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(54) NMR QUANTIFICATION OF TMAO

NMR-QUANTIFIZIERUNG VON TMAO

QUANTIFICATION PAR RMN DE TMAO

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Description

Field of the Invention

[0001] The present invention relates generally to analysis of *in vitro* biosamples. The invention may be particularly suitable for NMR analysis of human urine, blood plasma and serum.

Background of the Invention

[0002] Researchers have described the use of trimethylamine containing compounds, and in particular trimethylamine-N-oxide ("TMAO" or "TMANO"), as risk predictors for cardiovascular disease. *See,* U.S. Patent Application Publication 2010/0285517 and Wang et al, Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease, Nature, Vol. 472, pp. 57-63 (April, 2011).

[0003] An article "Nuclear magnetic resonance studies of blood plasma and urine from subjects with chronic renal failure: identification of trimethylamine-N-oxide", Bell J D et al, Biochimica et Biophysica Acta, vol. 1096 (1991), pages 101-107, describes NMR spectroscopy studies of patients with chronic renal failure. The resonances include TMAO.

[0004] An article "The Effect of pH on the Analysis of 1H MRS Data of Urine in Biomedical Applications", Ijare O. B. et al, describes NMR spectroscopy of urine samples. The article describes adjusting pH to a predetermined value.

Summary

[0005] Embodiments of the invention provide methods, systems and computer program products for NMR quantification of TMAO. In accordance with the invention the method of determining a measure of TMAO in an *in vitro* biosample comprises electronically identifying a pH-stable reference peak region in a proton NMR spectrum of the biosample; electronically identifying a defined calibration peak region in the NMR spectrum of the biosample, wherein the calibration peak region has a location that changes based on pH of the biosample; electronically calculating a distance between the reference peak region and the calibration peak region; electronically determining a location of a single TMAO peak within a defined TMAO peak region residing between about 3.2 and about 3.4 ppm of the proton NMR spectrum, the identification of the TMAO peak being based on the calculated distance between the reference peak region and the calibration peak region; then electronically determining a level of TMAO in the biosample using the single TMAO peak.

[0006] The electronic determination of the TMAO peak region location can be carried out using a defined relationship of a location of the reference peak region to the calibration peak region with a location of the TMAO peak.

[0007] The method can include, before the determining step, calculating a position of a TMAO peak region using a fitting region having first size between about 50-100 data points based on a location of a citrate reference peak or peaks, then reducing the fitting region to about 30 data points centered about the calculated location of the TMAO peak, and electronically curve fitting the TMAO peak region with a defined curve fitting function or functions.

[0008] The defined TMAO peak can be at about 3.3 ppm. The electronic determination of the level of TMAO can be carried out by identifying an expected TMAO peak location using a defined (e.g., linear or other) relationship between a location of a reference peak or peaks and a location of a pH sensitive calibration peak or peaks, and location of an expected TMAO peak. A probable actual TMAO peak location can be identified by: first electronically weighting a region around the expected TMAO peak location with a defined function; then electronically identifying a highest weighted data point of the weighted region; then electronically identifying a probable actual TMAO peak location corresponding to location of the highest weighted data point.

[0009] The method can include, after the identification of the probable actual TMAO peak location, applying a curve fitting function or functions to a curve fitting region of about 30 to about 50 data points centered about the identified probable actual TMAO peak location to determine the level of TMAO.

[0010] The curve fitting function or functions can be configured to selectively allow for one or more neighbors on either side of the probable actual TMAO peak location to account for small misalignments to determine the level of TMAO.

[0011] The calibration peak multiplet can be a citrate quartet. The electronic curve fitting of the defined peak region with a defined curve fitting function or functions that can be applied to selectively use, zero, one, two or three peak neighbors of the TMAO peak to determine the level of TMAO.

[0012] Determining the measure of TMAO can be carried out to generate a measurement that is substantially linear in a typical biological range of between about 1-50 μM, and may be provided in a lower range of between about 1-10 μM (for blood plasma or serum). Larger ranges may be used for other biosamples, such as between about 0-1000 μM for urine.

[0013] The reference peak region can be a glucose peak region.

[0014] The reference glucose peak region can be associated with anomeric glucose at about 5.20 ppm.

[0015] The calibration peak region can be associated with one or more peaks of a citrate peak multiplet (e.g., quartet).

[0016] The biosample can be a human blood plasma or serum sample. The biosample can include an acidic pH buffer so that the biosample has a pH between about 5.15 and 5.53.

[0017] The reference peak region can be associated with anomeric glucose at about 5.20 ppm and the calibration peak region can include one peak of the citrate multiplet (centered) at about 3.7 ppm.

[0018] The method may also include: (i) providing containers holding respective biosamples with a solution of citrate acid and sodium dibasic phosphate in a defined ratio, with the pH being between about 5.15 and 5.53; (ii) positioning a respective biosample in an NMR probe of an NMR spectrometer; and (iii) obtaining NMR signal to generate the NMR proton spectrum for determining the level of TMAO.

[0019] The electronic determination can be carried out by applying curve fitting functions NMR signal associated with the TMAO peak to determine a first level of TMAO, then subtracting a known concentration of the TMAO standard that was added to the biosample to generate a patient-specific level of TMAO.

[0020] The ratio of the solution can be between 25:75 to about 50:50 (buffer:serum) by volume, but other ratios can be used.

[0021] In some embodiments, the method may include (i) providing containers holding small volumes (e.g., about 50 μL or less) of respective biosamples with a solution of citrate acid and sodium dibasic phosphate in a defined ratio with the pH being between about 5.15 and 5.53.

[0022] The biosample can be human serum and the obtaining step can be carried out with and acquisition time (on average) of about 4 seconds per scan with a plurality of scans per biosample (typically ≥ 16 scans, and more typically ≥ about 96 scans per biosample, with between about 3- 7 minutes, on average, of total acquisition time per biosample).

[0023] The method can include generating an output of the level of TMAO with an indication of whether the level is considered normal, high or low and/or with visual (graphic and/or numerical) indicia of a continuum of risk (e.g., a color graphic of increased risk and/or a TMAO risk score going from low to high), and indicating whether a subject is at risk of a complication of atherosclerotic cardiovascular disease, and wherein a subject whose TMAO is above a value associated with a defined - percentile of a reference population is at risk of experiencing a complication of atherosclerotic cardiovascular disease. It is anticipated that the at-risk population would be at or above about the 75th or about the 80th percentile.

[0024] Other embodiments are directed to computer program products for evaluating *in vitro* biosamples. The computer program product in accordance with the invention is defined in claim 12 and includes a non-transitory computer readable storage medium having computer readable program code embodied in the medium. The computer-readable program code includes computer readable program code that evaluates NMR signal in a defined peak region residing between about 3.2 and 3.4 of a proton NMR spectrum of an *in vitro* biosample to determine a level of trimethylamine-N-oxide ("TMAO").

[0025] Still other embodiments are directed to an analysis system in accordance with claim 13.

[0026] Further features, advantages and details of the present invention will be appreciated by those of ordinary skill in the art from a reading of the figures and the detailed description of the preferred embodiments that follow, such description being merely illustrative of the present invention. Features described with respect with one embodiment can be incorporated with other embodiments although not specifically discussed therewith. That is, it is noted that aspects of the invention described with respect to one embodiment, may be incorporated in a different embodiment although not specifically described relative thereto. That is, all embodiments and/or features of any embodiment can be combined in any way and/or combination, provided the combination falls within the scope of the appended claims. The foregoing and other aspects of the present invention are explained in detail in the specification set forth below.

[0027] As will be appreciated by those of skill in the art in light of the present disclosure, embodiments of the present invention include methods, systems and computer program products.

Brief Description of the Figures

[0028]

Figure 1A is an NMR spectrum of human serum with an expansion of the spectrum showing a location of an NMR peak region for TMAO according to embodiments of the present invention.

Figure 1B is an NMR spectrum of human urine with an expansion of the region containing the TMAO peak.

Figure 2A shows a stacked plot of NMR spectra showing the change in the location of the TMAO peak (indicated by the asterisk) changes with changes in the sample pH according to embodiments of the present invention. **Figure 2B** is an expansion of a stacked plot of NMR spectra showing the changes in the TMAO peak location across a narrow pH range from 5.15 to 5.53 according to embodiments of the present invention. Across this range the TMAO peak moves by about 20 Hertz (0.05 ppm at about 400MHz).

Figure 2C is an overlay of the TMAO region of NMR spectra collected, one with endogenous TMAO only and one with the addition of a defined amount of TMAO added to the buffer solution according to embodiments of the present invention.

Figure 3A is a schematic illustration of an NMR spectrum with a reference peak region and a calibration peak region used to determine a location of the TMAO peak according to embodiments of the present invention.

Figure 3B is a schematic illustration of an NMR spectrum with a reference peak region and a calibration peak region used to determine a location of the TMAO peak according to other embodiments of the present invention.

Figure 3C is a schematic illustration of an NMR spectrum with a pH-variable calibration peak region used to determine pH and/or a location of the TMAO peak according to embodiments of the present invention.

Figure 3D is a graph of a weighted function that can be used to identify a probable actual TMAO peak location according to some embodiments of the present invention.

Figure 4A is a graph of distance between citrate and glucose peak (delta citrate) across the pH range from 5.15 to 5.45. The data shows the defined mathematical relationship between the distance between glucose and TMAO peak (delta TMA) according to embodiments of the present invention.

Figure 4B is a graph of distance between citrate and glucose peak (delta citrate) in a set of patient samples that have a narrow pH range. The data shows the defined mathematical relationship between the distance between glucose and TMAO peak (delta TMA) according to embodiments of the present invention.

Figure 5 is an NMR spectrum illustrating alternative locations of glucose peak regions according to embodiments of the present invention.

Figure 6A-6F are graphs of curve fitting for different amounts of TMAO in a sample according to embodiments of the present invention. **Figures 6A and 6B** illustrate concentrations in an estimated 1st quartile. **Figures 6C** and **6D** correspond to measurements in an estimated 2nd quartile. **Figures 6E** and **6F** correspond to measurements in an estimated 4th quartile.

Figure 7A is a graph showing curve fitting of the actual TMAO peak with multiple TMAO and protein basis functions according to embodiments of the present invention.

Figure 7B is a flow chart of an intelligent TMAO curve fitting function according to embodiments of the present invention.

Figure 8 is an exemplary pulse sequence using a standard "one-pulse" protocol with WET solvent suppression according to embodiments of the present invention.

