed control group.

Figure 43 shows the results of PCA (scores plot, 43A; IR spectra, 43B) of amoxicillin susceptible *S. salivarious* exposed to amoxicillin for 120 minutes compared to an unexposed control group.

Figure 44 shows the results of PCA (scores plot, 44A; IR spectra, 44B) of amoxicillin resistant *S. salivarious* exposed to amoxicillin for 120 minutes compared to an unexposed control group.

Figure 45 shows the results of PCA (scores plot, 45A; IR spectra, 45B) of vancomycin susceptible *S. salivarious* exposed to vancomycin for 120 minutes compared to an unexposed control group.

Figure 46 shows the results of PCA (scores plot, 46A; IR spectra, 46B) of vancomycin resistant *S. salivarious* exposed to vancomycin for 120 minutes compared to an unexposed control group.

Figure 47 shows the results of PCA (scores plot, 47A; IR spectra, 47B) of ceftriaxone susceptible *S. salivarious* exposed to ceftriaxone for 120 minutes compared to an unexposed control group.

Figure 48 shows the results of PCA (scores plot, 48A; IR spectra, 48B) of ceftriaxone resistant *S. salivarious* exposed to ceftriaxone for 120 minutes compared to an unexposed control group.

Figure 49 shows the results of PCA (scores plot, 49A; IR spectra, 49B) of ceftriaxone susceptible *K. pneumoniae* exposed to ceftriaxone for 120 minutes compared to an unexposed control group.

Figure 50 shows the results of PCA (scores plot, 50A; IR spectra, 50B) of ceftriaxone resistant *K. pneumoniae* exposed to ceftriaxone for 120 minutes compared to an unexposed control group.

Figure 51 shows the results of PCA (scores plot, 51A; IR spectra, 51B) of ciprofloxacin susceptible *K. pneumoniae* exposed to ciprofloxacin for 120 minutes compared to an unexposed control group.

Figure 52 shows the results of PCA (scores plot, 52A; IR spectra, 52B) of ciprofloxacin resistant *K. pneumoniae* exposed to ciprofloxacin for 120 minutes compared to an unexposed control group.

Figure 53 shows the results of PCA (scores plot, 53A; IR spectra, 53B) of gentamicin susceptible *K. pneumoniae* exposed to gentamicin for 60 minutes compared to an unexposed control group.

Figure 54 shows the results of PCA (scores plot, 54A; IR spectra, 54B) of gentamicin resistant *K. pneumoniae* exposed to gentamicin for 60 minutes compared to an unexposed control group.

Figure 55 shows the results of PCA (scores plot, 55A; IR spectra, 55B) of ceftriaxone susceptible *A. baumannii* exposed to ceftriaxone for 60 minutes compared to an unexposed control group.

Figure 56 shows the results of PCA (scores plot, 56A; IR spectra, 56B) of ceftriaxone resistant *A. baumannii* exposed to ceftriaxone for 60 minutes compared to an unexposed control group.

Figure 57 shows the results of PCA (scores plot, 57A; IR spectra, 57B) of ciprofloxacin susceptible *A. baumannii* exposed to ciprofloxacin for 60 minutes compared to an unexposed control group.

Figure 58 shows the results of PCA (scores plot, 58A; IR spectra, 58B) of ciprofloxacin resistant *A. baumannii* exposed to ciprofloxacin for 60 minutes compared to an unexposed control group.

Figure 59 shows the results of PCA (scores plot, 59A; IR spectra, 59B) of gentamicin susceptible *A. baumannii* exposed to gentamicin for 60 minutes compared to an unexposed control group.

Figure 60 shows the results of PCA (scores plot, 60A; IR spectra, 60B) of gentamicin resistant *A. baumannii* exposed to gentamicin for 60 minutes compared to an unexposed control group.

DETAILED DESCRIPTION

Embodiments disclosed herein relate to systems and methods of detecting a pathogen's susceptibility or resistance to a given treatment or substance, such as a drug or other medicine, via spectroscopy. The minimum inhibitory concentration and/or breakpoint concentration of the substance may be determined. The pathogens detected may include

bacteria and fungi. Bacteria may be Gram positive or Gram negative. Some pathogens may be involved in sepsis and may include *Candida parapsilosis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Hafnia aivaris*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus capitus*, *Staphylococcus epidermidis*, *Stenotrophomonas maltophila*, and *Streptococcus dysgalactiae*. Other pathogens may include *Acinetobacter baumannii*, *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Neisseria meningitides, Staphylococcus pyogenes, Listeria monocytogenes, and Streptococcus agalactaie*. The pathogens may be associated with one or more of community-acquired pneumonia, meningitis, or sepsis. The pathogen may be present in a bodily fluid such as whole blood, serum, plasma, urine, cerebrospinal fluid, milk, saliva, or tears; in or on a bodily tissue such as hair, skin, or nails; in or on liquid or solid consumables such as water, juice, milk, or solid food; or sources of drinking water such as lakes, rivers, streams, reservoirs, and water treatment facilities.

The systems and methods disclosed herein may include detection of the phenotypic response of a pathogen to drug exposure. The phenotypic response may be reflected in one or more metabolic changes or changes in chemical composition. Changes in chemical composition may in turn be detected as spectroscopic changes. Pathogens that are susceptible to the drug to which they are exposed may demonstrate changes in chemical composition and/or spectral data compared to the same pathogen not exposed to the drug. Pathogens that are not susceptible (resistant) to the drug to which they are exposed may demonstrate few or no changes in chemical composition and/or spectral data compared to the same pathogen not exposed to the drug.

The spectroscopy may be infrared or Raman spectroscopy. Examples of infrared spectroscopy include infrared-attenuated total reflection (IR-ATR) spectroscopy, Fourier-transform infrared-attenuated total reflection (ATR-FTIR) spectroscopy, transmission spectroscopy, transflection spectroscopy, reflectance spectroscopy, near infrared (NIR) spectroscopy, and/or far infrared (FIR) spectroscopy. Spectral data may include a spectrum in the UV, visible, or infrared wavelength regions (e.g., 1-50000cm^{-1}), spectral components of spectra, and/or wavenumber, absorbance, and/or Raman intensity values associated with the components.

Systems for determining or helping to determine drug susceptibility or resistance of pathogens in samples are also disclosed. In some embodiments, a system includes a computer readable storage medium for storing in non-transient form an application for executing a method of classifying drug susceptibility or resistance of a pathogen in a sample,

including recording spectral data representative of the sample, and comparing the sample spectral data to spectral data representative of one or more internal references or controls to determine if a pathogen is present in the sample. In some implementations, the application is stored in the cloud or other computing equivalent.

In some embodiments, a system includes a memory and a controller with a processor and a predetermined instruction set to record spectral data from a sample, compare the sample spectral data to data obtained from an internal reference, and determine if a pathogen in the sample in susceptible or resistant to a given treatment based on a difference between sample spectra data and reference spectral data.

The presently disclosed systems and methods may be automated and/or may employ software for quality control of spectral data and/or its analysis without requiring user input or interpretation. Antibiotic sensitivity or resistance may be classified by artificial intelligence systems, which may be subject to continuous refinement with continuing data input. The quality and classification systems may be cloud-based, which may enhance protection of software code and/or data.

The presently disclosed methods of determining drug susceptibility or resistance of a pathogen employ an internal control, which may be the same pathogen not exposed to the drug. Using such an internal control may help the methods be largely or completely insensitive to potential preparation or processing differences across various sites.

The presently disclosed methods may be applied to more than one drug for a given pathogen, which may provide a partial or complete drug resistance profile of the investigated pathogen.

The presently disclosed methods may be used to test the efficacy and/or mode of action of new treatments, such as new antibiotics.

The disclosed methods may isolate a pathogen directly from a source, such as a patient's blood or other bodily fluid, without culturing the pathogen. Compared to known methods of determining drug susceptibility or resistance of a pathogen, which generally include culturing the pathogen, which in some instances is a slow-growing pathogen, the presently disclosed methods may be faster. Compared to known methods, the presently disclosed methods of determining drug susceptibility or resistance of a pathogen may be easier and/or less expensive.

Overview of Methods

Figure 1A schematically illustrates a method 100 of determining the susceptibility or resistance of a pathogen to a given drug. The method 100 includes providing a sample 102;

identifying the sample 104; culturing the sample 106; preparing the sample 108; measuring the sample 110; analyzing the sample 112; and supplying a result 114, and optionally stressing the sample 107. The providing 102, identifying 104, culturing 106, stressing 107, preparing 108, measuring 110, analyzing 112, and supplying 114 may be performed in any order, including concurrently.

In some implementations, the method 100 is performed in a laboratory, hospital, clinic, or other research, laboratory, or medical setting. The entire method 100 may not be performed in a single location.

The sample may be of a bodily fluid such as whole blood, serum, plasma, urine, cerebrospinal fluid, milk, saliva, or tears; a bodily tissue such as hair, skin, or nails; a liquid or solid consumable such as water, juice, milk, or solid food; or a source of drinking water such as lakes, rivers, streams, reservoirs, and water treatment facilities. The sample may be from a pathogen culturing device such as an agar plate or blood culture bottle, or may be directly sampled from a patient. The pathogen of the culturing device may have originated from any of the preceding sample sources. In one implementation, providing the sample 102 is as depicted in Figure 2A. In one implementation, providing the sample 102 is as depicted in Figure 2B.

Identifying the sample 104 may ascertain the type or types of pathogens in a sample, such as Gram positive bacteria, Gram negative bacteria, or fungi. Identification may be performed via, for example, Gram staining or spectroscopy, which may be any type of spectroscopy described above.

Culturing the sample 106 may be with or without a drug of interest. In one example, multiple concentrations of a drug (including 0 μg/mL) may be tested. In one example, multiple drugs may be tested. Each test condition may be segregated, such as in a well of a multi-well plate. In one implementation, culturing the sample 106 is as depicted in Figure 3.

In some implementations, the sample may be stressed, such as after culturing the sample 106. Stressing the sample 107 may be by intense and/or repeated centrifugation. In one implementation, stressing the sample 107 is as described in Example 7. Preparing the sample 108 for spectral analysis may include centrifuging the sample, removing the supernatant from the sample, rinsing the sample, and mixing the sample. In one implementation, preparing the sample 108 is as depicted in Figure 4A.

Measuring the sample 110 may be via spectroscopy, which may be any type of spectroscopy described above. Spectroscopy may be, for example, vibrational, electronic, or rotational. Examples include, but are not limited to, Raman spectroscopy,infrared-attenuated

total reflection (IR-ATR) spectroscopy, Fourier-transform infrared-attenuated total reflection (ATR-FTIR) spectroscopy, near infrared (NIR) spectroscopy, and far infrared (FIR) spectroscopy. In one implementation, measuring the sample 110 is as depicted in Figure 4B. In one implementation, measuring the sample 110 is as depicted in Figure 4C.

Analyzing the sample 112 may include comparing spectral data from a sample treated with a drug to the same sample not exposed to the same drug to determine if spectral differences exist. The analysis may be performed by a software program and/or may otherwise be automated. The analysis may be performed by a cloud-based system, as described below.

In some examples, the spectroscopy data is pre-processed to generate wavenumber values and then Principal Component Analysis is applied to the data. The principal components are then selected based on group differences and the selected component may be incorporated into a linear regression model based on goodness-of-fit. A classifier based on one or more principal component thresholds, cutoffs, or ranges is provided, or a classifier function, comprising one or more principal components and corresponding coefficient, and an intercept.

Supplying a result 114 may include reporting that a tested pathogen is susceptible or resistant to a tested drug. The result may be supplied to user, such as health care provider.

Figure 1B illustrates a method 150 similar to the method 100 disclosed in Figure 1A, but in the form of a flow diagram. The method 150 includes providing a sample 152; identifying the sample 154; culturing the sample 156; preparing the sample 158; measuring the sample 160; analyzing the sample 162; and supplying a result 164, and optionally stressing the sample 157. Each of providing a sample 152; identifying the sample 154; culturing the sample 156; stressing the sample 157; preparing the sample 158; measuring the sample 160; analyzing the sample 162; and supplying a result 164 are as described above for providing a sample 102; identifying the sample 104; culturing the sample 106; stressing the sample 107; preparing the sample 108; measuring the sample 110; analyzing the sample 112; and supplying a result 114, respectively.

Providing a Sample

With reference to Figure 2A, a method 200 of providing a sample is disclosed. The method 200 may be performed as part of one of the methods 100, 150 described above (i.e., to provide a sample 102, 152) or may be performed independently. The sample may be from any source described above. Performing the method 200 may help to isolate and/or purify a pathogen from the remainder of the sample. The method 200 includes centrifuging a sample

202, 212; removing the supernatant from the sample 204, 214; rinsing the sample 206; and mixing the sample 208. Some or all of the foregoing centrifuging 202, removing 204, rinsing 206, and mixing 208 may be repeated 210.

Centrifuging the sample 202, 212 may be sufficient to pellet a pathogen in the sample. The centrifugation may performed at about 2,000 RPM to about 10,000 RPM for about 2 minutes to about 10 minutes. In one example, the sample is centrifuged at about 10,000 RPM for about 2 minutes.

Rinsing the sample 206 may help to re-suspend the pellet and/or to remove impurities from the sample. In some examples, about 1 mL to about mL, such as about 3 mL, of ultrapure water is added.

Mixing the sample 208 may help to re-suspend the pellet and/or to remove impurities from the sample. In some examples, the sample is vortexed for about 1 minute to about 4 minutes, or about 2 minutes.

The optional repetition 210 of the centrifuging 202, removing 204, adding 206, and mixing 208 may help to re-suspend the pellet and/or to remove impurities from the sample. The repeating 210 may be performed at least once, such as about 2 times to about 10 times, about 4 times to about 8 times, or about 6 times.

With reference to Figure 2B, a method 250 of providing a sample is disclosed. The method 250 may be performed as part of one of the methods 100, 150 described above (i.e., to provide a sample 102, 152) or may be performed independently. The sample may be from a culturing device, such as an agar plate. Performing the method 250 may help to isolate and/or purify a pathogen from the remainder of the plate. The method 250 includes collecting a sample 252; diluting the sample 254; adjusting the concentration of the sample 256; mixing the sample 258, 266; centrifuging the sample 260, 270; removing the supernatant from the sample 262, 272; and rinsing the sample 264. Some or all of the foregoing centrifuging 260, removing 262, rinsing 264, and mixing 266 may be repeated 268.

Collecting the sample 252 may include, for example, removing a colony from a culturing device, such as an agar plate, such as with a sterile loop or sterile pipette tip.

Diluting the sample 254 may be with, for example, ultrapure water.

Adjusting the concentration of the sample 256 may include adjusting the concentration of a pathogen within the sample. The concentration may be adjusted to any desired concentration. The concentration may be adjusted while using a McFarland standard, such as a 0.5 standard, for reference. The concentration may be adjusted using measurements of optical density at a selected wavelength, such as about 625 nm.

Mixing the sample 258, 266 may be as described above for mixing the sample 208.

Centrifuging the sample 260, 270 may be as described above for centrifuging the sample 202, 212.

Removing the supernatant from the sample 262, 272 may be as described above for removing the supernatant from the sample 204, 214.

Rinsing the sample 264 may be as described above for rinsing the sample 206.

The optional repetition 268 may be as described above for the optional repetition 210.

Identifying a Sample

Identifying a sample may ascertain the type or types of pathogens in a sample, such as Gram positive bacteria, Gram negative bacteria, or fungi. Identification may be performed via, for example, Gram staining or spectroscopy, which may be infrared or Raman spectroscopy. The spectroscopy may be any type described above. Methods for identifying pathogens via spectroscopy are disclosed in U.S. Patent No. 9,983,130, U.S. Patent No. 10,145,839, and PCT Patent Application Publication No. WO 2018/033894, which are incorporated by reference herein in their entirety.

Identifying a sample may be performed as part of one of the methods 100, 150 described above (i.e., identifying a sample 104, 154) or may be performed independently. The sample may be a sample obtained from one or more of the methods 200, 220 of providing a sample described above or from any other method of providing a sample.

Culturing a Sample

With reference to Figure 3, a method 300 of culturing a sample is disclosed. The method 300 may be performed as part of one of the methods 100, 150 described above (i.e., culturing a sample 106, 156) or may be performed independently. The sample may be a sample obtained from one or more of the methods 200, 220 of providing a sample described above or from any other method of providing a sample. Performing the method 300 may help enable the determination of a pathogen's susceptibility or resistance to a given test substance. The method 300 includes preparing control and/or test substances 302; preparing a sample 304; combining the sample with the control or test substances 306; and incubating the sample 308.

A test substance may be any drug, such as an antibiotic or antifungal, or other treatment of interest. An antibiotic may be bactericidal (i.e., it kills bacteria) and/or bacteriostatic (i.e., it prevents the growth of bacteria. Examples of antibiotic classes include, but are not limited to, β-lactams, glycopeptides, lincomycins, cephalosporins,

fluoroquinolones, and aminoglycosides. Examples of β-lactam antibiotics include methicillin, oxacillin, and amoxicillin. Examples of glycopeptide antibiotics include vancomycin. Examples of lincomycin antibiotics include clindamycin. Examples of cephalosporin antibiotics include ceftriaxone. Examples of fluoroquinolones include ciprofloxacin. Examples of aminoglycoside antibiotics include gentamicin. More than one test substance may be prepared when preparing control and/or test substances 302. Each test substance may be prepared at one or more concentrations, which may be prepared in any appropriate diluent. The diluent may be, for example, water or growth medium for a pathogen. The control substance may be the diluent alone. Any desired volume of diluent may be used. The control or test substance and diluent may be mixed together, such as by vortexing.

Preparing the sample 304 may include resuspending one or more pellets obtained from one or more of the methods 200, 220 of providing a sample described above. The pellet may be resuspended in any appropriate diluent. The diluent may be, for example, water or growth medium. The sample may be prepared at any desired concentration. Concentration may be determined by, for example, optical density and may be adjusted as needed.

Combining the sample with the control or test substances 306 may include mixing, such as by pipetting or vortexing. The combining 306 be performed in an incubation device or in a separate vessel outside of the incubation device. When combining 306 outside of the incubation device, the combination may be later transferred to the incubation device. Growth medium appropriate for the sample may be added to the incubation device. In some examples, the incubation device is a plate such as a culture plate, which may be a multi-well culture plate. The plate may include any number of wells, such as from about 6 wells to about 96 wells, such as 12 wells or 96 wells. In one example of using a multi-well plate, one test substance is tested, a different testing condition is assigned to each column, and each row represents a testing replicate number. A testing condition may be a given concentration of the test substance, including no test substance (i.e., a control). Examples of concentrations include, but are not limited to, 0 μg/mL (i.e., control), about 0.25 μg/mL, about 0.5 μg/mL, about 0.75 μg/mL, about 1.0 μg/mL, about 1.5 μg/mL, about 2.0 μg/mL, about 2.5 μg/mL, about 3.0 μg/mL, about 3.5 μg/mL, about 4.0 μg/mL, about 4.5 μg/mL, about 5.0 μg/mL, about 5.5 μg/mL, about 6.0 μg/mL, about 6.5 μg/mL, about 7.0 μg/mL, about 7.5 μg/mL, about 8.0 μg/mL, about 10.0 μg/mL, about 12.0 μg/mL, about 14.0 μg/mL, about 16.0

μg/mL, about 32.0 μg/mL, or about 64.0 μg/mL. Test substance concentration may be the minimum inhibitory concentration (MIC) for a given substance.

Incubating the sample 308 may be under any condition appropriate for the pathogen in the sample. For example, the sample may be incubated at about 37°C with about 5% CO_2. The sample may be incubated in an incubator. The sample may be incubated for any desired amount of time, such as about 15 minutes to about 360 minutes, about 15 minutes to about 300 minutes, about 15 minutes to about 240 minutes, about 15 minutes to about 180 minutes, about 15 minutes to about 120 minutes, about 15 minutes to about 90 minutes, about 15 minutes to about 60 minutes, about 15 minutes to about 30 minutes, about 30 minutes to about 360 minutes, about 60 minutes to about 360 minutes, about 90 minutes to about 360 minutes, about 120 minutes to about 360 minutes, about 180 minutes to about 360 minutes, about 240 minutes to about 360 minutes, or about 30 minutes to about 240 minutes.

In one implementation, a multi-well plate is provided with one or more of control substances, test substances, and growth medium preloaded in the wells. The substances and/or medium may be dry and may be reconstituted upon addition of a sample.

Stressing a Sample

Stressing a sample may help enhance the response of a pathogen susceptible to a test substance following exposure to the test substance. Following test substance exposure, susceptible cells may be weakened. Upon exposure to a low-to-mid external stress factor, the cells may become more vulnerable, may experience reduced integrity, and/or may rupture. By comparison, cells unexposed to the test substance (e.g., a control) or unaffected by the test substance (e.g., resistant) maintain their integrity when subjected to the same conditions. The differential response may help enhance the detectable differences in chemical composition between susceptible-exposed and unsusceptible-exposed or unexposed cell populations.

A sample may be stressed by one or more of centrifugation, temperature changes, pressure changes, UV exposure, and the like. In one implementation, the stressor is centrifugation, which may be performed more than once, such as about 5 to about 10 times, about 7 to about 10 times, about 9 to about 10 times, about 5 to about 8 times, or about 5 to about 6 times.

Each centrifugation may be performed at from about 10,000 g to about 16,000 g, about 11,000 g to about 15,000 g, or about 12,000 g to about 14,000 g. Each centrifugation may last about 1 minute to about 4 minutes, about 2 minutes to about 4 minutes, or about 1 minute to about 3 minutes. In one implementation, centrifugation is performed 6 times, each time at about 15,000 g for about 2 minutes.

Stressing a sample may be performed as part of one of the methods 100, 150 described above (i.e., stressing a sample 107, 157) or may be performed independently. The sample may have been obtained from the method 300 of culturing a sample described above or from any other method of culturing a sample.

Preparing a Sample for Spectroscopy

With reference to Figure 4A, a method 400 of preparing a sample for spectroscopy is disclosed. The method 400 may be performed as part of one of the methods 100, 150 described above (i.e., preparing a sample 108, 158) or may be performed independently. The sample may be from any pathogen that has been incubated with a control or test substance. The sample may have been obtained from the method 300 of culturing a sample described above or from any other method of culturing a sample. The sample may have been stressed such as described above for stressing a sample 107, 157. Performing the method 400 may help to isolate and/or purify a pathogen that has been incubated with or without a drug of interest. The method 400 includes centrifuging a sample 402, 412; removing the supernatant from the sample 404, 414; rinsing the sample 406; and mixing the sample 408. Some or all of the foregoing centrifuging 402, removing 404, rinsing 406, and mixing 408 may be repeated 410.

Each of centrifuging a sample 402, 412; removing the supernatant from the sample 404, 414; rinsing the sample 406; mixing the sample 408; and repeating 410 may be as described above for like-numbered activities 202, 212, 204, 214, 206, 208, and 210, respectively.

Measuring a Sample by Spectroscopy

With reference to Figure 4B, a method 420 of measuring a sample by spectroscopy is disclosed. The spectroscopy may be infrared spectroscopy, such as IR-ATR or ATR-FTIR spectroscopy, or any other type of infrared spectroscopy. The method 420 may be performed as part of one of the methods 100, 150 described above (i.e., measuring a sample 110, 160) or may be performed independently. The sample may have been obtained from the method 400 of preparing a sample for spectroscopy described above or from any other method of preparing a sample for spectroscopy. The method 420 includes collecting background spectral data 422 and collecting test spectral data 424, which may occur in any order.

An ATR crystal may be cleaned, dried, and/or equilibrated to room temperature before collecting background spectral data 422.

A sample may be from a pathogen-containing pellet produced in method 400. The sample, such as about 0.5 µl to about 3 µl, or about 1 µl, may be transferred to an ATR

crystal. The sample may be dried before collecting test spectral data 424. Collecting test spectral data 424 may include collecting data for at least 1, such as about 2 to about 10, or about 3 to about 5, spectrum.

ATR-FTIR data collection parameters may include about 64 to about 512 scans for background, about 32 to about 512 scans for sample, spectral resolution of about 4 cm^{-1} to about 8 cm^{-1} , and/or one or more spectral ranges (e.g., about 4000 cm^{-1} – about 600 cm^{-1}, about 4000 cm^{-1} – about 900 cm^{-1}, about 1800 cm^{-1} – about 600 cm^{-1}, about 1800 cm^{-1} – about 900 cm^{-1}). In one example, the ATR-FTIR data collection parameters are as follows: number of scans for background, 128; number of scans for sample, 64; spectral resolution, 8 cm^{-1}; and spectral range, 4000 cm^{-1} – 600 cm^{-1}. In one example, the ATR-FTIR data collection parameters are as follows: number of scans for background, 64; number of scans for sample, 32; spectral resolution, 8 cm^{-1}; and spectral range, 1800 cm^{-1} – 900 cm^{-1}.

Wavenumber values (reciprocal centimeters) in the preceding paragraph and throughout the application are approximations. In some instances, wavenumber values are preceded by the term "about" to indicate such approximation. Wavenumber values should be understood as approximations even in the absence of the term "about." The provided wavenumber values may range +/- (0.5 x the spectral resolution of the measurement).

In some implementations, the spectroscopy is NIR spectroscopy (about 20000-4000 cm^{-1}) and utilizes overtones and/or combination bands from mid infrared marker bands. In some implementations, NIR may utilize lattice or phonon modes in the far infrared range (about 1000-1 cm^{-1})

With reference to Figure 4C, a method 450 of measuring a sample by spectroscopy is disclosed. The spectroscopy may be Raman spectroscopy. The method 450 may be performed as part of one of the methods 100, 150 described above (i.e., measuring a sample 110, 160) or may be performed independently. The method 450 includes preparing a sample 442 and collecting test spectra data 444.

A sample may be from a pathogen-containing pellet produced in method 400. Preparing a sample 442 may include transferring a volume of the sample, such as about 0.5 µl to about 3 µl, or about 1 µl, to a separate vessel, such as a microcentrifuge tube. The sub-sample may be diluted to a desired concentration, such as by adding any suitable diluent (e.g., ultrapure water). In one example, about 9 µL of ultrapure water is added to about 1 µL of sub-sample and then the diluted sub-sample is mixed, such as by vortexing for about 2 minutes. The diluted sub-sample may be transferred to a slide, such as a Raman-grade slide

or an aluminum-coated slide. The slide may be air dried, such as for about 10 to about 30 minutes, or about 20 minutes, or actively dried.

Collecting test spectral data 444 may include collecting data for at least 1, such as about 5 to about 15, or about 8 to about 12, spectrum.

Raman data collection parameters may include a spectral resolution of about 6 cm^{-1} to about 1 cm^{-1}, one or more spectral ranges (e.g., about 3800 cm^{-1} – about -50 cm^{-1}, about 1800 cm^{-1} – about -50 cm^{-1}, about 3800 cm^{-1} – about 200 cm^{-1}, about 1800 cm^{-1} – about 200 cm^{-1}), about 1 to about 100 accumulations for each spectrum, and/or an integration time of about 0.1 second to about 10 seconds for each spectrum. In one example, the Raman data collection parameters are as follows: spectral resolution, 3 cm^{-1}; spectral range, 3800 cm^{-1} – (-50) cm^{-1}; number of accumulations for each spectrum, 50; and integration time for each spectrum, 1 second.

Analyzing a Sample

Analyzing a sample may be performed as part of one of the methods 100, 150 described above (i.e., analyzing a sample 112, 162) or may be performed independently. The spectral data may be spectral data obtained in the methods 420, 440 described above or in any other method of spectral data collection.

Analyzing a sample may include comparing spectral data obtained from a control pathogen incubated in the absence of a test substance to spectral data obtained from the same pathogen incubated in the presence of the test substance. The spectral data may reflect the biochemical composition of the pathogens such that differences between the control and test spectral data can be used to determine the impact, if any, of a test substance on the metabolism, growth, multiplication, and/or death of the pathogens. The differences in biochemical composition may reflect metabolic changes and/or may precede, for example, inhibition of growth and may be detectable before any of inhibition of growth, inhibition of multiplication, or cell death is observable. The current standard of care, antibody susceptibility testing (AST), measures inhibition of growth and may require at least 24 hours to return results. In contrast to the current standard of care, the presently disclosed methods can detect early chemical changes and thereby return results much faster, such as in about 2 hours or less.

Comparing differences between spectral data obtained from a control pathogen incubated in the absence of a test substance and spectral data obtained from the same pathogen incubated in the presence of the test substance may be enhanced following exposure of the pathogens to an external stress factor. The chemical composition of a susceptible

pathogen may be altered in response to test substance exposure. Subsequent exposure to an external stress factor may result in decreased integrity, such as cell wall integrity, and the vulnerable cells may lyse. Samples of lysed cells are likely to include more cell wall fragments than samples of unlysed cells. Cell wall fragments include peptidoglycan, which is a polymer of sugars and amino acids detectable via spectroscopy. Peptidoglycan markers include bands at about 1622-1624 cm^{-1} and about 1515 cm^{-1}. (Figures 14A and B.) Such bands can be indicative of exposure of a pathogen to a test substance to which the pathogen is susceptible. Other bands are indicative of molecular changes that may be unique to the mode of action of a given test substance.

The spectral data may be pre-processed, which may be by smoothing, baseline correction, calculation of second derivatives and/or normalization, which may be standard normal variate (SNV) normalization. The spectral data may be averaged for a given treatment, and may be averaged prior to and/or following pre-processing.

Chemometric tools, such as Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLSDA), and/or Soft Independent Modelling by Class Analogy (SIMCA), may be employed in the methods disclosed herein. PCA may focus on selected spectral regions that may be associated with disturbances in nucleic acids, amino acids, proteins, carbohydrates, and/or combinations thereof. In one example, spectral regions of interest in ATR-FTIR spectroscopy, transmission spectroscopy, transflection spectroscopy, or reflection spectroscopy may include about 3100 cm^{-1} to about 2800 cm^{-1}, about 1750 cm^{-1} to about 1500 cm^{-1}, about 1450 cm^{-1} to about 1350 cm^{-1}, about 1300 cm^{-1} to about 1200 cm^{-1}, and/or about 1100 cm^{-1} to about 900 cm^{-1}. In one example, spectral regions of interest in Raman spectroscopy may include about 3100 cm^{-1} to about 2800 cm^{-1}, about 1750 cm^{-1} to about 1500 cm^{-1}, about 1450 cm^{-1} to about 1350 cm^{-1}, about 1300 cm^{-1} to about 1200 cm^{-1}, and/or about 1100 cm^{-1} to about 600 cm^{-1}. In one example, spectral regions of interest may include a range that covers major bands of peptidoglycan. In ATR-FTIR spectroscopy, transmission spectroscopy, transflection spectroscopy, or reflection spectroscopy, major bands of peptidoglycan may include bands present in the spectrum at about 1622 cm^{-1} and about 1515 cm^{-1}.

The analysis may include determining the presence of changes between control and test spectral data, such as a distance between spectra, with respect to the distance to the model center or cluster centroid. A large change or distance may reflect a different biochemical composition, which may be the result of the effect of a tested drug on a pathogen. A threshold may be established and a distance above the threshold may indicate significant

differences in biochemical composition and/or phenotypic response between pathogens subjected to control and test conditions. A distance above the threshold may identify or help identify the tested pathogen as susceptible to the test substance. A distance calculation may be performed by, for example, a k-nearest neighbor algorithm.

In another example, a threshold absorbance value or values for a band or bands of interest may be calculated from the mean absorbance value or values for the control spectra +/- up to three (i.e., one, two, or three) standard deviations of the mean absorbance value or values, which may depend on the desired level of significance and whether the band, characteristically, increases or decreases in intensity in response to a test substance.

The analysis may be performed by a software program and/or may otherwise be automated. The analysis may be performed by a cloud-based system, as described below.

In one example, a classifier is developed using a training set comprising a plurality of testing samples that each comprising a set of subsamples or otherwise divided into one or more control samples and one or more incubation samples with one or more concentrations of an antibiotic, anti-fungal, or other anti-infective agent, and wherein the sensitivity, resistance, and/or minimum inhibitory concentration are known. This training set may be used to train a Soft Independent Modeling by Class Analogy (SIMCA) classifier, PLS-DA, Support Vector Machine, or other machine learning or deep learning algorithm wherein the input of the spectra of the control(s) and incubation samples are used in a SIMCA model and classified as resistant or sensitive (susceptible), and a MIC value is determined, where a difference between an incubation sample spectra and a control sample spectra exceeds a predetermined distance or value. The MIC value may be determined as the lowest drug concentration where the incubation spectra has a model distance great than the predetermined distance, for example. The predetermined distance or value may depend on the number of different incubation samples and/or number of control samples specified for the procedure.

In a further example, the classifier may be configured to test for susceptibility or resistance in the presence of one or more concentrations of a test substance (e.g., oxacillin at 0, 0.25. 0.5, 2, 4 and 8 µg/mL. PLS-DA or SIMCA may be used to discriminate the MIC. For PLS-DA, for example, a Y variable of +1 may be denoted for bacteria inhibited by the drug and -1 to spectra that are not exposed to the drug, e.g. the control samples. Scores ranging from -1 to +4 are achieved, and values of +3 or more may be classified as sensitive to the test substance. In examples utilizing SIMCA, PCA models developed for control spectra and spectra from pathogens exposed to inhibitory concentrations of an antibiotic, anti-fungal, or other an anti-infective would be used.

Quality control and performance measurement could also be implemented, where the Y value of the control would have to be less than zero. For SIMCA classification, the sample to model distance is used to classify a control sample to a control class rather than the inhibited or sensitive class to confirm validity. The SIMCA comparison may be provided with a predetermined significance limit, e.g. 1%, 2.5%, 5% or 10%.

The determination of the MIC value may be determined by the identification of the minimum drug concentration of the incubation samples where the spectra was classified by the algorithm, e.g. PLS-DA or SIMCA, as a drug inhibited spectra. In examples utilizing PLS-DA, the spectra would be the lowest concentration spectra having a Y variable greater than zero, or some other predetermined threshold value. In the examples utilizing SIMCA, the sample to model distance is used to classify the incubation samples as belong to the inhibited class and the lowest concentration of a sample classified into the inhibited class is used to identify the MIC. determined and based on the model distance, classified into either a control class rather than the inhibited or sensitive class to confirm validity. This SIMCA comparison may so be provided with a predetermined significance limit, e.g. 1%, 2.5%, 5% or 10%.

Supplying a Result

Supplying a result may include reporting that a tested pathogen is susceptible or resistant to a test substance. The result may be supplied by a software program, which may reside in the cloud. The result may be supplied to user, such as health care provider. Supplying a result may help guide treatment of the patient from whom the tested pathogen was obtained.

Supplying a result may be performed as part of one of the methods 100, 150 described above (i.e., supplying a result 114, 164) or may be performed independently. The result may be a result obtained from a method of analyzing a sample described immediately above or from any other method of analyzing a sample.

Systems

Systems for providing a sample, identifying a sample, culturing a sample, optionally stressing a sample, preparing a sample for spectroscopy, measuring a sample, analyzing a sample, and/or supplying a result are disclosed herein. The systems are designed to determine or help determine the drug susceptibility or resistance of a pathogen in the samples.

A system may include one or more devices for providing a sample. The system may include at least one vessel in which samples are collected, at least one pipette, at least one mixer for mixing sample, and at least one centrifuge for centrifuging samples.

The vessel may be a tube such as an Eppendorf tube or volumetric tube. The mixer may be a vortexer. The centrifuge may be a microcentrifuge or tabletop centrifuge.

A system may include one or more devices for identifying a sample. The system may include at least one Gram staining kit and/or at least one spectrometer. The spectrometer may be, for example, an IR-ATR spectrometer, an ATR-FTIR spectrometer, a Raman spectrometer, or a spectrometer suitable for any other type of spectrometry described above.

A system may include one or more devices for culturing a sample. The system may include at least one vessel, test substance, incubation device, and incubator. In some embodiments, the system includes a test kit, as described below.

The vessel may be a tube such as an Eppendorf tube or volumetric tube. The samples and/or test substances may be placed in the vessels. The test substance may be any drug, such as an antibiotic or antifungal, or other treatment of interest.

The diluent may be any appropriate vehicle for diluting a sample and/or a test substance. The diluent may be, for example, water or growth medium for a pathogen. The mixer may be, for example, a pipette or a vortexer. The incubation device may be, for example, a multi-well plate.

A system may include one or more devices for stressing a sample. The system may include at least one of a centrifuge, incubator, or UV-light.

A system may include one or more devices for preparing samples for spectroscopy. The system may include at least one vessel in which samples are prepared, at least one pipette, and at least one centrifuge for centrifuging samples.

The vessel may be a tube such as an Eppendorf tube or volumetric tube. The centrifuge may be a microcentrifuge or tabletop centrifuge.

A system may include one or more devices for measuring a sample. The device may be a spectrometer, such as any spectrometer described above, which may generate spectral data, such as a spectrum, from a sample. The system may also include a computer or a subsystem for processing the data, as described immediately below.

A system may include one or more devices for analyzing a sample, which may include processing spectral data. A processing system may include a controller, which may be implemented consistent with numerous general purpose or special purpose computing systems or configurations. Various exemplary computing systems, environments, and/or

configurations that may be suitable for use with the systems and devices disclosed herein may include, but are not limited to, software or other components within or embodied on personal computing devices, network appliances, servers, or server computing devices such as routing/connectivity components, portable (e.g., hand-held) or laptop devices, multiprocessor systems, microprocessor-based systems, and distributed computing networks. Examples of portable computing devices include smartphones, personal digital assistants (PDAs), cell phones, tablet PCs, phablets (personal computing devices that are larger than a smartphone, but smaller than a tablet), wearable computers taking the form of smartwatches, portable music devices, and the like, and portable or wearable augmented reality devices that interface with an operator's environment through sensors and may use head-mounted displays for visualization, eye gaze tracking, and user input.

A system may include a processor, which may be any suitable processing device configured to run and/or execute a set of instructions or code and may include one or more data processors, image processors, graphics processing units, physics processing units, digital signal processors, and/or central processing units. The processor may be, for example, a general-purpose processor, Field Programmable Gate Array (FPGA), an Application Specific Integrated Circuit (ASIC), and the like. The processor may be configured to run and/or execute application processes and/or other modules, processes, and/or functions associated with a system and/or a network associated therewith. The underlying device technologies may be provided in a variety of component types, e.g., metal-oxide semiconductor field-effect transistor (MOSFET) technologies like complementary metal-oxide semiconductor (CMOS), bipolar technologies like emitter-coupled logic (ECL), polymer technologies (e.g., silicon-conjugated polymer and metal-conjugated polymer-metal structures), mixed analog and digital, and the like.

In some implementations, one or more processors may execute the methods described herein in a cloud computing environment or as a Software as a Service (SaaS). For example, at least some of the steps of the methods described herein may be performed by a group of computers in communication via a network (e.g., the Internet) and via one or more appropriate interfaces (e.g., APIs). The cloud computing system may include clients and servers. A client and server are generally remote from each other and typically interact through a communication network. The relationship of client and server arises by virtue of computer programs running on the respective computers and having a client-server relationship to each other.

A system may include a memory, which may include a database and may be, for example, a random access memory (RAM), a memory buffer, a hard drive, an erasable programmable read-only memory (EPROM), an electrically erasable read-only memory (EEPROM), a read-only memory (ROM), Flash memory, and the like. As used herein, "database" refers to a data storage resource. The memory may store instructions to cause the processor to execute modules, processes, and/or functions associated with a spectroscopy processing system, such as spectroscopy data processing, communication, display, and/or user settings. In some variations, storage may be network-based and accessible for one or more authorized users. Network-based storage may be referred to as remote data storage or cloud data storage. Spectroscopy data stored in cloud data storage (e.g., database) may be accessible to respective users via a network, such as the Internet. In some variations, database may be a cloud-based FPGA.

Some disclosed implementations relate to a computer storage product with a non-transitory computer-readable medium, which may also be referred to as a non-transitory processor-readable medium, having instructions or computer code thereon for performing various computer-implemented operations. The computer-readable medium (or processor-readable medium) is non-transitory in the sense that it does not include transitory propagating signals per se (e.g., a propagating electromagnetic wave carrying information on a transmission medium such as space or a cable). The media and computer code, which may also be referred to as code or algorithm, may be those designed and constructed for a specific purpose or purposes. Examples of non-transitory computer-readable media include, but are not limited to, magnetic storage media such as hard disks, floppy disks, and magnetic tape; optical storage media such as Compact Disc/Digital Video Discs (CD/DVDs); Compact Disc-Read Only Memories (CD-ROMs); holographic devices; magneto-optical storage media such as optical disks; solid state storage devices such as a solid state drive (SSD) and a solid state hybrid drive (SSHD); carrier wave signal processing modules; and hardware devices that are specially configured to store and execute program code, such as Application-Specific Integrated Circuits (ASICs), Programmable Logic Devices (PLDs), Read-Only Memory (ROM), and Random-Access Memory (RAM) devices. Other embodiments described herein relate to a computer program product, which may include, for example, the instructions and/or computer code disclosed herein,

The systems, devices, and/or methods described herein may be performed by software (executed on hardware), hardware, or a combination thereof. Hardware modules may include, for example, a general-purpose processor (or microprocessor or microcontroller), an FPGA)

and/or an ASIC. Software modules (executed on hardware) may be expressed in a variety of software languages (e.g., computer code), including C, C++, JAVA®, Python, Ruby, VISUAL BASIC®, and/or other object-oriented, procedural, or other programming language and development tools. Examples of computer code include, but are not limited to, micro-code or micro-instructions, machine instructions, such as produced by a compiler, code used to produce a web service, and files containing higher-level instructions that are executed by a computer using an interpreter. Additional examples of computer code include, but are not limited to, control signals, encrypted code, and compressed code.

A user interface may permit an operator to interact with and/or control the disclosed systems directly and/or remotely. For example, the user interface may include an input device for an operator to input commands and an output device for an operator and/or other observers to receive output (e.g., view sample or patient data on a display device) related to operation of the system. In some variations, the user interface may comprise an input device and output device (e.g., touch screen and display) and be configured to receive input data and output data from one or more of the spectrometers, input device, and output device. For example, spectroscopy data generated by spectrometers may be processed by a controller and displayed by the output device (e.g., monitor display). As another example, operator control of an input device (e.g., joystick, keyboard, touch screen) may be received by a user interface and then processed by a controller for user interface to output a control signal to one or more of the systems and spectrometers.

An output device of a user interface may output spectroscopy data corresponding to a sample or patient, and may comprise one or more display devices. The display device may be configured to display a graphical user interface (GUI). A display device may permit an operator to view spectroscopy data and/or other data processed by the controller. In some implementations, an output device may include a display device having one or more of a light emitting diode (LED), liquid crystal display (LCD), electroluminescent display (ELD), plasma display panel (PDP), thin film transistor (TFT), organic light emitting diodes (OLED), electronic paper/e-ink display, laser display, and holographic display.

An input device may include at least one switch configured to generate a control signal. For example, an input device may comprise a touch surface for an operator to provide input (e.g., finger contact to the touch surface) corresponding to a control signal. An input device including a touch surface may be configured to detect contact and movement on the touch surface using any of a plurality of touch sensitivity technologies including capacitive,

resistive, infrared, optical imaging, dispersive signal, acoustic pulse recognition, and surface acoustic wave technologies.

A system, such as a processing system, described herein may communicate with one or more networks and spectrometers through a network interface. In some variations, the processing system may be in communication with other devices via one or more wired and/or wireless networks. For example, the network interface may permit the processing system to communicate with one or more of a network (e.g., Internet), remote server, and database. The network interface may facilitate communication with other devices over one or more external ports (e.g., Universal Serial Bus (USB), multi-pin connector) configured to couple directly to other devices or indirectly over a network (e.g., the Internet, wireless LAN).

A network interface may comprise radiofrequency (RF) circuitry (e.g., RF transceiver) including one or more of a receiver, transmitter, and/or optical (e.g., infrared) receiver and transmitter configured to communicate with one or more devices and/or networks. RF circuitry may receive and transmit RF signals (e.g., electromagnetic signals). The RF circuitry converts electrical signals to/from electromagnetic signals and communicates with communications networks and other communications devices via the electromagnetic signals. The RF circuitry may include one or more of an antenna system, an RF transceiver, one or more amplifiers, a tuner, one or more oscillators, a digital signal processor, a CODEC chipset, a subscriber identity module (SIM) card, memory, and the like.

A wireless network may refer to any type of digital network that is not connected by cables of any kind. Examples of wireless communication in a wireless network include, but are not limited to, cellular, radio, satellite, and microwave communication. The wireless communication may use any of a plurality of communications standards, protocols, and technologies, including but not limited to Global System for Mobile Communications (GSM), Enhanced Data GSM Environment (EDGE), high-speed downlink packet access (HSDPA), wideband code division multiple access (W-CDMA), code division multiple access (CDMA), time division multiple access (TDMA), Bluetooth, Wireless Fidelity (Wi-Fi) (e.g., IEEE 802.1 1 a, IEEE 802.1 1 b, IEEE 802.1 1 g and/or IEEE 802.1 1η), voice over Internet Protocol (VoIP), Wi-MAX, a protocol for email (e.g., Internet Message Access Protocol (IMAP) and/or Post Office Protocol (POP)), instant messaging (e.g., extensible Messaging and Presence Protocol (XMPP), Session Initiation Protocol for Instant Messaging and Presence Leveraging Extensions (SIMPLE), and/or Instant Messaging and Presence Service (IMPS)), and/or Short Message Service (SMS), or any other suitable communication

protocol. Some wireless network deployments combine networks from multiple cellular networks or use a mix of cellular, Wi-Fi, and satellite communication.

In some implementations, a wireless network may connect to a wired network in order to interface with the Internet, other carrier voice and data networks, business networks, and personal networks. A wired network is typically carried over copper twisted pair, coaxial cable, and/or fiber optic cables. Suitable wired networks include wide area networks (WAN), metropolitan area networks (MAN), local area networks (LAN), Internet area networks (IAN), campus area networks (CAN), global area networks (GAN), like the Internet, and virtual private networks (VPN). As used herein, "network" refers to any combination of wireless, wired, public, and private data networks that are typically interconnected through the Internet and provide a unified networking and information access system.

Processing of spectroscopy data may be performed using the hardware described herein using a wired or wireless communication link with the spectrometers at the site where the sample or patient is located. The communication between the processing system and the spectrometers may or may not be performed in real-time as the spectroscopy data is received or recorded. The processing may be performed in the same housing as the spectrometers, or in a separate housing in the same room or building as the spectrometers. The processing system may also be located in a remote location from the spectrometers (e.g., a different building, city, country).

Figure 5 is a schematic of a spectroscopy processing system 500 according to an embodiment. The processing system 500 may include a controller 502 in communication with one or more spectrometers 506. The controller 502 may include one or more processors 504 and one or more machine-readable memories 508 in communication with the one or more processors 504. The processor 504 may incorporate data received from memory and operator input to control the spectroscopy processing system 500. The inputs to the controller 502 may be received from one or more machine-generated (e.g., spectrometer 506) and/or human-generated, such as user input, sources. The memory 508 may further store instructions to cause the processor 504 to execute modules, processes, and/or functions associated with a processing device, such as the methods described herein. The controller 502 may be connected to the one or more spectrometers 506 by wired or wireless communication channels. The controller 502 may be configured to control one or more components of the spectroscopy processing system 500 including a network interface and/or a user interface.

The controller 502 may be configured to perform processing and/or analysis of spectroscopy data, such as to determine if a pathogen is present in a sample, as described

above. The controller 502 may be configured to import and selectively store data from a spectrometer 506. The processing system 500 may provide centralized data collection and standardized spectroscopy signal processing across a plurality of remote locations. The processing system 500 may also allow an authorized user to access and review patient study results and perform additional analysis. For instance, different levels of patient results may be available to one or more patients, caretakers, healthcare providers, health plans, and authorized internal and/or external users via a web-based interface. Record keeping, security, and consistency may thus be improved when data processing and data storage are centralized at the spectroscopy processing system 500. This may allow trained personnel such as infectious disease specialists or laboratory technicians that manually process and review spectroscopy data to be provided access at a central location, further increasing efficiency and cost savings.

Figure 6 schematically illustrates an architecture of a spectroscopy computing system 600 according to an embodiment. The system 600 may include a local computing network 602 and a remote computing network 620, which may be a cloud-based system. The local network 602 may be local in the sense that one or more users 604, such as patients, technicians, or health care providers, may provide a sample to a spectrometer 606. The spectrometer 606 may be coupled to a control system 608 such as a computer system or computing device. The control system 608 may be located in the same room as the spectrometer 606, in an adjacent or nearby room, or tele-operated from a remote location in a different building, city, or country. In some implementations, a plurality of spectrometers 606 and/or control systems 608 may be provided. The control system 608 may include RF circuitry to communicate with the remote network 620. In some variations, a secure token service 622, such as a security token, and/or a user authentication service 624 may be used to secure communication between the local network 602 and remote network 620 and prevent unauthorized access to collected data. The token service 622 may include cryptographic keys, passwords, digital signatures, or the like. The user authentication service 624 may include, for example, desktop single sign-on (SSO) and username/password verification.

Spectral data may be transmitted to a remote server 626 for processing. For example, server 626 may perform pre-processing, comparative processing, and other processing as described above on spectral data. A server database 628 may store spectral data, control or reference data, or other data. In some variations, the server database 628 may receive processed spectral data from the remote server 626 and may transmit control or reference data to the server 626. The servers 626, 628 may be provided on the same or different networks.

Communication between the servers 626, 628 and the control system 608 may be secured using a unique identifier 610 such as a username/password or biometric authentication. In some implementations, a secure notification service 630 may be used to help ensure communication between the local network 602 and remote network 620 is secure.

Kits

Kits for incubating a sample are disclosed herein. The kits may be designed to test the resistance of a pathogen to different concentrations of a test substance; to test the resistance of a pathogen to different test substances, such as to produce a resistance profile of the pathogen; and/or to test the efficiency of a new test substance.

In some embodiments, a kit includes one or more of at least one vessel, at least one test substance, at least one diluent for the test substances, a growth medium, water, an incubation device, Parafilm for covering the incubation device; and at least one instruction.

The vessel may be a tube such as an Eppendorf tube or volumetric tube.

The test substance may be any drug, such as an antibiotic or antifungal, or other treatment of interest. The test substance may be any antibiotic class, or member of a class, described above. The diluent for the test substance may be any suitable vehicle for the test substance, such as water or growth medium.

The incubation device may be a plate such as a culture plate, which may be a multi-well culture plate.

The instructions may be for preparing a sample, preparing test substances, and/or incubating the samples.

Kits for performing one or more of identifying a sample, preparing a sample for spectroscopy, and measuring a sample may include one or more of an incubator; a centrifuge, which may be a microcentrifuge; a pipette; pipette tips; a spectrometer, which may be one or more of, for example, a UV-VIS spectrometer for measuring optical density, an ATR-FTIR spectrometer, and a Raman spectrometer; a sample substrate, which may be a UV-VIS cuvette, an ATR-FTIR crystal, or a Raman-grade substrate (e.g. CaF_2, Al-coated plastic slide); and a Gram staining kit.

In one embodiment, a kit for determining breakpoint concentration of a test substance is provided. The kit includes at least one multi-well plate. Growth medium may be included in a plurality of the wells of the plate or may be provided separately. A test substance, such as an antibiotic, may be included in the wells, such as at varying concentrations across a row or a column of wells, or the test substance may be provided separately.

Spectral data may be collected from a plurality of the wells directly or from the contents of the wells. A spectrometer for acquiring spectra may be included in the kit or may be provided separately.

In one example, ATR-FTIR spectra from a plurality of the wells of the plate may be acquired by passing each well through the infrared beam of an ATR-FTIR spectrometer. ATR crystal elements may be embedded in the bottoms of the wells.

In another example, spectra from a plurality of the wells of the plate may be acquired by extracting suspended matter from each well and spraying the matter onto an ATR element before exposing the ATR element to a spectrometer. In another example, the suspended matter is sprayed onto a transparent IR substrate before exposing the IR substrate to a spectrometer and acquiring spectra. In further examples, spectra from a plurality of the wells of the plate may be acquired by one or more of transmission, transflection, or reflection spectroscopy, such as by utilizing mirrored or coated wells or substrates. In yet further examples, spectra from a plurality of the wells of the plate may be acquired by NIR spectroscopy. The wells may have an NIR-compatible transparent bottom surface.

The concentration of the test substance in the well from which spectral marker bands are above a given threshold indicates the breakpoint dosage of the test substance.

In one embodiment, a kit for determining the response of a pathogen to more than one test substance is provided. The kit includes at least one multi-well plate. Growth medium may be included in a plurality of the wells of the plate or may be provided separately. At least two test substances, such as two different antibiotics, may be included in the wells, such as at varying concentrations across a row or a column of wells, or the test substances may be provided separately. In one example, a different test substance may be provided in or added to each row of a multi-well plate at a variety of concentrations. Spectra may be collected from the wells or the contents of the wells by any method described for above for the kit for determining breakpoint concentration.

In one embodiment, a kit for determining the response of a particular type of pathogen to a test substance is provided. The kit is as described for any kit above, except that a given multi-well plate is dedicate to a type of pathogen, such as Gram positive bacteria or Gram negative bacteria or fungus.

EXAMPLES

Example 1 – Spectral Markers of *Staphylococcus aureus* Growth

A 600 mL volume of sterile heart infusion medium was prepared in five 2L flasks. Each flask contained a different concentration of oxacillin, which is a β-lactam antibiotic and

an analogue of methicillin: 0 µg/mL (control); 0.25 µg/mL (<minimum inhibitory concentration [MIC]); 0.5 µg/mL (MIC), 2 µg/mL (>MIC), or 8 µg/mL (>>MIC). An overnight culture of a methicillin-sensitive *Staphylococcus aureus* ("MSSA") strain (AP308) or a methicillin-resistant *Staphylococcus aureus* ("MRSA") strain (A8090) was used to inoculate each flask to reach a bacterial density at UV 625 nm (OD_{625}) of 0.2. Cultures were incubated at 37 °C with aeration. Approximately 90 mL of samples were collected from each culture at time points 0 minutes, 30 minutes, 60 minutes, 90 minutes, 120 minutes, 240 minutes, and 360 minutes. 1 mL from each sample was taken to record the optical density (OD_{625}) for growth analysis. The remaining material was subjected to sample preparation, as described in detail in Example 2 below.

The average growth curves are presented in Figure 7A (MSSA) and Figure 7B (MRSA). Each OD_{625} value for each growth curve was obtained from an average of three independent repetitions of the experiment for each of MSSA and MRSA.

The results show that MSSA and MRSA incubated with 0 µg/mL oxacillin (control) went through a lag growth phase during 0 minutes to 120 minutes and entered an exponential (log) growth phase after 2 hours. Oxacillin had a dose-dependent effect on MSSA and MRSA growth. Oxacillin at 2 µg/mL and 8 µg/mL inhibited growth of MSSA cultures by 60 minutes; lower concentrations (0.25 µg/mL and 0.5 µg/mL) inhibited growth by 120 minutes. Oxacillin at 2 µg/mL and 8 µg/mL inhibited growth of MRSA by 120 minutes or earlier. Lower concentrations (0.25 µg/mL and 0.5 µg/mL) had a lesser effect on growth, which started at 120 minutes.

Example 2 – Spectral Characterization of *S. aureus* Growth

Sample Preparation

The remaining culture material from Example 1 was prepared for spectral analysis. For Raman spectroscopy, about 1 mL of the collected material was centrifuged at 10,000 × g for 2 minutes, then washed in sterile, deionized water three times. The final pellet was resuspended to achieve a slightly turbid suspension. 20 µL of the obtained solution of bacteria in water was placed on a Raman grade CaF_2 window, air-dried for about 30 minutes, and measured directly afterwards.

For ATR-FTIR spectroscopy, the remaining volume of each sample was pelleted by centrifugation at 5,000 × g for 5 minutes at 4 °C. The supernatant was then removed and the pellet was resuspended in sterile, deionized water. The procedure was repeated 3 times to ensure complete removal of any residual media. A portion of the final pellet was used directly for ATR-FTIR analysis.

Data Collection

ATR-FTIR spectra were recorded directly after sample preparation using a Bruker Alpha FTIR (Ettlingen, Germany) spectrometer with an Attenuated Total Reflection (ATR) sampling device containing a single bounce diamond internal reflection element (IRE) and equipped with a globar source, KBr beam splitter, and a deuterated triglycine sulfate (DTGS) detector. All spectra were collected in the range of 4000 cm^{-1} – 600 cm^{-1} with spectral resolution of 8 cm^{-1} and 64 scans co-added. Samples were dried directly on the ATR crystal for approximately 3 minutes until no changes were visible in the live view of the spectra. Prior to collecting sample measurements, background measurements were collected using 128 scans. The collection of background was repeated after every 3 individual sample spectra were recorded. For each experimental group, 3 technical replicates were collected ($n_{experiment}$=93 spectra, n_{total}=558 spectra: n_{MSSA}=279 spectra, n_{MRSA}=279 spectra). Data collection was randomized not only on the group level, but also on the technical replicate level, in order to avoid consecutive collection of all technical replicates from the same group.

Raman spectra were recorded directly after sample preparation, using WITec confocal CRM alpha 300 Raman microscope, equipped with an air–cooled solid–state laser operating at 532 nm, a CCD detector cooled to –60 °C, and 600 grooves/mm grating. The laser was coupled to the microscope by an optical fiber with a diameter of 50 µm. For data collection, a dry Olympus MPLAN (100x/0.90NA) objective was used. Each time prior to data collection the monochromator of the spectrometer was calibrated using a Raman scattering line produced by a silicon plate (520.5 cm^{-1}). Spectra were recorded with spectral resolution of 3 cm^{-1} in the spectral range of -44 cm^{-1} to 3700 cm^{-1}, with the integration time of 1 second and using 50 accumulations. For each experimental group in each experiment, 9 individual spectra were recorded ($n_{experiment}$=279 spectra, n_{total}=1674 spectra: n_{MSSA}=837 spectra, n_{MRSA}=837 spectra).

Data Analysis

MATLAB 8.6 2015b (Mathworks, Natick, USA), PLS toolbox v8.2 (Eigenvector research, Manson, USA), Witec Project Plus, and Origin Pro 9.1 were used for data pre-processing, analysis, and presentation. ATR-FTIR spectra were pre-processed using second derivative (Savitzky-Golay algorithm, 15 smoothing points) and Standard Normal Variate (SNV). Raman spectra prior to analysis were subjected to comic spike removal (CRR). Subsequently, they were pre-processed using second derivative (Savitzky-Golay algorithm, 17 smoothing points) and SNV. Spectra of all collected standards were pre-processed in the same manner.

Presented average spectra of bacterial pellets were obtained by a two-step process. Firstly, technical replicates (n_{ATR} = 3, n_{Raman}= 9) were averaged to obtain spectra representative for each biological replicate. Secondly, the spectra of biological replicates were averaged. The patterns of spectral behaviors and integral area of bands (see Example 3 below and Figures 10A and 10B) were calculated in MATLAB, using 2nd derivatives. For the MSSA and MRSA drug exposure, datasets of ATR-FTIR and Raman spectra were analyzed independently and in combination (multimodal analysis). Analysis was performed within each time point (0, 30, 60, 90, 120, 240, and 360 minutes) and for each concentration over time. For multimodal analysis, the number of Raman spectra for each sample was reduced by a factor of 3 through averaging in order to have the same number of ATR-FTIR and Raman spectra per sample. Multimodal Principal Component Analysis (PCA) was performed on combined datasets using spectral ranges 1800 cm^{-1} – 900 cm^{-1} (ATR-FTIR) and 1800 cm^{-1} – 600 cm^{-1} (Raman). In addition to described pre-processing, data was mean centered prior to PCA.

Results

The 2nd derivatives of the average ATR-FTIR or Raman spectra obtained from MSSA incubated without oxacillin for 0, 30, 60, 90, 120, 240, and 360 minutes are presented in Figures 8A-8H (ATR-FTIR) and 9A-9H (Raman). Arrows in Figures 8A and 9A indicate distinctive bands between spectra of bacteria from lag and log phase. The star indicates a band distinctive for the beginning of the experiment.

Figures 8A-8H and 9A-9H show that the spectra are highly reproducible and consistent across replicates of the experiment.

The results indicate that different growth phases can be reflected in spectral differences. The growth curves (Figures 7A and 7B) revealed that samples collected after 240 minutes and 360 minutes of incubation contained bacteria in the log phase, whereas samples collected in the prior time points include bacteria at the beginning (0 minutes) or during the lag phase (up to 240 minutes) of growth.

The 2nd derivatives of the spectra at the beginning of the experiment and after 240 minutes and 360 minutes are similar to each other and are different from the remaining 2nd derivatives (after 30, 60, 90, and 120 minutes). Examples of this include the ATR-FTIR bands at 1394 cm^{-1} ($\delta(CH_3)$) and 1215 cm^{-1} ($\nu_{as}PO_2^-$) (Figure 8A) and the Raman bands at 1453 cm^{-1} ($\delta(CH_3$ & $CH_2)$) and 1480 cm^{-1} (Figure 9A). The Raman band at 1480 cm^{-1}, related to nucleic acids, is almost invisible in spectra of bacteria in log phase but more pronounced in spectra of bacteria from the lag phase (Figures 9A-9H). The ATR-FTIR bands

at 1215 and 965 cm^{-1} are also assigned to nucleic acids, and Figures 8A-8H reveal a decrease in relative nucleic acid content in bacteria in log phase compared to bacteria in lag phase.

Although data at 0, 240, and 360 minutes is similar, the 2nd derivatives of spectra recorded at the beginning of incubation (0 minutes) remain distinguishable (e.g. through the ATR-FTIR band at 1335 cm^{-1}) from other time points.

Example 3 – Spectral Patterns of Chemical Changes in *S. aureus* During Growth

The ATR-FTIR and Raman spectra of untreated MSSA collected in Example 2 were further analyzed for patterns of changes. Several bands showing notable variability were selected and changes in their intensity relative to amide I intensity, over the course of the experiment, are presented in Figures 10A (ATR-FTIR) and 10B (Raman). Statistical significance is indicated by "***" for $p < 0.01$ and by "**" for $p < 0.05$.

The results show that several Raman markers (Figure 10B) were attributable to nucleic acids (i.e., 1480 cm^{-1}, 1244 cm^{-1}, and 784 cm^{-1}). Bands at these wavenumbers were increased through the lag phase of growth compared to log phase. These changes were accompanied by a decrease in relative intensity of the band at 1453 cm^{-1}. Within the log phase, the nucleic acid markers were more pronounced after 90 minutes (1480 cm^{-1}) or after 60 and 90 minutes (1244 cm^{-1} and 784 cm^{-1}).

The results show that ATR-FTIR markers (Figure 10A) were also predominately associated with nucleic acids (i.e., 1215 cm^{-1}, 1082 cm^{-1}, and 965 cm^{-1}). The band intensities were higher in the lag phase of growth than in the log phase, and one of them (i.e., 1082 cm^{-1}) showed a distinct increase within the lag phase after 90 minutes and 120 minutes. A substantial increase is also seen for the band located at 1035 cm^{-1}, but at earlier time points (30 minutes and 60 minutes). This 1035 cm^{-1} band can be assigned to carbohydrates and/or nucleic acids. In this case, the pattern of behavior does not correspond to the observed pattern of changes in nucleic acids. Instead, 1035 cm^{-1} band resembles the pattern of changes observed for the 1045 cm^{-1} Raman band, which is assigned to carbohydrates and/or proteins.

The intra-lag phase spectral changes were pronounced and consistent between replicates. Multimodal PCA enabled discrimination between individual time points (data not shown).

The spectral characterization of MRSA growth in the absence of oxacillin (data not shown) resembles the spectral characterization for MSSA shown in Figures 8A-10B. The spectral characterization shown in Figures 8A-10B represents changes in chemical composition related to undisturbed growth of *S. aureus*, which occurs in a similar manner, under the same growth conditions, regardless of the resistance profile.

Example 4 – Spectral Signatures of Chemical Changes Following Exposure of Methicillin-sensitive *S. aureus* to Oxacillin for 120 Minutes

Results of multimodal PCA performed on combined ATR-FTIR and Raman data, collected as described in Example 2, are presented in Figures 11A-E. Similar results are presented in Figure 13A, which was derived from five strains of MSSA. Figures 11A and 13A are scores plots. Figures 11B and 11D show portions of the ATR-FTIR spectra, and Figures 11C and 11E show portions of the Raman spectra, obtained from loading 1 (Figures 11B, 11C) and loading 2 (11D, 11E) with prominent bands marked. The Figures demonstrate a clear discrimination between spectra of bacteria exposed to oxacillin and bacteria not exposed oxacillin, occurring along PC1. Specifically, Figure 11A shows a threshold PC1 score greater than -3.5 for MSSA cells treated with oxacillin; PC1 > -2.0, -1.5, -1.0, -0.8 and PC2 > -3.0, -1.5, -2.0, -1.5, -1.0, -0.5, 0, 0.1 for cells treated with 0.25 μg/mL or 0.5 μg/mL oxacillin. With reference to Figure 13A, PC1 < 0, -0.2, -0.4, -0.6, -0.8, -1.0, -1.2, -1.4 for MSSA cells treated with oxacillin. The discriminatory bands can be generally associated with three major groups of components, each described below.

First, multimodal PCA loadings (Figures 11B and 11C) demonstrate a higher relative content of nucleic acids in the untreated (control) bacteria, compared to the treated ones, as indicated by the following bands: 1710, 1215, 1084, 1051, and 965 cm^{-1} (ATR-FTIR, Figure 11B) and 785 cm^{-1} (Raman, Figure 11C).

Second, the control group demonstrates (via PC1) a higher relative amount of β-sheet proteins than all treated groups, as indicated by an amide I band at 1634 cm^{-1} (Figure 11B). Amide I position is indicative of the secondary structure of proteins, with its maximum at approximately 1656 cm^{-1} for α-helix structures and at approximately 1635 cm^{-1} for β-sheet structures.

Third, another difference in chemical composition between control and treated bacteria is visible in the ATR-FTIR spectra at 1029 cm^{-1}. Spectra of control bacteria exhibited a low intensity band at 1035 cm^{-1}. For all treated bacteria, this band shifted to 1029 cm^{-1} and became more pronounced. The 1029 cm^{-1} band may be associated with intact bacteria, rather than cell wall fragments. Also, the appearance and intensity of the 1029 cm^{-1} band in spectra of treated bacteria shows no correlation to, for example, the band at 1624 cm^{-1}, which is a marker of fully formed peptidoglycan (PG) found in intact cell walls. Because the 1029 cm^{-1} band is associated with intact bacteria and the pellets collected for bacteria exposed to a high concentration of oxacillin are made up only partially from intact bacteria, the observed decrease of intensity of the band at 1029 cm^{-1} relative to spectra of pellets

collected from bacteria exposed to a low dose of oxacillin may result from lower absolute content of intact organisms. The change in the 1029 cm^{-1} band may not reflect a reduced amount of the compound assigned to the 1029 cm^{-1} band within intact organisms. A continuous shift from 1035 cm^{-1} to 1029 cm^{-1} with time was also observed (data not shown).

All groups for which a shift and increase of the intensity of the band at 1029 cm^{-1} was observed exhibited increased thickness in the layer external to cell membrane. In undisturbed bacteria, this layer is occupied primarily by cell wall, containing predominantly fully formed PG. The major marker band of fully formed PG is located at 1622 cm^{-1} and is accompanied by a band located at 1515 cm^{-1}, but no band at 1029 cm^{-1}. In addition, the observed increased intensity of the band at 1029 cm^{-1} in bacteria exposed to oxacillin at low concentrations was not associated with the presence or increased intensity of marker bands of fully formed PG (1622 cm^{-1} and 1515 cm^{-1}). The band at 1029 cm^{-1} may not represent increased content of fully formed PG. Increased thickness of the layer external to the cytoplasmic membrane in drug-exposed MSSA may not be associated with increased content of fully formed PG.

Example 5 – Effects of Oxacillin Exposure on Methicillin-resistant *S. aureus*

Spectra were collected from the MRSA cultures of Example 1 (at 0, 30, 60, and 120 minutes and all oxacillin concentrations) according to the methods of Example 2, including multimodal PCA. Spectra were similarly collected from five strains of MRSA and processed.

PCA scores plots are presented in Figure 12A. Portions of the ATR-FTIR (Figure 12B) and Raman (Figure 12C) spectra obtained from PC1 loading are also shown, with prominent bands marked.

The results show that no discrimination between groups is visible after 120 minutes. Oxacillin treatment, even at 8 μg/mL, did not have an effect on spectral data collected from MRSA cultured for up to 120 minutes.

Example 6 – Further Testing of MSSA and MRSA to Oxacillin Exposure

Five strains each of MSSA (AP308, AP309, AP310, AP311, and AP312) and MRSA (A8090, A8819, A6300, A9719, and A224) were tested. Each strain was tested in triplicate such that n=30 for the total number of independent repetitions of the experiment. In each experiment, the same strain was incubated for up to 120 minutes without (0 μg/mL; "control") and with oxacillin, in two concentrations: minimal inhibitory concentration ("MIC"; 0.5 mg/L) and "cut-off" concentration (i.e., the drug concentration considered as the clinical breakpoint, used to define the resistance to β-lactam antibiotics in *S. aureus*; 4 mg/L).

PCA results are presented in Figures 15A and 15B for the five MSSA strains after 120 minutes of control or oxacillin treatment, in Figures 16A and 16C after 90 minutes, and in

Figures 16B and 16D after 60 minutes. Figures 15A, 16A, and 16B are scores plots. Figures 15B, 16C, and 16D show portions of the ATR-FTIR spectra with prominent bands marked. Scores plots are also presented in Figures 17A-C, which were derived from five strains of MRSA after 120 minutes (17A), 90 minutes (17B), or 60 minutes (17C) of control or oxacillin treatment.

The scores plots (Figures 15A, 16A, and 16B) demonstrate a clear, dose-dependent discrimination between spectra of MSSA strains exposed to oxacillin and MSSA strains not exposed to oxacillin, occurring along PC1, where PC1 < 2.25 for MSSA strains exposed to 0.5 mg/L oxacillin for 120 minutes or PC1 < 2.25, 2.0, 1.5, 1.0, 0.5, or 0 for MSSA strains exposed to 4 mg/L oxacillin for 120 minutes. The discrimination is in part driven changes in by peptidoglycan bands (1624 and 1515 cm^{-1}), as shown in Figures 15B, 16C, and 16D. No such discrimination between drug and control treatments was observed for MRSA strains, as shown in Figures 17A-17C.

Example 7 – Effects of External Stress Factor on Positive Drug Response

To test whether an external stress factor (e.g. centrifugation) could enhance the detectable response of drug-susceptible cells to drug exposure, an MSSA strain was exposed to oxacillin (MIC) or not exposed (control) and probed every 30 minutes for 120 minutes. Each bacterial pellet collected was divided into two portions: one subjected to a delicate centrifugation protocol of 15,000 g for 2 minutes 3 times and the other for an intense centrifugation protocol of 15,000 g for 2 minutes 6 times. Spectra were then obtained and compared.

Results are shown in Figure 18 for MSSA cells exposed to oxacillin (MIC) for 120 minutes. Figure 18A is a scores plot that shows a clear discrimination between cells subjected to the delicate ("three washes") and the intense ("six washes") stressor protocols. Figure 18B is a PCA loadings plot (IR spectra) demonstrating that cells subjected to intense centrifugation show a substantially higher intensity of the band at 1622 cm^{-1} (peptidoglycan) than cells subjected to delicate centrifugation. This demonstrates that susceptible bacteria weakened by exposure to a drug to which they are susceptible lose integrity upon exposure to an external stress factor. Loss of integrity, manifested as cell lysing, produces detectable peptidoglycan. The external stress factor thereby enhances the detectability of the cells' drug response.

No differences in control (cells unexposed to oxacillin) groups were observed (data not shown). Cells unexposed to or unaffected by a drug can maintain their integrity following exposure to an external stress factor.

Example 8 – Predictive Models

Five strains each of MSSA and MRSA were tested in triplicate such that n=30 for the total number of independent repetitions of the experiment. In each experiment, the same strain was incubated for 60 minutes without oxacillin (0 μg/mL; "control") and with oxacillin at 4 μg/mL. After post-incubation processing (i.e., centrifugation) as described in Example 7, ATR spectra were collected.

Data pre-processing was performed, and the spectral characteristic of the drug response were extracted. The spectral characteristic may include a plurality of marker bands indicative of a positive or a negative drug response. The dataset was then divided into a Calibration set (66% of spectra) and a Validation set.

The spectral characteristic of the positive and negative drug response in the Calibration set was used to build predictive models *via* each of PLS-DA and SVM-C. The models were subsequently applied to the Validation set to predict drug susceptibility (i.e., MSSA or MRSA group membership).

The results of the modeling and its validation for prediction of susceptibility towards β-lactam antibiotics for *S. aureus* (MSSA or MRSA) are presented in Figures 19 (SVM-C model) and 20 (PLS-DA) model for the Calibration set (19A-C and 20A-C) and the Validation set (19D-F and 20D-F). Group membership for the Calibration set is shown in Figures 19A and 20A. Prediction of group membership (MSSA/MRSA) based on the model for the Validation set is provided in Figures 19D and 20D, with 1 corresponding to MSSA and 2 corresponding to MRSA. The legend (light circles for MSSA; dark squares for MRSA) is based on susceptibility profiles determine via AST (the current standard of care). Figures 19B and 20B show the probability of each sample in the Calibration set being MSSA and Figures 19E and 20E show the same for the Validation set. Figures 19C and 20C show the probability of each sample in the Calibration set being MRSA and Figures 19F and 20F show the same for the Validation set. In both models, all samples were classified correctly, with high probabilities.

Both models were subjected to independent double-blind testing. Four unknown strains of *S. aureus* were tested after 60 min. of incubation with oxacillin. Their susceptibility profiles were determined using the SVM-C and PLS-DA models. Results are presented in Figure 21, which shows that both models correctly predicted blind sample strains 1 and 4 as MSSA and blind sample strains 2 and 3 as MRSA. The probabilities for each sample strain are presented in Table 1.

Table 1

	Probabilities of prediction for double blind test					
	PLS-DA			SVM-C		
	Predicted group membership	MSSA [%]	MRSA [%]	Predicted group membership	MSSA [%]	MRSA [%]
Sample 1	MSSA	100	5.39E-11	MSSA	95.91836	4.081644
Sample 2	MRSA	5.36E-10	100	MRSA	2.320713	97.67929
Sample 3	MRSA	1.33E-10	100	MRSA	1.919856	98.08014
Sample 4	MSSA	100	8.42E-14	MSSA	95.91836	4.081644

Example 9 – Application of Models to *S. aureus* Exposed to Vancomycin

Vancomycin sensitive *S. aureus* (VSSA) (strain A8090) and vancomycin intermediate-resistant *S. aureus* (VISA) (strain A8094) were subjected to testing as described above. Briefly, each strain was incubated with and without vancomycin (probing points: 60 and 120 min; drug concentration: 4 µg/mL). Subsequently, PCA was conducted for VSSA, comparing the drug-exposed to the control group, and for VISA, again comparing the drug-exposed to the control group.

The outcomes of the analyses for VSSA and VISA incubated for 120 minutes are shown in Figures 22 and 23, respectively. A clear discrimination in PCA scores plots is visible between drug-exposed and control VSSA (Figures 22A and 22B), driven mainly by changes in peptidoglycan bands observed in PCA loadings plots. In scores plots, PC1 < 1, 0.5, 0, or -0.25 for drug-exposed VSSA. No such discrimination was observed for VISA in PCA scores plots (Figures 23A and 23B).

Because the main markers used for discrimination between MSSA and MRSA were also present in the analysis of VSSA and VISA, the models constructed for MSSA and MRSA (Example 8) could be used to predict vancomycin resistance. The outcomes of the prediction for the 120-minute time point and each model are presented in Figure 24. The models were successfully employed to determine vancomycin resistance in *S. aureus*.

Without being limited to any mechanism or mode of action, the similarity of markers of effective drug action for oxacillin/amoxicillin (β-lactam antibiotics) and vancomycin (a glycopeptide antibiotic) may be attributable to the drugs shared bactericidal activity, despite the different modes of action of each drug.

Example 10 – Application of Models to *S. aureus* Exposed to Clindamycin

The approach described in Example 9 was adapted to clindamycin susceptible and resistant *S. aureus* (susceptible strain: AH19I050; resistant strain: AH19F068). Both strains were incubated with and without clindamycin (drug concentration: 2 mg/L) for 120 minutes.

PCA of the clindamycin susceptible strain is shown in Figures 25A and 25B. A clear discrimination between treated and control cells was observed in PCA scores plots (PC1 < 2.5, 2.0, 1.0, 0.5, 0, -0.5, -1.0, -1.5, -2.0, or -2.5 for treated cells). Such discrimination demonstrates the presence of molecular changes marking a positive drug response. For the clindamycin resistant strain, no discrimination was observed in PCA scores plots (Figures 26A and 26B).

The changes between the drug-exposed and control group for the clindamycin sensitive strain (Figures 25A and 25B), although clear, were not the same as those observed following treatment with a β-lactam antibiotic (Figures 15A-16D) or vancomycin (Figures 22A and 22B). Without being limited to any mechanism or mode of action, the differences may be attributable to the bacteriostatic—rather than bactericidal—effect of clindamycin at the tested concentration. Clindamycin may not cause cell damage, as indicated by the less pronounced changes in peptidoglycan bands in ATR-FTIR spectra over the same time course compared to treatment with oxacillin or vancomycin.

As the spectral markers of effective clindamycin action on a sensitive *S. aureus* strain were different from the ones for oxacillin and vancomycin, the models of Example 8 were not tested here. However, the sharp distinction between drug-exposed and control groups observed for the sensitive strain, and the lack of discrimination between groups in the resistant strain, clearly demonstrated that spectral markers of a positive/negative drug response are observable.

Example 11 – Effects of Vancomycin Exposure on *E. faecium*

The approach described in Example 9 was adapted to vancomycin susceptible and resistant *E. faecium* (susceptible strain: AH19D045; resistant strain: AH17B030). Both strains were incubated with and without vancomycin (drug concentration: 4 mg/L) for 60 minutes.

PCA of the vancomycin susceptible strain is shown in Figures 27A and 27B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 > 0, 0.25, 0.5, or 0.75 for treated cells). Such discrimination demonstrates the presence of molecular changes marking a positive drug response, including the presence of peptidoglycan bands as well as other marker bands (e.g., 921 and 1113 cm^{-1}). For the vancomycin resistant strain, no discrimination was observed in PCA scores plots (Figures 28A and 28B).

Example 12 – Effects of Amoxicillin Exposure on *E. faecium*

Amoxicillin susceptible and resistant *E. faecium* (susceptible strain: AH19J037; resistant strain: AH19D045) were incubated with and without amoxicillin (drug concentration: 4 mg/mL) for 120 minutes.

PCA of the amoxicillin susceptible strain is shown in Figures 29A and 29B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 0.5, 0, -0.5, -1.0, or -1.5 for treated cells). Such discrimination demonstrates the presence of molecular changes marking a positive drug response, including the presence of peptidoglycan bands (1624 cm^{-1}) and other marker bands (963, 1246, 1398, 1640, 1724 cm^{-1}). For the amoxicillin resistant strain, no discrimination was observed in PCA scores plots (Figures 30A and 30B).