Figure 9 illustrates the first pulse sequence in **Figure 8** modified to include a CPMG sequence to attenuate signals associated with macromolecules and large aggregates such as proteins and lipoproteins s according to particular embodiments of the present invention.

Figures 10A and **10B** are NMR spectra acquired using the pulse sequence of **Figure 8** (**Figure 10A**) and that of **Figure 9 (Figure 10B).** The broad signals from macromolecules are reduced in the CPMG pulse sequence according to embodiments of the present invention.

Figure 11 is a graph of concentration versus CV% for LOQ for 64, 128 and 192 scans according to embodiments of the present invention.

Figure 12A is a graph of %CV versus TMAO concentration (μM) from spiked dialyzed serum according to embodiments of the present invention.

Figure 12B is a graph of %CV versus TMAO concentration (μM) from patient blood serum samples according to embodiments of the present invention.

Figure 13A is a graph of the correlation between the NMR determined TMAO concentrations (μM) in dialyzed serum spiked with known concentrations of TMAO (μM). The ordinate of the graph indicates the TMAO concentration bases on gravimetrically determined spiking of the dialyzed plasma. The abscissa indicates the NMR measurement of the concentration.

Figure 13B is a graph of correlation between the NMR determined TMAO concentrations (μM) and those determined by hyphenated liquid-chromatography mass spectrometry measurements (μM) in patient samples to assess accuracy of the assay according to embodiments of the present invention.

Figure 14A is a flow chart of exemplary operations.

Figure 14B is a flow chart of an exemplary pre-analytical quality control evaluation that can be carried out according to embodiments of the present invention.

Figure 14C is a multiplet (reference) peak region of an NMR spectrum that can be used for the quality control evaluation shown in **Figure 14B** according to embodiments of the present invention.

Figure 14D is an enlarged view of one peak shown in **Figure 14C** that can be used to evaluate skew according to embodiments of the present invention.

Figure 15A is a schematic illustration of an NMR measurement system according to embodiments of the present invention.

Figure 15B is a schematic illustration of a container with a patient sample and buffer solution for analysis to assess TMAO level according to embodiments of the present invention.

Figure 16 is a schematic illustration of an NMR analyzer according to embodiments of the present invention.

Figure 17 is a block diagram of a data processing system according to embodiments of the present invention.

Figure 18 is a schematic illustration of a patient report with examples of visual risk indicia associated with different levels of TMAO according to embodiments of the present invention.

Figure 19 is a stacked plot of NMR spectra (chemical shift/ppm) of urine according to embodiments of the present invention (the pH goes from 4.62 at the bottom to 6.83 at the top).

Figures 20A and **20B** are graphs of a TMAO peak versus reference peak **(Figure 20A** shows Creatinine and **Figure 20B** shows Citrate) illustrating a relationship between TMAO and the reference peak according to embodiments of the present invention.

Figure 21 is a graph of a series of Lorentzian and Gaussian basis functions used to model the TMAO signal to determine TMAO concentration according to embodiments of the present invention. **Figure 21** shows a set of the functions with constant height and differential linewidths Quadratic and linear functions are included to model baseline offsets.

Figure 22 is a graph of a series of Lorentzian and Gaussian basis functions used to model baseline TMAO signal to determine TMAO concentration according to embodiments of the present invention. These basis functions have constant area but have differential peak heights and linewidths.

Figures 23-26 are graphs of additional examples of basis sets (Lorentzian alone, **Figures 23, 25** and Gaussian alone, **Figures 24, 26**) according to embodiments of the present invention.

[0029] The foregoing and other objects and aspects of the present invention are explained in detail in the specification set forth below.

Detailed Description of Embodiments of the Invention

[0030] The present invention now is described more fully hereinafter with reference to the accompanying drawings, in which embodiments of the invention are shown. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

[0031] Generally stated, embodiments of the invention are directed to NMR assays that can measure the concentration of TMAO in biosamples, typically urine, serum or plasma samples. The concentration can be measured by determining the peak area of a defined region in the NMR proton spectra of the NMR signal and translating this into concentration units of micromoles (μmol) with a calibration based on TMAO standard solutions. The concentration of TMAO in the sample can be related to the subject’s risk of developing cardiovascular disease and may also be associated with other diseases or pathologies.

[0032] Like numbers refer to like elements throughout. In the figures, the thickness of certain lines, layers, components, elements or features may be exaggerated for clarity. Broken lines illustrate optional features or operations unless specified otherwise.

[0033] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the terms "comprises" and/or "comprising," when used in this specification, specify the presence of stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof. As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items. As used herein, phrases such as "between X and Y" and "between about X and Y" should be interpreted to include X and Y. As used herein, phrases such as "between about X and Y" mean "between about X and about Y." As used herein, phrases such as "from about X to Y" mean "from about X to about Y."

[0034] Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the specification and relevant art and should not be interpreted in an idealized or overly formal sense unless expressly so defined herein. Well-known functions or constructions may not be described in detail for brevity and/or clarity.

[0035] It will be understood that, although the terms first, second, etc. may be used herein to describe various elements, components, regions, layers and/or sections, these elements, components, regions, layers and/or sections should not be limited by these terms. These terms are only used to distinguish one element, component, region, layer or section from another region, layer or section. Thus, a first element, component, region, layer or section discussed below could be termed a second element, component, region, layer or section without departing from the teachings of the present invention. The sequence of operations (or steps) is not limited to the order presented in the claims or figures unless specifically indicated otherwise.

[0036] The term "programmatically" means carried out using computer program and/or software, processor or ASIC directed operations. The term "electronic" and derivatives thereof refer to automated or semi-automated operations carried out using devices with electrical circuits and/or modules rather than via mental steps and typically refers to operations that are carried out programmatically. The terms "automated" and "automatic" means that the operations can be carried out with minimal or no manual labor or input. The term "semi-automated" refers to allowing operators some input or activation, but the calculations and signal acquisition as well as the calculation of the concentrations of the ionized constituent(s) are done electronically, typically programmatically, without requiring manual input.

[0037] The term "about" refers to +/- 10% of a specified value or number (which can the mean or average value). The term "about" with respect to a chemical shift ppm value for a particular peak location means +/-0.1 as chemical shifts can change with different sample conditions (e.g. salt and protein concentration, etc.).

[0038] The terms" CAD" and "CHD" are used interchangeably to correspond to a patient or subject's risk of developing or having coronary artery and/or coronary heart disease, respectively. The term "cardiovascular disease" (CVD) refers to a combined outcome that is typically CHD plus stroke.

[0039] The term "biosample" refers to *in vitro* blood, serum, urine, CSF, saliva, bronchoalveolar lavage, fecal or tissue samples of humans or animals. The biosamples can be from any target subject. Subjects, according to the present invention, can be any animal subject, and are preferably mammalian subjects (e.g., humans, canines, felines, bovines, caprines, ovines, equines, rodents (mice, rats, hamsters, guinea pigs or others), porcines, primates, monkeys, and/or lagomorphs). The animals can be laboratory animals or non-laboratory animals, whether naturally occurring, genetically engineered or modified, and/or whether being laboratory altered, lifestyle and/or diet altered or drug treated animal variations. Embodiments of the invention may be particularly suitable for evaluating human urine and/or human blood plasma or serum biosamples. The samples may be fasting or non-fasting. In some embodiments, the urine and/or blood plasma or serum sample is a fasting sample, at least about 12 hours of fasting time. In other embodiments, the sample can be obtained after a prescribed diet challenge.

[0040] The term "patient" is used broadly and refers to an individual that provides a biosample for testing or analysis.

[0041] The NMR analysis can be carried out using a small sample size, typically about 500μL or less, such as between about 100-250μL. The samples can be diluted with a defined diluent, such as a pH-changing buffer or buffers.

[0042] The term "exponential function" refers to a mathematical transformation in which the "FID" is multiplied by an exponential function. Typically decaying exponentials are used to provide a defined increase in the linewidth with commensurate increase in signal-to-noise. The term "FID" refers to free induction decay. The time-domain signal is detected and digitized by the spectrometer after application of the read pulse. Gaussian Multiplication refers to a mathematical transformation in which the FID is multiplied by a Gaussian function in order to narrow the linewidths and increase resolution.

[0043] The term "linearity" refers to the ability (within a given range) to provide results that are directly proportional to the concentration of the analyte (here TMAO) in the test sample. The term "limit of detection" ("LoD") refers to the lowest actual concentration at which the analyte is reliably detected. The term "limit of quantification" ("LoQ") refers to the lowest actual concentration at which the analyte is reliably detected (LoD) and at which the uncertainty of the observed results is less than or equal to the error set for uncertainty. The term "precision" refers to the closeness of agreement between independent test results obtained under stipulated conditions.

[0044] The term "WET" refers to a solvent suppression scheme in which a series of radiofrequency and pulsed field gradients are used to reduce the water signal. *See,* Ogg, R. J.; Kingsley, R. B.; Taylor, J. S. J. Magn. Reson., Ser. B 1994, 104, 1-10; and Smallcombe, S. H.; Patt, S. L.; Keifer, P. A. J. Magn. Reson., Ser. A 1995, 117, 295-303.

[0045] The term "CPMG" refers to a Carr-Purcel-Meiboom-Gill pulse sequence. This is a series of phase defined radiofrequency pulses that provide means to attenuate signals from large, rapidly relaxing molecules such as proteins and lipoprotein particles.

[0046] The term "AT" refers to acquisition time associated with the length of time that the FID is digitized in seconds. The term "D1" refers to a component of a pulse sequence denoting the delay time prior to the read pulse. The term "Ernst Angle" refers to a read pulse angle for a particular resonance that yields the maximum signal in a given amount of time.

[0047] The term "clinical" with respect to data measurements means qualitative and/or quantitative measurements that can be used for therapeutic or diagnostic purposes, and typically for diagnostic purposes and meets the appropriate regulatory guidelines for accuracy, depending on the jurisdiction or test being performed.

[0048] Embodiments of the invention can measure TMAO by NMR over an expected biological range of between about 1 to 50 μM, typically 1-30 μM, and more typically about 1-10 μM, for human plasma and/or serum samples. The NMR assay may quantify other expected biological ranges for other sample types, such as urine, for example, which may have an increased amount of TMAO over plasma or serum. The urine range may be much larger than the range for human plasma and/or serum such as between 0-1000 μM. The assay can be linear over the larger urine range of values.