Example 13 – Effects of Ciprofloxacin Exposure on *E. coli*

Ciprofloxacin susceptible and resistant *E. coli* (susceptible strain: AH19I003; resistant strain: AH19H037) were incubated with (1 mg/L) and without ciprofloxacin for 120 minutes. PCA of the ciprofloxacin susceptible strain is shown in Figures 31A and 31B. A clear discrimination between treated and control cells is observable in PCA scores plots after 120 minutes of drug exposure (PC1 > -1.5, 1.0, 0.5, 0. 0.5, 1.0, 1.5, 2.0, or 2.25 for treated cells), and was observable at least by 60 minutes of drug exposure (data not shown). Such discrimination demonstrates the presence of molecular changes indicating a positive drug response, including the presence of peptidoglycan bands in PCA loadings plots. For the ciprofloxacin resistant strain, no discrimination was observed in PCA loadings plots (Figures 32A and 32B).

Example 14 – Effects of Gentamicin Exposure on *E. coli*

Gentamicin susceptible and resistant *E. coli* were incubated with (16 mg/L) and without gentamicin for 120 minutes. PCA of the gentamicin susceptible strain is shown in Figures 33A and 33B. A clear discrimination between treated and control cells is observable in PCA scores plots after 120 minutes of drug exposure (PC1 < 0.5, 0, -0.5, -0.75, -1.0, or -1.25 for treated cells), and was observable at least by 60 minutes of drug exposure (data not shown). For the gentamicin resistant strain, no discrimination was observed (Figures 34A and 34B).

Example 15 – Effects of Ceftriaxone Exposure on *E. coli*

Ceftriaxone susceptible and resistant *E. coli* (susceptible strain: AH19I003; resistant strain: AH19H037) were incubated with (1 mg/mL) and without ceftriaxone for 120 minutes. PCA of the ceftriaxone susceptible strain is shown in Figures 35A and 35B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 0, -

0.25, or -0.50 for treated cells). Such discrimination demonstrates the presence of molecular changes indicative of a positive drug response, including the presence of peptidoglycan bands (1622 and 1515 cm^{-1}) and other marker bands (960, 980, 1006, 1057, 1092, 1540, and 1640 cm^{-1}) in PCA loadings plots. For the ceftriaxone resistant strain, no discrimination was observed in PCA scores plots (Figures 36A and 36B).

Example 16 – Effects of Ceftriaxone Exposure on *P. aeruginosa*

Ceftriaxone susceptible (strain E-009) and resistant (strain E-0023) *P. aeruginosa* were incubated with (4 mg/L) and without ceftriaxone for 120 minutes. PCA of the ceftriaxone susceptible strain is shown in Figures 37A and 37B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 0.5, 0, -0.25, or -0.5 for treated cells). For the ceftriaxone resistant strain, no discrimination was observed in PCA scores plots (Figures 38A and 38B).

Example 17 – Effects of Ciprofloxacin Exposure on *P. aeruginosa*

Ciprofloxacin susceptible (strain E-009) and resistant (strain E-0023) *P. aeruginosa* were incubated with (0.5 mg/L) and without ciprofloxacin for 120 minutes. PCA of the ciprofloxacin susceptible strain is shown in Figures 39A and 39B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 2, 1.5, 0.5, 0, -0.25, -0.5, -0.75, or -1.0 for treated cells). For the ciprofloxacin resistant strain, no discrimination was observed in PCA scores plots (Figures 40A and 40B).

Example 18 – Effects of Gentamicin Exposure on *P. aeruginosa*

Gentamicin susceptible (strain E-009) and resistant (strain E-0023) *P. aeruginosa* were incubated with (2 mg/L) and without gentamicin for 120 minutes. PCA of the gentamicin susceptible strain is shown in Figures 41A and 41B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 0 for treated cells). For the gentamicin resistant strain, no discrimination was observed in PCA scores plots (Figures 42A and 42B).

Example 19 – Effects of Amoxicillin Exposure on *S. salivarious*

Amoxicillin susceptible (strain AH19D090) and resistant (strain AH19JO46) *S. salivarious* were incubated with (8 mg/L) and without amoxicillin for 120 minutes. PCA of the amoxicillin susceptible strain is shown in Figures 43A and 43B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 1.0. 0.5, 0, -0.5, -1.0, -1.5, or -2.0 for treated cells). Such discrimination demonstrates the presence of molecular changes indicative of a positive drug response, including the presence of peptidoglycan bands (1624 cm^{-1}) and other marker bands (1022, 1064, 1092, 1394, and 1638

cm^{-1}) in PCA loadings plots. For the amoxicillin resistant strain, no discrimination was observed in PCA scores plots (Figures 44A and 44B).

Example 20 – Effects of Vancomycin Exposure on *S. salivarious*

Vancomycin susceptible (strain AH19D090) and resistant (strain AH19JO46) *S. salivarious* were incubated with (2 mg/L) and without vancomycin for 120 minutes. PCA of the vancomycin susceptible strain is shown in Figures 45A and 45B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 1.0, 0.5, 0. -0.25, -0.5, -0.75, or -1.0 for treated cells). The discriminatory bands include peptidoglycan bands (1622 cm^{-1}) as well as other marker bands (e.g. 1021, 1061, 1078, 1394, and 1640 cm^{-1}) in PCA loadings plots. For the vancomycin resistant strain, no discrimination was observed in PCA scores plots (Figures 46A and 46B).

Example 21 – Effects of Ceftriaxone Exposure on *S. salivarious*

Ceftriaxone susceptible (strain AH19D090) and resistant (strain AH19JO46) *S. salivarious* were incubated with (4 mg/L) and without ceftriaxone for 120 minutes. PCA of the ceftriaxone susceptible strain is shown in Figures 47A and 47B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 1.5, 1.0, 0.5, 0, -0.25, -0.5, -0.75, -1.0, -1.25, -1.5, -1.75, or -2.0 for treated cells). The discriminatory bands include peptidoglycan bands (1622 cm^{-1}) as well as other marker bands (e.g., 1022, 1063, 1154, 1396, and 1638 cm^{-1}) in PCA loadings plots. For the ceftriaxone resistant strain, no discrimination was observed in PCA scores plots (Figures 48A and 48B).

Example 22 – Effects of Ceftriaxone Exposure on *K. pneumonia*

Ceftriaxone susceptible (strain AH19I055) and resistant (strain 2017-A-0001) *K. pneumonia* were incubated with (4 mg/L) and without ceftriaxone for 120 minutes. PCA of the ceftriaxone susceptible strain is shown in Figures 49A and 49B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 2.0, 1.5, 1.0, 0.5, 0, -0.25, -0.5, -0.75, or -1.0 for treated cells), driven by changes in bands at 967, 1022, 1082, 1640, and 1655 cm^{-1}, seen in PCA loadings plots. For the ceftriaxone resistant strain, no discrimination was observed in PCA scores plots (Figures 50A and 50B).

Example 23 – Effects of Ciprofloxacin Exposure on *K. pneumonia*

Ciprofloxacin susceptible (strain AH19I055) and resistant (strain 2017-A-0001) *K. pneumonia* were incubated with (1 mg/L) and without ciprofloxacin for 120 minutes. PCA of the ciprofloxacin susceptible strain is shown in Figures 51A and 51B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 0.5, 0, -0.25, -0.5, or -0.75 for treated cells), driven by changes in bands at 1022, 1063, 1078, and 1154 cm^{-1},

observed in PCA loadings plots. For the ciprofloxacin resistant strain, no discrimination was observed in PCA scores plots (Figures 52A and 52B).

Example 24 – Effects of Gentamicin Exposure on *K. pneumonia*

Gentamicin susceptible (strain AH19I055) and resistant (strain 2017-A-0001) *K. pneumonia* were incubated with (16 mg/L) and without gentamicin for 60 minutes. PCA of the gentamicin susceptible strain is shown in Figures 53A and 53B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 0.5, 0, -0.25, -0.5, -0.75, or -1.0 for treated cells), driven by changes in bands at 964, 989, 1548, 1624, and 1655 cm^{-1}, observed in PCA loadings plots. For the gentamicin resistant strain, no discrimination was observed in PCA scores plots (Figures 54A and 54B).

Example 25 – Effects of Ceftriaxone Exposure on *A. baumannii*

Ceftriaxone susceptible (strain AH19E021) and resistant (strain AYP-A2) *A. baumannii* were incubated with (64 mg/L) and without ceftriaxone for 60 minutes. PCA of the ceftriaxone susceptible strain is shown in Figures 55A and 55B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < -0.1 or -0.2 for treated cells), driven by changes in the peptidoglycan band (at 1624 cm^{-1}) and other marker bands (1640 and 1655 cm^{-1}) observed in PCA loadings plots. For the ceftriaxone resistant strain, no discrimination was observed in PCA scores plots (Figures 56A and 56B).

Example 26 – Effects of Ciprofloxacin Exposure on *A. baumannii*

Ciprofloxacin susceptible (strain AH19E021) and resistant (strain AYP-A2) *A. baumannii* were incubated with (1 mg/L) and without ciprofloxacin for 60 minutes. PCA of the ciprofloxacin susceptible strain is shown in Figures 57A and 57B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 0.5, 0, -0.1, -0.2, or -0.3 for treated cells), driven by changes in the peptidoglycan band (at 1624 cm^{-1}) and other marker bands (1640 and 1655 cm^{-1}) observed in PCA loadings plots. For the ciprofloxacin resistant strain, no discrimination was observed in PCA scores plots (Figures 58A and 58B).

Example 27 – Effects of Gentamicin Exposure on *A. baumannii*

Gentamicin susceptible (strain AH19E021) and resistant (strain AYP-A2) *A. baumannii* were incubated with (4 mg/L) and without gentamicin for 60 minutes. PCA of the gentamicin susceptible strain is shown in Figures 59A and 59B. A clear discrimination between treated and control cells is observable in PCA scores plots (PC1 < 2.0, 1.5, 1.0, 0.5, 0, -0.5, -1.0, or -1.5 for treated cells), driven by changes in bands at 969, 988, 1004, 1028,

1057, 1088, 1375, and 1396 cm^{-1}, observed in PCA loadings plots. For the gentamicin resistant strain, no discrimination was observed in PCA scores plots (Figures 60A and 60B).

Although various representative embodiments and implementations have been described above with a certain degree of particularity, those skilled in the art could make numerous alterations to the disclosed embodiments without departing from the spirit or scope of the inventive subject matter set forth in the specification and claims. In some instances, in methodologies directly or indirectly set forth herein, various steps and operations are described in one possible order of operation, but those skilled in the art will recognize that steps and operations may be rearranged, replaced, or eliminated without necessarily departing from the spirit and scope of the present disclosure. It is intended that all matter contained in the above description or shown in the accompanying drawings shall be interpreted as illustrative only and not limiting. Changes in detail or structure may be made without departing from the spirit of the disclosure as defined in the appended claims.

Although the present disclosure has been described with reference to preferred embodiments, persons skilled in the art will recognize that changes may be made in form and detail without departing from the spirit and scope of the disclosure.

CLAIMS

1. A method of determining if a pathogen is susceptible or resistant to a treatment, the method comprising:

providing a sample including the pathogen;

identifying the pathogen in the sample;

culturing the sample in each of the presence of the treatment and the absence of the treatment;

preparing the cultured samples for spectral analysis;

measuring spectral data of the sample cultured in each of the presence of the treatment and the absence of the treatment;

analyzing the spectral data by comparing the spectral data of the sample cultured in the presence of the treatment to the spectral data of the sample cultured in the absence of the treatment to determine if at least one difference in the spectral data is present; and

supplying a result that the pathogen is susceptible to the treatment or resistant to the treatment.

2. The method of claim 1, wherein the sample is from a bodily fluid, a bodily tissue, a liquid consumable, a solid consumable, or a water source..

3. The method of claim 1, wherein providing a sample includes centrifuging the sample, removing the supernatant from the sample, rinsing the sample, and mixing the sample.

4. The method of claim 1, wherein identifying the pathogen includes determining whether the pathogen is a bacterium or a fungus.

5. The method of claim 1, wherein the treatment is an antibiotic or an antifungal.

6. The method of claim 1, wherein the sample is cultured for about 15 minutes to about 120 minutes.

7. The method of claim 1, wherein the sample is cultured in the presence of the treatment at a plurality of treatment concentrations.

8. The method of claim 1, wherein preparing the sample for spectral analysis includes centrifuging the sample, removing the supernatant from the sample, rinsing the sample, and mixing the sample.

9. The method of claim 1, wherein spectral data is measured by infrared-attenuated total reflection (IR-ATR) spectroscopy, Fourier-transform infrared-attenuated total reflection (ATR-FTIR) spectroscopy, transmission spectroscopy, transflection spectroscopy, reflectance spectroscopy, near infrared (NIR) spectroscopy, far infrared (FIR) spectroscopy, or Raman spectroscopy.

10. The method of claim 1, wherein the analyzing is performed by a cloud-based system.

11. The method of claim 1, wherein the differences in spectral data are distances, compared to a distance to a model center or cluster centroid, between the spectral data of the sample cultured in the presence of the treatment and the spectral data of the sample cultured in the absence of the treatment.

12. The method of claim 1, wherein the presence of at least one difference between the spectral data of the sample cultured in the presence of the treatment and the spectral data of the sample cultured in the absence of the treatment indicates a treatment-susceptible pathogen.

13. The method of claim 1, wherein the absence of a difference between the spectral data of the sample cultured in the presence of the treatment and the spectral data of the sample cultured in the absence of the treatment indicates a treatment-resistant pathogen.

14. The method of claim 1, comprising stressing the sample before preparing the cultured sample for spectral analysis.

15. The method of claim 14, wherein stressing is at least one of centrifuging, heating, cooling, increasing pressure, decreasing pressure, and exposing to UV light.

16. A method of determining if a pathogen is susceptible or resistant to a treatment, the method comprising:

culturing the pathogen in the presence of the treatment and in the absence of the treatment;

collecting a plurality of spectral data from the cultured pathogens; and

transmitting the plurality of spectral data to a processor configured to:

process the plurality of spectral data from the pathogen cultured in the absence of the treatment to produce a control average spectra,

process the plurality of spectral data from the pathogen cultured in the presence of the treatment to produce a test average spectra,

compare the test average spectra to the control average spectra to determine if at least one difference between the test average spectra and the control average spectra is present, and

generate a result that the pathogen is susceptible to the treatment or resistant to the treatment.

17. The method of claim 16, wherein the spectral data are processed in wavenumber regions associated with disturbances in nucleic acids, amino acids, proteins, carbohydrates, or a combination thereof.

18. The method of claim 16, wherein the spectral data are collected by ATR-FTIR spectroscopy, transmission spectroscopy, transflection spectroscopy, or reflection spectroscopy and the spectral data are processed in wavenumber regions of about 3100 cm^{-1} to about 2800 cm^{-1}, about 1750 cm^{-1} to about 1500 cm^{-1}, about 1450 cm^{-1} to about 1350 cm^{-1}, about 1300 cm^{-1} to about 1200 cm^{-1}, about 1100 cm^{-1} to about 900 cm^{-1}, or a combination thereof.

19. The method of claim 16, wherein the spectral data are collected by Raman spectroscopy and the spectral data are processed in wavenumber regions of about 3100 cm^{-1} to about 2800 cm^{-1}, about 1750 cm^{-1} to about 1500 cm^{-1}, about 1450 cm^{-1} to about 1350 cm^{-1}, about 1300 cm^{-1} to about 1200 cm^{-1}, about 1100 cm^{-1} to about 600 cm^{-1}, or a combination thereof.

20. The method of claim 16, wherein a spectral data threshold is calculated and a distance or spectral change above the threshold indicates a difference in biochemical composition

between the pathogen cultured in the presence of the treatment and the same pathogen cultured in the absence of the treatment.

21. The method of claim 16, wherein a spectral data threshold is calculated and a distance above the threshold indicates a difference in phenotypic response between a pathogen cultured in the presence of a treatment and the same pathogen cultured in the absence of the treatment.

22. The method of claim 16, wherein the presence of at least one difference between the test average spectra and the control average spectra indicates a treatment-susceptible pathogen.

23. The method of claim 22, wherein the difference is at about 1708 cm^{-1}, 1638 cm^{-1}, 1653 cm^{-1}, 1622 cm^{-1}, 1515 cm^{-1}, 1215 cm^{-1}, 1082 cm^{-1}, 1035 cm^{-1}, 1029 cm^{-1}, or 965 cm^{-1} in ATR-FTIR spectra or about 784 cm^{-1} or 1483 cm^{-1} in Raman spectra.

24. The method of claim 16, wherein the plurality of spectral data includes at least one absorbance value for at least one spectral band of interest, and a threshold absorbance value for the at least one spectral band of interest is calculated from a mean absorbance value or values for the control average spectra +/- up to three standard deviations of the mean absorbance value or values.

25. The method of claim 16, wherein the absence of a difference between the test average spectra and the control average spectra indicates a treatment-resistant pathogen.

26. A method of determining if a pathogen is susceptible to a treatment, the method comprising:

receiving each of a test sample exposed to a treatment and a control sample not exposed to the same treatment;

acquiring at least one spectrum from the control sample, the spectrum having a plurality of absorbance values and wavenumbers;

calculating a threshold value from a wavenumber region of the spectrum of the control sample that would display absorbance values indicative of biochemical composition changes to the control sample;

acquiring at least one spectrum from the control sample, the spectrum having a plurality of absorbance values and wavenumbers;

calculating a test value from a wavenumber region of the spectrum of the test sample that would display absorbance values indicative of biochemical composition changes to the test sample; and

comparing the test value to the threshold value, wherein a difference between the test value and the threshold value indicates the treatment susceptibility of the pathogen.

27. The method of claim 26, wherein acquiring at least one spectrum from the control sample is by IR-ATR spectroscopy, ATR-FTIR spectroscopy, transmission spectroscopy, transflection spectroscopy, reflectance spectroscopy, NIR spectroscopy, FIR spectroscopy, UV-visible spectroscopy, or Raman spectroscopy.

28. The method of claim 26, wherein acquiring at least one spectrum from the test sample is by IR-ATR spectroscopy, ATR-FTIR spectroscopy, transmission spectroscopy, transflection spectroscopy, reflectance spectroscopy, NIR spectroscopy, FIR spectroscopy, UV-visible spectroscopy, or Raman spectroscopy.

29. The method of claim 26, wherein the wavenumber region of the spectrum of the control sample that would display absorbance values indicative of biochemical composition changes to the control sample and the wavenumber region of the spectrum of the test sample that would display absorbance values indicative of biochemical composition changes to the test sample at least partially overlap.

30. The method of claim 26, wherein a plurality of spectra are acquired for each of the control sample and the test sample.

31. A system for determining if a pathogen is susceptible or resistant to a treatment, the system comprising:

a remote processing network configured to:

receive spectral data from a control system, the spectral data including a plurality of test spectra gathered from the pathogen cultured in the presence of the treatment and a plurality of control spectra gathered from the pathogen cultured in the absence of the treatment, each of the spectra having a plurality of absorbance values and wavenumbers;

process the spectral data by calculating a test average spectra from a wavenumber region of the test spectra that would include absorbance values indicative of the biochemical composition of the pathogen, calculating a control average spectra from the control spectra in the wavenumber region, and comparing the control average spectra to the test average spectra to produce a result describing the susceptibility or resistance of the pathogen to the treatment; and

return the result to the control system.

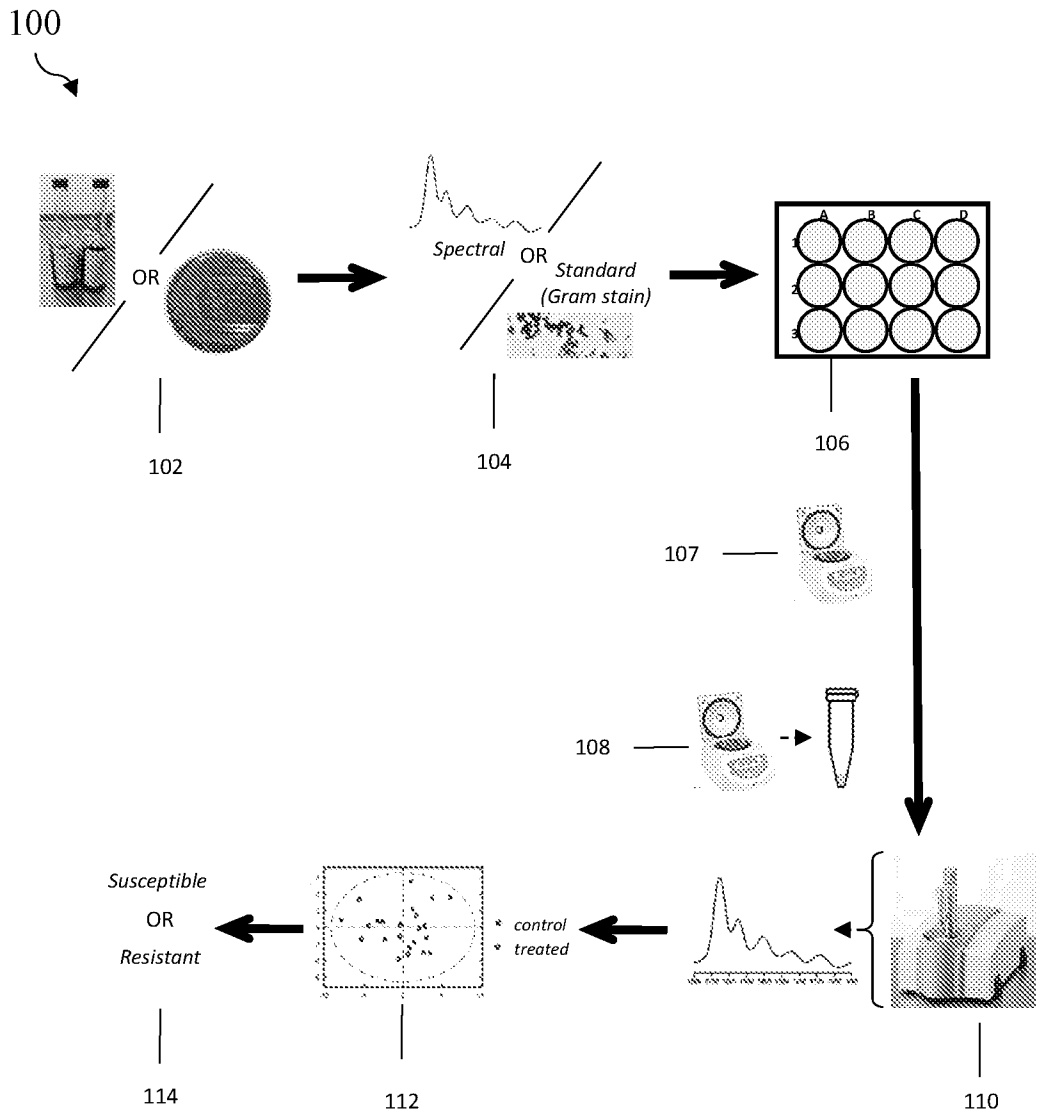

100
OR
Spectral
OR
Standard
(Gram stain)
A
B
C
D
1
2
3
102
104
106
107
108
Susceptible
OR
Resistant
control
treated
114
112
110

FIG. 1A

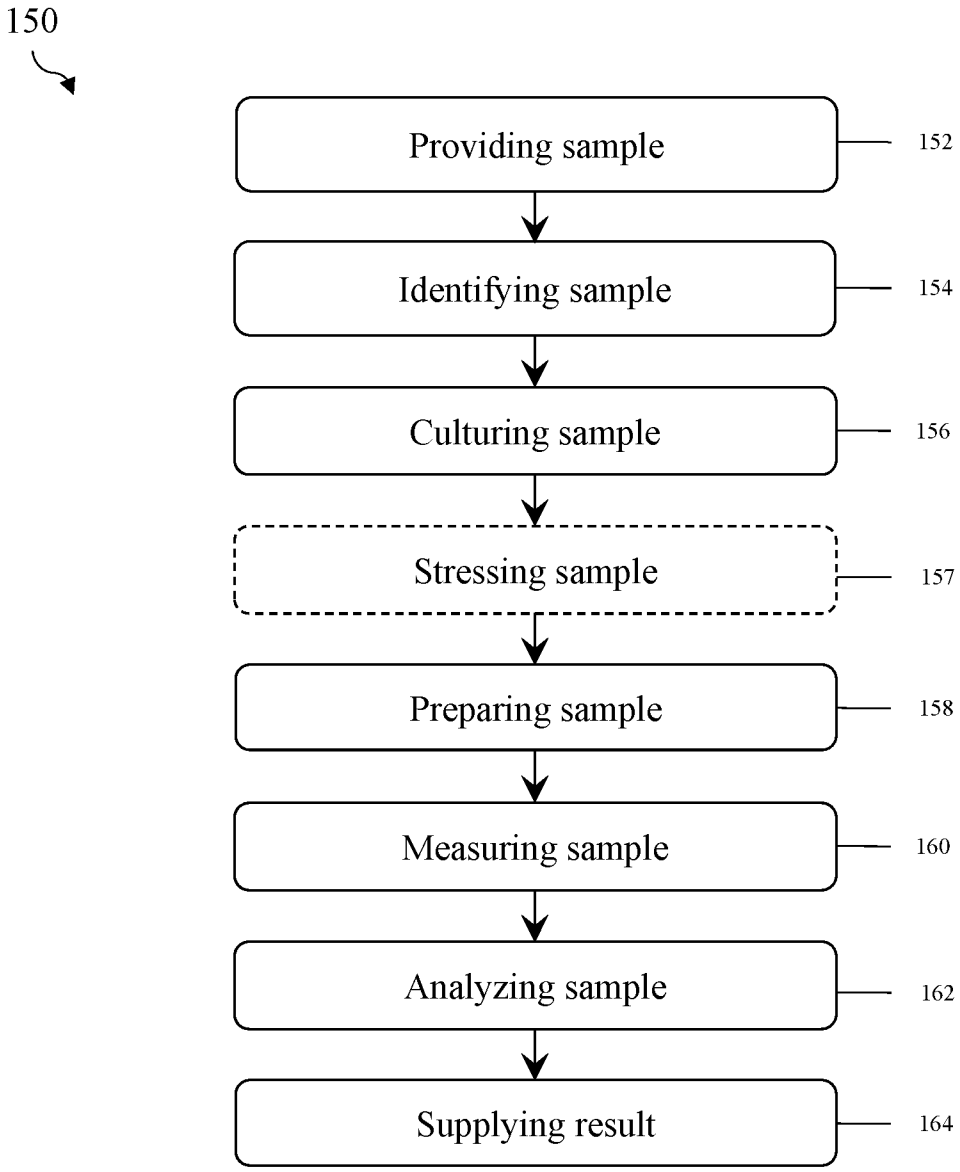

150
Providing sample
152
Identifying sample
154
Culturing sample
156
Stressing sample
157
Preparing sample
158
Measuring sample
160
Analyzing sample
162
Supplying result
164

FIG. 1B

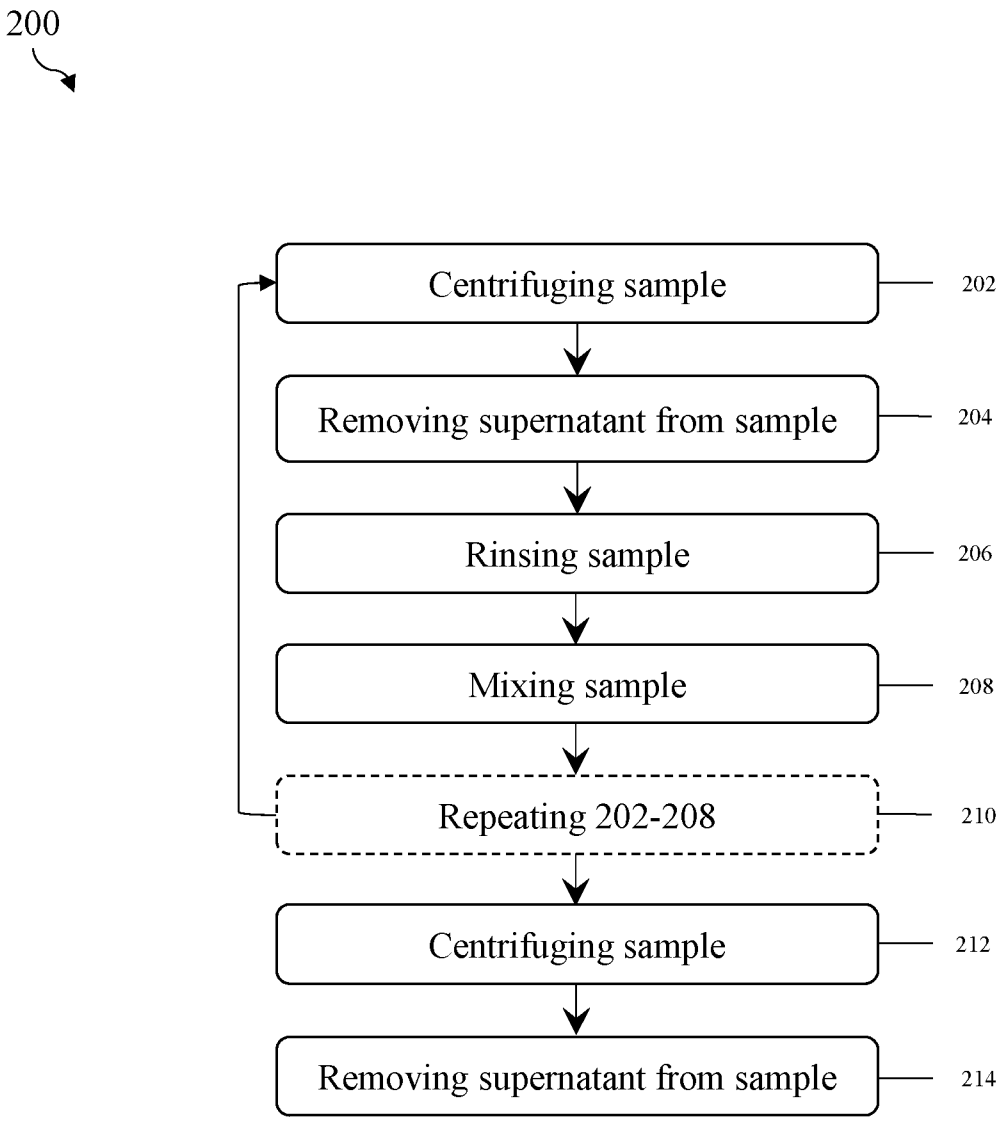

200
Centrifuging sample
202
Removing supernatant from sample
204
Rinsing sample
206
Mixing sample
208
Repeating 202-208
210
Centrifuging sample
212
Removing supernatant from sample
214

FIG. 2A

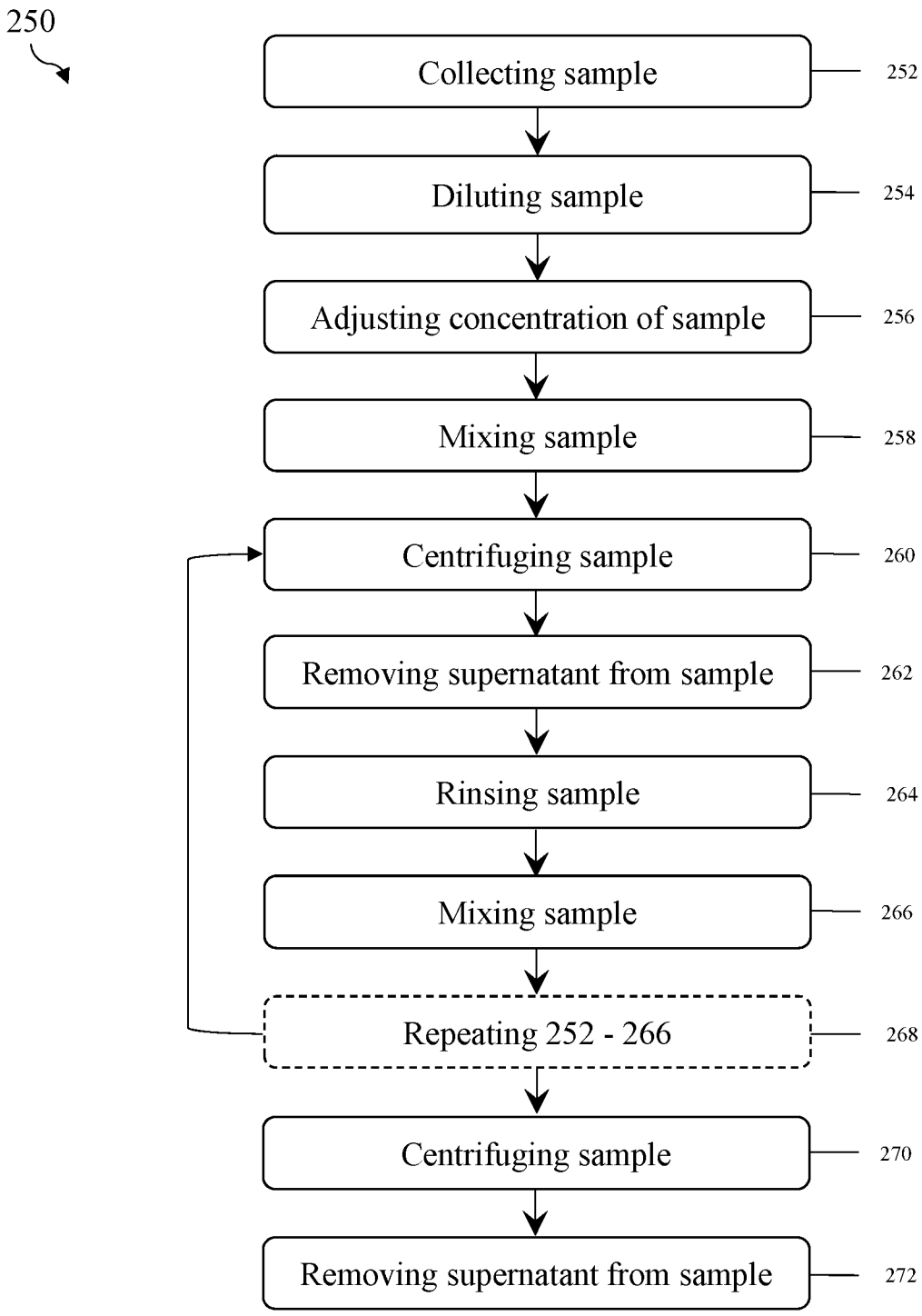

250
Collecting sample
252
Diluting sample
254
Adjusting concentration of sample
256
Mixing sample
258
Centrifuging sample
260
Removing supernatant from sample
262
Rinsing sample
264
Mixing sample
266
Repeating 252 - 266
268
Centrifuging sample
270
Removing supernatant from sample
272

FIG. 2B

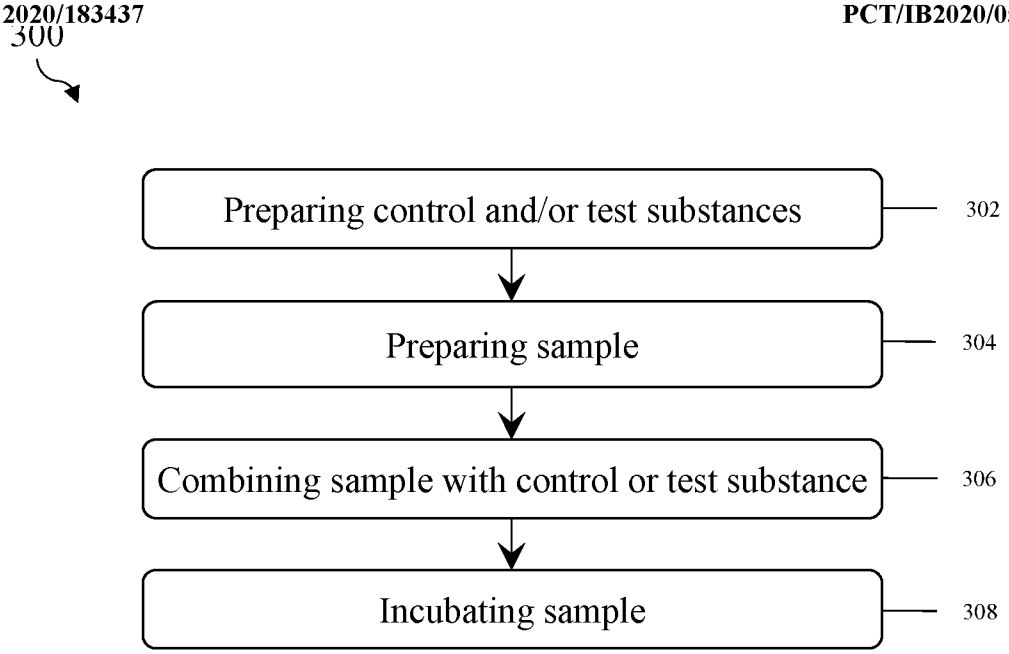

300
Preparing control and/or test substances
302
Preparing sample
304
Combining sample with control or test substance
306
Incubating sample
308

FIG. 3

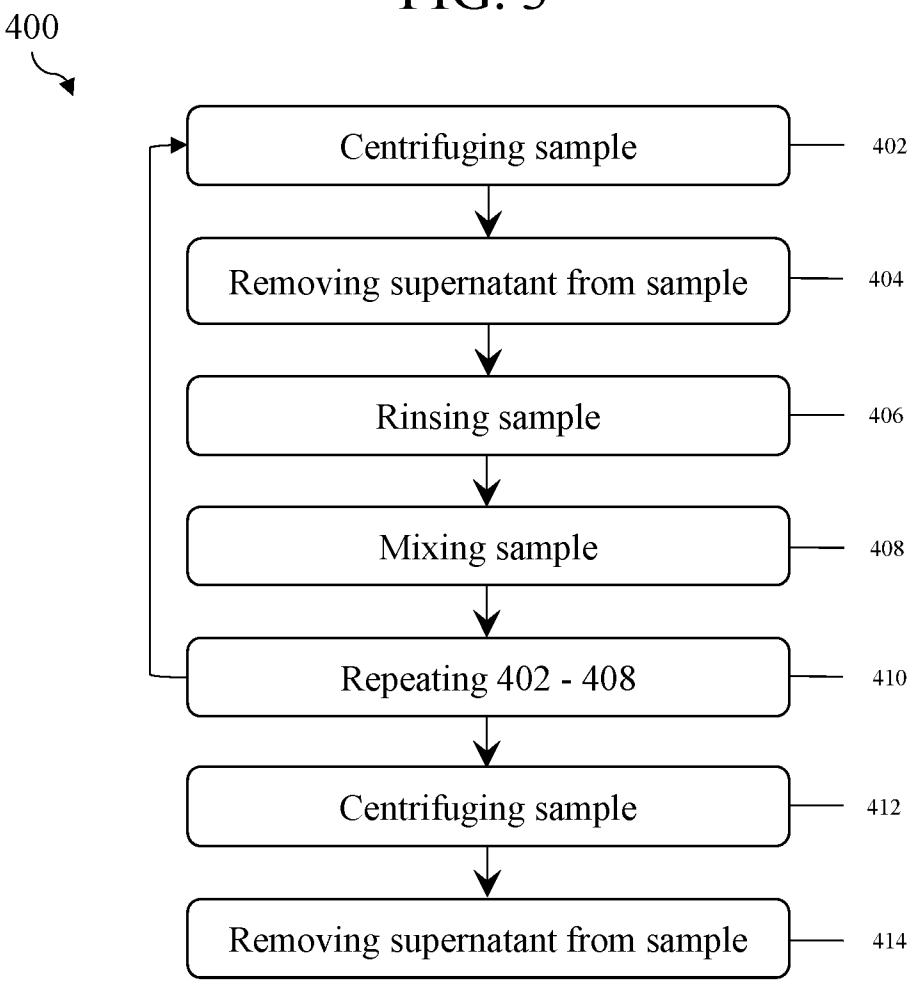

400
Centrifuging sample
402
Removing supernatant from sample
404
Rinsing sample
406
Mixing sample
408
Repeating 402 - 408
410
Centrifuging sample
412
Removing supernatant from sample
414

FIG. 4A

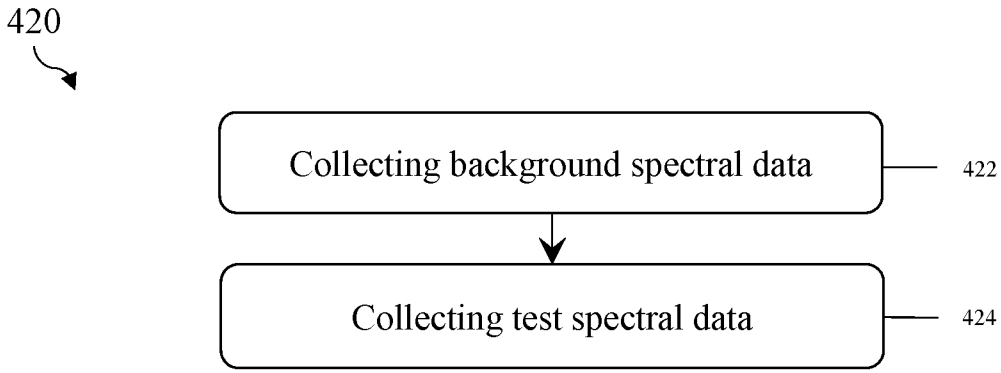


FIG. 4B

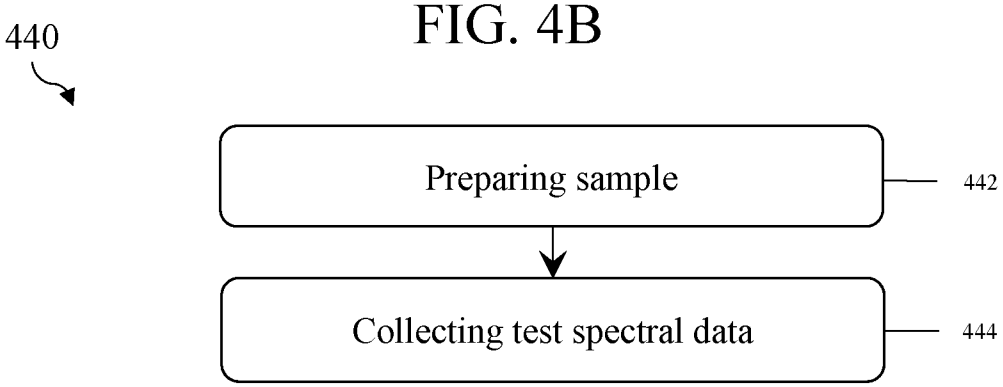


FIG. 4C

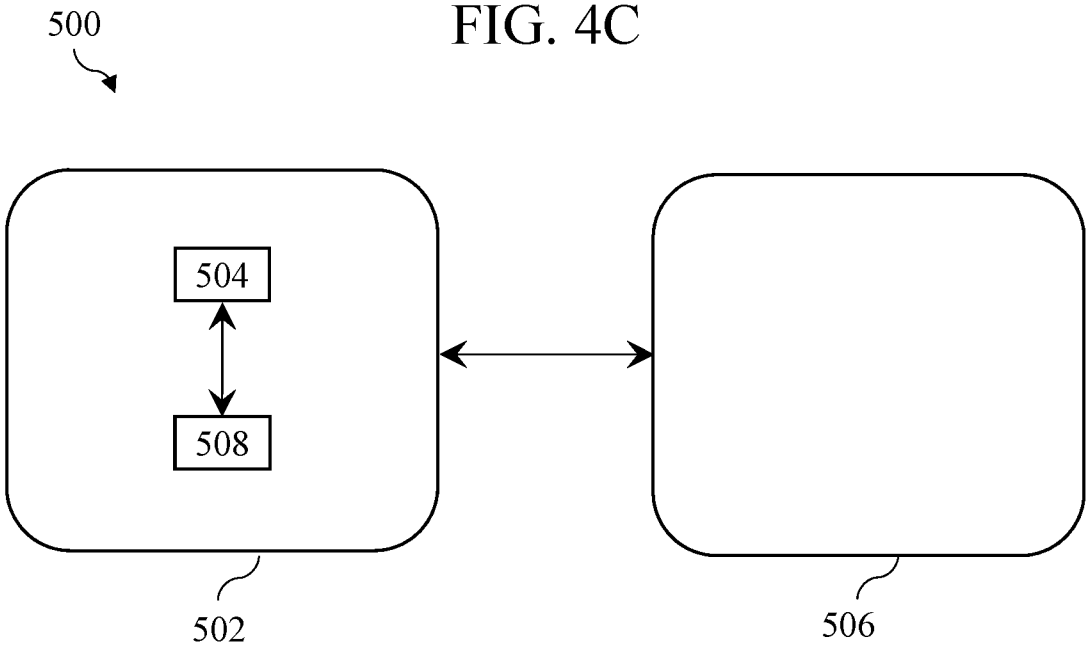


FIG. 5

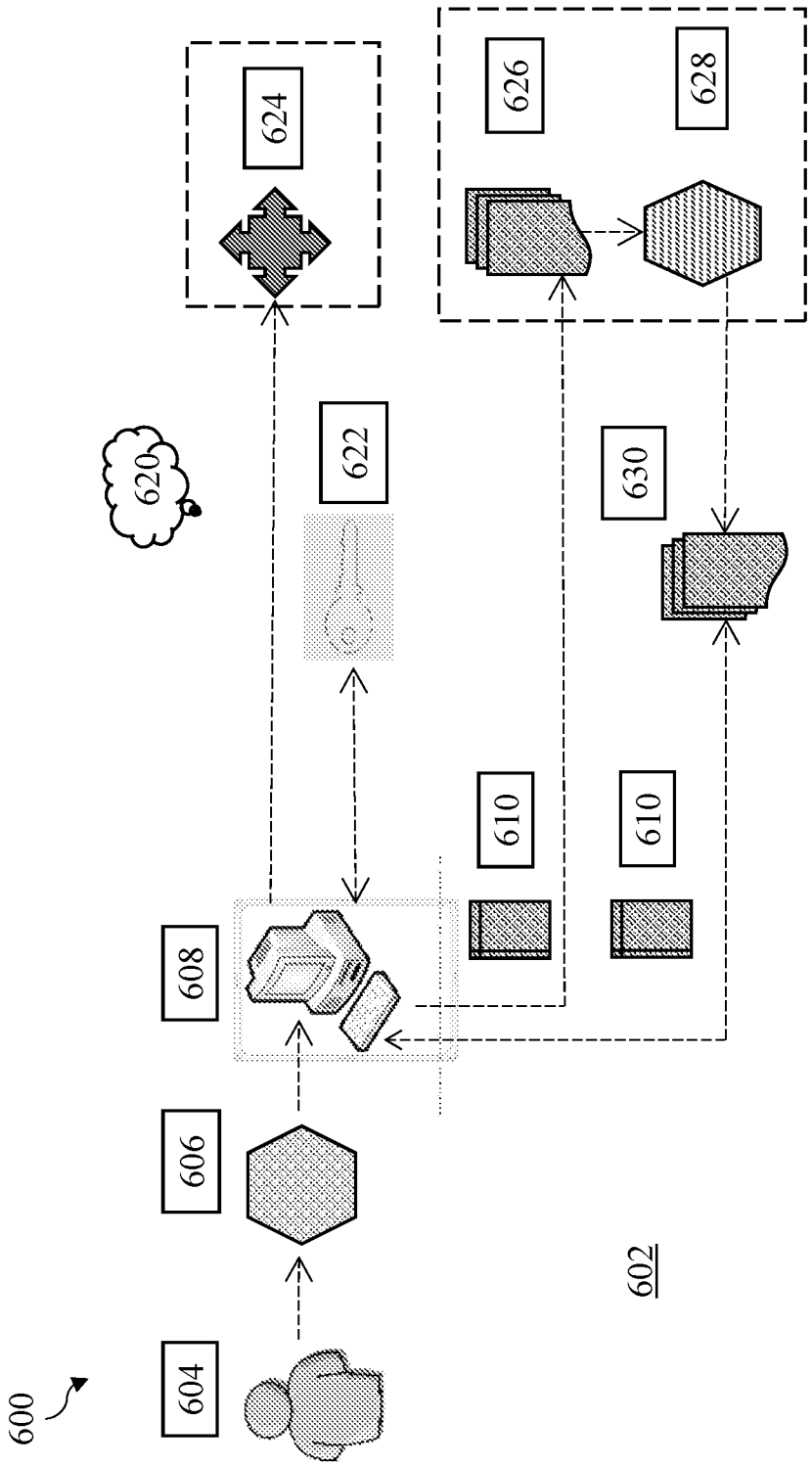


FIG. 6

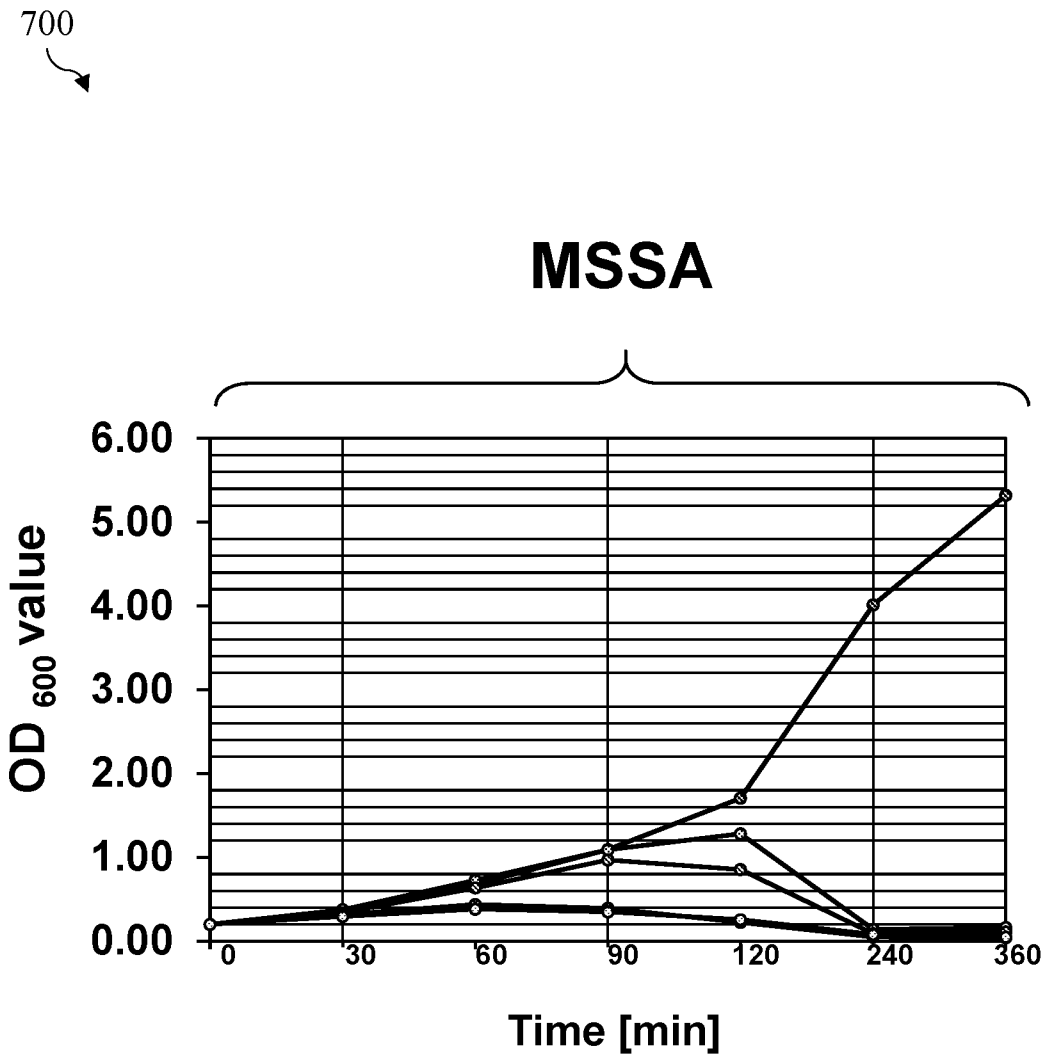

700
MSSA
6.00
5.00
4.00
3.00
2.00
1.00
0.00
OD 600 value
0
30
60
90
120
240
360
Time [min]

FIG. 7A

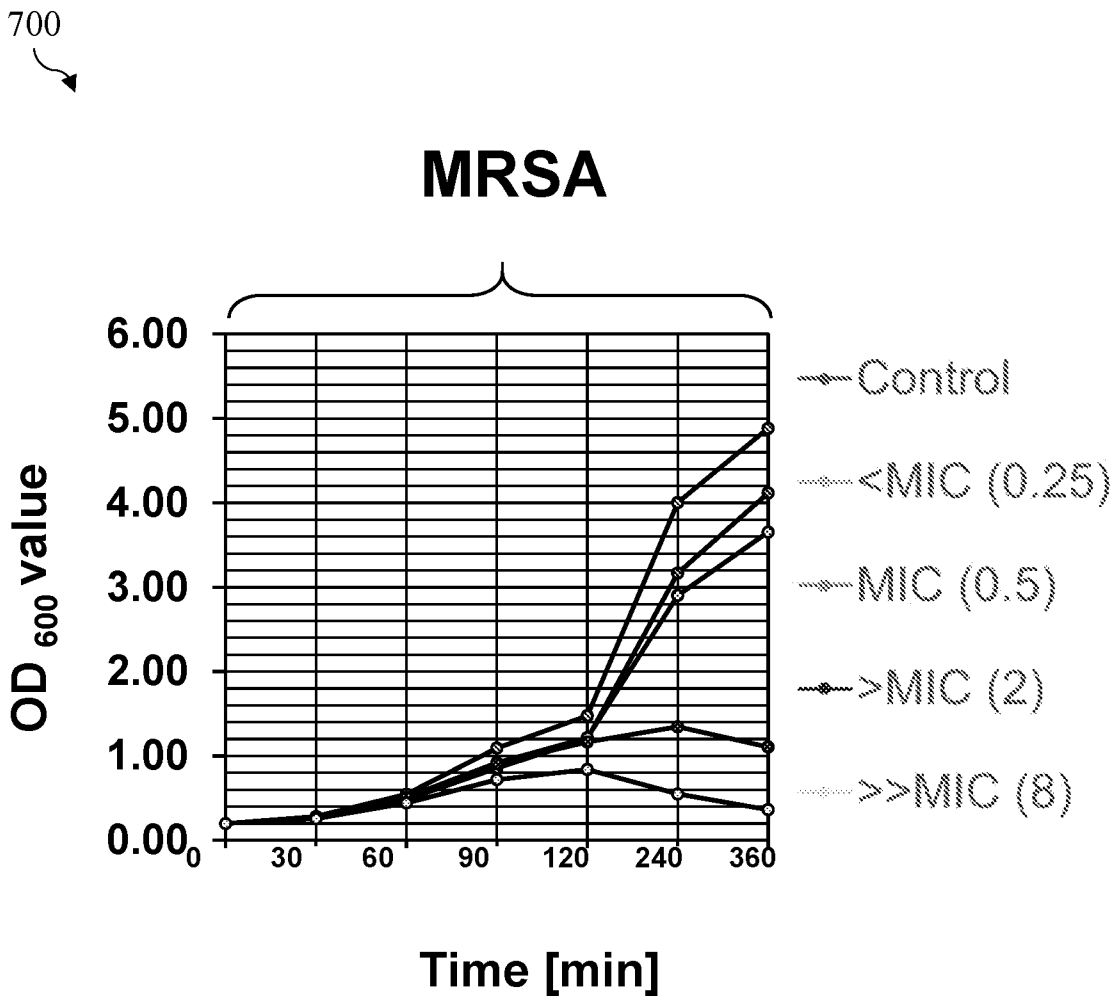

700
MRSA
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
30
60
90
120
240
360
OD 600 value
Time [min]
Control
<MIC (0.25)
MIC (0.5)
>MIC (2)
>>MIC (8)

FIG. 7B

FIG. 8A

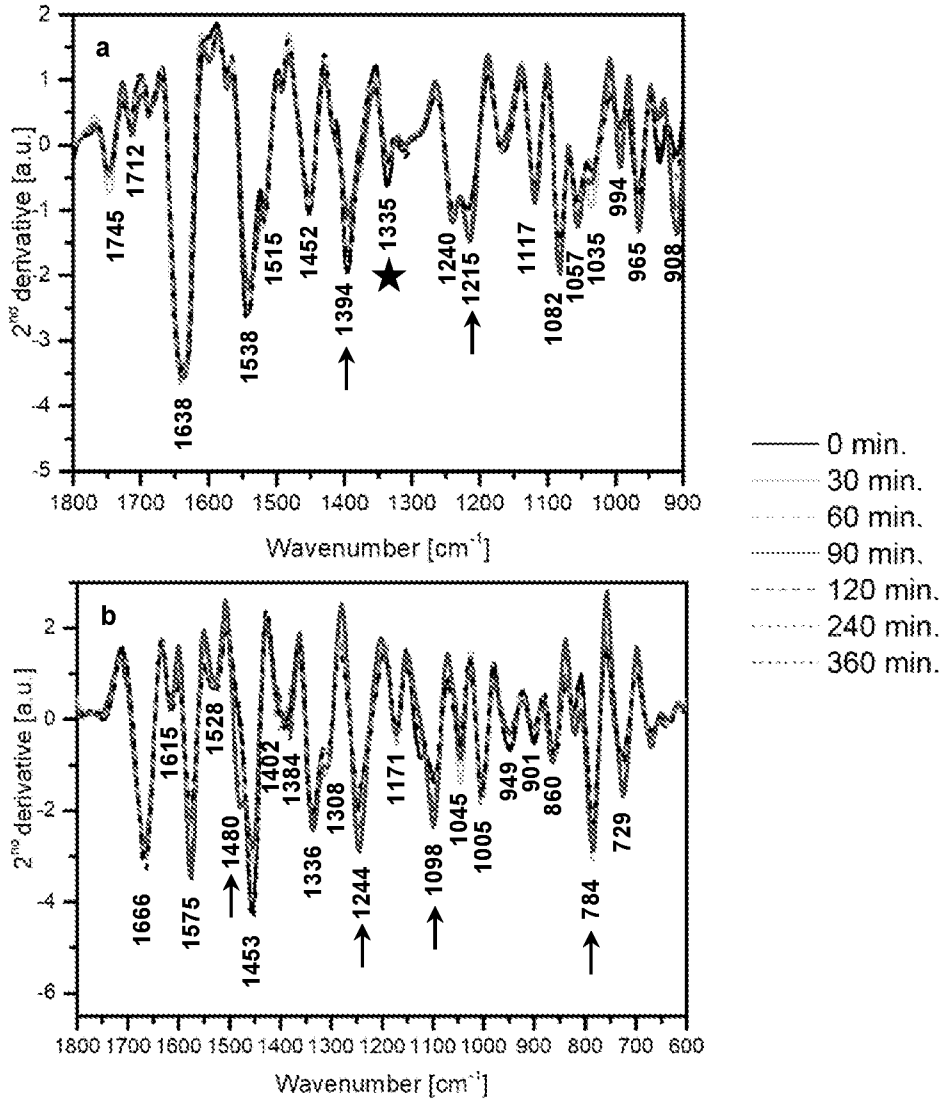

a
b
2nd derivative [a.u.]
Wavenumber [cm-1]
1745
1712
1638
1538
1515
1452
1394
1335
1240
1215
1117
1082
1057
1035
994
965
908
1666
1615
1575
1528
1480
1453
1402
1384
1336
1308
1244
1171
1098
1045
1005
949
901
860
784
729
0 min.
30 min.
60 min.
90 min.
120 min.
240 min.
360 min.

FIG. 9A

FIG. 8B

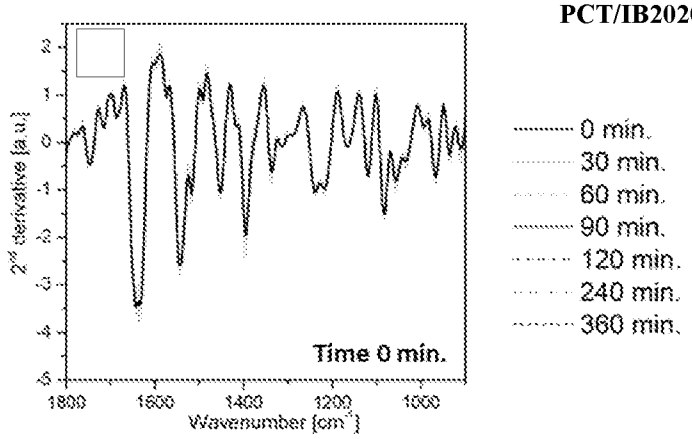


FIG. 8C

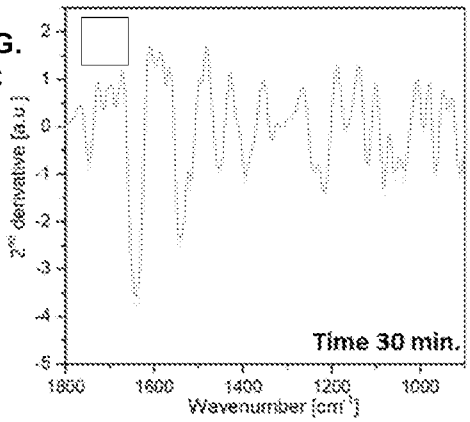


FIG. 8D

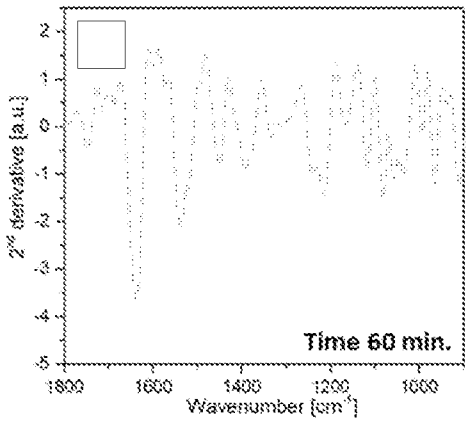


FIG. 8E

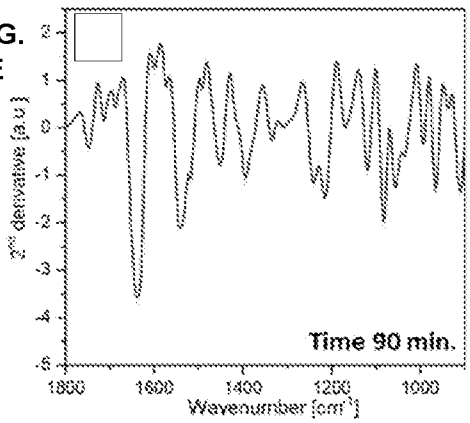


FIG. 8F

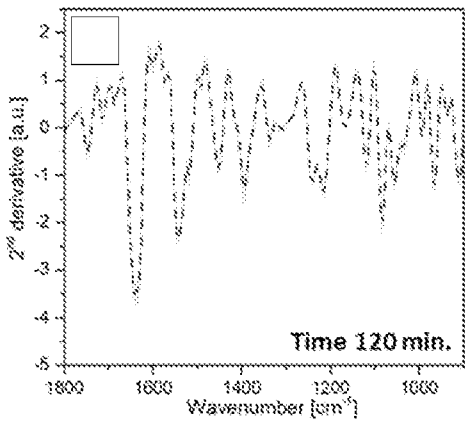


FIG. 8G

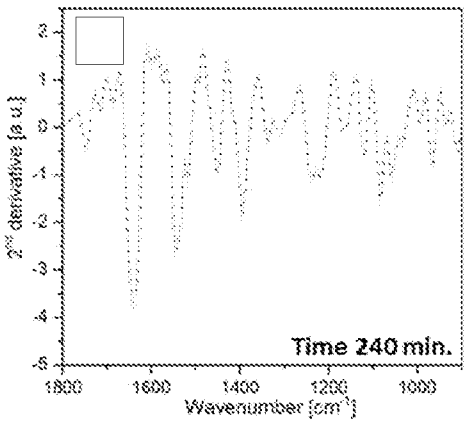


FIG. 8H

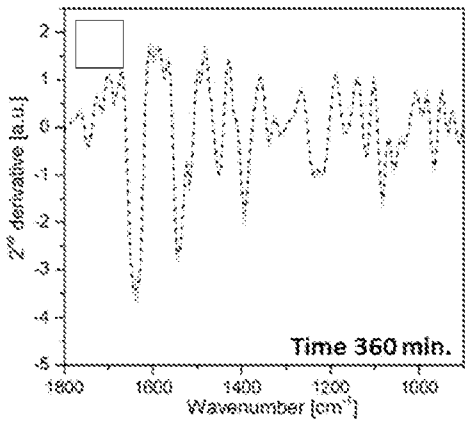


FIG. 9B

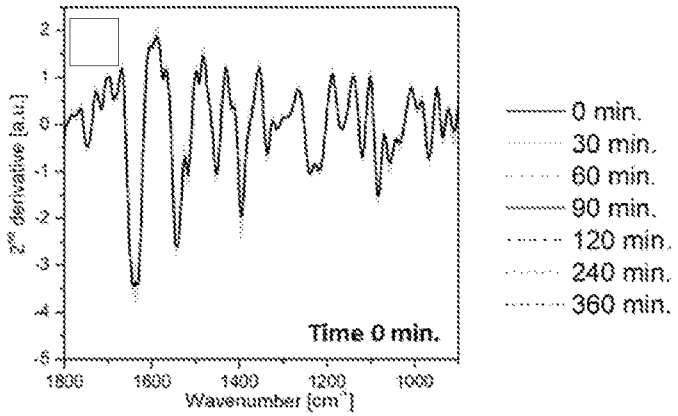


FIG. 9C

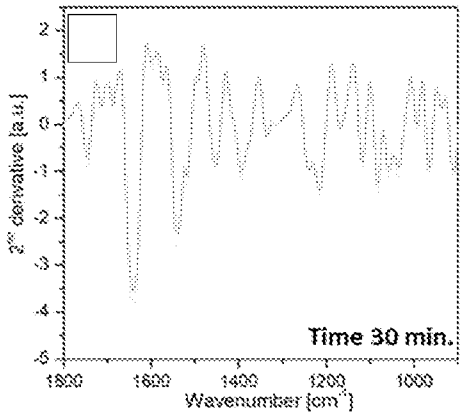


FIG. 9D

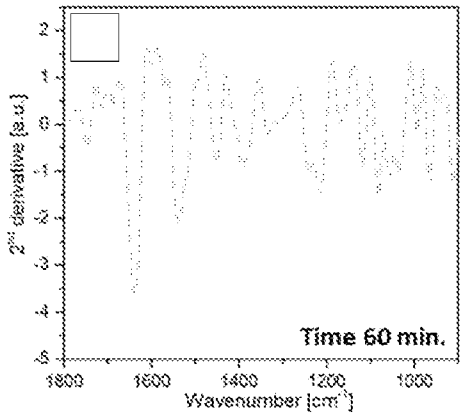


FIG. 9E

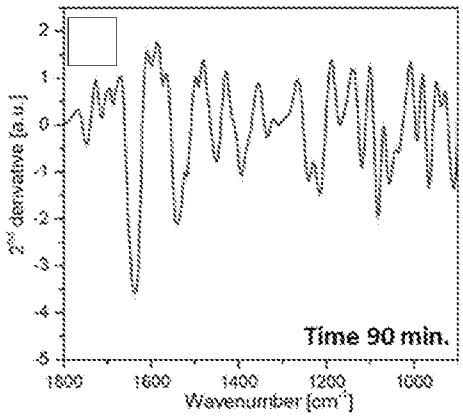


FIG. 9F

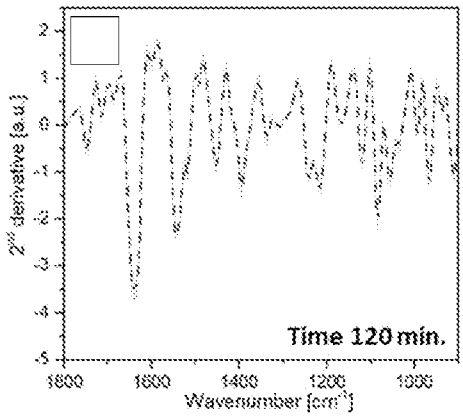


FIG. 9G

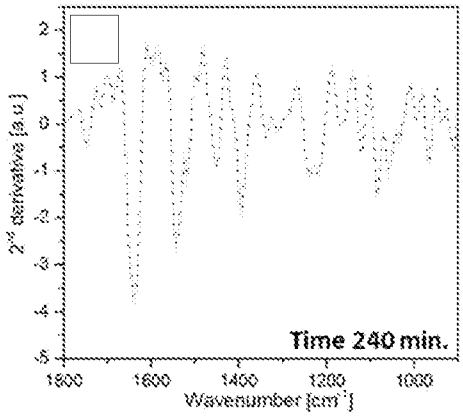
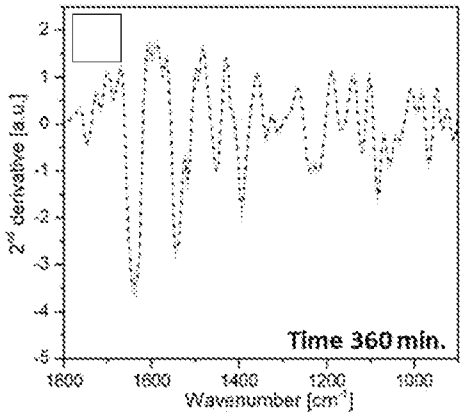


FIG. 9H



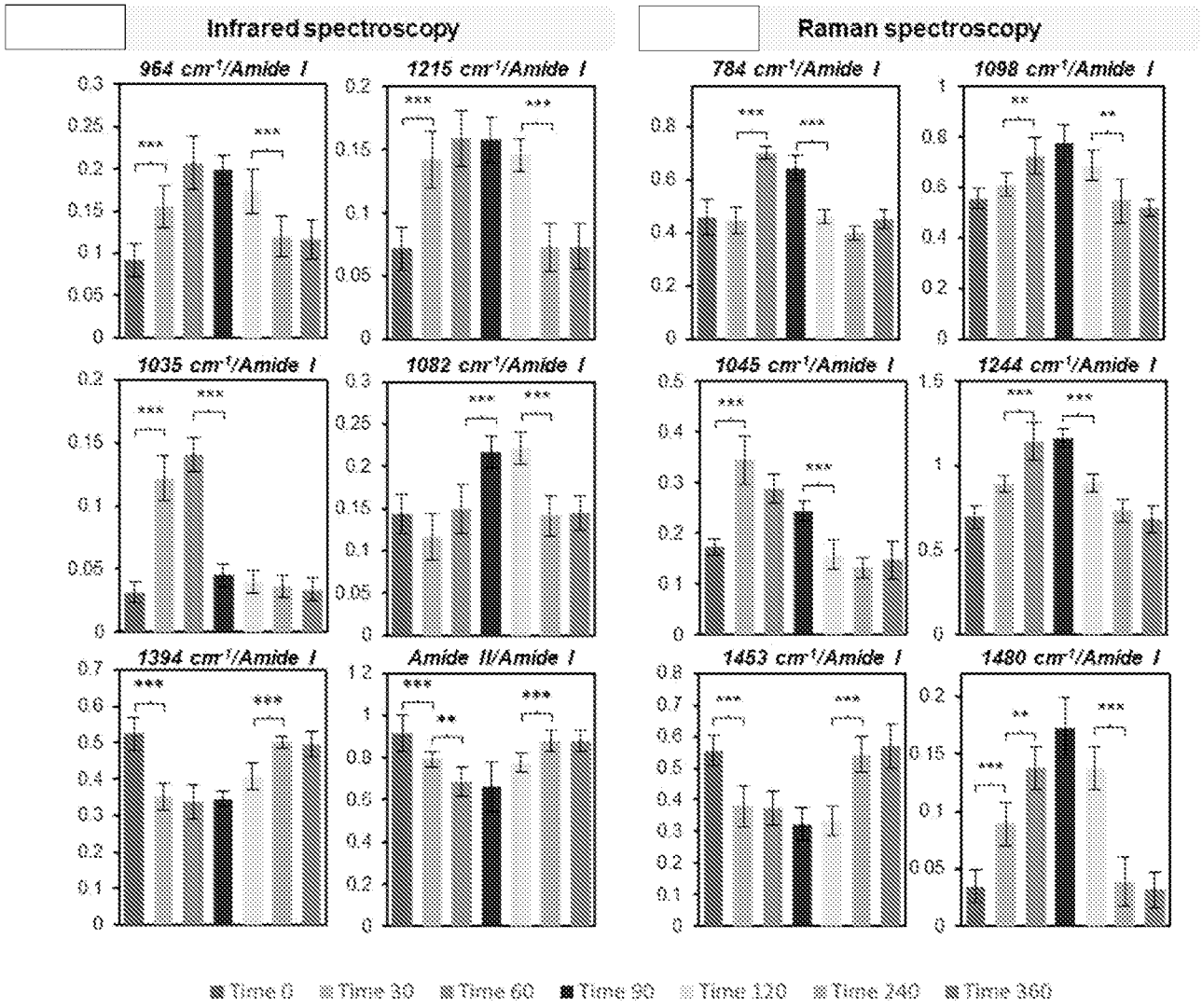


FIG. 10A FIG. 10B

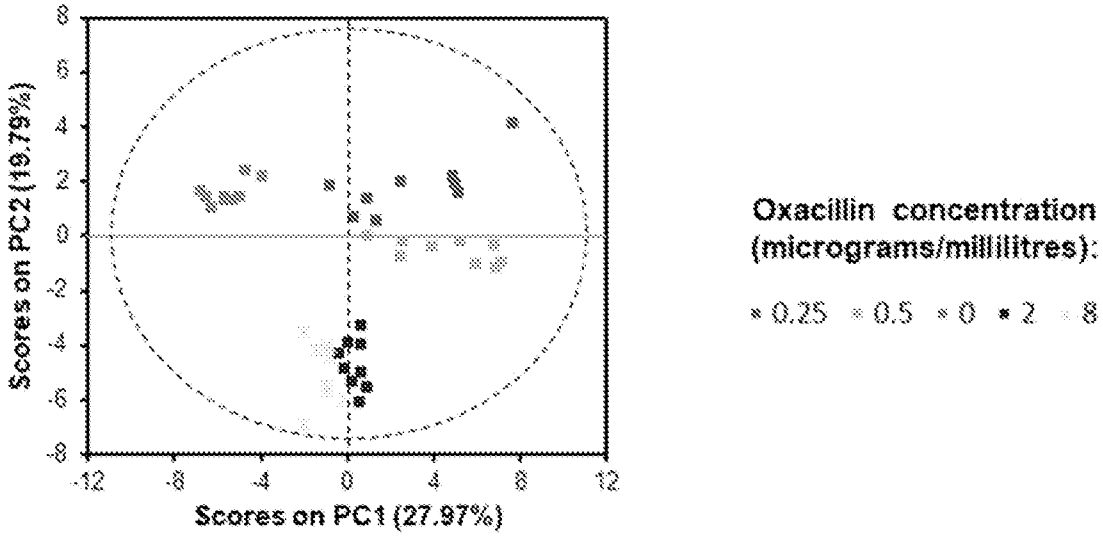

Scores on PC2 (19.79%)
Scores on PC1 (27.97%)
-12
-8
-4
0
4
8
12
Oxacillin concentration (micrograms/millilitres):
0.25
0.5
0
2
8

FIG. 11A

FIG. 11B

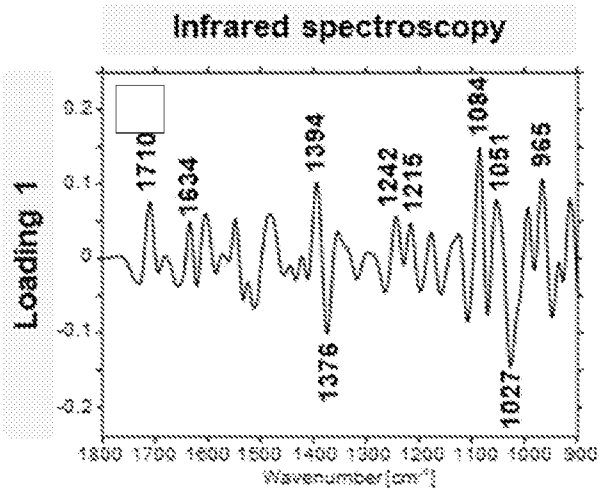
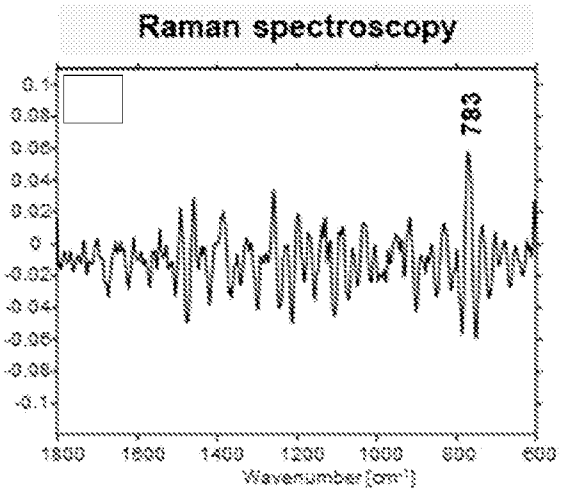


FIG. 11C



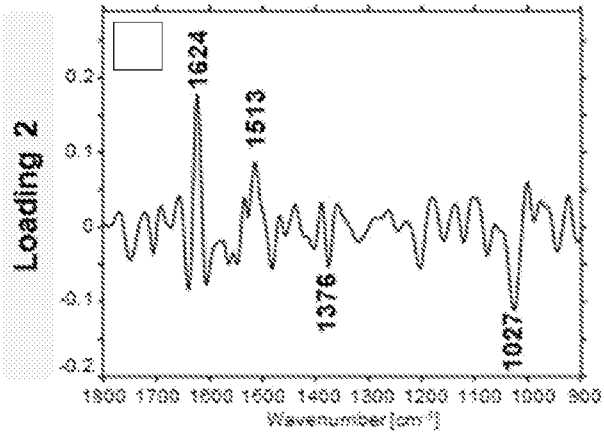


FIG. 11D

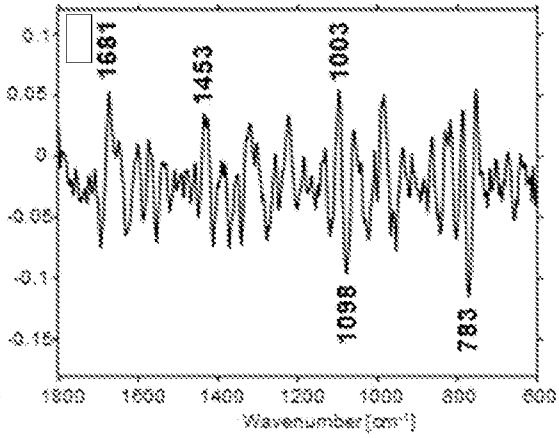


FIG. 11E

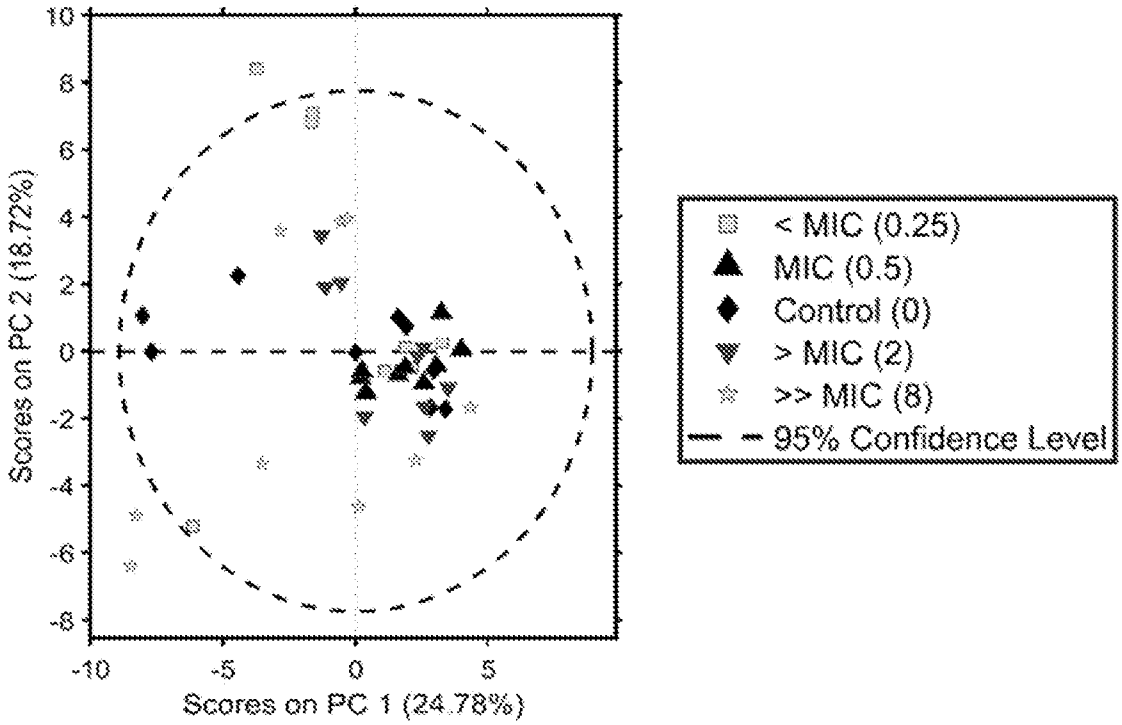

Scores on PC 2 (18.72%)
Scores on PC 1 (24.78%)
10
8
6
4
2
0
-2
-4
-6
-8
-10
-5
0
5
< MIC (0.25)
MIC (0.5)
Control (0)
> MIC (2)
>> MIC (8)
95% Confidence Level