[0049] The term "pH buffer" refers to a chemical added to the biosample to create a defined pH-induced NMR peak shift in the NMR spectrum. The buffer can be any suitable acidic buffer such as acetate and/or citrate. As will be discussed below, one particularly suitable buffer is citrate phosphate buffer (e.g., citric acid and sodium dibasic phosphate, e.g., $C_6H_8O_7 \cdot H_2O$ and $Na_2HPO_4 \cdot 7H_2O$).

[0050] Embodiments of the invention provide an NMR assay with sufficient accuracy, precision and linearity to provide clinically beneficial measures of TMAO.

[0051] It is understood that the chemical shift described herein for NMR signals and peaks are with respect to a spectrometer having an operating frequency of about 400 Hz. As is well-known, peak locations measured in ppm should remain constant at different field strengths, but the features of the spectrum may differ due to the different resolution and altered appears of scalar coupling. **Figure 1A** shows an example of a full NMR spectrum of serum taken under standard conditions on the Vantera® clinical NMR Analyzer by LipoScience, Inc., Raleigh, N.C. The expansion shows that TMAO is in a very crowded region which can confound the quantitation. The normal biological range of TMAO in human serum or plasma is between about 1 to about 50 μM, which may normally be between about 1-10 μM, but dietary spikes can raise the upper value, typically to between about 30-**50 μM** or even higher. This amount is very low for NMR detection, but this metabolite benefits from the fact that the only signal is a singlet that results from 9 magnetically degenerate protons; thus, the ^1H signal concentration is 9 times higher than the absolute chemical concentration.

[0052] Urinary TMAO is thought to be highly correlated with serum TMAO levels, after correcting for concentration using the creatinine concentration. **Figure 1B** shows the NMR spectrum of urine with an expansion around the TMAO signal. TMAO is present in urine at a much higher concentration. In **Figure 1B,** the TMAO level is 540 uM, which is more than about 100 times greater than the normal concentrations in serum. Typical urine ranges of TMAO can be between about 0-1000 μM as noted above.

[0053] It is contemplated that the normal biological variability may be sufficiently large that only a semi-quantitative test is necessary, e.g., quantitative measures of values associated with a fourth quartile or fifth quintile of a hazard ratio, e.g., the 75th percentile or 80th percentile, which may be associated with a concentration of about 6.2μm or greater. In some embodiments, the NMR assay can reliably quantify to at least the 50th percentile, e.g., about 3.7 μm or greater for human blood plasma or serum samples. That is, where TMAO is associated with increased risk or abnormal conditions or disease, the amounts of TMAO in a sample can be greater than normal ranges/values and can be more precisely measurable than low levels.

[0054] Urinary TMAO levels will likely be more influenced by acute dietary influences whereas the serum assay is likely to more reflective of the chronic TMAO levels. An NMR urinary assay has higher concentrations of TMAO, than is present in serum or plasma, which may allow a similar-high volume throughput. While discussion of diluents, buffers and sample preparation discussed below are applicable to multiple biosample types, particular evaluation protocols for urine biosamples for TMAO will be discussed further below, *see, e.g.,* **Figures 19, 20A** and **20B**.

[0055] Typically, the NMR analyzer **22 (Figures 13A, 14)** can diagnostically analyze at least about 400, and more typically at least about 600, samples per twenty-four hours. *See,* e.g., U.S. Patent No. 8,013,602 for a description of a suitable NMR analyzer **22** (**Figures 13A** and **14**). **Figure 2A** shows an expansion of the serum spectrum shown in **Figure 1A** with the pH adjusted. Thus, referring to **Figure 2A**, in order to resolve the TMAO peak, marked with an asterisk, from the overlapping metabolites, the pH of the biosample is altered over pH ranges from 2.82 at the top to 7.9 at the bottom. It is clear that there is a significant shift of the peak between pH 4.6 and 5.6. **Figure 2B** shows the chemical shift behavior of this TMAO peak over the narrower pH range from 5.15 to 5.53. The region in the NMR spectrum between 3.2 and 3.4 ppm is relatively open, i.e., with fewer significant interferences to TMAO concentration. Thus, in some embodiments, a target pH for the sample can be set to position the NMR TMAO peak between about 3.2 and 3.4, which can be carried out, for example, by adjusting the pH to be between about 5.15 and 5.53. In some particular embodiments, the pH of the sample undergoing analysis can be set to about 5.3 to put the TMAO peak in about the center of the open region.

[0056] When analyzing biosamples, such as urine or serum, for example, other peaks may overlap with the TMAO peak but they can be modeled by a peak finding and quantitation algorithm so that accurate measurements can be obtained.

[0057] **Figure 2C** is an overlay of the TMAO region of two NMR spectra collected, one with endogenous TMAO and one with the addition of a defined (10 uM) amount of TMAO added to the buffer solution. This region displays a number of other background signals which could in some instances confound the quantitation of the TMAO. The addition of a known quantity of TMAO in the buffer solution insures that the TMAO peaks will be the largest peak in this region and therefore can be unequivocally identified. The standard addition also aides in the fitting of the TMAO peak such that the effects of overlapping resonances are minimized in the peak fitting routine. As a known amount of the standard TMAO (e.g., "standard" in a known concentration is used), once the TMAO concentration is measured from the enhanced peak, the concentration added by the TMAO standard diluent is subtracted to yield the TMAO concentration resident in the sample. It is contemplated that a TMAO standard concentration between about 1-100 μM can be used for TMAO biosample evaluations, typically between about 5-20 μM, and more typically about 10 μM.

[0058] As will be discussed below, the diluents and/or buffer (including the TMAO standard) can be provided with a defined final concentration to yield a defined blood plasma or serum to buffer ratio, typically of 50:50 or greater, and more typically 75% serum and 25% buffer but more buffer than serum can also be used. However, other final concentration values and ratios may be used as discussed below.

[0059] Referring now to **Figure 3A**, in accordance with the invention, to facilitate locating the TMAO peak region **10,** recognizing that commercial application may be such that some samples may have slight variations in pH, a pH stable reference peak region **20** in the **NMR** spectrum of the biosample is utilized. In some embodiments, the reference peak region is associated with glucose which is in the biosample. This does not require that a reference analyte be added to the sample to create the reference peak **20.** However, it is contemplated that other reference peak regions may be used using added reference material or other pH stable constituents in the biosample. For some biosamples, such as urine, other added or natural (internal shift standards) or reference compounds can be used, e.g. TSP (the sodium salt of trimethylsilylpropionic acid (including deuterated version), DSS (4, 4-dimethyl-4-silapentane-1-sulfonic acid), and the like can be used as well as other pH stable chemicals and/or compounds.

[0060] As shown in **Figure 3A,** a defined calibration reference peak region **30** with signal intensity greater than TMAO is used. The location of the calibration peak region varies or changes with pH. The distance "d1" between the stable reference peak region **20** and the calibration peak region **30** (one or more of the citrate quartet peaks) can be calculated. Then, based on a defined relationship between the location of the calibration peak region **30** and the relative position of the TMAO peak region **10,** the distance "d2" can be determined which identifies the location of the TMAO peak region **10.** The calibration peak region can be one or more peaks of a citrate quartet peak region. However, other pH varying calibration peak regions may also be used.

[0061] **Figure 3B** illustrates a method similar to that shown in **Figure 3A,** but the TMAO peak location can be calculated based on a distance d2 between glucose and TMAO rather than citrate and TMAO.

[0062] In the example shown in **Figure 3C** (which is not an embodiment of the invention), the location of the TMAO peak region can be determined without the use of the pH-stable reference peak region. As shown, two or more peaks of a pH-variable calibration signal, e.g., the individual peaks of the citrate multiplet **30** can also give pH information, as the distance between these peaks varies as a function of pH. The distance between peaks can be used to determine the TMAO peak location. The distance can be calculated based on leading edges, trailing edges or a center of the peak. This embodiment does not require a separate reference compound/reference peak region **20** as the calibration peak region can be used to define pH that is used to define the TMAO peak location. As shown, there are four peaks associated with the calibration multiplet, shown as P1-P4 (right to left), and a distance "Δ" between each adjacent peak and a distance Δ between other combinations of the peaks, e.g., between any or combinations of P1-P2, P2-P3, P3-P4, P1-P3, P1-P4, P2-P4 and the like including a summative distance between each or combinations of peaks that may be used to identify a pH level. This distance changes as the chemical structure bends in response to pH level of the biosample. Thus, one or more distances between one or more of the peaks in the citrate multiplet can be used to identify pH level, which can then be used to locate the TMAO peak. The location of the TMAO peak **10** can be based on a look-up table or other computational model that correlates spacing distance to pH level and pH level to TMAO peak location, or may be identified by calculating a distance Δ2 between one or more of the citrate peaks and the projected TMAO peak location.

[0063] In some embodiments, recognizing that across the pH range from 5.15 to 5.45, the downfield peak of the citrate shifts downfield by about 52 points (14 Hz), the spectra can be acquired with a sufficient digital resolution, such as, for example, about (16384 pt)/(4496.4Hz) = (3.64 pt/Hz).

[0064] In particular embodiments, the reference peak region can be an anomeric glucose peak region **20** at about 5.20 ppm with glucose peaks that are highly stable to pH and display substantially no shift across this range. The distance between the citrate peak region **30** is linearly correlated to the distance between one or more of the citrate peaks and the TMAO peak. This relationship is shown in **Figures 4A** and **4B.** The TMAO peak **10** can be accurately located based on the location of the citrate peak(s) or the glucose peak(s). The locations of the three peak regions **10, 20, 30** and the defined mathematical relationship is believed to be independent of temperature of the sample during analysis and spectrometer field strength.

$$Y=1.5449x+2819.9 \quad \textbf{EQUATION (1A)}$$

where Y is the distance of the TMAO peak from glucose, and x is the distance between the calibration (citrate) peak and the reference (glucose) peak.

$$Y=1.4924x+2626 \quad \textbf{EQUATION (1B)}$$

In summary, a set of samples around the expected range can be prepared. The spectra can be analyzed and the distance between the invariant glucose and the pH sensitive citrate can be measured. The distance from the invariant glucose and TMAO can be measured and a defined mathematical relationship between the two can be determined. Equations 1A and 1B are examples of equations for determining TMAO peak location using glucose. However, it is noted that experimental conditions (pH buffers, NMR spectrometers and the like) can vary and the TMAO peak distance can be calculated from one or more citrate peaks rather than glucose. Thus, these Equations are by way of example only and any similar equation that results in an R^2 of ≥ 0.9 will be considered equivalent to these defined mathematical relationships.