FIG. 12A

FIG. 12B

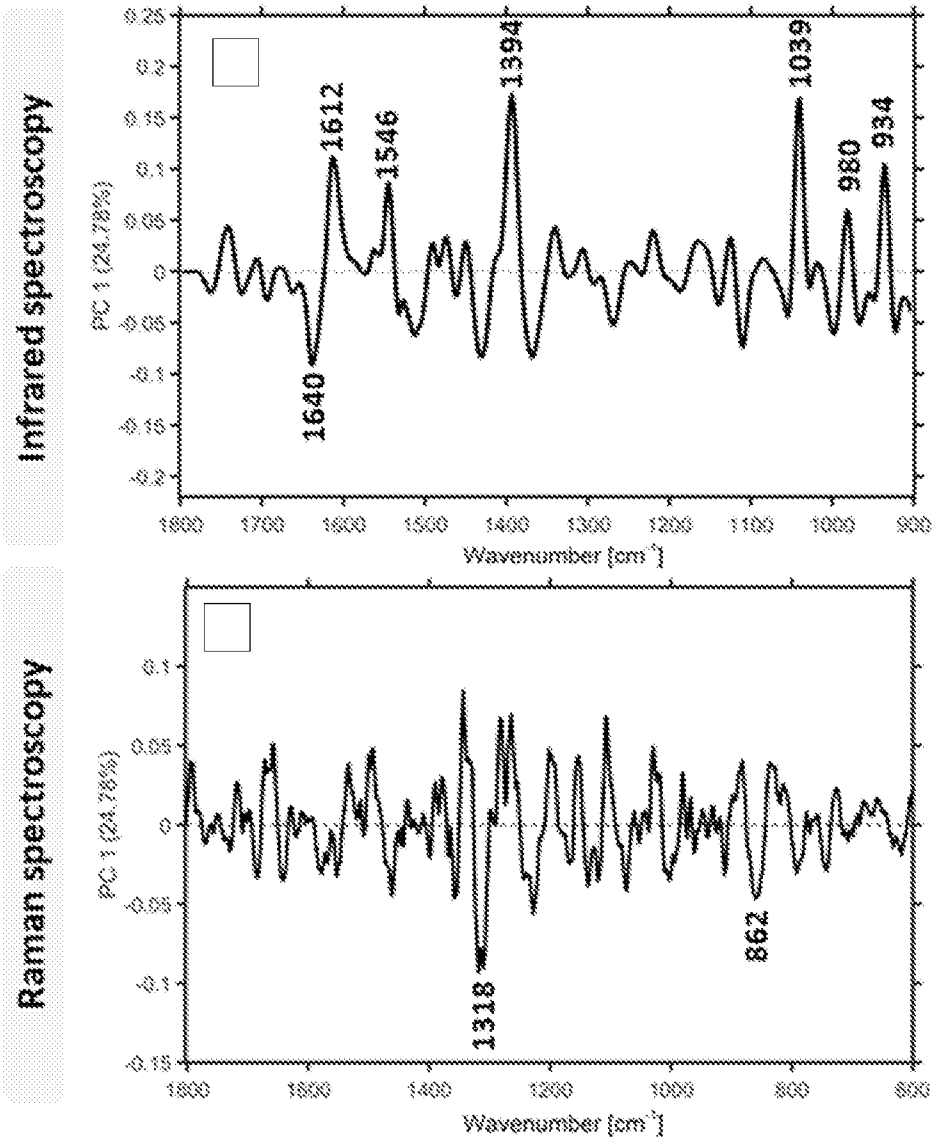


FIG. 12C

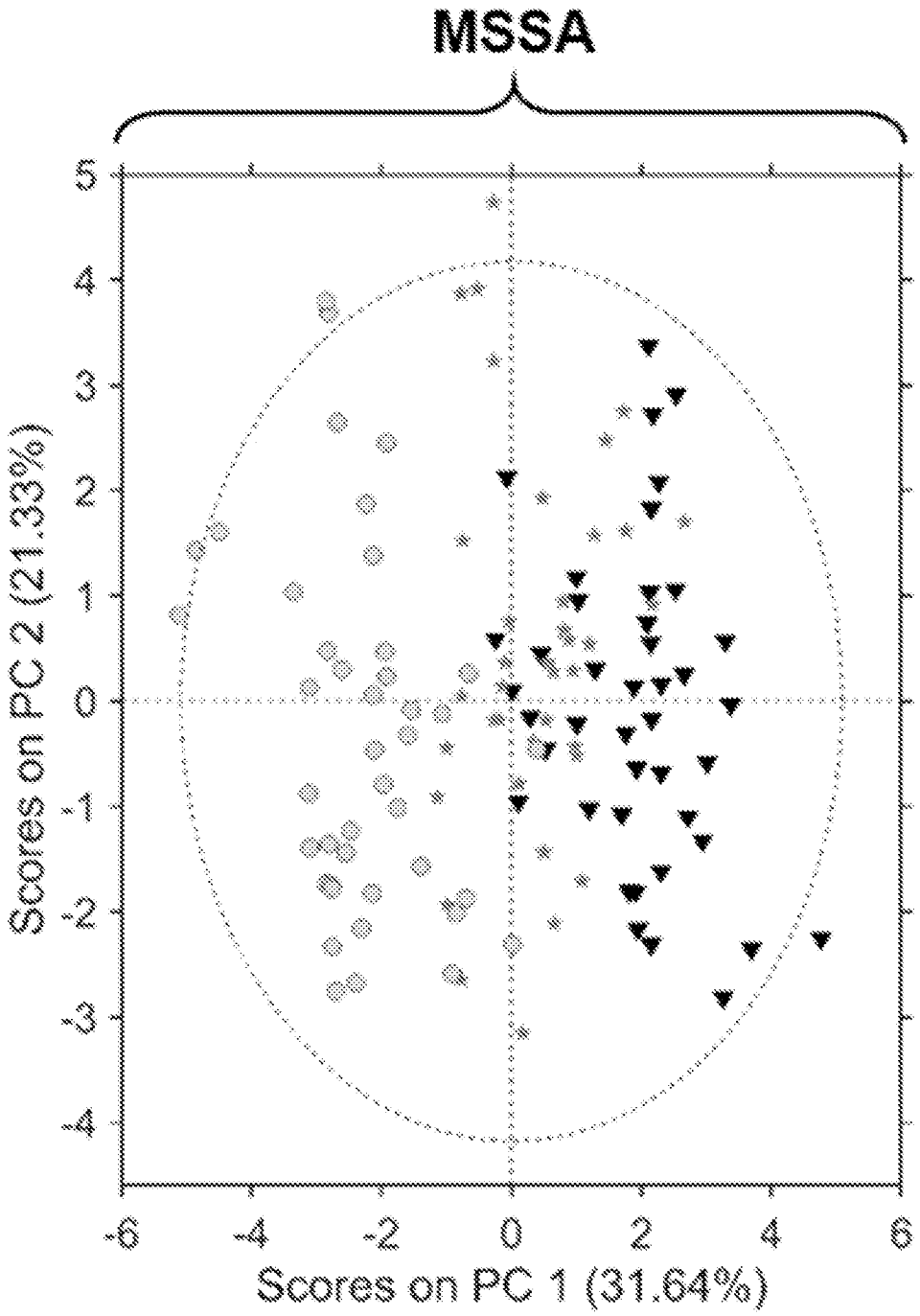

MSSA
Scores on PC 2 (21.33%)
Scores on PC 1 (31.64%)
5
4
3
2
1
0
-1
-2
-3
-4
-6
-4
-2
0
2
4
6

FIG. 13A

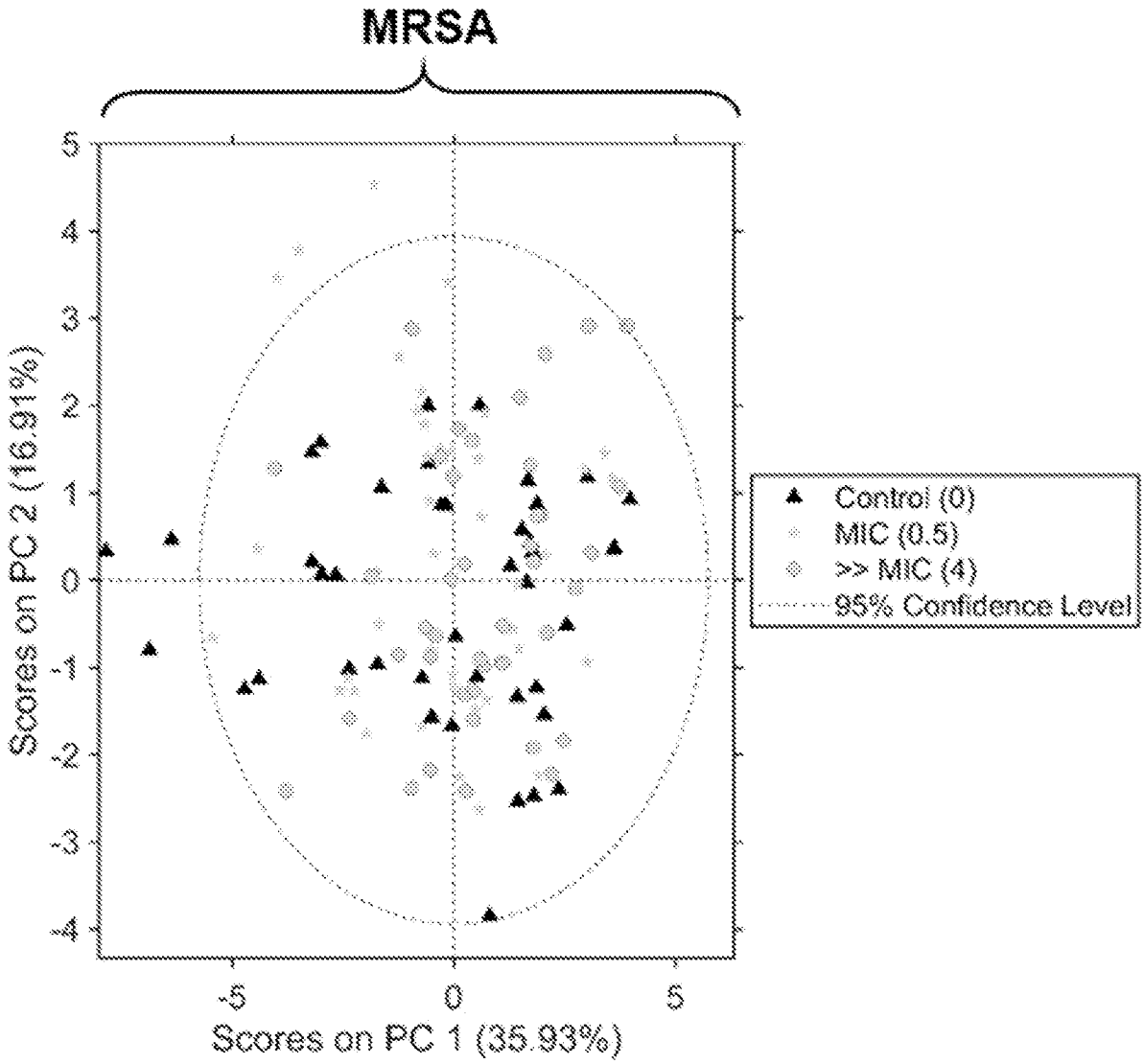

MRSA
Scores on PC 2 (16.91%)
Scores on PC 1 (35.93%)
5
4
3
2
1
0
-1
-2
-3
-4
-5
0
5
Control (0)
MIC (0.5)
>> MIC (4)
95% Confidence Level

FIG. 13B

FIG. 14

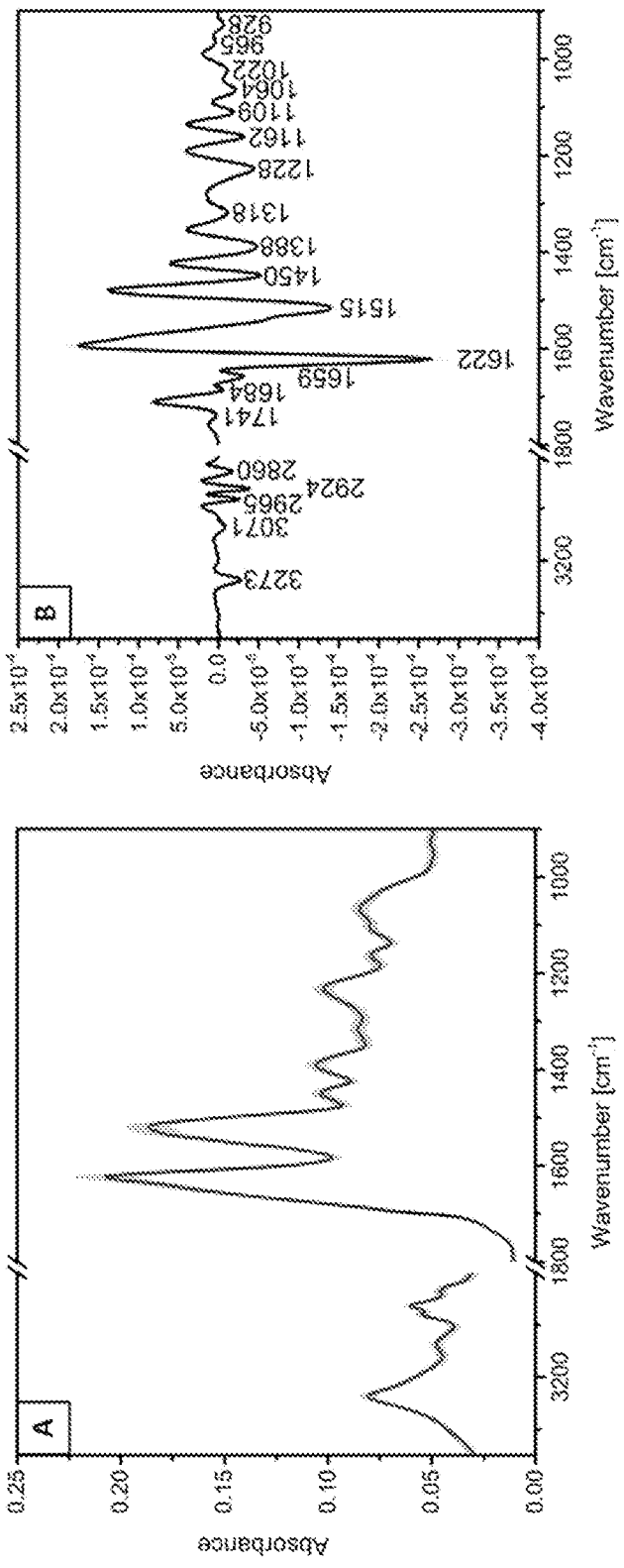
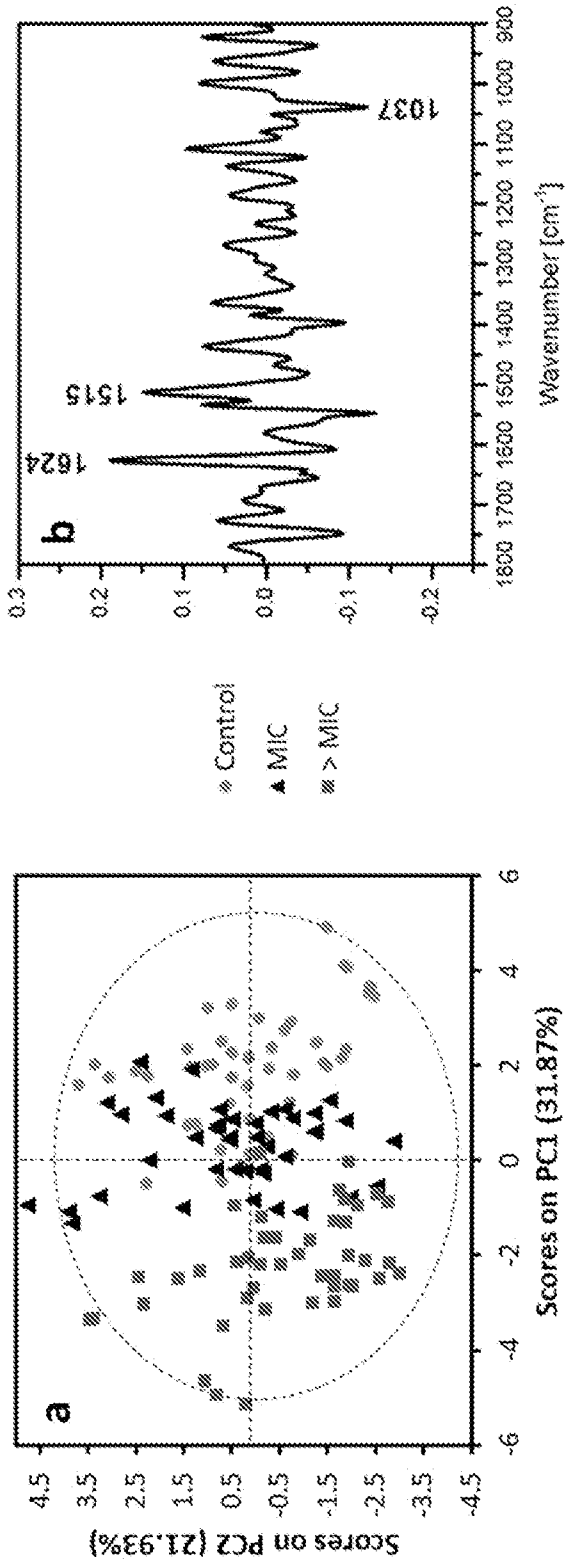

A
Absorbance
Wavenumber [cm-1]
B
Absorbance
Wavenumber [cm-1]
3273
3071
2965
2924
2860
1741
1684
1659
1622
1515
1450
1368
1318
1228
1162
1109
1064
1022
965
928

FIG. 15


a
Scores on PC2 (21.93%)
Scores on PC1 (31.87%)
Control
MIC
> MIC
b
Wavenumber [cm-1]
1624
1515
1037

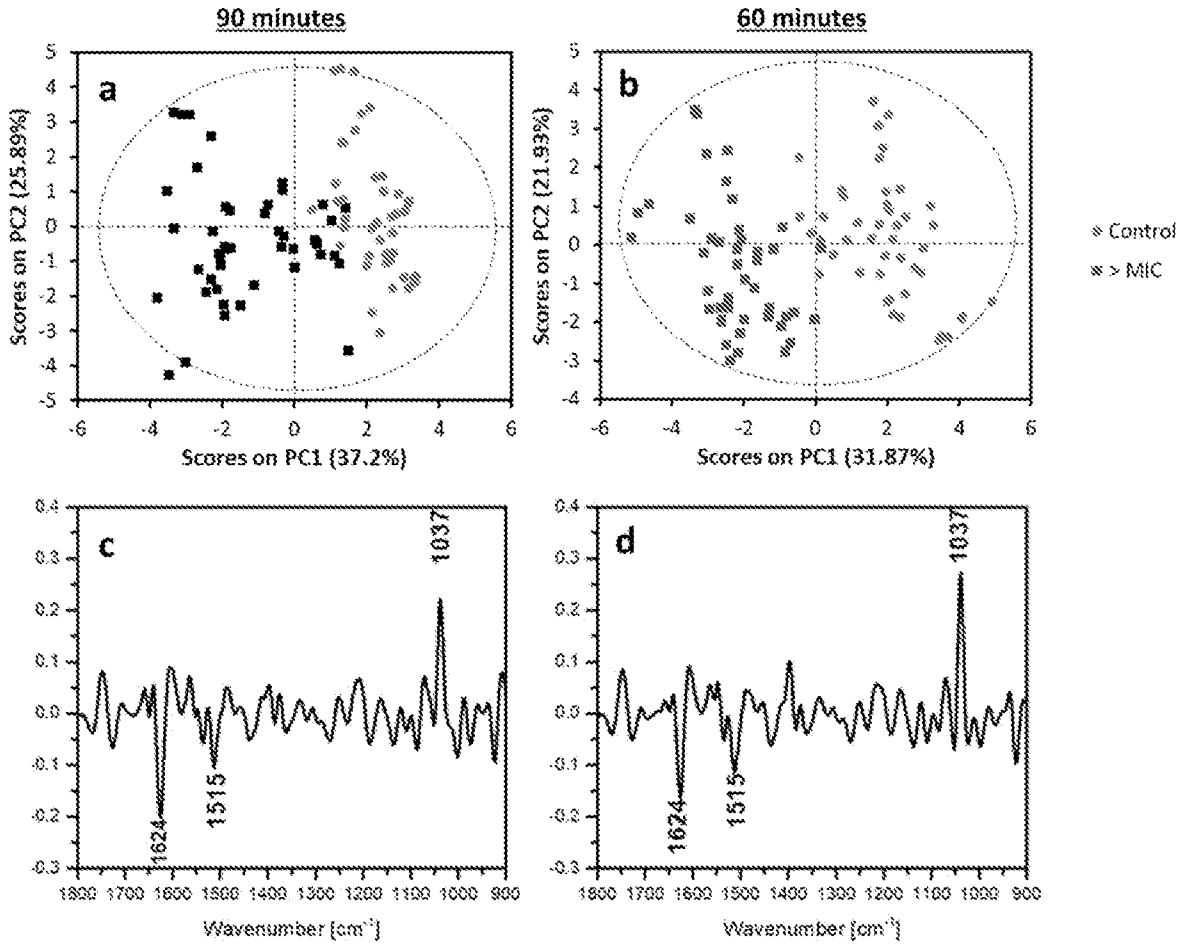

90 minutes
60 minutes
a
b
c
d
Scores on PC2 (25.89%)
Scores on PC1 (37.2%)
Scores on PC2 (21.83%)
Scores on PC1 (31.87%)
Control
> MIC
1624
1515
1037
Wavenumber [cm⁻¹]

FIG. 16

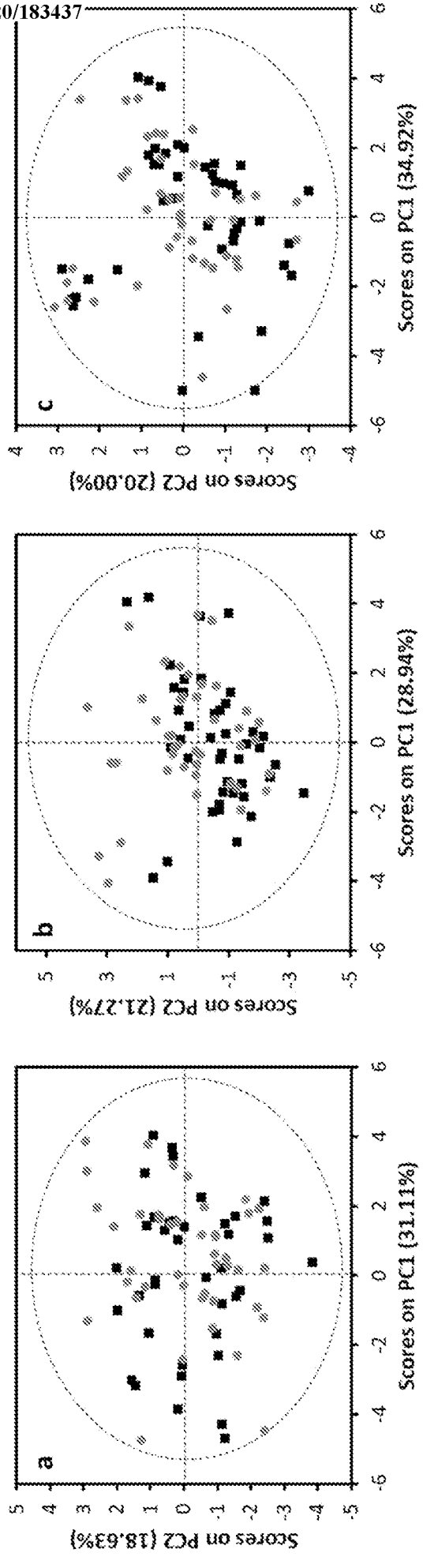

a
Scores on PC2 (18.63%)
Scores on PC1 (31.11%)
b
Scores on PC2 (21.27%)
Scores on PC1 (28.94%)
c
Scores on PC2 (20.00%)
Scores on PC1 (34.92%)

FIG. 17

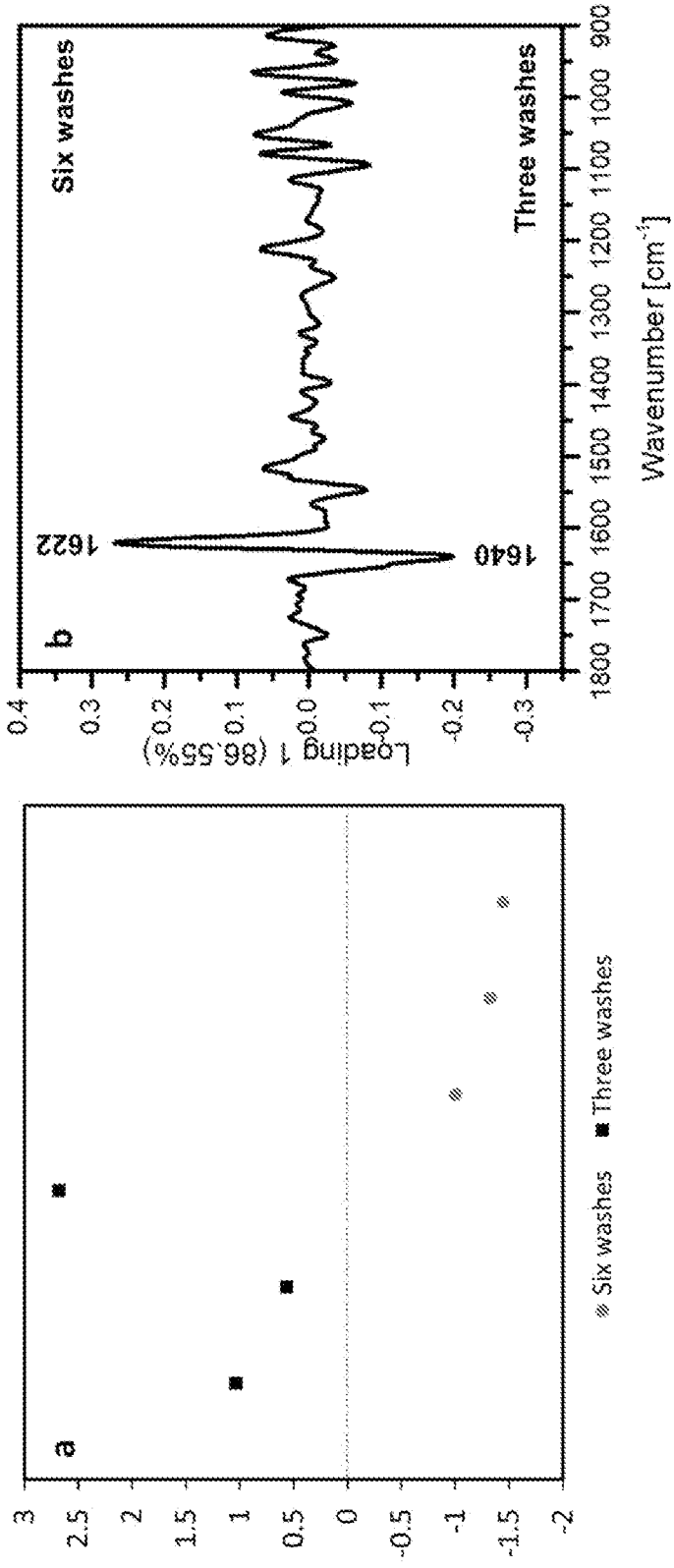

a
b
Six washes
Three washes
1622
1640
Loading 1 (86.55%)
Wavenumber [cm-1]
Six washes
Three washes

FIG. 18

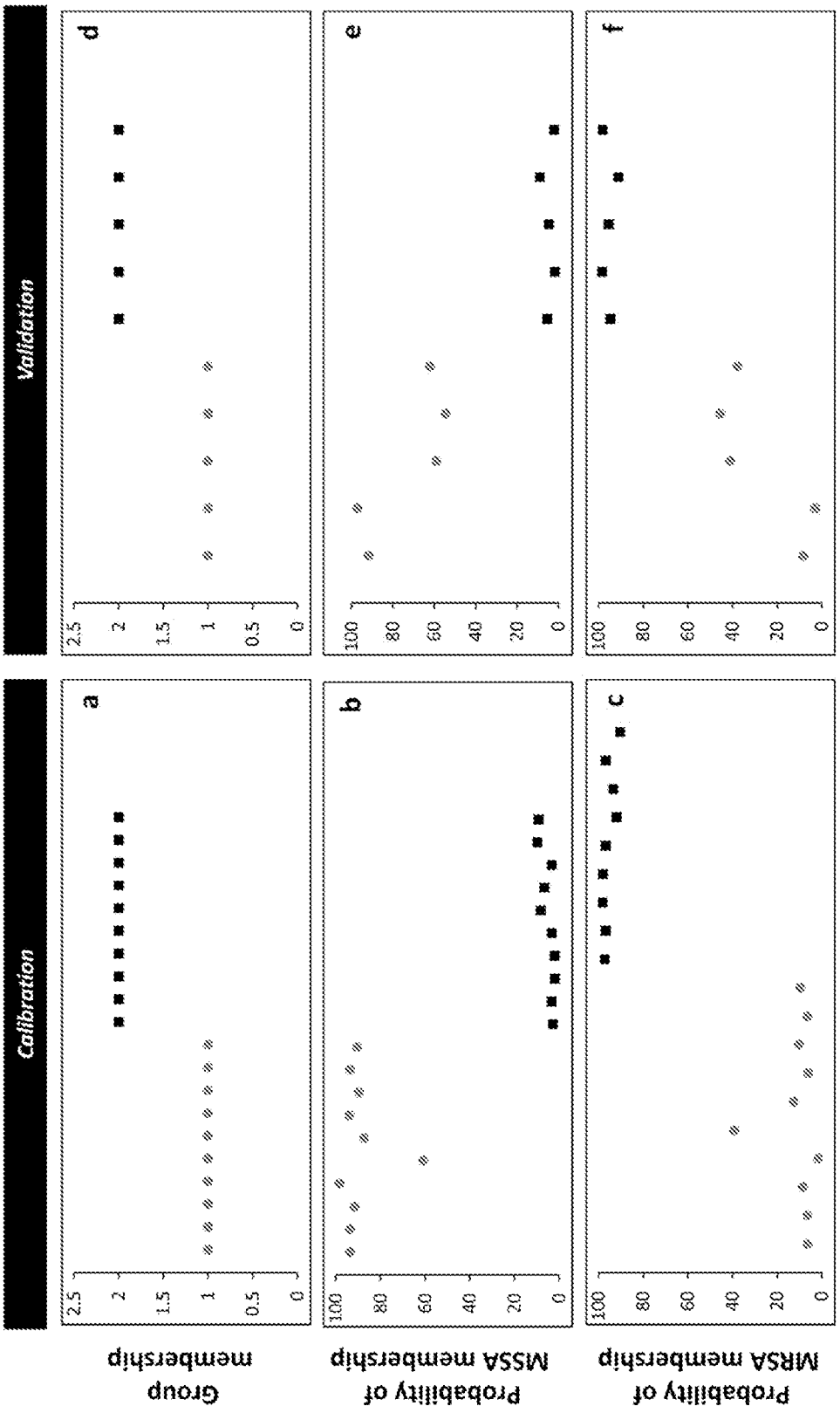

Calibration
Validation
a
b
c
d
e
f
Group membership
Probability of MSSA membership
Probability of MRSA membership
MSSA
MRSA

FIG. 19

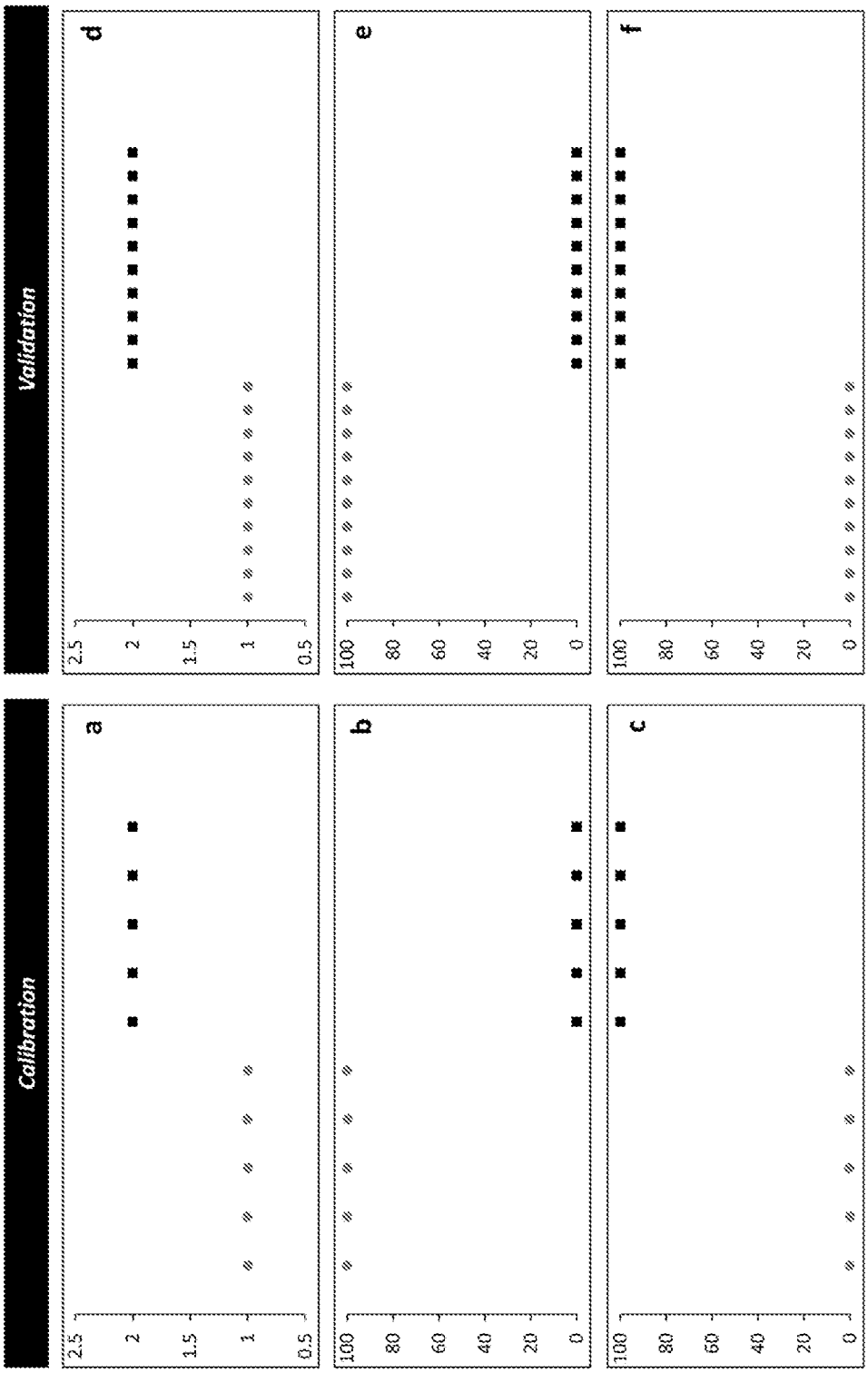

Calibration
Validation
a
b
c
d
e
f
Group membership
Probability of MSSA membership
Probability of MRSA membership
2.5
2
1.5
1
0.5
100
80
60
40
20
0
MSSA
MRSA

FIG. 20

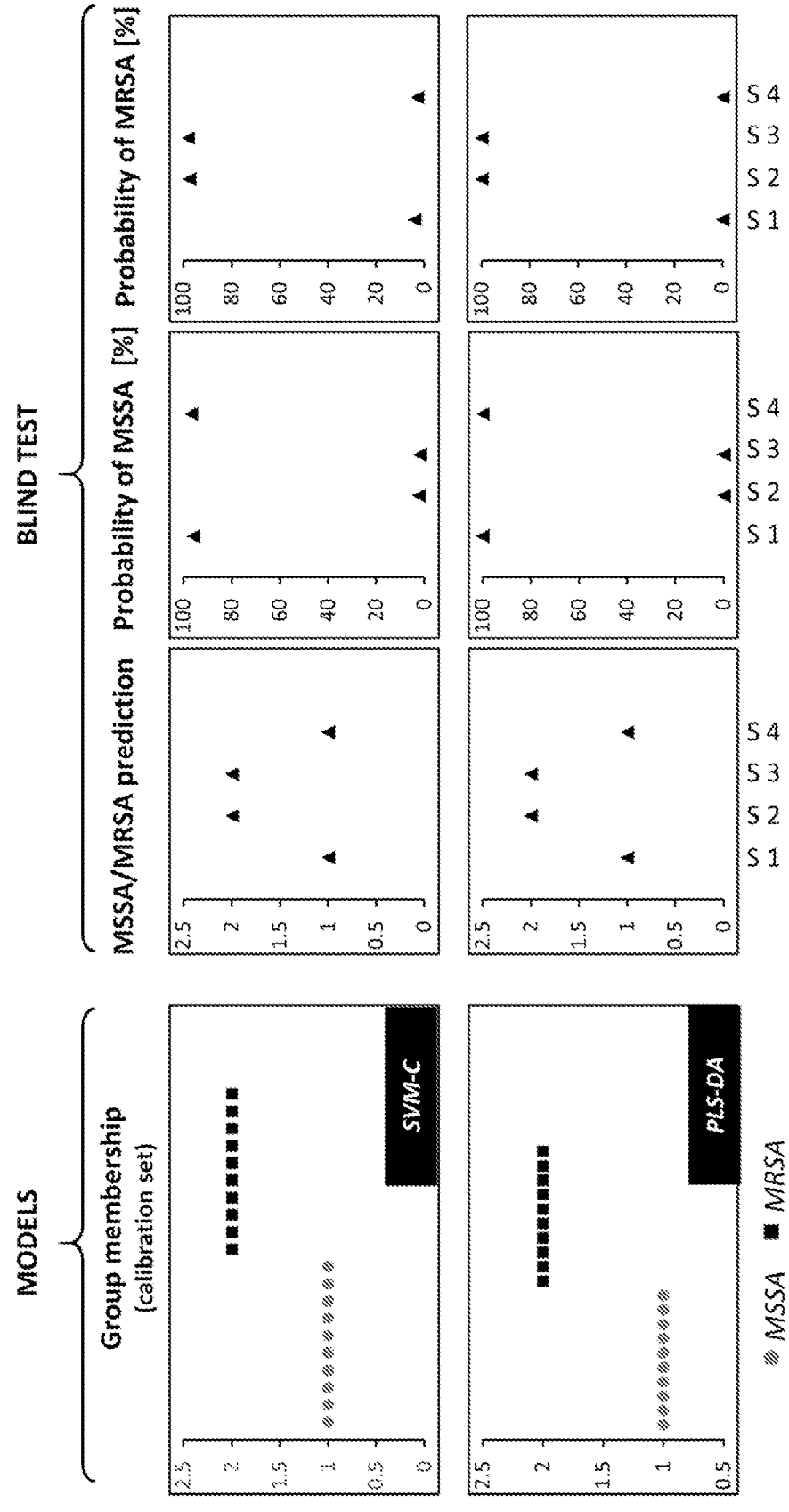

MODELS
BLIND TEST
Group membership
(calibration set)
MSSA/MRSA prediction
Probability of MSSA [%]
Probability of MRSA [%]
SVM-C
PLS-DA
MSSA
MRSA
S 1
S 2
S 3
S 4