[0065] While the glucose peak region **20** (multiplets that are centered) at 5.20 ppm was used in this example, other reference peak or peak regions may be used. In some embodiments, one or more other glucose peak regions may be used such as one or more peaks of glucose multiplets "G" centered at one or more of about 4.6, 3.9, 3.8, 3.7, 3.5, 3.4 and 3.2 ppm as shown by the lower darker lines in **Figure 5.**

[0066] As noted above, in some embodiments, the location of the TMAO peak region 10 can be determined using one or more of the citrate peaks as the calibration reference peak/peak region. In some embodiments, one peak of the citrate multiplet is found at 3.7 ppm. Given that the typical biosample with the added pH buffer contains a large concentration of citrate (e.g., typically the buffer is at least about 25% by volume), these citrate peaks are easy to find electronically as they are among the largest peaks in the spectrum. The distance between the anomeric glucose peaks **20** and the citrate peaks **30** is related to the distance between the citrate **30** and TMAO peaks **10.** This defined mathematical relationship has been shown to robustly determine the location of the TMAO peak within approximately 10 data points.

[0067] Given the low signal to noise of the TMAO at the low concentration, finding the actual TMAO peak can be challenging. In some embodiments, the TMAO peak 10 can be determined to be the 1st peak maximum that is found near the starting location determined by the calibration peak evaluation. However, it is contemplated that other protocols or algorithms can be used to effectively and efficiently determine the location of the TMAO peak, especially with low TMAO concentrations where the noise and low concentration interferences are more confounding. For example, when the signal to noise is quite low, the peaks **10** may not be readily distinguishable from the noise. In some embodiments, as described above, TMAO can be added to a buffer to "amplify" the signal and/or insure that TMAO will be the largest peak in the region of interest.

[0068] In some embodiments, the electronic determination of the expected TMAO peak location can be carried out using a defined linear relationship between the location of the reference peak, the location of the pH sensitive calibration peak, and the location of the TMAO peak. The (probable) actual TMAO peak location can be identified by weighting the region around the expected TMAO peak location with a Gaussian or similar function such as, but not limited to, triangular and parabolic functions.

[0069] The probable actual TMAO peak location can then identified as the highest weighted data point. The algorithm can mathematically emphasize the search for the actual peak around that location. **Figure 3D** shows an example function in which the highest point on the curve corresponds to the probable actual TMAO peak location. The search for the actual TMAO peak will therefore be weighted toward that location. Thus, the highest weighted data point of the weighted region is defined as the probable actual TMAO peak location which can be used as the center point of the fitting region to determine the level of TMAO.

[0070] The fitting region can include between about 30 to about 50 data points centered about the calculated (probable) actual location of the TMAO peak. The fitting region can be electronically curve fit with a defined curve fitting function or functions. The curve fit can selectively allow for one or more neighbors on either side to account for small misalignments to determine the level of TMAO.

[0071] **Figures 6A-6F** illustrate curve fitting of TMAO peaks from patient biosamples with concentrations in the 1st (**6A**, **6B**), 2nd **(6C, 6D)** and 4th (**6E, 6F**) quartiles. The quartile ranges are by way of example only and are estimates from literature values. See, e.g., Wang et al., Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease, 58 Nature, Vol. 472, April 7, 2011, the contents of which are hereby incorporated by reference as if recited in full herein. These values may change with additional evaluation of clinical data or over time. The TMAO peaks can have a narrow width of less than 2 Hz, more typically about 1.5 Hz or less, e.g., about 1.2 Hz to about 0.6 Hz, and the peak shape can vary and may have an asymmetric shape, e.g., a non-Lorentzian shape. In order to account for small differences in the linewidth of the TMAO peak, a curve fitting technique can be used. The line shape and linewidth can vary which can make quantitation difficult. The fits in **Figures 6A** and **6B** are not ideal and quantitative measures of TMAO at this low end may be unreliable. However, the fits in **Figures 6A** and **6B** yield low values that would accurately categorize the patient as being in the 1st quartile or quintile, for example.

[0072] It is contemplated that patients having high TMAO values (in the 4th quartile or 5th quintile, for example) relative to a defined population are considered to be "at-risk" or as having an elevated risk relative to the population norm.

[0073] In order to account for small differences in the linewidth of the TMAO peak, a curve fitting technique can be used. The curve fitting may use different sets of basis functions that can vary biosample to biosample, which can include none, or one or more neighboring TMAO peaks that reside on adjacent the main TMAO peak.

[0074] Once the TMAO peak **10** has been found, it can be computationally fit using one or more defined fitting functions as shown in **Figure 7A.** The two composite lines shown at the upper portion represent an actual line, marked "actual" and a fitted line, marked "fitted" and shown in broken line. As shown on the bottom of **Figure 7A,** the TMAO fitting functions can include a primary function F1 and at least one secondary function F2 which may be programmatically used selectively if the curve fitting is not sufficiently accurate for a particular biosample. The basis functions can be pre-defined and selected for use programmatically based on certain defined measurement or curve fitting decisions and can be configured to include none (only the primary basis function F1), or one or more of different pre-defined secondary curve fitting or secondary basis functions which may use one, two or three neighboring peaks of the TMAO peak.

[0075] The analysis circuit or module (e.g., at least one digital signal processor) can be programmed or otherwise configured to decide whether one or more secondary curve fitting functions is appropriate for any particular biosample. Thus, the analysis may vary biosample to biosample based on a defined set of alternate curve fitting functions. The one or more secondary curve fitting functions F2 may use one or more neighboring TMAO peaks to help more accurately or reliably fit this region.

[0076] The fitting can include a set of basis functions that include a TMAO peak (e.g., the primary function F1 and optionally one or more secondary functions F2) as well as a quadratic function that accounts for the residual protein baseline interferences (bottom of **Figure 7A**) that survive the pulse sequence. The TMAO basis function can be an experimentally acquired spectrum of TMAO processed with consistent parameters to the actual spectrum. Computationally derived TMAO basis functions, i.e., specified functions comprised of Lorentzians, Gaussians or some combination of both can also be used..

[0077] The lineshape deconvolution can be achieved with a non-negative least squares fitting program (Lawson, CL, Hanson RJ, Solving Least Squares Problems, Englewood Cliffs, NJ, Prentice-Hall, 1974). This is avoids the use of negative concentrations which will lead to error due especially in low signal to noise spectra. Mathematically, the lineshape analysis was described in detail for lipoproteins in the paper by Otvos, JD, Jeyarajah, EJ and Bennett, DW, Clin Chem, 37, 377, 1991. Referring particularly to the equation in the left column of page 379. In this equation, Vji can represent the TMAO peaks (including main peak and optionally one or more neighbors) and Vki can be the protein components. A synthetic baseline correction function may also be used to account for baseline offsets from residual protein components. This can take the form of a quadratic or other polynomial function. Weighting factors are determined and the fit can be optimized by minimizing the root mean squared deviation between the experimental and calculated spectrum. *See also,* U.S. Patent No. 7,243,030.

[0078] The relative TMAO concentrations determined have no physical meaning. A linear calibration function can be determined which relates the integral units from the spectrometer to micromolar concentration values. The calibration function is determined by measuring the signals of samples with known concentrations of TMAO. These are typically samples prepared by spiking TMAO into extensively dialyzed plasma which has all of the small molecule metabolites dialyzed away.

[0079] **Figure 7B** is a flow chart that illustrates exemplary operations or steps that can be used for selecting basis functions for the TMAO curve fitting analysis. The boxes shown with a broken line perimeter indicates that these operations or steps can be generated prior to analysis of respective biosamples. As shown, the basis functions for the protein background and target analytes are defined (block **60, 62**) and the design matrix from each basis function is also defined (block **65**). A biosample spectrum is obtained (electronically read or otherwise provided or obtained) (block **70**). The location of the analyte (e.g., TMAO) peak is determined using either blocks **71, 73** (the reference peak and calibration peaks are determined, then a pH dependent relationship is used to determine the location of the analyte peak), or a calibration multiplet is used with distance between peaks varying according to pH (block **75**). The basis functions and baseline functions are used in non-negative least squares fitting of the analyte peak region (block **80**) which can selectively include the use of one or more of several defined basis functions for a single analyte to compensate for non-ideal lineshapes that maybe present in a particular biosample (block **82**). A polynomial function can be applied for a baseline correction (block **84**). An analyte conversion factor can be applied to determine a molar concentration of the biosample (block **86**). This conversion factor can be based on a calibration correlation defied using known analyte samples of different concentrations (block **87**). The final concentration of the analyte can be output, such as to a patient report (block **88**).

[0080] It is technically challenging to fit peaks in experimental spectra from complex mixtures such as biofluids, where signals from other components of the sample can interfere with the signal of interest and peaks can have non-ideal lineshapes. Factors including, but not limited to, differential protein binding, ionic composition and field inhomogenity can lead to non-Lorentzian, sometimes asymmetric peak shapes. To fit these types of peaks, embodiments of the invention provide the option to use additional analyte basis functions that are placed on either side of the main peak. Where used, the neighbors can be placed in one point increments on either side of the main peak, with up to 3 neighbors on each side. The number of neighbors allowed in a fitting protocol can be set prior to the analysis and is dependent upon the spectral characteristics of the assay including signal to noise ratio and potentially confounding signals. The contribution of the main peak plus the neighbors as well as the protein basis functions and baseline correction function can be evaluated using the non-negative linear least squares algorithm (block **80**). Thus, deconvolution of the small, single, pH-dependent TMAO peak typically takes place after determining its exact (or substantially exact) location based on a defined mathematical relationship between the pH-dependent reference and TMAO.

[0081] In order to deconvolute the TMAO peak located upfield from the water peak, a sixty data point search window can be established from predetermined parameters in the program setup menu. The search window covers all the possible TMAO locations across all (normal) patient samples. The analysis can use a pH-independent reference present in patient samples, such as the anomeric glucose peaks, to determine the location of this 60 data point window in the spectrum. The approximate location of the glucose doublet (located downfield of the water peak) is specified within the program and a least squares fit is performed to find an exact match between the doublet and a Lorentzian lineshape. However, as noted above, a calibration reference multiplet can alternatively or also be used.