FIG. 21

FIG. 22

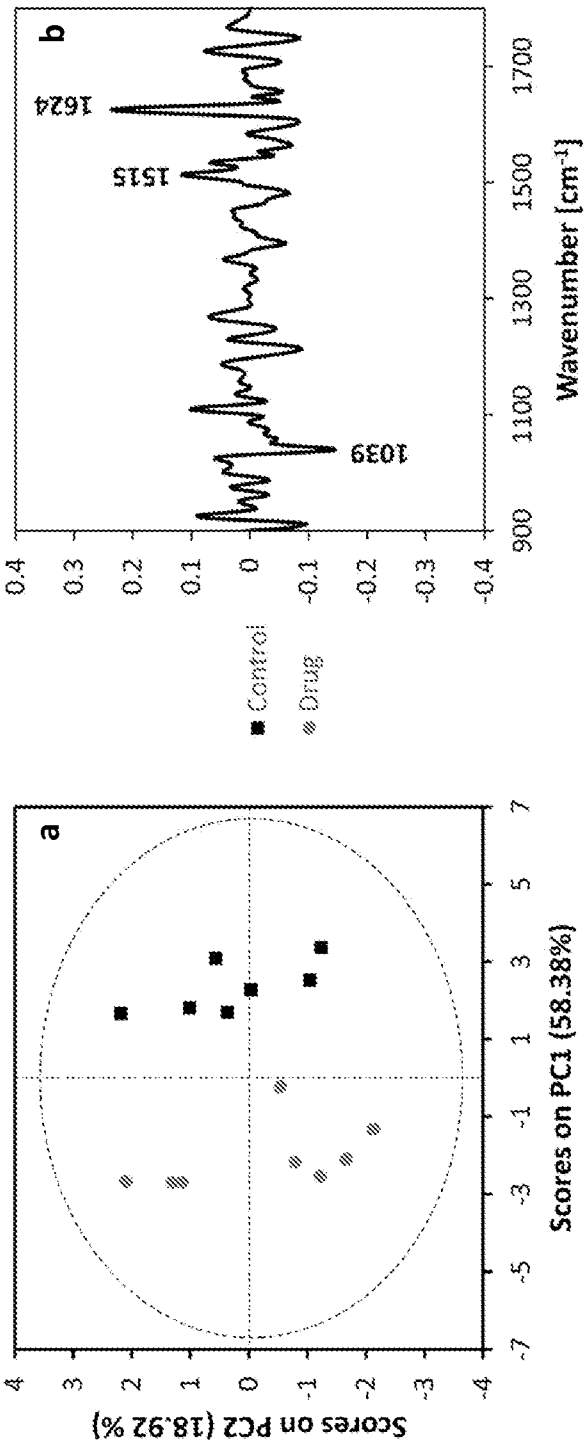
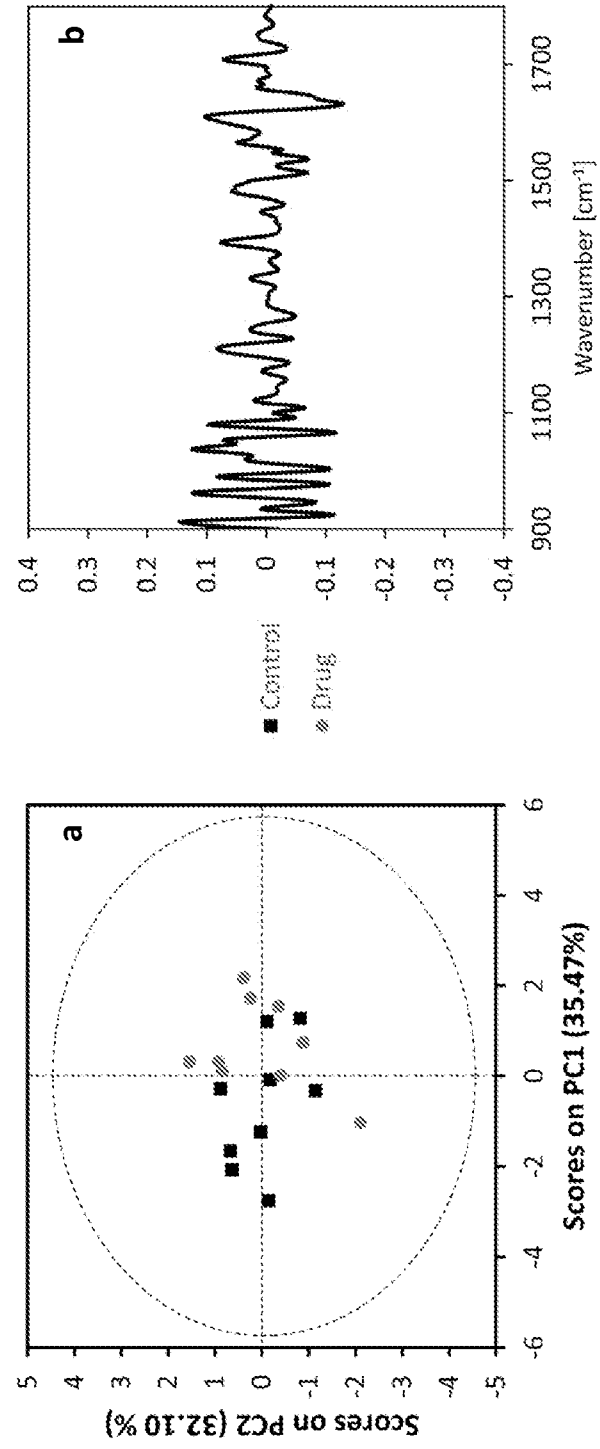

a
Scores on PC2 (18.92 %)
Scores on PC1 (58.38%)
b
1624
1515
1039
Wavenumber [cm-1]
Control
Drug

FIG. 23


a
Scores on PC2 (32.10 %)
Scores on PC1 (35.47%)
b
Wavenumber [cm-1]
Control
Drug

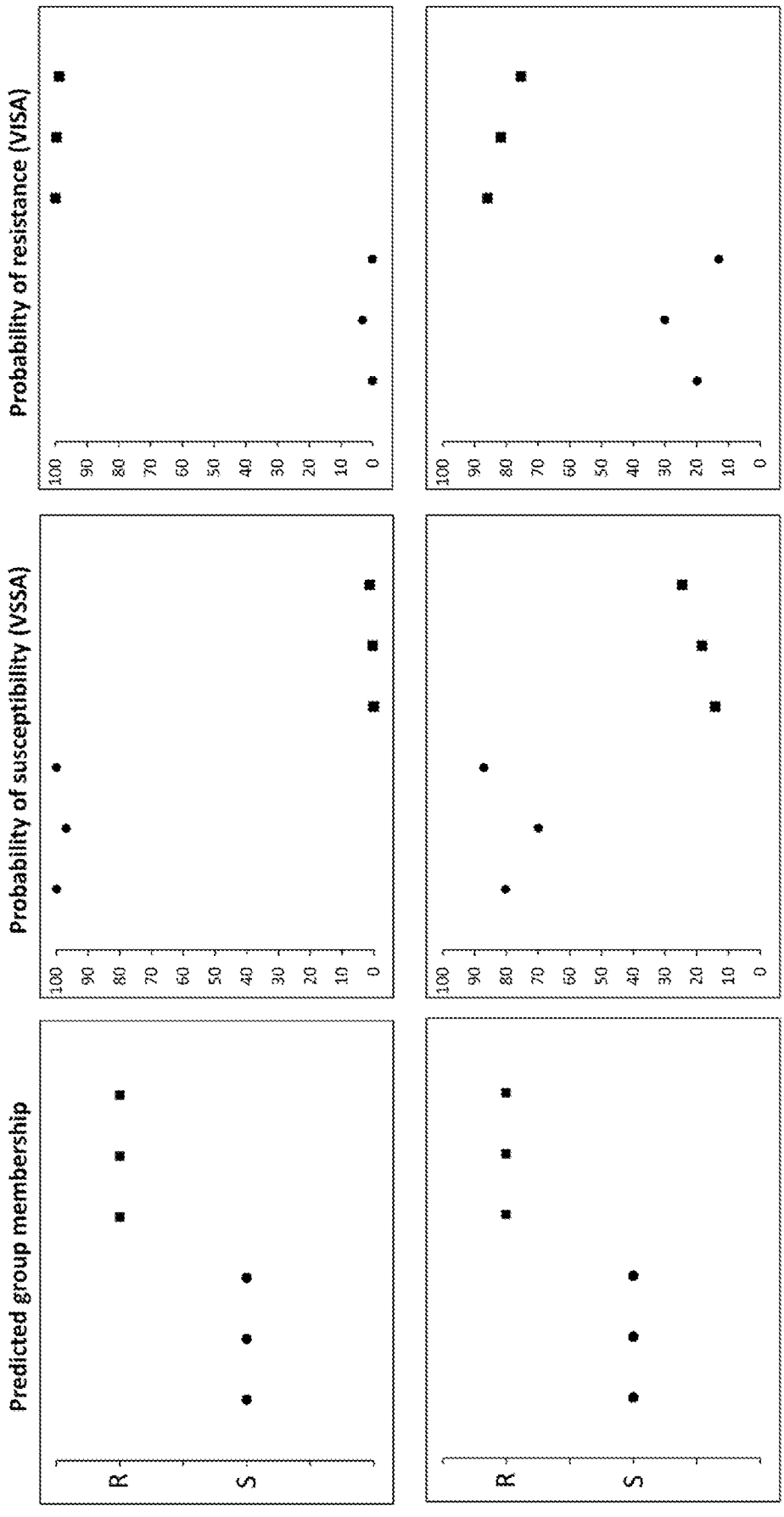

PLS-DA
SVM-C
Predicted group membership
R
S
Probability of susceptibility (VSSA)
Probability of resistance (VISA)
100
90
80
70
60
50
40
30
20
10
0
VSSA
VISA

FIG. 24

FIG. 25

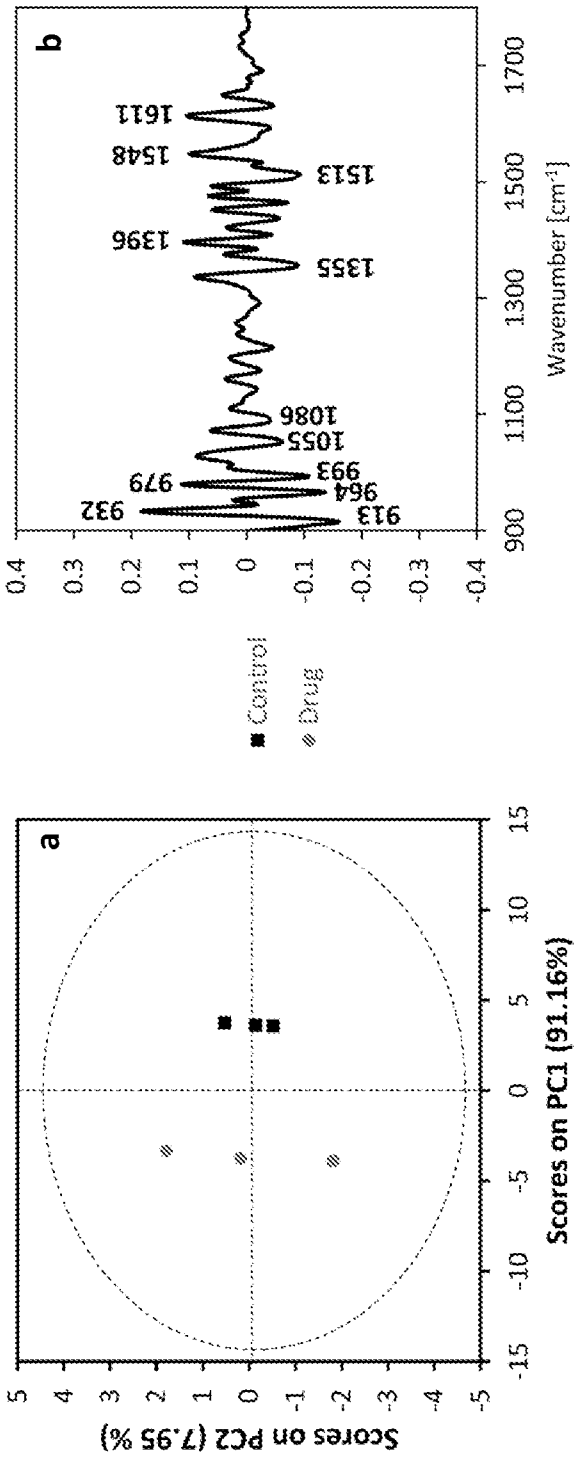

a
Scores on PC1 (91.16%)
Scores on PC2 (7.95 %)
b
Wavenumber [cm-1]
Control
Drug
913
932
964
979
993
1055
1086
1355
1396
1513
1548
1611

FIG. 26

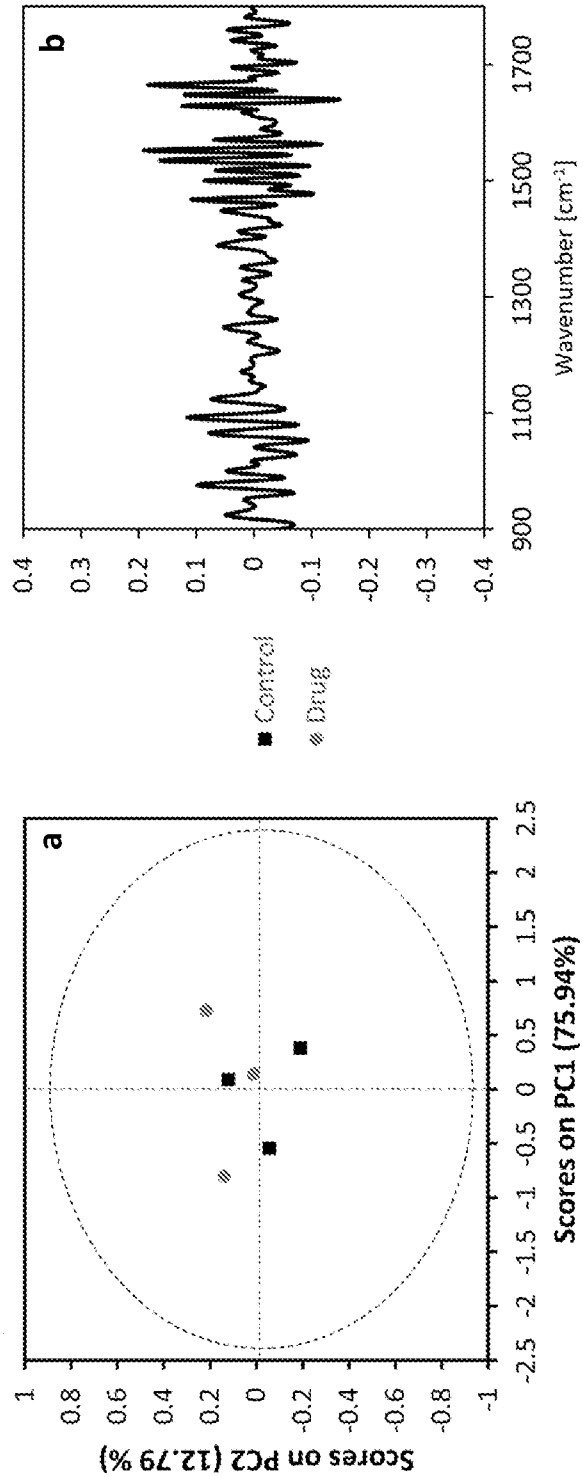

a
Scores on PC1 (75.94%)
Scores on PC2 (12.79 %)
b
Wavenumber [cm-1]
Control
Drug

FIG. 27

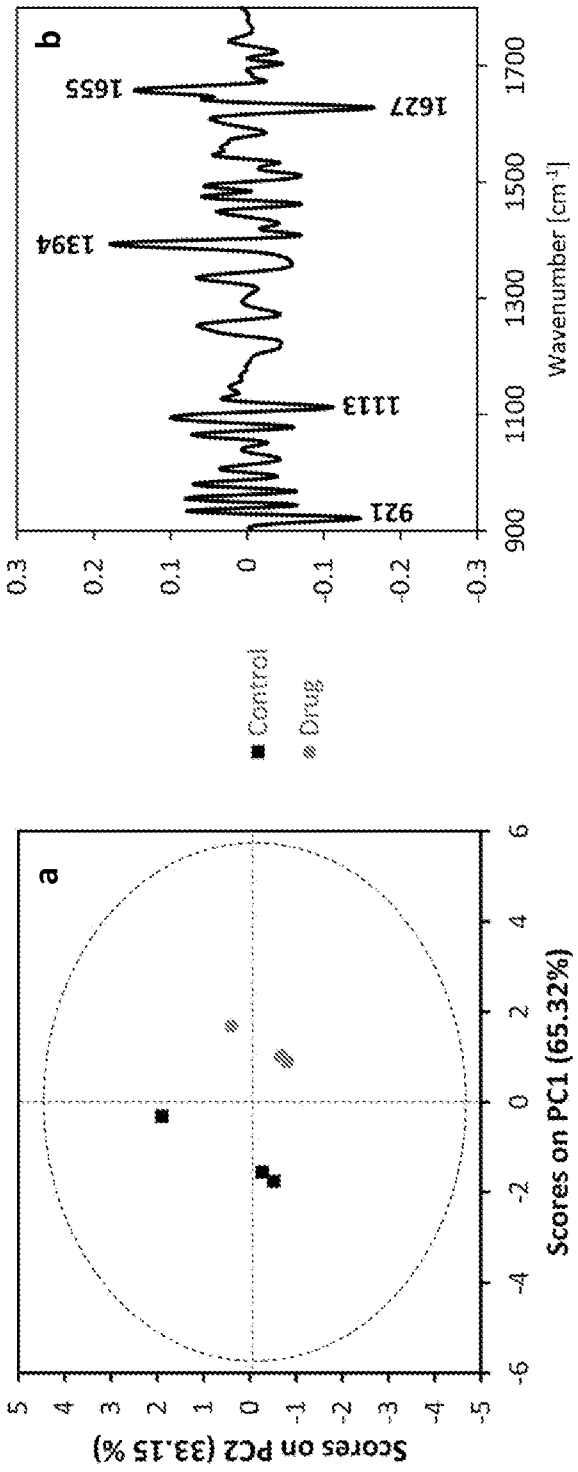

a
Scores on PC1 (65.32%)
Scores on PC2 (33.15 %)
Control
Drug
b
Wavenumber [cm-1]
921
1113
1394
1627
1655

FIG. 28

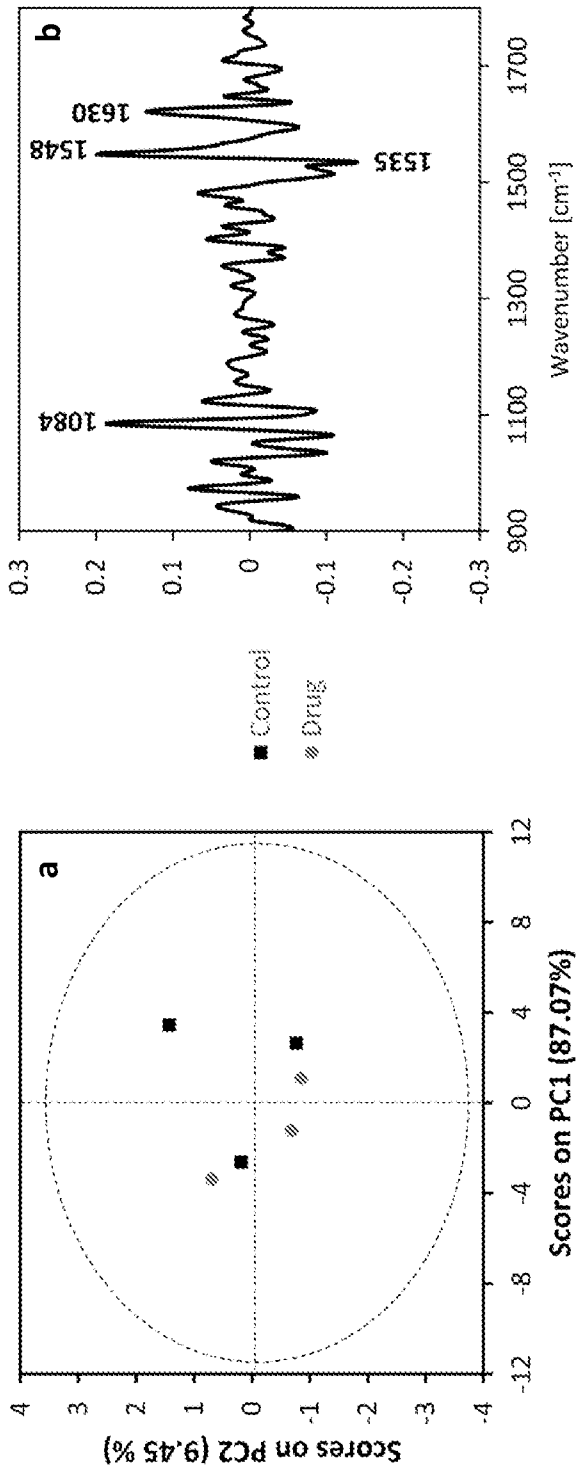

a
Scores on PC1 (87.07%)
Scores on PC2 (9.45 %)
Control
Drug
b
Wavenumber [cm-1]
1084
1535
1548
1630

FIG. 29

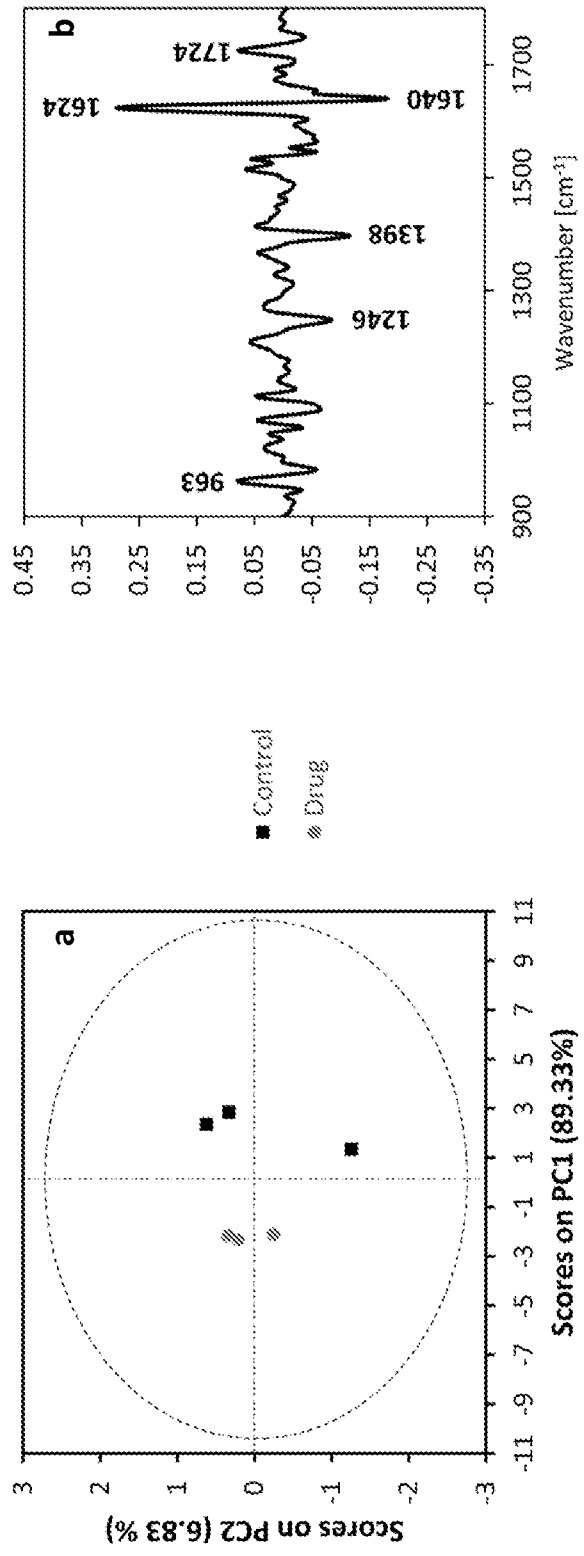

a
Scores on PC2 (6.83 %)
Scores on PC1 (89.33%)
Control
Drug
b
Wavenumber [cm-1]
963
1246
1398
1624
1640
1724

FIG. 30

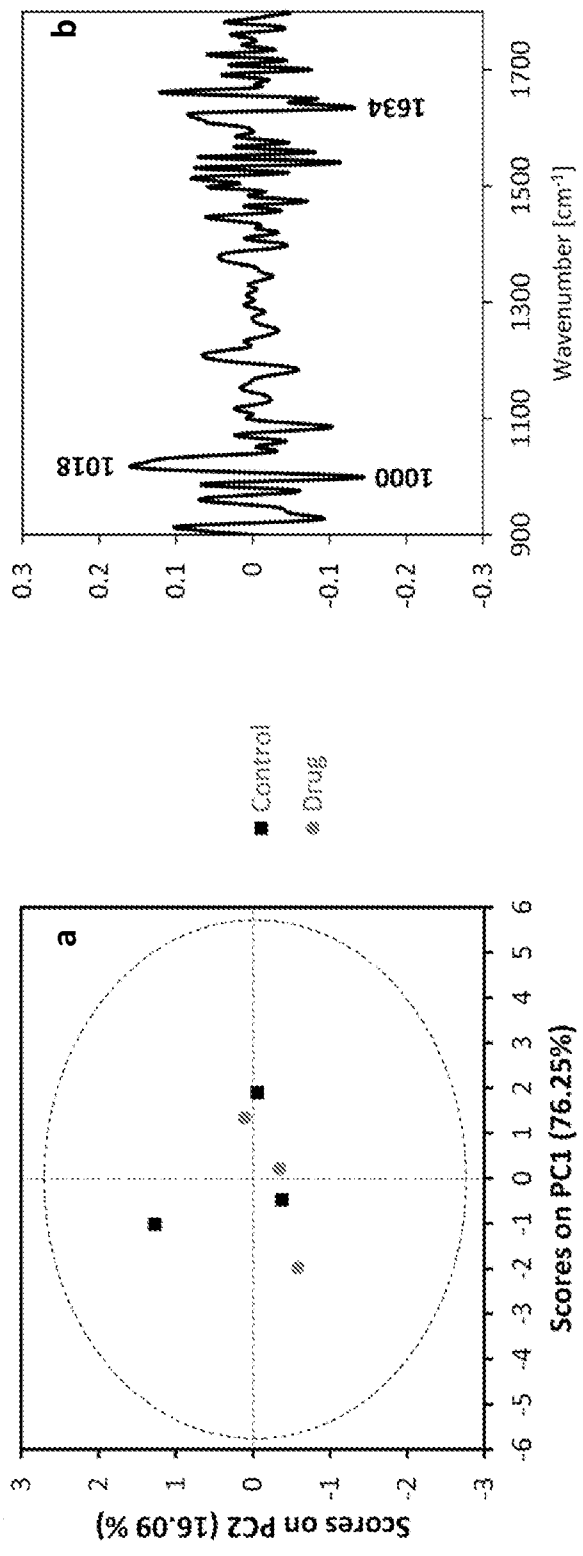

a
Scores on PC2 (16.09 %)
Scores on PC1 (76.25%)
Control
Drug
b
Wavenumber [cm-1]
1000
1018
1634

FIG. 31

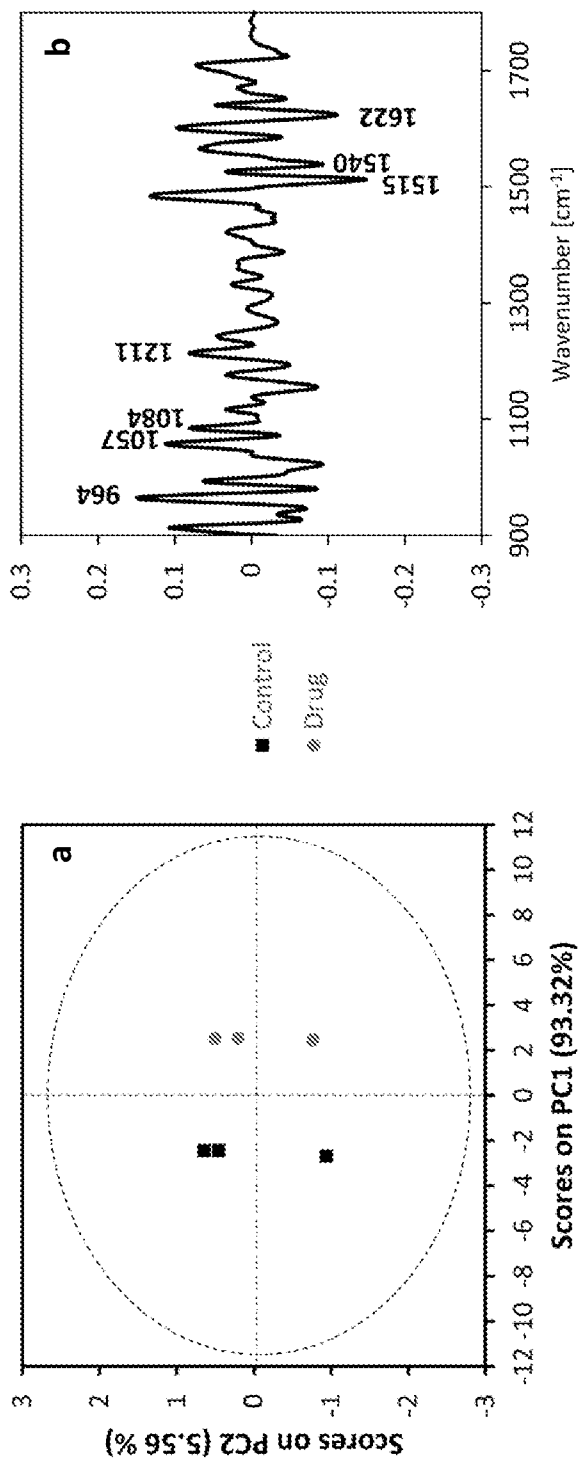

a
Scores on PC1 (93.32%)
Scores on PC2 (5.56 %)
b
Wavenumber [cm-1]
964
1057
1084
1211
1515
1540
1622
Control
Drug

FIG. 32

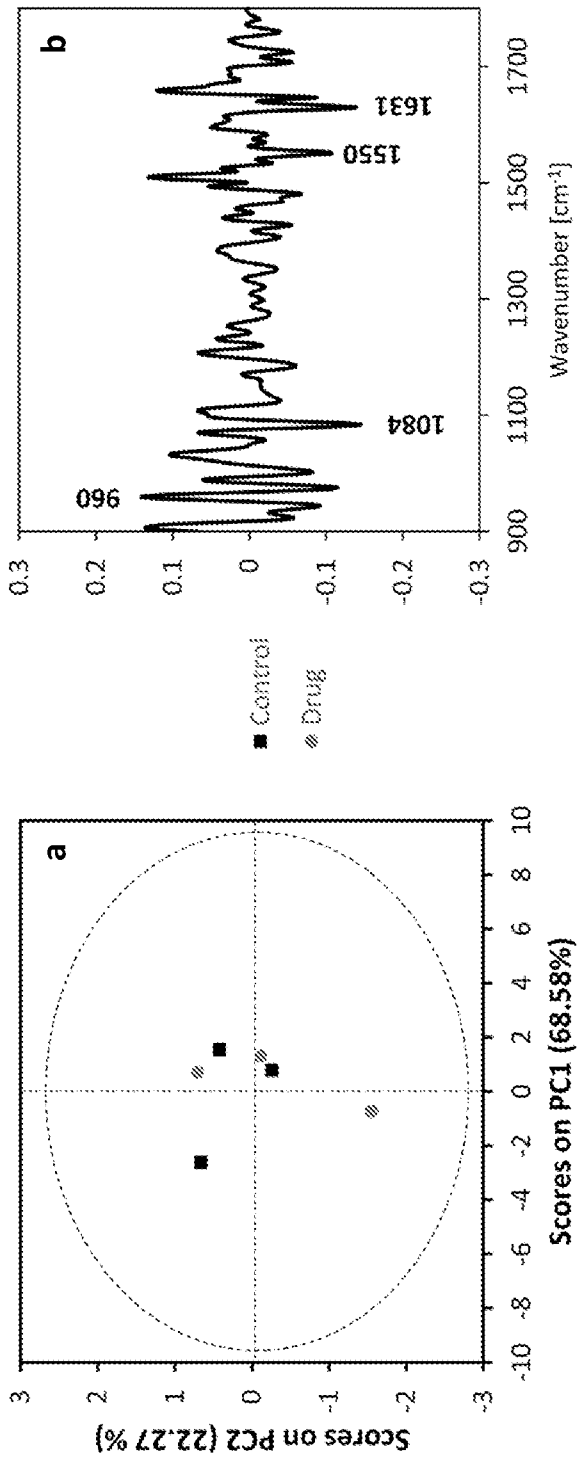

a
Scores on PC1 (68.58%)
Scores on PC2 (22.27 %)
b
Wavenumber [cm-1]
960
1084
1550
1631
Control
Drug

FIG. 33

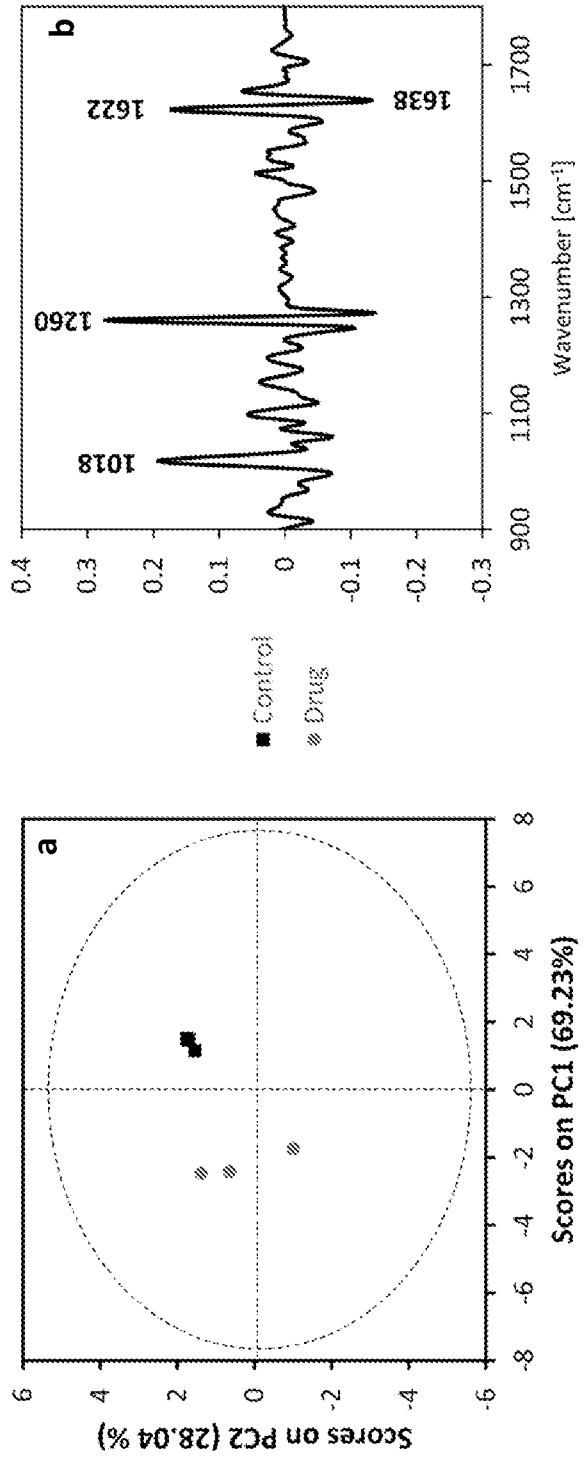

a
Scores on PC1 (69.23%)
Scores on PC2 (28.04 %)
b
1018
1260
1622
1638
Wavenumber [cm-1]
Control
Drug

FIG. 34

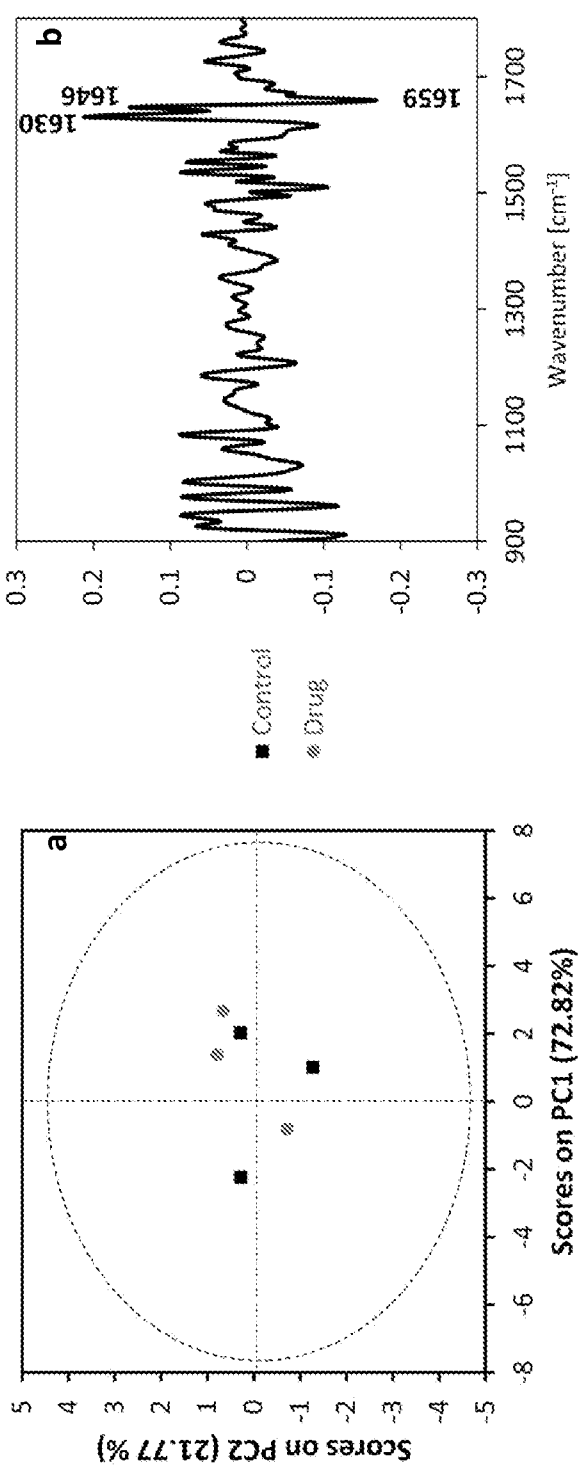

a
Scores on PC1 (72.82%)
Scores on PC2 (21.77 %)
b
1630
1646
1659
Wavenumber [cm-1]
Control
Drug