[0082] Because the TMAO peak (located upfield from the water peak) often has a very small amplitude, it is difficult to locate its position accurately, especially in the presence of other analytes with similar concentrations. However, it is possible to determine the exact location of the TMAO peak relative to a citrate reference peak located upfield from the TMAO resonance, since there is a defined mathematical relationship relating the location of the pH dependent downfield citrate resonance and the separation of the citrate and TMAO peaks. The analysis locates the position of one or more of the citrate peaks, again using a least squares fit with a Lorentzian lineshape. This location can be entered into an empirically determined function that calculates the position of the TMAO peak.

[0083] After locating the position of the TMAO peak, the size of the fitting region can be reduced, typically to about 30-50 data points, centered around the calculated location of the TMAO peak. The size reduction from the larger search window to the smaller fitting window can diminish potential interferences from other metabolites. Finally, the analysis model can employ a single real TMAO basis component to deconvolute the peak while also allowing for one neighbor on either side to account for small misalignments. In addition, the minimal protein baseline present in the TMAO fitting region can be modeled with three quadratic equations: positive, negative and zero (a line). The least squares fit can be performed with a 30 data point analyte vector (the spectrum in the 30 point window) and a 30 x 6 design matrix, consisting of three TMAO basis vectors (the TMAO basis component and its neighbors - shifted by a single data point to each side) and the three baseline correction vectors. The fitting coefficients are generated from a Lawson-Hansen non-negative least squares QR fit (on just the thirty data point fitting region), resulting in coefficients that are then multiplied by concentration factors and combined to generate the final TMAO concentration.

[0084] **Figures 3** and **4** illustrate one embodiment that can find the location of the TMAO peak by calculating the distance between glucose and citrate versus the distance between glucose and TMAO. In other embodiments, the TMAO location can be based on the distance between citrate and TMAO. In the embodiment discussed with respect to **Figures 3** and **4,** for example, the following protocol can be used.

1. Find the pH stable glucose
2. Determine distance to citrate
3. Plug distance into defined linear equation (e.g., **Equation 1A**, **1B**) as "x"
4. Calculate distance from glucose to TMAO

[0085] As noted above, a predefined established relationship for the Δ between glucose and citrate vs. the Δ between glucose and TMAO can be used to calculate TMAO location. For example, as shown in **Figures 3, 4,** the citrate position relative to glucose can be determined by subtracting the position of citrate from that of glucose per Equation 2.

a.

$$7272-10908=-3636 \qquad \textbf{Equation 2}$$

b. The 'citrate position' can be inserted into the defined linear equation (along the lines of Equation 1A or 1B), e.g.: y=1.4924(-3636) + 2626. This value, y=-2800 defines the TMAO distance from glucose.

c. The actual TMAO position can then be calculated.

$$7272-x=-2800 \qquad \textbf{Equation 3}$$

Thus, in this example, x=10072, which is the TMAO position (peak center).

[0086] The temperature for this assay can be any appropriate temperature, typically between about 20 degrees C to about 47 degrees C. However, measurement of TMAO does not require an elevated temperature. Some preliminary examinations have indicated a very slight increase in sensitivity when the NMR assay is run at 25 degrees C. This small improvement is not likely to have a significant impact in overall assay performance. If this assay were to be run at a different temperature, then all TMAO assays can be run at one time i.e., in batches, to avoid any potential need for frequent and time consuming temperature changing and equilibration when performing tests at other temperatures.

[0087] The pulse sequence parameters can include any appropriate parameters including solvent suppression scheme, pulse angle and acquisition time. However, generally stated, in some particular embodiments, the NMR signal acquisition time per scan, for any one biosample, can be between about 2- 4 seconds (on average) and typically between about 3-4 seconds (on average), such as about 3.07 seconds (on average). The NMR analyzer may be configured to obtain at least 16 scans per biosample, typically between 16-256 scans, such as ≥ 64 scans, and more typically ≥ 96, such as 96 scans or 128 scans with at least about 16K data points collected over a 4400 Hz sweep width, per sample, to obtain the NMR data used to measure TMAO.

[0088] One element in the pulse sequence is the solvent suppression scheme. A WET solvent suppression scheme uses a series of shaped pulses and pulsed field gradients over the course of 80 ms. The ID NOESY-presat scheme uses the first increment of a 2D Nuclear Overhauser Effect Spectroscopy (NOESY) experiment (Beckonert, O.; Keun, H. C.; Ebbels, T. M. et. al. Nat. Protoc. 2007, 2, 2692-2703). In this scheme, a continuous low power, frequency selective pulse on water resonance is applied during D1 and 'mixing' time. The PURGE solvent suppression scheme (Simpson, A. J.; Brown, S. A. J. Magn. Reson. 2005, 175, 340-346) uses a continuous low power, frequency selective pulse on water resonance, relaxation gradients and echoes to attenuate the water signal.

[0089] The performance of all three sequences (and potentially other sequences known to those of skill in the art) is sufficient to achieve consistent spectra. One advantage of the WET sequence is that it does not involve any low power saturation period which could perturb the protein baseline via spin diffusion. It also does not have any significant delays which could lead to signal attenuation via relaxation.

[0090] As is well known, a standard presaturation ("Presat") pulse sequence can be used to obtain the NMR spectrum for analyzing the TMAO signal. This pulse sequence involves a selective low power pulse targeting the water resonance and lasting several seconds. This is well established in NMR practice and is a robust and reliable method to attenuate the water signal.

[0091] In some embodiments, the WET water suppression scheme can be used. The WET sequence involves a series of short selective pulses targeting the water resonance. The entire scheme is prefixed to the pulse sequence as is the Presat, but only requires 80 ms. The other advantage of the WET sequence is the fact that this sequence imposes only a minimal perturbation on the protein signals. Due to the length of a typical Presat sequence, some of the solvent saturation can be transferred to the protein which can lead to inconsistent contributions of the protein to the baseline. Other solvent presaturation schemes can be used, e.g., a PURGE sequence.

[0092] **Figure 8** illustrates a standard "one-pulse" sequence with WET solvent suppression. This leads to a spectrum in which the signals from the high concentrations of proteins and macromolecular aggregates, e.g. lipoprotein particles, dominate the spectra (**Figure 10,** upper profile line). The lower concentration, small molecule metabolites are greatly obscured in these spectra. As shown in **Figure 8,** the WET solvent suppression scheme (Smallcombe, S. H.; Patt, S. L.; Keifer, P. A. J. Magn. Reson., Ser. A 1995, 117, 295-303) includes a series of solvent-directed selective pulses and pulsed field gradients followed by a read pulse and an acquisition time during which the signal is digitized for a fixed amount of time.

[0093] The second sequence shown in **Figure 9** incorporates a CPMG pulse train or sequence. This pulse train includes a series of refocusing π pulses (180 degrees) during which the signals from large, rapidly relaxing molecules are attenuated. The duration of this pulse train can be optimized to minimize the background signals from macromolecules, e.g. proteins and lipoprotein particles, while maintaining most of the signal intensity from the small molecules such as TMANO. This value is set to 100 ms. Thus, the CPMG pulse train shown in **Figure 9** (for CPMG spectrum in **Figure 10B,** upper profile line) is designed to attenuate the signals from the macromolecules which facilitate the detection of many more small molecule metabolites. This pulse sequence relies on the fact that the signals from macromolecules relax much faster than small molecules. The spin-echo train occurring can occur after the read pulse and is designed to maintain the small molecule magnetization while the signals from the macromolecules relax back to equilibrium. The duration of this spin echo train relates to the degree of attenuation of the macromolecule signals. This delay is typically set to around 100 ms. It is expected that there will be little perceptible change in performance over the range from approximately 60 ms to 150 ms. However, below 60 ms the protein attenuation may be compromised and above 150 ms the overall signal intensity loss from relaxation may be unsatisfactory.

[0094] Comparisons of signal to noise and assay performance were marginally better with the CPMG over the WET. However, it is contemplated that the use of a CPMG sequence can allow the detection of many more metabolites should the assay composition expand in the future. CPMG sequences are well known to those of skill in the art.

[0095] In order to obtain an increased (e.g., maximum) signal from a molecule, a 90 degree pulse can be used and the time between these pulses should be in excess of 10 times the longitudinal relaxation time (T1) of the signal. The T1 for TMAO in dialyzed plasma was determined to be about 2.4 seconds. This is not a time efficient means to maximize the signal intensity so a compromise between pulse length and inter-pulse delay can be made. The relationship between signal intensity, T1, and pulse length is given by the Ernst angle equation. The first step in using this equation is to define the length of the entire pulse sequence. The length is defined by the required solvent suppression period, the CPMG delay and the length of the data acquisition period needed to provide the required digitization of the FID. The Ernst angle equation is as follows:

$$\text{Cos (theta)} = \exp -(\text{total delay})/T1 \quad \textbf{EQUATION (4)}$$

[0096] In equation (4), the total delay equals the d1 delay (including solvent suppression), the CPMG time, plus the acquisition time. T1 represents the longitudinal relaxation time of the analyte signal of interest. It is noted that in front of the parentheses containing "total delay" is a negative sign. Solving for theta will give an optimal flip angle.

[0097] Current results indicate that the optimal pulse flip angle for TMAO is about 70 degrees. The equation is relatively insensitive in this region so it is unlikely that small errors in calibration or small differences in the T1 due to specific sample composition will lead to significant inefficiency.

[0098] As noted above, the acquisition time (AT) is the time that the FID is digitized. The duration of AT is determined by both the relaxation time of the signal(s) being quantified and the required digitization. If the relaxation time of the signal being examined is longer than the AT then the FID will become truncated resulting in a signal with poor shape. As mentioned above, the T1 relaxation time for TMAO is about 2.4 seconds in serum and thus the acquisition time should be at least that long. The digitization rate of the spectrometer, i.e. the number of points taken per second of acquisition time, is determined by the sweep width of the spectrum. In some embodiments, a desired digital resolution uses at least 16K data points that are collected over the 4400Hz sweep width. This can employ between about 2-4 seconds, typically about 3.07 seconds, of AT per scan and a plurality of scans can be used per biosample such as between 16-384 scans, typically $\geq$ 16 scans, more typically $\geq$ 64 scans, such as $\geq$ about 96 scans, such as 96 scans, 128 scans, and 192 scans.

[0099] The most direct way to increase the detection sensitivity is to increase the number of scans. In some embodiments, the TMAO assay can be carried out on samples at about 47 degrees C so that this assay can be easily interleaved with the current LipoProfile® assay. However, as the number of scans increases, the residence time of the sample in the probe at 47 degrees increases and the samples may become denatured. The challenge is to achieve the requisite signal-to-noise ratio for the TMAO peak to allow accurate and precise quantification over a desired biological range (at least those with adverse clinical association).