FIG. 35

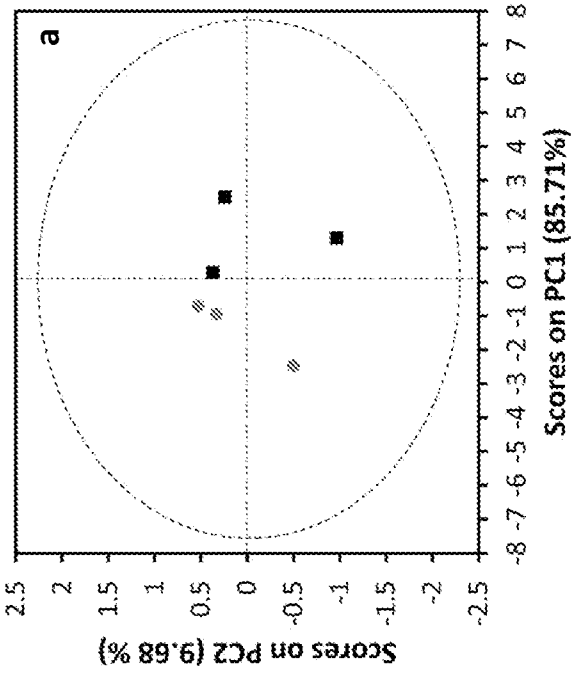

a
Control
Drug
Scores on PC1 (85.71%)
Scores on PC2 (9.68 %)
b
Wavenumber [cm-1]
960
980
1006
1057
1092
1515
1540
1622
1640

FIG. 36

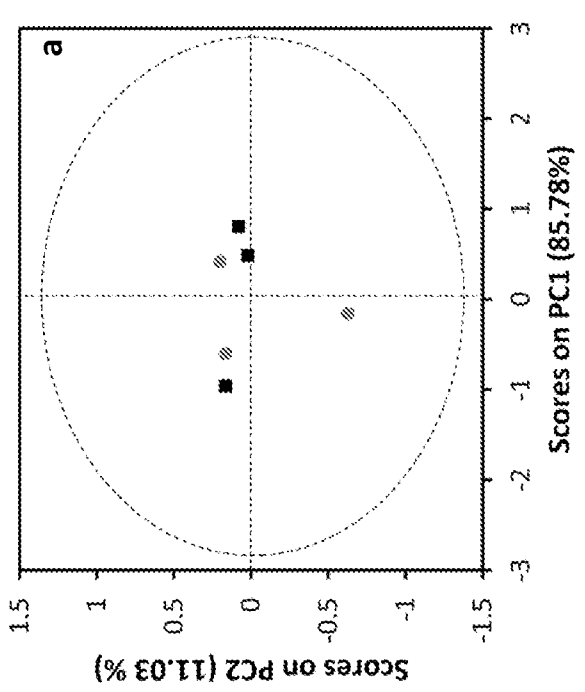

a
Control
Drug
Scores on PC1 (85.78%)
Scores on PC2 (11.03 %)
b
Wavenumber [cm-1]
960
1088
1210
1508
1550
1630

FIG. 37

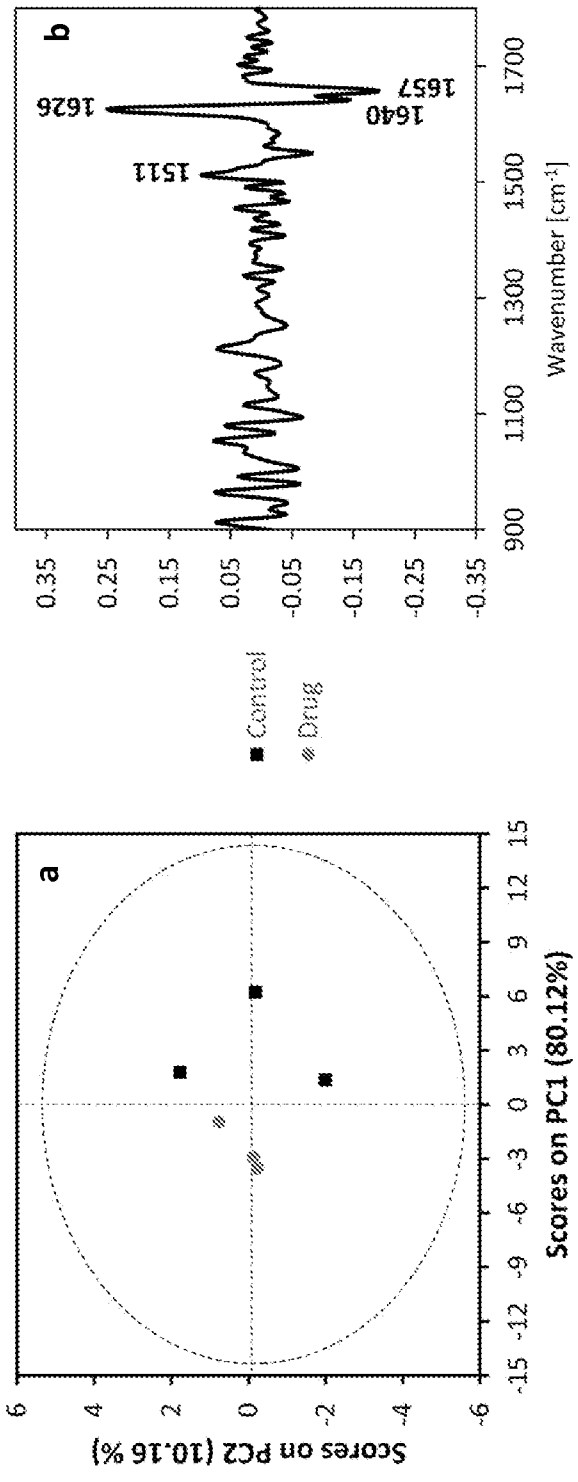

a
Scores on PC2 (10.16 %)
Scores on PC1 (80.12%)
b
1626
1640
1657
1511
Wavenumber [cm-1]
Control
Drug

FIG. 38

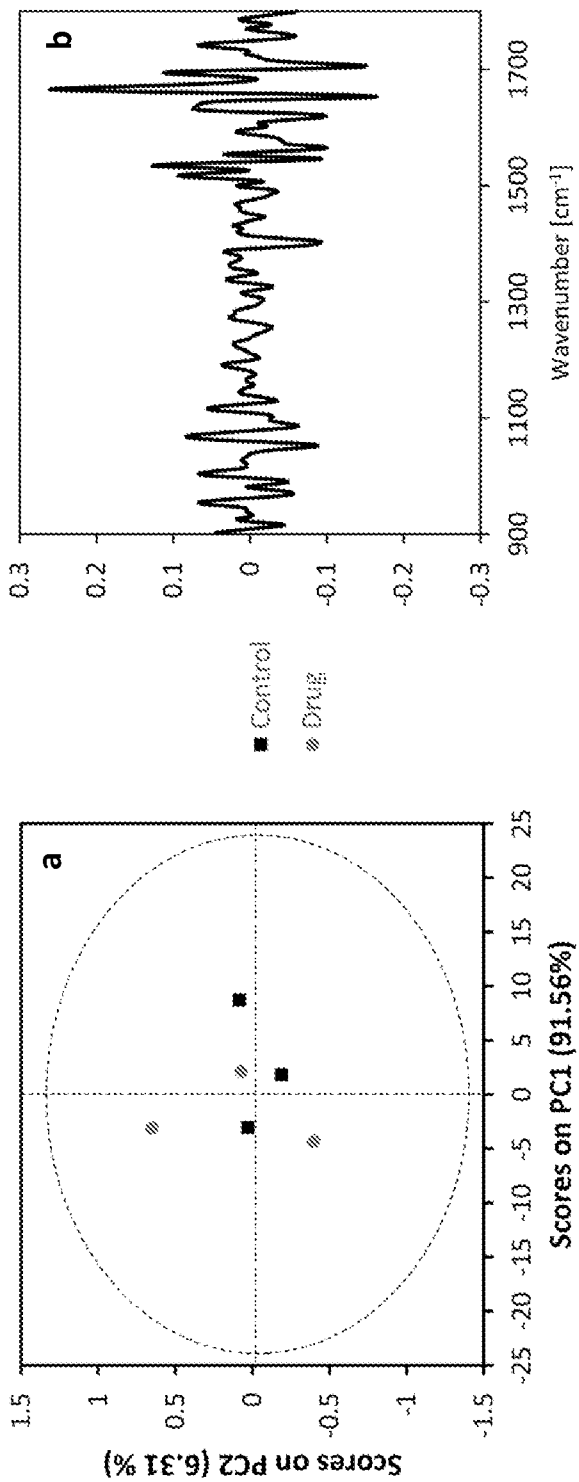

a
Scores on PC2 (6.31 %)
Scores on PC1 (91.56%)
b
Wavenumber [cm-1]
Control
Drug

FIG. 39

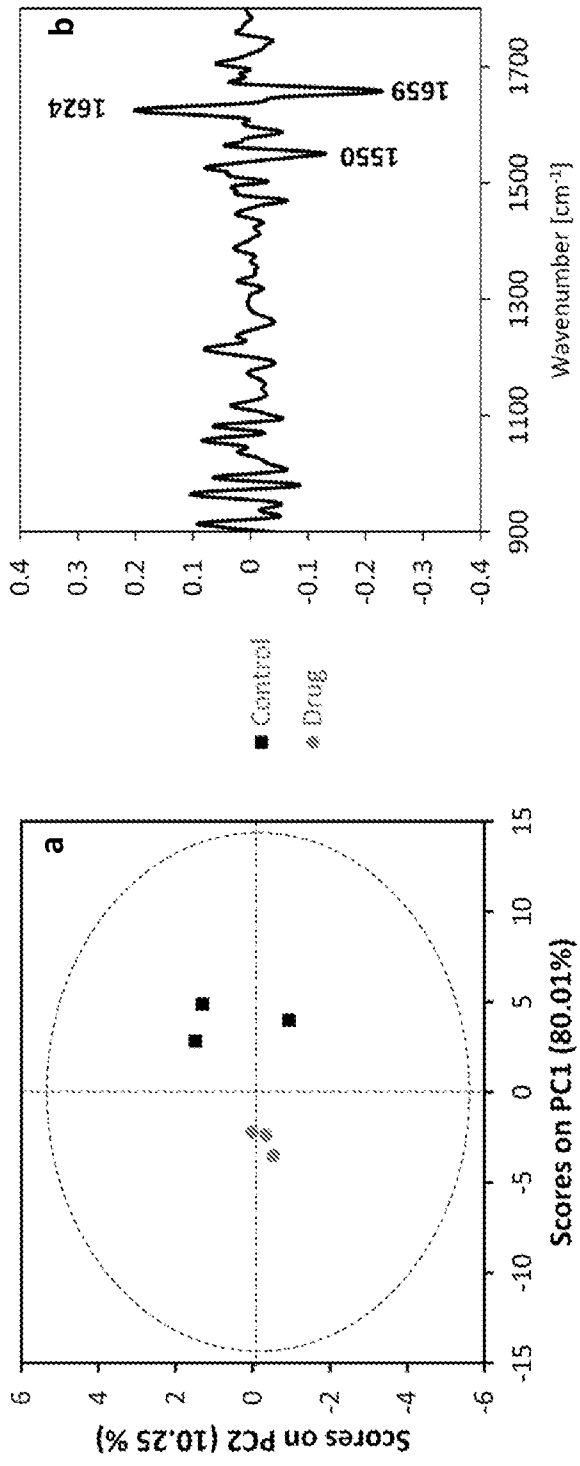

a
Scores on PC1 (80.01%)
Scores on PC2 (10.25 %)
Control
Drug
b
1659
1624
1550
Wavenumber [cm-1]

FIG. 40

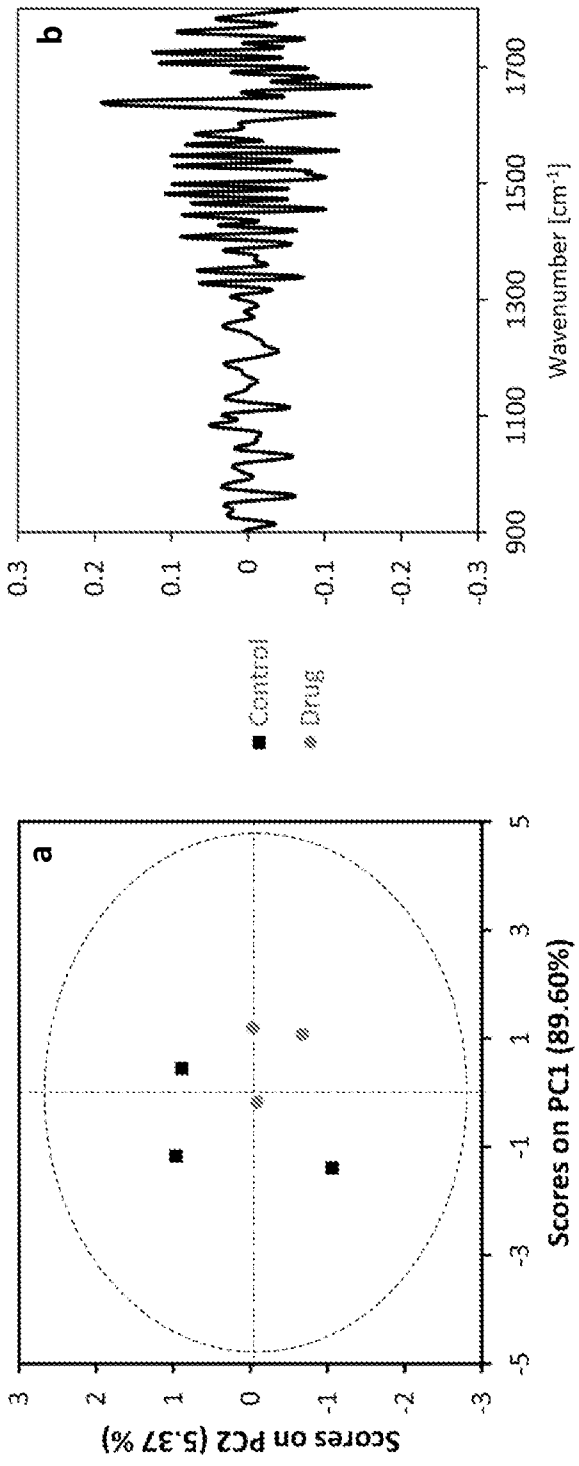

a
Scores on PC1 (89.60%)
Scores on PC2 (5.37 %)
Control
Drug
b
Wavenumber [cm-1]

FIG. 41

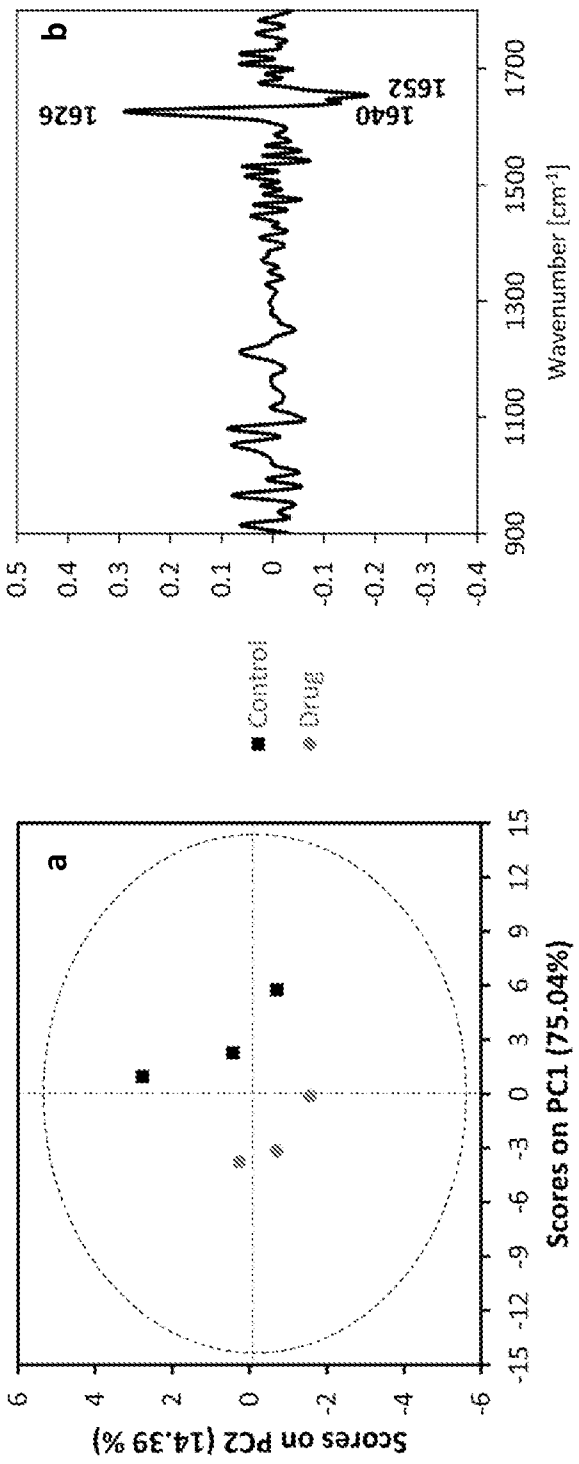

a
Scores on PC1 (75.04%)
Scores on PC2 (14.39 %)
Control
Drug
b
Wavenumber [cm-1]
1626
1640
1652

FIG. 42

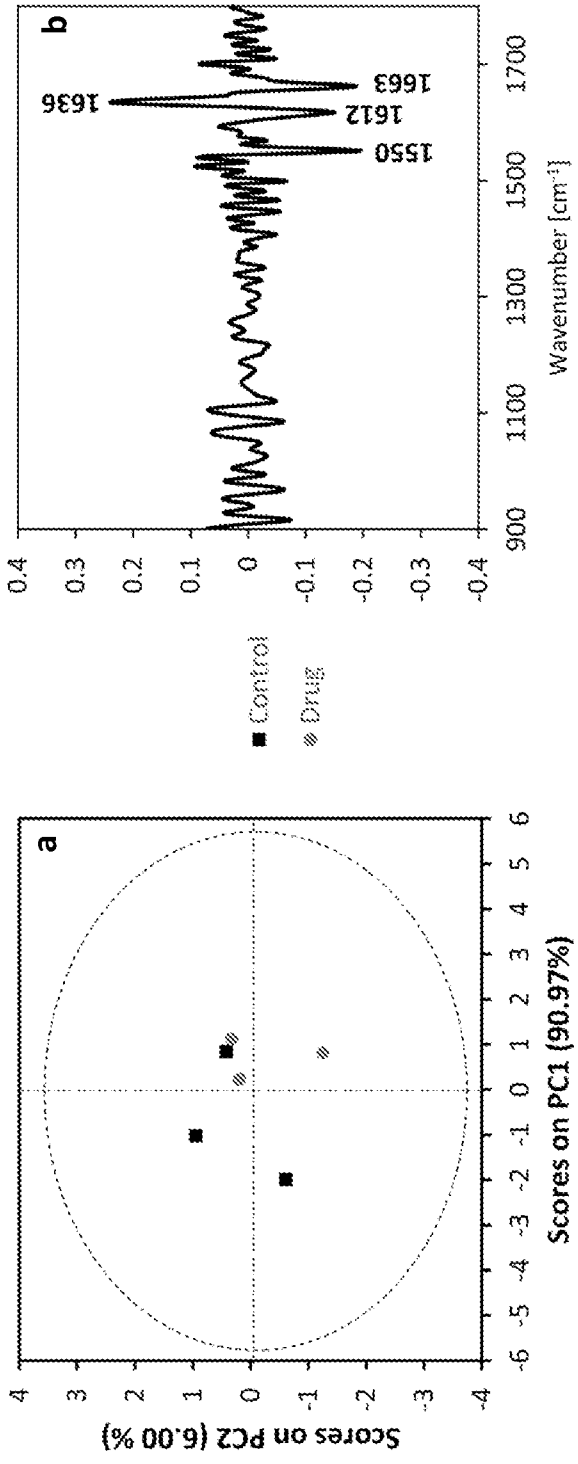

a
Scores on PC1 (90.97%)
Scores on PC2 (6.00 %)
Control
Drug
b
Wavenumber [cm-1]
1636
1612
1550
1663

FIG. 43

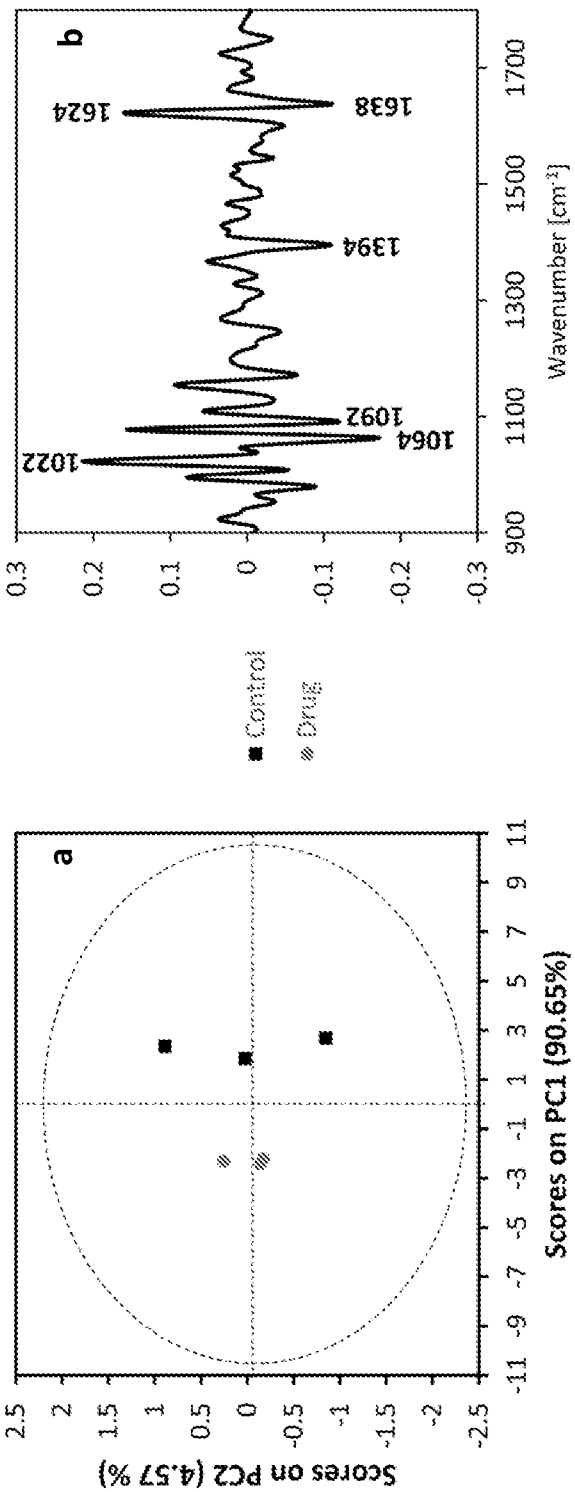

a
Scores on PC1 (90.65%)
Scores on PC2 (4.57 %)
Control
Drug
b
Wavenumber [cm-1]
1022
1064
1092
1394
1624
1638

FIG. 44

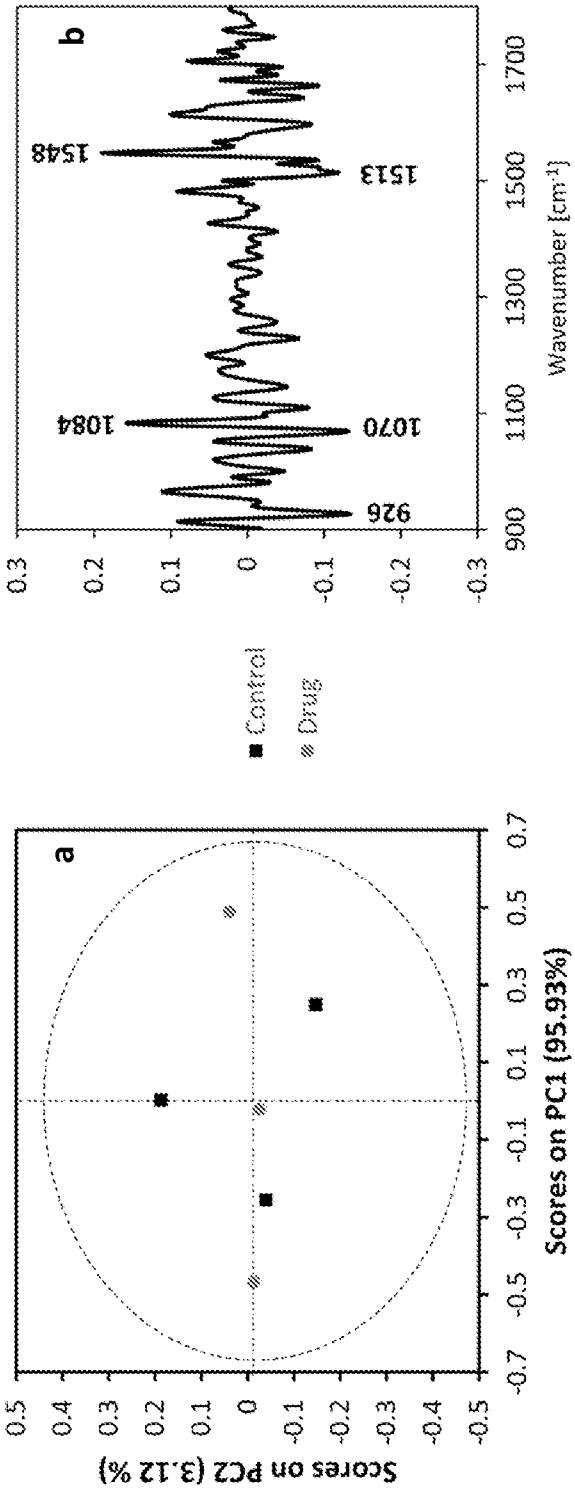

a
Scores on PC1 (95.93%)
Scores on PC2 (3.12 %)
Control
Drug
b
Wavenumber [cm-1]
926
1070
1084
1513
1548

FIG. 45

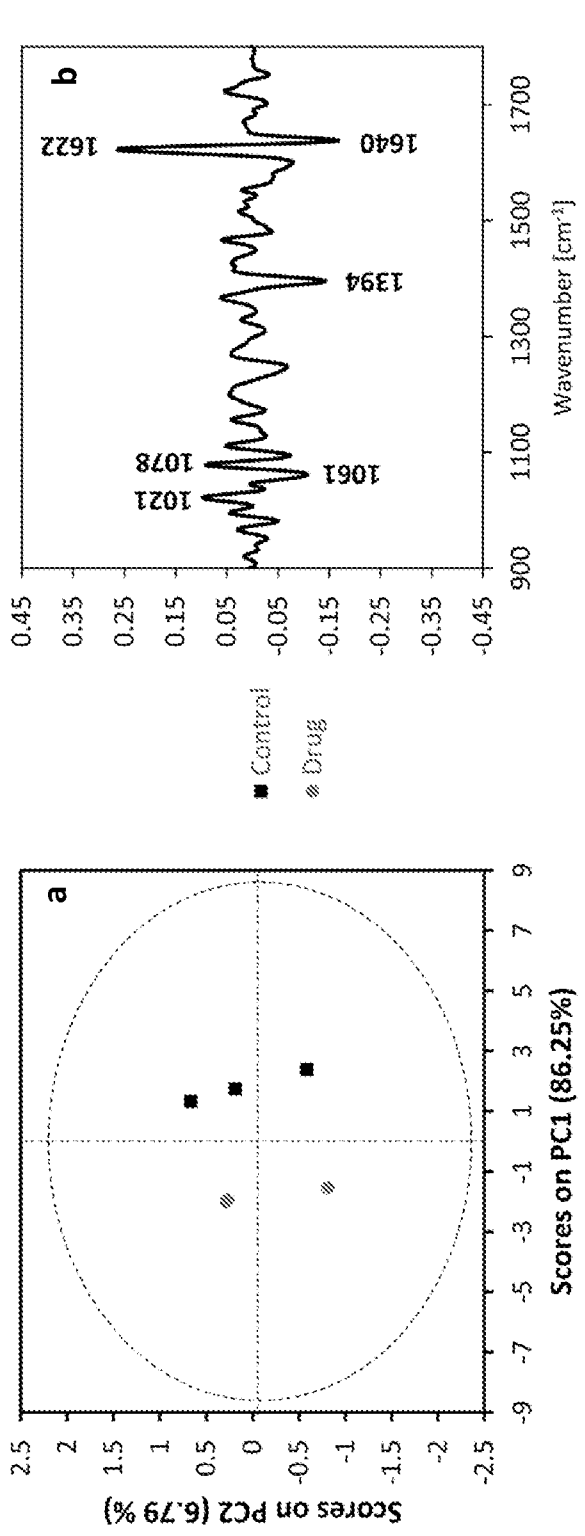

a
Scores on PC1 (86.25%)
Scores on PC2 (6.79 %)
Control
Drug
b
1622
1640
1394
1061
1078
1021
Wavenumber [cm-1]

FIG. 46

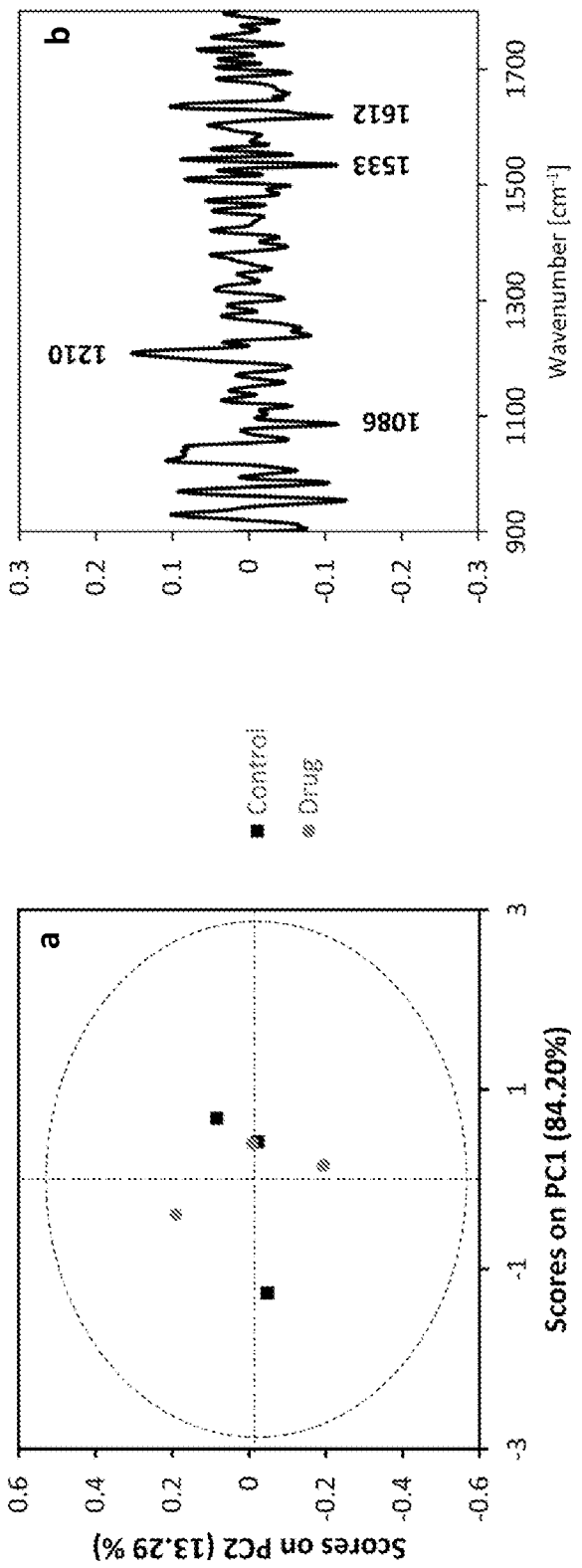

a
Scores on PC1 (84.20%)
Scores on PC2 (13.29 %)
Control
Drug
b
1612
1533
1210
1086
Wavenumber [cm-1]

FIG. 47

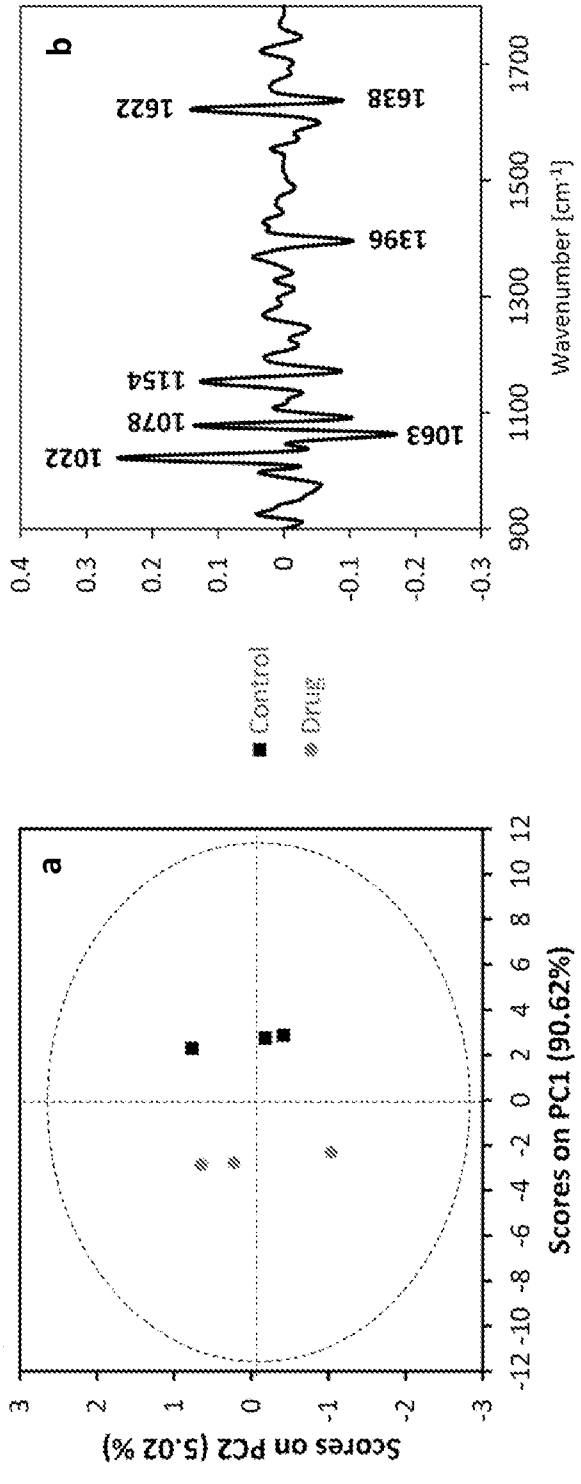

a
Scores on PC1 (90.62%)
Scores on PC2 (5.02 %)
Control
Drug
b
Wavenumber [cm-1]
1022
1078
1063
1154
1396
1622
1638

FIG. 48

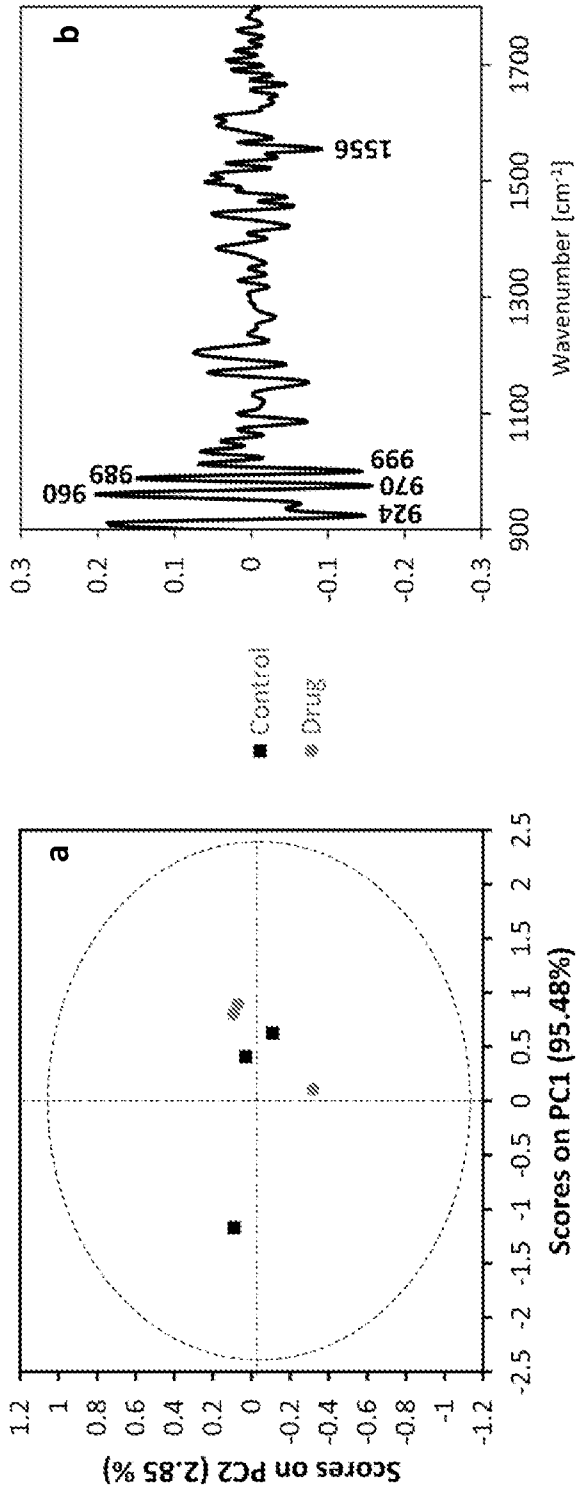

a
Scores on PC1 (95.48%)
Scores on PC2 (2.85 %)
Control
Drug
b
Wavenumber [cm-1]
924
960
970
989
999
1556