[0100] The data can optionally be collected in blocks of 8 so the assay performance can be evaluated considering scans in multiples of 8. **Figure 11** shows the assay performance using spiked dialyzed serum with 64, 128 and 192 scans. Dialyzed serum is an "ideal" matrix in that there are no confounding small molecule metabolites.

[0101] The current data shown in **Figure 12A** and **12B** indicate that with 96 scans the LoQ is near 2.0 μM. The LoQ for actual patient samples is near 3.5 μM. The medical decision limit, indicated in the Nature paper by Hazen (incorporated by reference above), is approximately 6.1 μM. The performance with 96 scans is sufficient to achieve a quantitative assay that can quantify TMAO in the clinically relevant range. Lower numbers of scans may also be suitable such as $\geq$ 16 scans, particularly depending on the relevant clinical decision levels and/or curve fitting performance. Currently, the LoQ of the 128 scan assay is close to the 25th percentile (2.43 μM) and the LoQ for the 96 scan assay is close to the 50th percentile (3.67 μM). Given the biological variability and the fact that a current medical decision point is at the 75th percentile (6.18 μM), a 96 scan assay may be sufficient. This data is plotted as the % CV versus concentration. The data are fit with a simple power function as indicated on the plot and, from this, concentration yielding a 20% CV is calculated. This value is the accepted value for the LoQ. Concentrations below the LoQ cannot currently be quantitatively reported with a high degree of confidence (but may be qualitatively reported). The values shown in **Figures 12A** and **12B** may change as the assay is optimized.

[0102] The biosample can comprise any suitable pH-buffer alone or with other buffers as noted above. The buffer(s) can be present with a buffer to serum or plasma ratio of any one of the following (or any number there between) 10:90, 15:85; 20:80, 25:75, 30:70; 35:65, 40:60, 45:55, 50:50 and even 60:40 (or other values where there is more buffer or buffers than sample). However, embodiments of the invention use more sample by volume than buffers or other additives, e.g., reference or calibration additives. In certain embodiments, a buffer can maximize the amount serum or plasma in the biosample (e.g., provide a buffer to serum or plasma ratio of 45:55 or greater). In some particular embodiments, the sample comprises a 25:75 (buffer:serum) sample composition that is easy to prepare and provides a significant increase in sensitivity over a 50:50 composition. It is contemplated that, in some embodiments, low volume biosamples may be analyzed, such as $\leq$ 50 μL. If so, it may be suitable to formulate the biosample to have a greater amount of buffer relative to serum or other biospecimen (e.g., saliva, CSF) such as, for example, 75:25 (buffer:biospecimen).

[0103] A buffer can include one or more of albumin, glucose, citrate, acetate or other acidic compounds as well any of the well established chemical shift or quantitation references such as formate, trimethylsilylpropionate (and isotopically labeled isomers), and EDTA for example.

[0104] Exemplary pH buffers include, but are not limited to, acidic buffers, such as, but not limited to, a citrate, a phosphate, and/or an acetate buffer. For example, the biosample can comprise a citrate buffer and/or citrate phosphate buffer having a pH from about 2.6 to about 5.5 and comprising citric acid, sodium citrate, and/or sodium phosphate. Other exemplary buffers include, but are not limited to, an acetate buffer and/or an acetate phosphate buffer having a pH from about 3.7 to about 5.5 and comprising acetic acid, sodium acetate, and/or sodium phosphate. In some embodiments, the biosample comprises a citrate phosphate buffer comprising citric acid and sodium dibasic phosphate. Typically, the buffers are mixed with deionized water and are added to the biosample to dilute the sample by a defined amount. However, the chemical buffer(s) may also be added directly into a liquid or tissue biosample.

[0105] In some embodiments, the biosample comprises a citrate phosphate buffer comprising citric acid monohydrate and sodium dibasic phosphate heptahydrate. Citric acid monohydrate can be present in a citrate phosphate buffer in an amount from about 160 mM to about 170 mM, or any range therein, such as, but not limited to, about 164 mM to about 167 mM, about 164.4 mM to about 165.6 mM, or about 165.6 mM to about 166.7 mM. Sodium dibasic phosphate heptahydrate can be present in a citrate phosphate buffer in an amount from about 260 mM to about 275 mM, or any range therein, such as, but not limited to, about 267 mM to about 272 mM, about 267.2 mM to about 269.6 mM, or about 269.6 mM to about 271.9 mM. In some embodiments, citrate phosphate buffer can comprise about 160 mM to about 170 mM citric acid and about 260 mM to about 275 mM sodium dibasic phosphate heptahydrate. In certain embodiments, the biosample comprises a citrate phosphate buffer comprising about 165.6 mM citric acid monohydrate, about 269.6 mM sodium dibasic phosphate heptahydrate, and deionized water. In some embodiments, a citrate phosphate buffer has a pH of about 4.65.

[0106] The sample may be prepared for analysis shortly before analysis by the NMR spectrometer (manually or automatically with a sample handler) or at a remote, preprocessing or collection site. For example, a TMAO analysis container can be pre-loaded with the amount and or processed for analysis. In other embodiments, the collection container itself can be pre-loaded with the buffer in a range and the container marked for sample collection level to form the desired buffer:serum volume so that the appropriate amount of biosample is collected in the container.

[0107] **Figures 13A** and **13B** are graphs assessing the accuracy of the assay by comparing values from gravimetric measure of TMAO into spiked dialyzed serum versus NMR and NMR versus MS determined TMAO in patient samples. The agreement of NMR in both graphs is acceptable with R2 values greater than 0.9.

[0108] **Figure 14A** is a flow chart of exemplary operations that can be used to carry out embodiments of the present invention. NMR spectra of a biosample having a pH between about 5.15 to 5.53 are electronically analyzed (block **160**). A curve-fitting function (that can selectively apply one or more of a plurality of predefined basis functions to use zero, one, two or three neighboring peaks) can be applied to a defined peak region in the NMR spectrum at about 3.2 to 3.4 (block **170**). An amount of TMAO in the biosample is calculated (block 175).

[0109] According to the invention, a defined pH stable reference peak region in the NMR spectrum can be identified (block **161**). A distance from the reference peak region to a calibration peak region can be calculated (block **163**). The TMAO peak location can be determined using a defined distance from the calibration peak (block **164).**

[0110] The reference peak region can be a (anomeric) glucose peak region (block 162).

[0111] In an example which does not represent an embodiment of the invention, a defined calibration peak multiplet region can be used to determine the location of the TMAO peak. The calibration peak multiplet has peaks that vary in distance apart based on pH of the biosample variable (block **169**).

[0112] The calibration peak region can be a citrate peak region (block **165**).

[0113] The method may include adding a pH buffer to the biosample, then acquiring FID signal of the sample in an NMR flow probe **(166)** with an AT of less than about 4 seconds per scan with a plurality of scans per biosample. The TMAO peak region can have a peak with a width, on average, of about 1.2 Hz (block **172**) (and can vary to be between about 0.6 Hz and 2 Hz per biosample). A fitting function can be applied to the TMAO peak region with a line width of under 2 **Hz,** typically about 1 Hz (block **174**).

[0114] The position of a TMAO peak region can be calculated using a fitting region having a size between 50-100 data points based on a location of a citrate reference peak or peaks, then reducing the fitting region to about 30-50 data points centered about the calculated location of the TMAO peak. TMAO peak region can be analyzed with a defined curve fitting function or functions that can selectively allow for one or more neighbors on either side to account for small misalignments to determine the level of TMAO.

[0115] As noted above, the probable actual TMAO peak location can be identified by weighting the region around the expected TMAO peak location with a Gaussian, triangular parabolic or similar function. The probable actual TMAO peak location can then be identified as the highest weighted data point used to center the curve fitting region for determining the level of TMAO.

[0116] **Figures 14B, 14C** and **14D** illustrate an exemplary (and optional) automated pre-analytical quality control evaluation **175** that can be carried out programmatically (e.g., by the analyzer **22** or a processor in communication with the analyzer **22**) before performing a clinical assay, e.g., the TMAO assay according to some embodiments. Thus, the

TMAO assay is not performed if the input spectra fail pre-analytical quality control checks. The pre-analytical quality control evaluation **175** evaluates input spectra to determine acquisition occurred or will occur under specified conditions:

- Sample is properly mixed and the injected sample completely fills the flow cell
- Magnetic field is homogeneous
- Water suppression is adequate
- Sample pH is within a defined range, e.g., 5.3 ± 0.1.

[0117] Evaluation of these conditions is based on characterization of a reference peak in the spectra. In the case of the TMAO assay, the citrate peak serves as the pH sensitive reference peak. The height, integral, linewidth and skew of one of the citrate peaks is evaluated. This information is used as shown in the flowchart shown in **Figure 14B** to identify problems with mixing, injection and shimming.

[0118] As shown in **Figure 14B,** a reference peak height is evaluated (shown as citrate) block **176.** For ease of discussion, the term "citrate" will be used for this evaluation, but other reference peaks may also be used. If it is high, this indicates "bad mixing" or too much serum. If low, the citrate peak integral is obtained (block **177**). If the integral is acceptable (within defined values), this indicates "bad" shimming and can alert an operator via an output **175o** (audio and visual or just visual) on the display **22D** to initiate a shim operation or generate an alert that identifies the problem. If low, a linewidth is taken at 50%, 20% and 10% (block **178**), if acceptable, this indicates "bad mixing" associated with the sample and again the analyzer **22** can alert an operator of the problem or flag via an ouput on the display **22D** and/or initiate a retest. If high, skew can be evaluated (block **179**). If it is right of a defined location, a "bad injection and bad shimming" alert can be generated to the operator (e.g., via display **22D**) and if it is to the left, only a "bad injection alert is generated.

[0119] The programmatic evaluation **175** can determine the following characteristics of the reference (e.g., citrate peak or peaks) for each input spectra. Height of one of the citric acid peaks, typically the second peak from the left (marked with asterisk * in **Figure 14C**) can be determined. Linewidth of one peak, typically the second citric acid peak from the left at 50%/20%/10% peak height **(Figure 14D)** and the integral of all four citric acid peaks **(Figure 14C)** can be determined. Skew can be determined at 10% of second citric acid peak from the left using skew =y/2-x **(Figure 14D).**

[0120] Bad shimming can identified if the citrate height is < 97 au and has an acceptable citrate peak integral between 3674 and 3314 for any of the input spectra. The low height indicates that the peak is too broad indicating poor field homogeneity.

[0121] Bad mixing can be identified if the citrate height is < 97 au, citrate integral < 3314 au, acceptable line-width of < 1.65/3.15/4.74 Hz (at 50%/20%/10% peak height, respectively) for any of the input spectra. Other threshold values may be appropriate under different sample and/or data acquisition conditions.

[0122] Bad injection or bad shimming can be identified if the citrate height is < 97 au, citrate integral < 3314 au, the line-widths are > 1.65/3.15/4.74 Hz (at 50%/20%/10% peak height, respectively), and 10% skew is to the right more than +0.09 to +0.32 Hz for any of the input spectra. Other threshold values may be appropriate under different sample and/or data acquisition conditions

[0123] Bad injection can be identified if the citrate height is < 97 au, citrate integral < 3314 au, the line-widths are > 1.65/3.15/4.74 Hz (at 50%/20%/10% peak height, respectively), and 10% skew is to the left more than -0.09 to -0.32 Hz for any of the input spectra. Other threshold values may be appropriate under different sample and/or data acquisition conditions

[0124] It is noted that the flowcharts and block diagrams of certain of the figures herein illustrate the architecture, functionality, and operation of possible implementations of analysis models and evaluation systems and/or programs according to the present invention. In this regard, each block in the flow charts or block diagrams represents a module, segment, operation, or portion of code, which comprises one or more executable instructions for implementing the specified logical function(s). It should also be noted that in some alternative implementations, the functions noted in the blocks might occur out of the order noted in the figures. For example, two blocks shown in succession may in fact be executed substantially concurrently or the blocks may sometimes be executed in the reverse order, depending upon the functionality involved.

[0125] Referring now to **Figure 15A,** it is contemplated that the TMAO analysis can be carried out using a system **100** with at least one NMR clinical analyzer **22** as described, for example, with respect to **Figure 15B** below and/or in U.S. Patent No. 8,013, 602.

[0126] The system **100** can include a NMR TMAO analysis module and/or circuit **200** that can be onboard the analyzer **22** or at least partially remote from the analyzer **22.** If the latter, the analysis module or circuit **200** can reside totally or partially on a server **150.** The server **150** can be provided using cloud computing which includes the provision of computational resources on demand via a computer network. The resources can be embodied as various infrastructure services (e.g. computer, storage, etc.) as well as applications, databases, file services, email, etc. In the traditional model of computing, both data and software are typically fully contained on the user's computer; in cloud computing, the user's computer may contain little software or data (perhaps an operating system and/or web browser), and may serve as little more than a display terminal for processes occurring on a network of external computers. A cloud computing service (or an aggregation of multiple cloud resources) may be generally referred to as the "Cloud". Cloud storage may include a model of networked computer data storage where data is stored on multiple virtual servers, rather than being hosted on one or more dedicated servers. Data transfer can be encrypted and can be done via the Internet using any appropriate firewalls to comply with industry or regulatory standards such as HIPAA. The term "HIPAA" refers to the United States laws defined by the Health Insurance Portability and Accountability Act. The patient data can include an accession number or identifier, gender, age and test data.

[0127] As shown in **Figure 15B,** the at least one analyzer **22** can be configured to evaluate containers **25** of biosamples **123** optionally including one or more defined diluents or buffers **124.** The containers **25** can have sample/buffer solutions and may be flowably introduced to the NMR probe using a flow cell or the containers can be placed in the NMR probe for evaluation. The containers **25** can include respective biosamples of human blood plasma or serum with a solution of citrate acid and sodium dibasic phosphate in a defined ratio, the ratio being between 25:75 to about 50:50 (buffer:serum) by volume, with the pH being between about 5.15 and 5.53.

[0128] The results of the analysis can be transmitted via a computer network, such as the Internet, via email or the like to a patient, clinician site **50,** to a health insurance agency or a pharmacy. The results can be sent directly from the analysis site (Site 1, Site 2) or may be sent indirectly via a central or distributed network (Site 3). The results may be printed out and sent via conventional mail. This information can also be transmitted to pharmacies and/or medical insurance companies, and/or respective patients. The results can be sent to a patient via email to a "home" computer or to a pervasive computing device such as a smart phone or notepad and the like. The results can be as an email attachment of the overall report or as a text message alert, for example.

[0129] The systems can be configured to measure different biosamples for assessing TMAO levels. For example, both urine and blood samples can be analyzed and measurements reported together or separately with any associated risk or as an independent measurement.

[0130] Referring now to **Figure 16,** a system **207** for acquiring and calculating the lineshape of a selected sample is illustrated. The system **207** includes an NMR spectrometer **22** for taking NMR measurements of a sample. In one embodiment, the spectrometer **22** is configured so that the NMR measurements are conducted at about 400 MHz for proton signals; in other embodiments the measurements may be carried out at between 200-900 MHz or other suitable frequency. Other frequencies corresponding to a desired operational magnetic field strength may also be employed. Typically, a proton flow probe is installed, as is a temperature controller to maintain the sample temperature at about 47 +/- 0.5 degrees C. However, as noted above, other sample temperatures may be employed. The spectrometer **22** is controlled by a digital computer **214** or other signal processing unit. The computer **211** should be capable of performing rapid Fourier transformations. It may also include a data link **212** to another processor or computer **213,** and a direct-memory-access channel **214** which can connects to a hard memory storage unit **215.**

[0131] The digital computer **211** may also include a set of analog-to-digital converters, digital-to-analog converters and slow device I/O ports which connect through a pulse control and interface circuit **216** to the operating elements of the spectrometer. These elements include an RF transmitter **217** which produces an RF excitation pulse of the duration, frequency and magnitude directed by the digital computer **211,** and an RF power amplifier **218** which amplifies the pulse and couples it to the RF transmit coil **219** that surrounds sample cell **220.** The NMR signal produced by the excited sample in the presence of a 9.4 Tesla polarizing magnetic field produced by superconducting magnet **221** is received by a coil **222** and applied to an RF receiver **223.** The amplified and filtered NMR signal is demodulated at **224** and the resulting quadrature signals are applied to the interface circuit **216** where they are digitized and input through the digital computer **211.** The TMAO analyzer circuit **200** and/or module **350 (Figures 15A** and **17)** can be located in one or more processors associated with the digital computer **211** and/or in a secondary computer **213** or other computers that may be on-site or remote, accessible via a worldwide network such as the Internet **227.**

[0132] The TMAO analyzer circuit **200** can include a database of experimental determinations of concentrations of TMAO to curves and/or areas of TMAO peak regions for different levels of expected TMAO values in biologic ranges as is known to those of skill in the art. Reference standards can be used for calibration or defining concentrations of NMR measurements. The TMAO experimental or reference samples can be obtained from known suppliers of "high purity" TMAO material (e.g., Sigma-Aldrich, LLC.). The TMAO analyzer circuit **200** can include a TMAO basis function that accounts for the residual protein baseline interferences that may survive the pulse sequence (e.g., the CPMG pulse sequence). The TMAO basis function can be an experimentally acquired spectrum of TMAO processed with defined (consistent) parameters to the actual spectrum. Computationally derived TMAO basis functions may also be used (e.g., specified Lorentzians, Gaussians or mixed functions) or combinations of same.

[0133] After the NMR data are acquired from the biosample in the measurement cell **220,** processing by the computer **211** produces another file that can, as desired, be stored in the storage database **215.** This second file is a digital representation of the chemical shift spectrum and it is subsequently read out to the computer **213** for storage in its storage **225** or a database associated with one or more servers. Under the direction of a program stored in its memory, or in another database or circuit in communication with the NMR analyzer **22** (or spectrometer), one or more processors, such as one associated with the computer **213,** which may be a personal, laptop, desktop, workstation, notepad, tablet or other computer, processes the chemical shift spectrum in accordance with the teachings of the present invention to generate a report which may be output to a printer **226** or electronically stored and relayed to a desired server, database(s), email address or URL. Those skilled in this art will recognize that other output devices, such as a computer display screen, notepad, smart phone and the like, may also be employed for the display of results.

[0134] It should be apparent to those skilled in the art that the functions performed by the computer **213** and its separate storage **225** may also be incorporated into the functions performed by the spectrometer's digital computer **211.** In such case, the printer **226** may be connected directly to the digital computer **211.** Other interfaces and output devices may also be employed, as are well-known to those skilled in this art.

[0135] Embodiments of the present invention may take the form of an entirely software embodiment or an embodiment combining software and hardware aspects, all generally referred to herein as a "circuit" or "module."

[0136] As will be appreciated by one of skill in the art, the present invention may be embodied as an apparatus, a method, data or signal processing system, or computer program product. Accordingly, the present invention may take the form of an entirely software embodiment, or an embodiment combining software and hardware aspects. Furthermore, certain embodiments of the present invention may take the form of a computer program product on a computer-usable storage medium having computer-usable program code means embodied in the medium. Any suitable computer readable medium may be utilized including hard disks, CD-ROMs, optical storage devices, or magnetic storage devices.

[0137] The computer-usable or computer-readable medium may be, but is not limited to, an electronic, magnetic, optical, electromagnetic, infrared, or semiconductor system, apparatus, device, or propagation medium. More specific examples (a non-exhaustive list) of the computer-readable medium would include the following: an electrical connection having one or more wires, a portable computer diskette, a random access memory (RAM), a read-only memory (ROM), an erasable programmable read-only memory (EPROM or Flash memory), an optical fiber, and a portable compact disc read-only memory (CD-ROM). Note that the computer-usable or computer-readable medium could even be paper or another suitable medium, upon which the program is printed, as the program can be electronically captured, via, for instance, optical scanning of the paper or other medium, then compiled, interpreted or otherwise processed in a suitable manner if necessary, and then stored in a computer memory.

[0138] Computer program code for carrying out operations of the present invention may be written in an object oriented programming language such as Java7, Smalltalk, Python, Labview, C++, or VisualBasic. However, the computer program code for carrying out operations of the present invention may also be written in conventional procedural programming languages, such as the "C" programming language or even assembly language. The program code may execute entirely on the user's computer, partly on the user's computer, as a stand-alone software package, partly on the user's computer and partly on a remote computer or entirely on the remote computer. In the latter scenario, the remote computer may be connected to the user's computer through a local area network (LAN), a wide area network (WAN), a secure area network (SAN) or the connection may be made to an external computer (for example, through the Internet using an Internet Service Provider).