FIG. 49

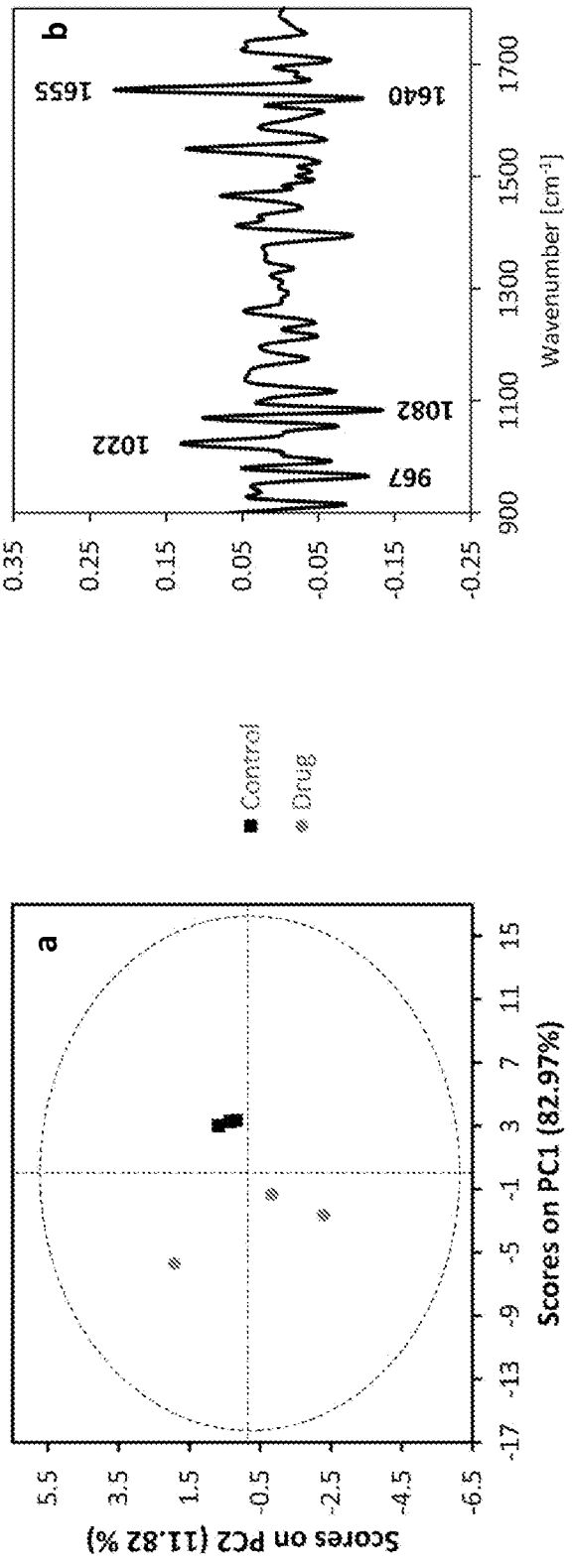

a
Scores on PC1 (82.97%)
Scores on PC2 (11.82 %)
Control
Drug
b
Wavenumber [cm-1]
1655
1640
1082
1022
967

FIG. 50

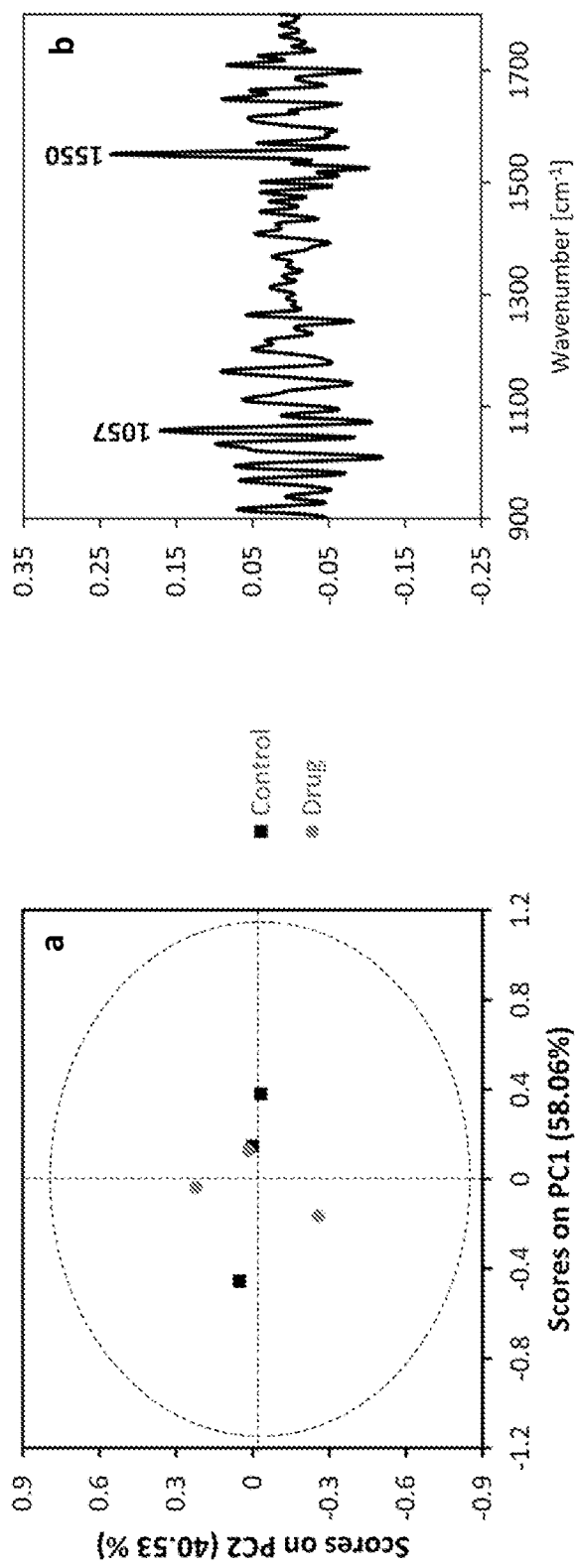

a
Scores on PC1 (58.06%)
Scores on PC2 (40.53 %)
Control
Drug
b
Wavenumber [cm-1]
1550
1057

FIG. 51

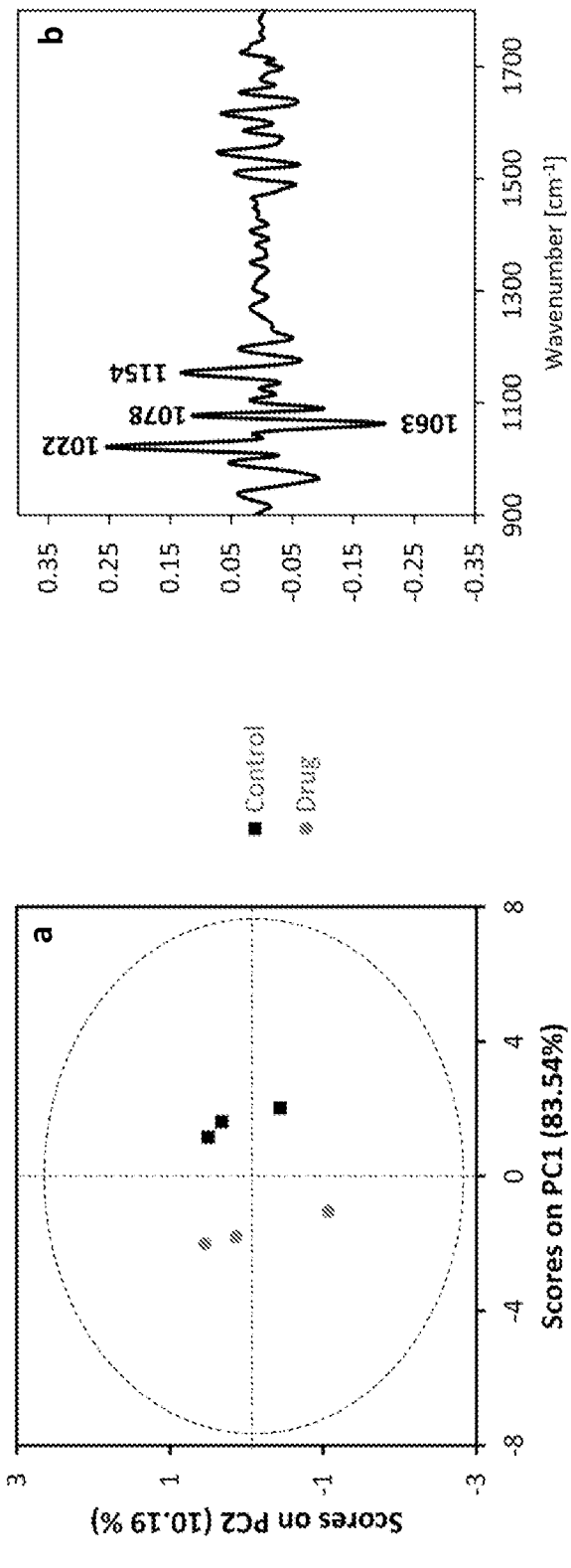

a
Scores on PC1 (83.54%)
Scores on PC2 (10.19 %)
Control
Drug
b
Wavenumber [cm-1]
1022
1078
1154
1063

FIG. 52

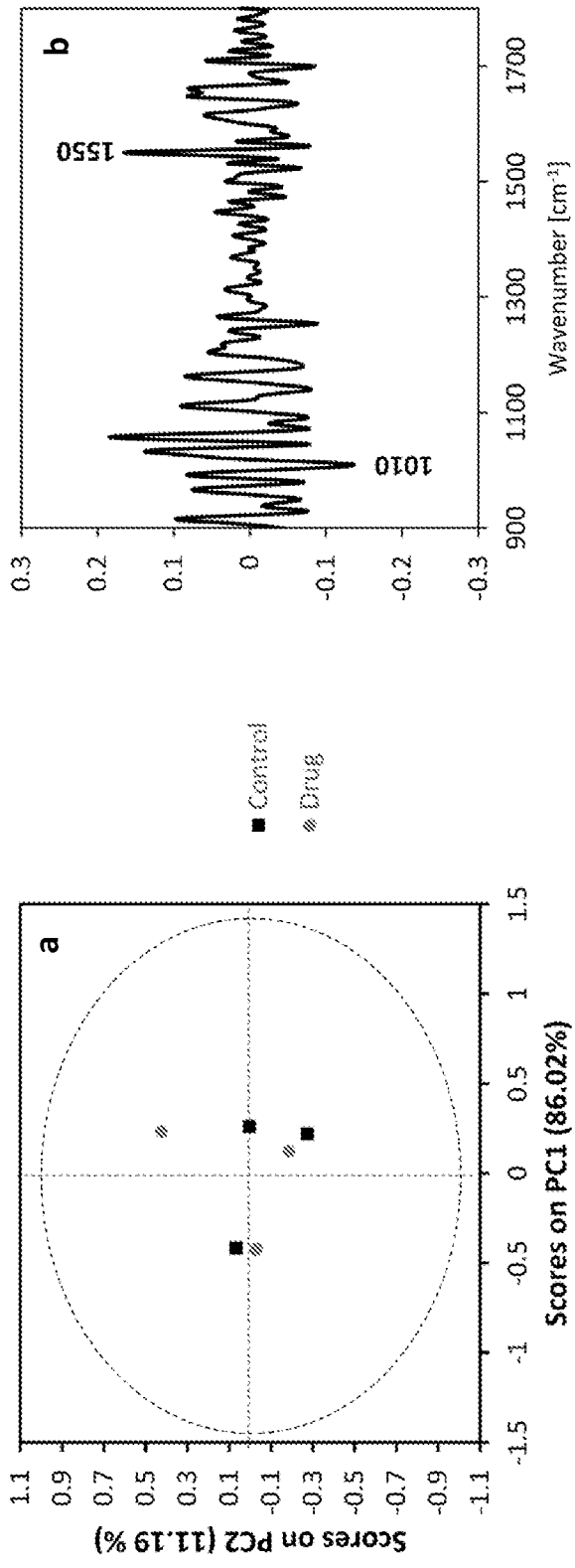

a
Scores on PC1 (86.02%)
Scores on PC2 (11.19 %)
Control
Drug
b
Wavenumber [cm-1]
1550
1010

FIG. 53

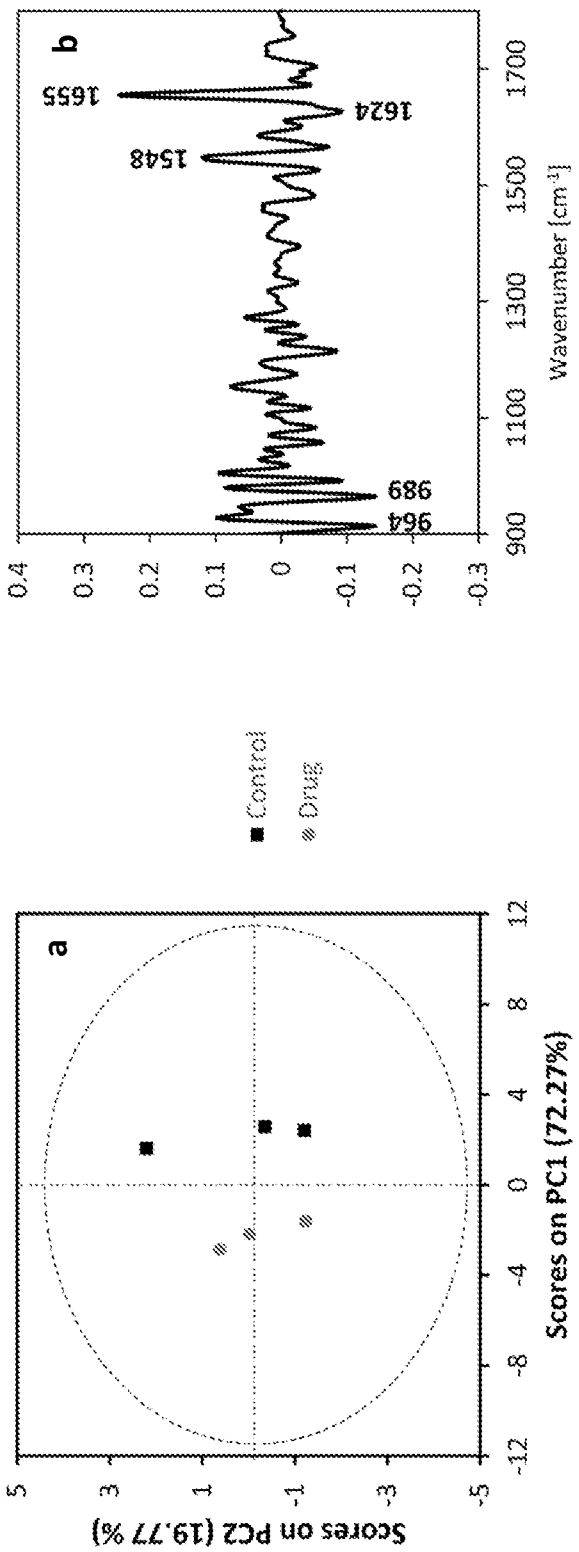

a
Scores on PC1 (72.27%)
Scores on PC2 (19.77 %)
Control
Drug
b
Wavenumber (cm-1)
964
989
1548
1624
1655

FIG. 54

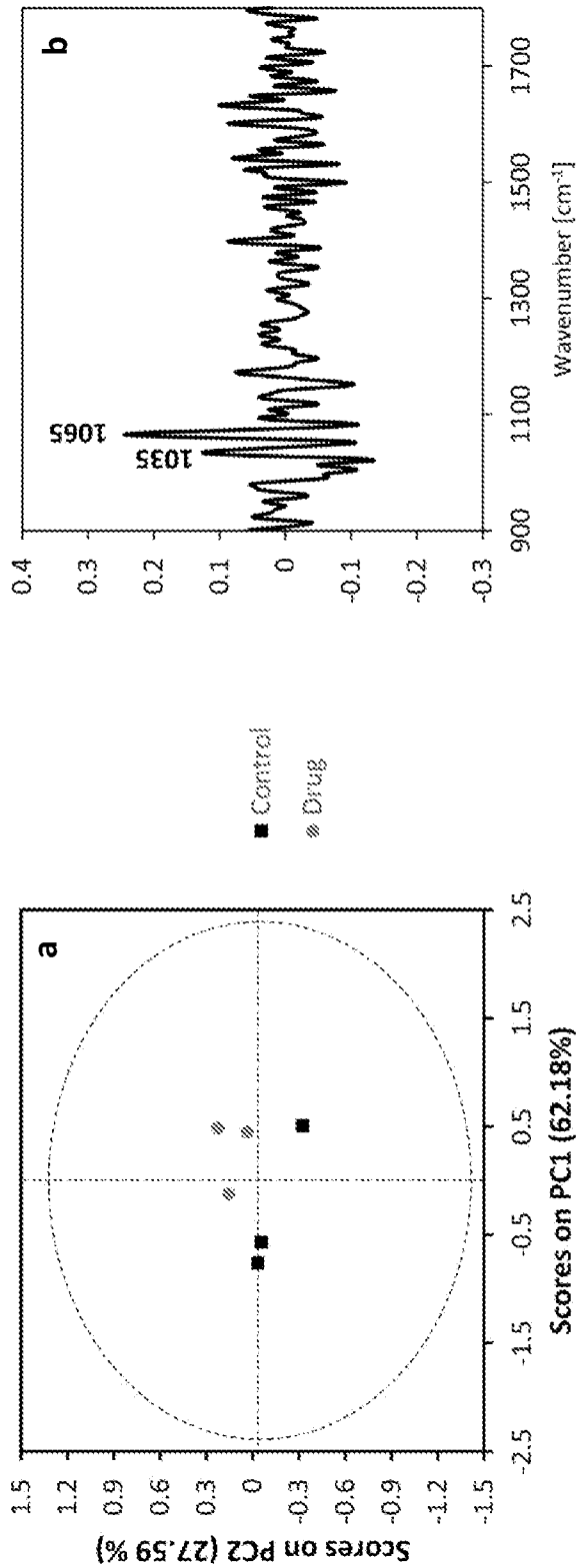

a
Scores on PC1 (62.18%)
Scores on PC2 (27.59 %)
Control
Drug
b
Wavenumber (cm-1)
1035
1065

FIG. 55

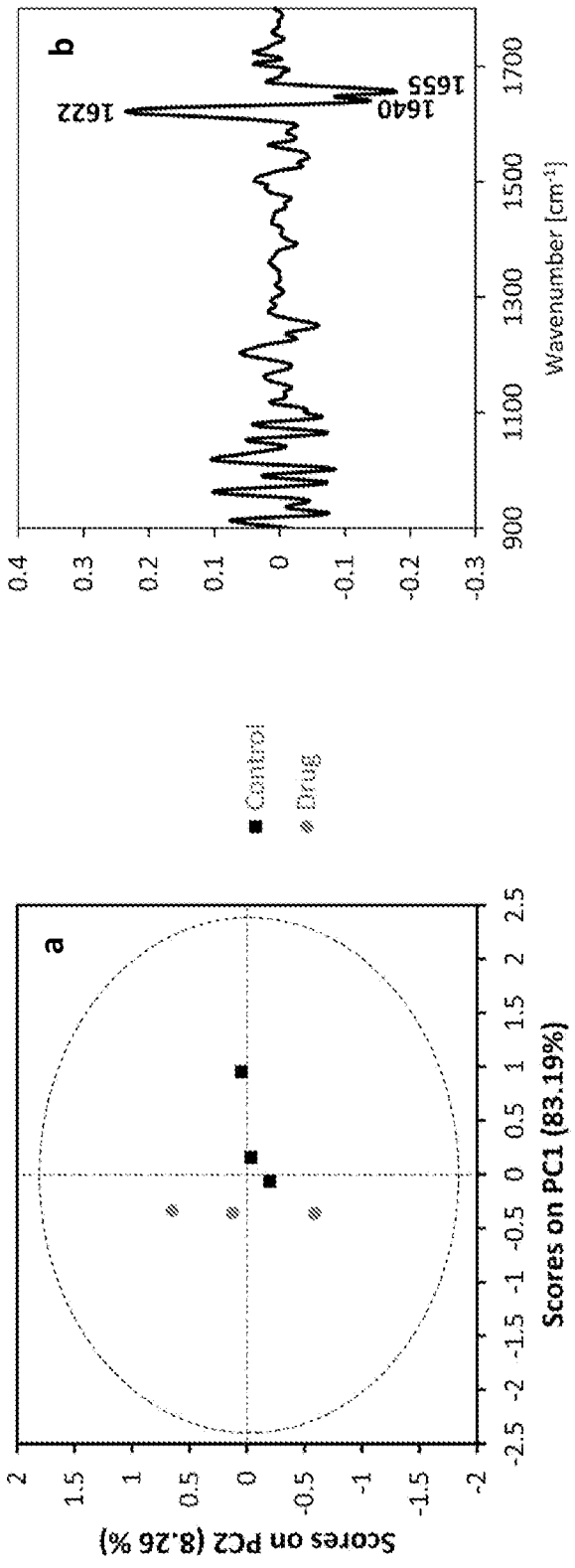

a
Scores on PC2 (8.26 %)
Scores on PC1 (83.19%)
Control
Drug
b
1622
1640
1655
Wavenumber [cm-1]

FIG. 56

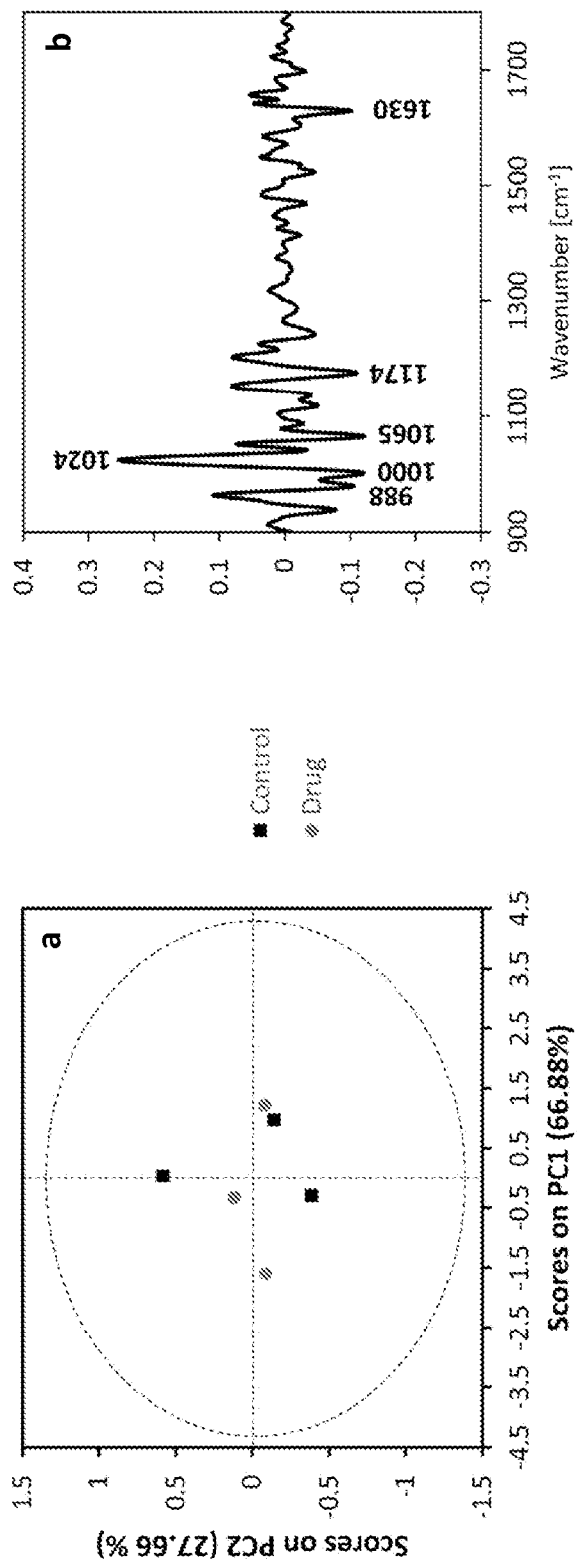

a
Scores on PC2 (27.66 %)
Scores on PC1 (66.88%)
Control
Drug
b
1024
1000
988
1065
1174
1630
Wavenumber [cm-1]

FIG. 57

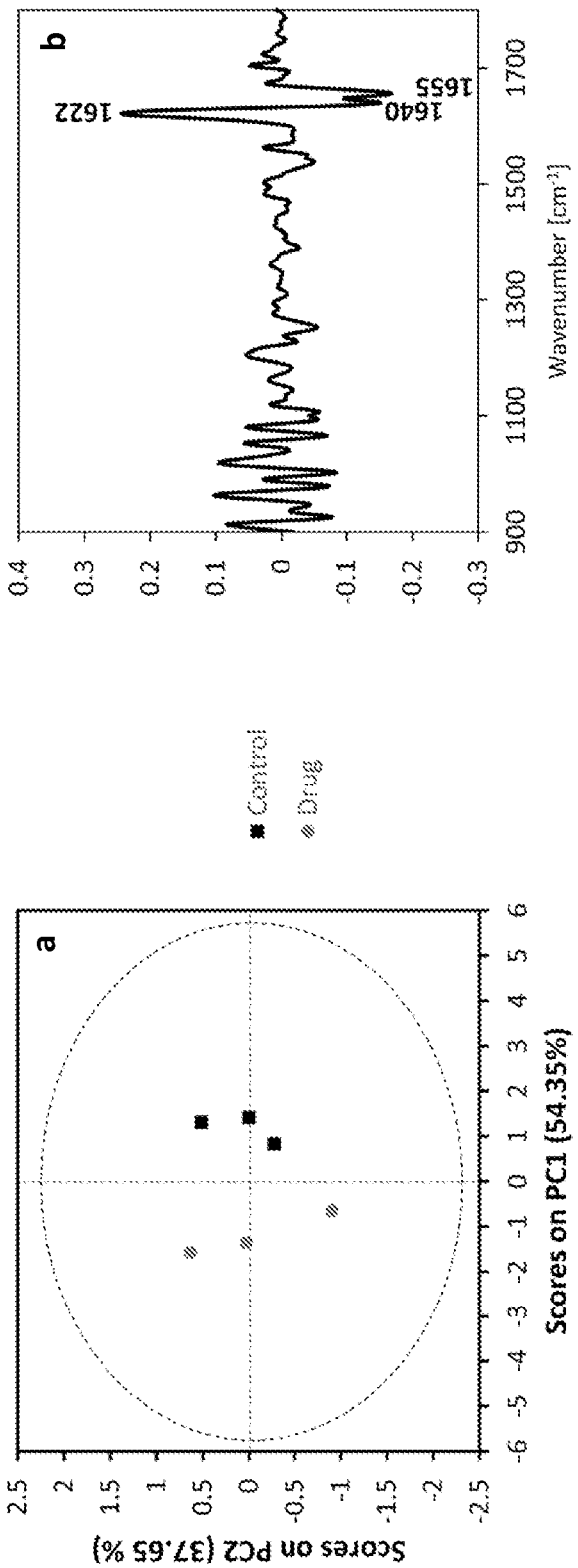

a
Scores on PC1 (54.35%)
Scores on PC2 (37.65 %)
Control
Drug
b
1622
1640
1655
Wavenumber [cm-1]

FIG. 58

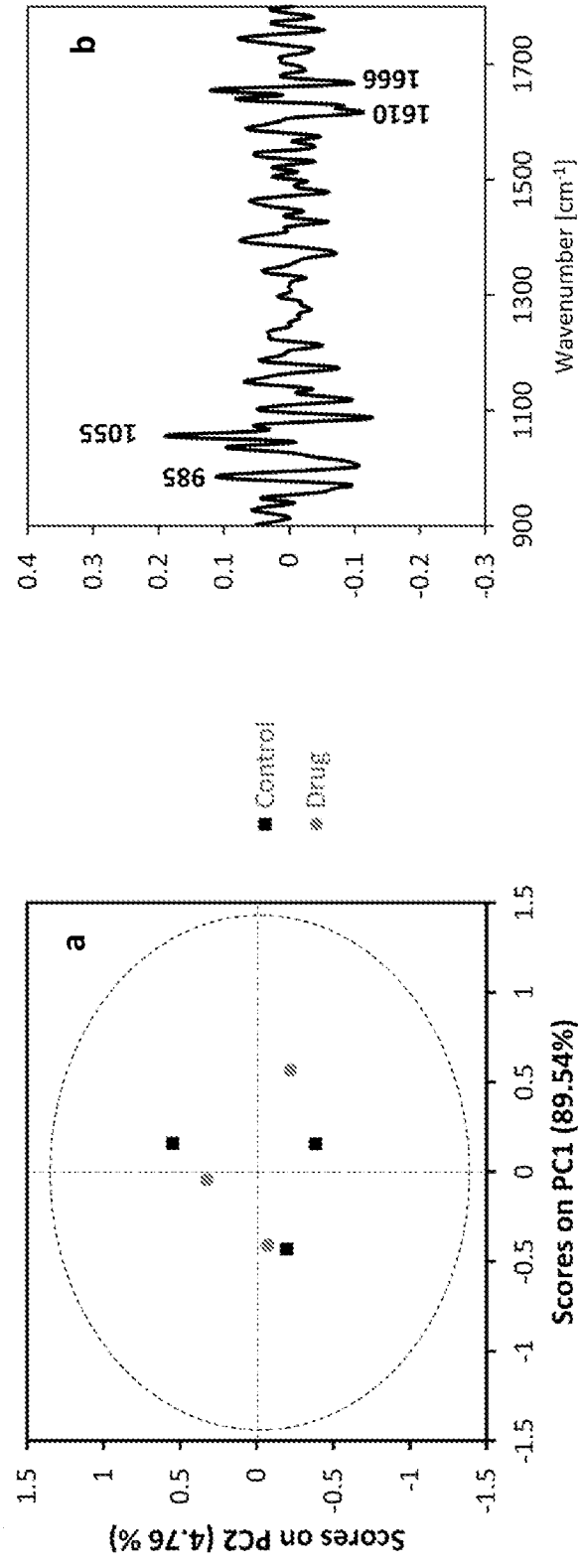

a
Scores on PC1 (89.54%)
Scores on PC2 (4.76 %)
Control
Drug
b
985
1055
1610
1666
Wavenumber [cm-1]

FIG. 59

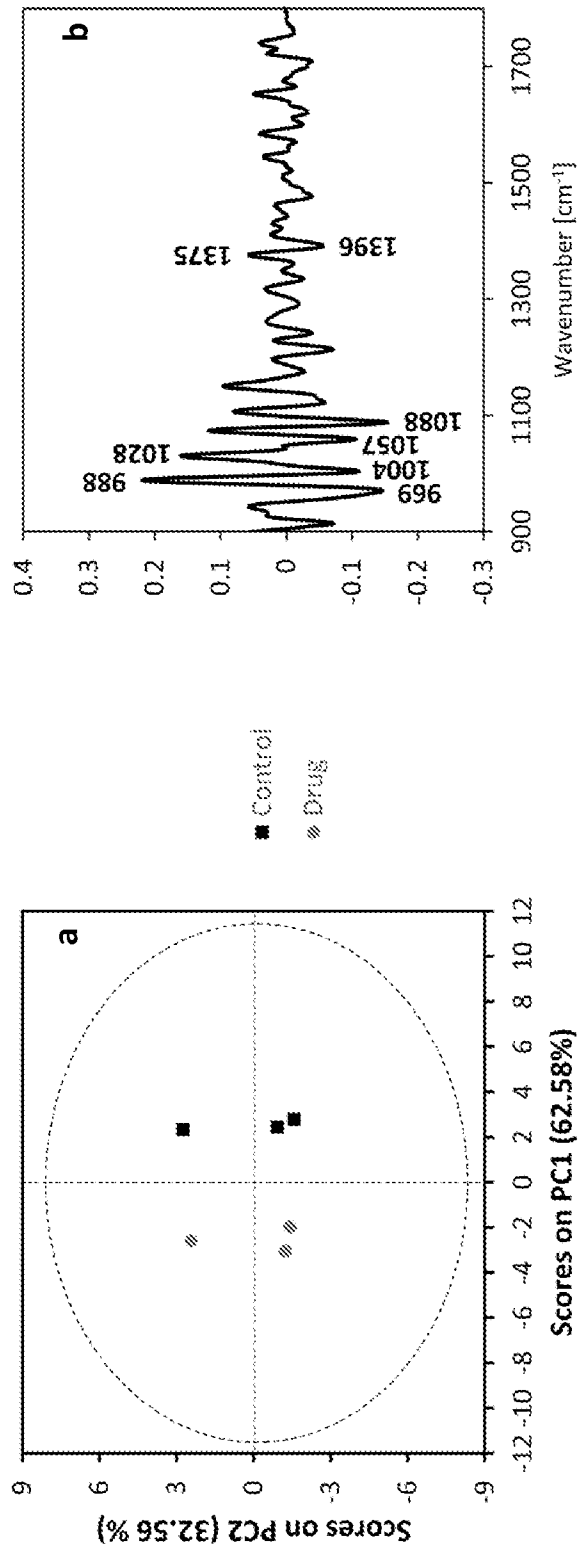
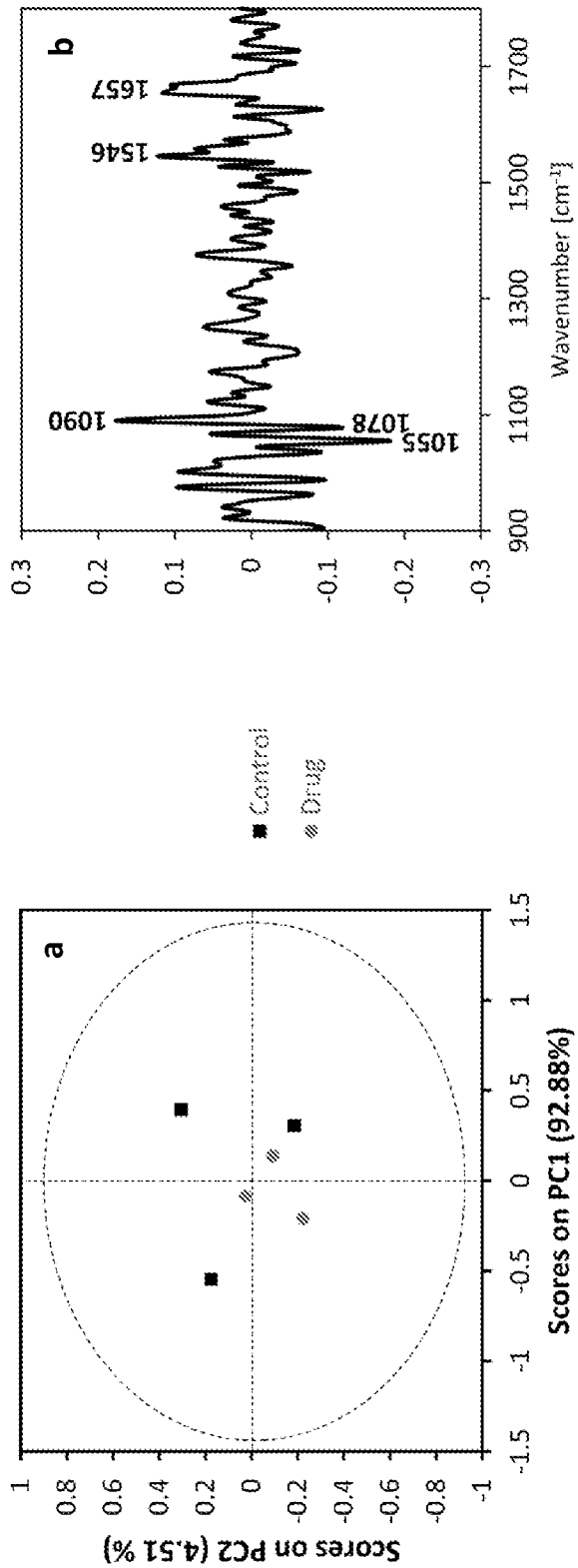

a
Scores on PC1 (62.58%)
Scores on PC2 (32.56 %)
Control
Drug
b
Wavenumber [cm-1]
988
1004
1028
1057
1088
1375
1396

FIG. 60


a
Scores on PC1 (92.88%)
Scores on PC2 (4.51 %)
Control
Drug
b
Wavenumber [cm-1]
1055
1078
1090
1546
1657

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2020/052339

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/18 (2006.01) G01N 21/00 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Google Patents, Google Scholar and ESPACENET using keywords and/ or marks: G01N21/25, C12Q1/18, C12Q1/02pathogen resistance identification, IR spectroscopy and like terms. **EPOQUE INTERNAL Databases:** EPODOC, WPIAP Cited/citing of most relevant patent documents found and their family members.CPC/IPC Marks: G01N21/25, G01N21/31, G01N21/552, G01N21/658, G01N21/65, G01N21/75, G01N21/84, G01N33/49, G01N2333/00, G01N2500/00, C12Q1/18, C12Q1/02, Keywords Spectroscopy, Raman, treatment, pathogen, antibiotic, antifungal, resistance, susceptibility, wavelength, comparison, control and like terms.
Applicant and inventor names searched in Google patents, Google scholar, Espacenet and EPOQUE:
Applicant: MONASH UNIVERSITY
Inventors: HERAUD,Philip Robert; KOCHAN, Kamila

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 June 2020	25 June 2020

Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Adam Palmer AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832205

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/IB2020/052339**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2018/0355399 A1 (bioMerieux, MARCY-L'ETOILE (FR); COMMISSARIAT A L'ENERGIE ATOMIQUE ET AUX ENERGIES ALTERNATIVES, PARIS (FR)) 13 December 2018 abstract, para [0012], para [0025], paras [0034-0035], para [0056], para [0064], para [0103], para [0112], para [0116], para [0120], paras [0126-0129], para [0136], para [0148], para [0151], Figure 9	1-31
X	Athamneh, A.I.M et al., "Phenotypic Profiling of Antibiotic Response Signatures in Escherichia coli Using Raman Spectroscopy", Antimicrobial Agents and Chemotherapy, March 2014, Volume 58, Number 3,p. 1302–1314. doi:10.1128/AAC.02098-13 abstract, pg 1304 col 2 paras 1-2, pg 1304 col 2 para 3, pg 1308 col 1, Fig 1 and caption, Fig 2, Fig 4, Table 1, Table 2.	1, 16, 26, 31
X	US 005/0123917 A1 (Labischinski et al.) 09 June 2005 Abstract, paras [0014-0015], para [0021], paras [0040-0041], para [0045], para [0056], para [0062], para [0064], claim 27, Figs 3,4 8, 9, 11 and 12.	1, 16, 26, 31
A	Quiles F. et al., "AFM combined to ATR-FTIR reveals Candida cell wall changes under caspofungin treatment", Nanoscale, vol 9, pgs 13731-13738, 2017. DOI: 10.1039/c7nr02170d	
A	Sharaha U. et al., "Using Infrared Spectroscopy and Multivariate Analysis to Detect Antibiotics' Resistant Escherichia coli Bacteria", Anal. Chem., vol 89, pgs. 8782−8790 (2017). DOI:10.1021/acs.analchem.7b01025	
A	US 2016/0177366 A1 (SERAPH BIOSCIENCES, LLC, Troy, MI (US); WAYNE STATE UNIVERSITY, Detroit, MI (US).) 23 June 2016	
A	WO 9009454 A1 (BRUKER ANALYTISCHE MESSTECHNIK [DE]) 08 February 1990	
A	Ajesh K. et al.,"Cryptococcus laurentii Biofilms: Structure, Development and Antifungal Drug Resistance", Mycopathologia, vol 174 pgs 409–419 (2012). DOI 10.1007/s11046-012-9575-2	
A	Premasiri W. R. et al., "Rapid urinary tract infection diagnostics by surface-enhanced Raman spectroscopy (SERS): identification and antibiotic susceptibilities", Anal Bioanal Chem, vol. 409:3043–3054, (2017). DOI:10.1007/s00216-017-0244-7	

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/IB2020/052339**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
US 2018/0355399 A1	13 December 2018	US 2018355399 A1	13 Dec 2018
		CN 108700521 A	23 Oct 2018
		EP 3380828 A1	03 Oct 2018
		EP 3380828 B1	06 Nov 2019
		FR 3044415 A1	02 Jun 2017
		FR 3044415 B1	01 Dec 2017
		JP 2019506590 A	07 Mar 2019
		WO 2017089727 A1	01 Jun 2017
US 005/0123917 A1	09 June 2005	None	
US 2016/0177366 A1	23 June 2016	US 2016177366 A1	23 Jun 2016
		US 10253346 B2	09 Apr 2019
		CN 105723195 A	29 Jun 2016
		CN 105723195 B	03 Sep 2019
		EP 3030870 A1	15 Jun 2016
		US 2019203255 A1	04 Jul 2019
		WO 2015021300 A1	12 Feb 2015
WO 9009454 A1	08 February 1990	WO 9009454 A1	23 Aug 1990
		DE 3903778 A1	16 Aug 1990
		EP 0457789 A1	27 Nov 1991
		EP 0457789 B1	11 Nov 1992

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.