[0139] **Figure 17** is a block diagram of exemplary embodiments of data processing systems that illustrates systems, methods, and computer program products in accordance with embodiments of the present invention. The processor **310** communicates with the memory **314** via an address/data bus **348.** The processor **310** can be any commercially available or custom microprocessor. The memory **314** is representative of the overall hierarchy of memory devices containing the software and data used to implement the functionality of the data processing system **305.** The memory **314** can include, but is not limited to, the following types of devices: cache, ROM, PROM, EPROM, EEPROM, flash memory, SRAM, and DRAM.

[0140] As shown in **Figure 17,** the memory **314** may include several categories of software and data used in the data processing system **305:** the operating system **352;** the application programs **354;** the input/output (I/O) device drivers **358;** a TMAO Evaluation Module **350;** and the data **356.** The TMAO Evaluation Module **350** can interrogate one or more defined NMR signal peak regions in proton NMR spectra of a respective biosample to identify a level of TMAO. As noted above, the TMAO Evaluation Module **350** may identify a reference peak region (pH stable) and a calibration peak region (pH variable) to more precisely locate the TMAO peak region based on a predetermined relationship of distance between the calibration, reference and TMAO peak regions. In some particular embodiments, the TMAO Module **350** can also subtract a known TMAO standard concentration from a calculated concentration of the TMAO based on the amount of TMAO standard added to the biosample to amplify the TMAO peak.

[0141] The data **356** may include signal (constituent and/or composite spectrum lineshape) data **362** which may be obtained from a data or signal acquisition system **320.** As will be appreciated by those of skill in the art, the operating system **352** may be any operating system suitable for use with a data processing system, such as OS/2, AIX or OS/390 from International Business Machines Corporation, Armonk, NY, WindowsCE, WindowsNT, Windows95, Windows98, Windows2000 or WindowsXP from Microsoft Corporation, Redmond, WA, PalmOS from Palm, Inc., MacOS from Apple Computer, UNIX, FreeBSD, or Linux, proprietary operating systems or dedicated operating systems, for example, for embedded data processing systems.

[0142] The I/O device drivers **358** typically include software routines accessed through the operating system **352** by the application programs **354** to communicate with devices such as I/O data port(s), data storage **356** and certain memory **314** components and/or the image acquisition system **320.** The application programs **354** are illustrative of the programs that implement the various features of the data processing system **305** and can include at least one application, which supports operations according to embodiments of the present invention. Finally, the data **356** represents the static and dynamic data used by the application programs **354,** the operating system **352,** the I/O device drivers **358,** and other software programs that may reside in the memory **314.**

[0143] While the present invention is illustrated, for example, with reference to the Module **350** being an application program in **Figure 17,** as will be appreciated by those of skill in the art, other configurations may also be utilized while still benefiting from the teachings of the present invention. For example, the TMAO Module **350** may also be incorporated into the operating system **352,** the I/O device drivers **358** or other such logical division of the data processing system **305.** Thus, the present invention should not be construed as limited to the configuration of **Figure 17,** which is intended to encompass any configuration capable of carrying out the operations described herein.

[0144] In certain embodiments, the Module **350** includes computer program code for providing a level of TMAO which may be used to assess CHD risk and/or to indicate whether therapy intervention is desired and/or track efficacy of a therapy. The TMAO test may be used in conjunction with clinical evaluation and other diagnostic tests as an aid in assessing a patients risk for developing cardiovascular disease (CVD) or coronary heart disease (CHD).

[0145] The NMR evaluated TMAO can be a useful companion diagnostic t nutritional guidance in the use of prebioticprobiotic containing foods or supplements or other types of functional foods.

[0146] TMAO may also provide valuable information for other clinical applications including therapeutic monitoring and/or management. TMAO measurements can be used for management associated with specific diet, probiotic or drug treatment(s). TMAO measurements can be used with clinical trials and/or drug development programs. The TMAO measurements can be used to contradict a planned or actual therapy.

[0147] TMAO may be used to monitor for signs or diagnosis of kidney transplant rejection. Metabolic profiling evaluations of kidney transplants have revealed biomarkers that include altered levels of trimethylamine-N-oxide (TMAO), dimethylamine, lactate, acetate and alanine. In many of these investigations, TMAO was increased by a factor of 3-4 compared to healthy controls. The increase in TMAO is believed to stabilize proteins when there is an increased concentration of protein denaturants such as urea and guanidine derivatives following a toxic insult to the kidney.

[0148] TMAO measurements may also be used to evaluate patients having Trimethylaminuria (TMAU), also known as fish odor syndrome or fish malodor syndrome. TMAU is a rare metabolic disorder that causes a defect in the normal production of the enzyme Flavin containing monooxygenase 3 (FMO3). When FMO3 is not working correctly or if not enough enzyme is produced, the body loses the ability to convert trimethylamine (TMA) from precursor compounds in food digestion into trimethylamine oxide (TMAO) through a process called N-oxygenation. Trimethylamine then builds up and is released in the person's sweat, urine, and breath, giving off a strong fishy odor or strong body odor. Measurement of urine for the ratio of trimethylamine to trimethylamine oxide is the standard screening test.

[0149] **Figure 18** illustrates patient reports **400** that may include visual indicia of risk 405 associated with TMAO measurements. **Figure 18** illustrates three potential embodiments of the TMAO risk report dependent upon the lower limit of quantitation that is achievable due to analytical consideration and/or what is appropriate based on biological variability, particularly associated with blood plasma or serum samples.

[0150] The visual indicia **405** can include a graphic with a degree of risk indicated in a defined color scale. The color scale may range from "green", "yellow" and "red or orange" for a continuum of risk from low (green) to higher risk (red/orange). The red or orange is indicated by the cross-hatch markings while the yellow is shown by the lighter gray scale. Green or another low risk color can be used for the Q1, T1 or H1 (lower half) values. Other colors may be used to visually denote risk. The report **400** may be generated with quantitative results only for the upper 3 quartiles, the upper two tertiles or the upper 2 quartiles. As shown in the three exemplary visual risk indicia formats, a demarcation line **410** can separate the lower range values from the upper ranges. In other embodiments, a quantitative result can be provided for all quartiles or tertiles of measurements.

[0151] **Figure 19** is an NMR (chemical shift/ppm) spectrum of urine according to embodiments of the present invention. The urinary TMAO concentration is typically much higher than in serum, but the range is quite large. Urine is also in a "crowded" matrix and there is a large number of potentially confounding metabolites. As in the serum matrix, adjustment of the pH can be used to move the TMAO peak into more or less crowded regions. **Figure 19** illustrates an expansion of the TMAO region of a ^{1}H NMR spectrum of urine. The samples were prepared with a 60:40 mixture of urine and NMR diluent with the pH starting at 4.62 at the bottom and going to 6.83 in the top; pH step size is approximately 0.15 units.

[0152] The location of the TMAO peak can be reliably determined by using either (or both) the creatinine peak labeled in the **Figure 19** or the citrate peak. Both creatinine and citrate are endogenous metabolites in urine and so this pH referencing could be carried out without the addition of corresponding compounds in the buffer or diluent. The relationship between the TMAO peak location and the respective citrate and creatinine peak locations are show in **Figures 20A** and

20B. Both graphs show a nearly linear correlation between the TMAO and the respective potential reference peak. It is noted that the exact nature of the relationship between the TMAO peak location and the creatinine and citrate can be dependent upon the sample composition, i.e., ratio of urine to buffer.

[0153] Quantitation of the TMAO concentration of the urine and other biosamples (e.g., blood plasma and serum) can be achieved by modeling the TMAO signal with computationally derived functions. The baseline can be modeled by DC offset, linear, and/or quadratic functions. The TMAO peak can be modeled by Lorentzian and/or Gaussian functions of varying line widths. These "mixed" basis set functions can have the identical height **(Figure 21)** or be normalized by area **(Figure 22).**

[0154] **Figure 21** shows a basis set of Lorentzian and Gaussian functions to model TMAO signal. The height of each function is identical. DC offset and quadratic functions can be used to model baseline signal.

[0155] **Figure 22** shows a normalized basis set of Lorentzian and Gaussian functions to model TMAO signal. The area of each function is identical. DC offset and quadratic functions used to model baseline signal are also normalized to the same area.

[0156] **Figures 23-26** are graphs of additional examples of basis sets. **Figure 23** shows Lorentzian functions with identical height and varying line width. **Figure 24** shows Gaussian functions with identical height and varying line width. **Figure 25** shows Lorentzian functions with normalized area and varying line width. **Figure 26** shows Gaussian functions with normalized area and varying line width.

[0157] The foregoing is illustrative of the present invention and is not to be construed as limiting thereof. Although a few exemplary embodiments of this invention have been described, those skilled in the art will readily appreciate that many modifications are possible in the exemplary embodiments without materially departing from the novel teachings and advantages of this invention. Accordingly, all such modifications are intended to be included within the scope of this invention as defined in the claims. Therefore, it is to be understood that the foregoing is illustrative of the present invention and is not to be construed as limited to the specific embodiments disclosed, and that modifications to the disclosed embodiments, as well as other embodiments, are intended to be included within the scope of the appended claims. The invention is defined by the following claims.

Claims

1. A method of determining a measure of trimethylamine-N-oxide (TMAO) in an *in vitro* biosample, comprising:

electronically identifying a pH-stable reference peak region in a proton NMR spectrum of the biosample;
electronically identifying a defined calibration peak region in the NMR spectrum of the biosample, wherein the calibration peak region has a location that changes based on pH of the biosample;
electronically calculating a distance between the reference peak region and the calibration peak region;
electronically determining a location of a single TMAO peak within a defined TMAO peak region residing between about 3.2 and about 3.4 ppm of the proton NMR spectrum, the identification of the TMAO peak being based on the calculated distance between the reference peak region and the calibration peak region; then
electronically determining a level of TMAO in the biosample using the single TMAO peak.

2. The method of claim 1 which determines a measure of TMAO in a biological range between 1 to 50 μM.

3. The method of Claim 1 or Claim 2, wherein the biosample is:

(i) a blood plasma or serum wherein the defined TMAO peak is at about 3.30 ppm; or
(ii) saliva.

4. The method of any preceding Claim, further comprising, before the electronically determining the level of TMAO step, electronically calculating a location of the defined TMAO peak region using a fitting region having a size between about 50-100 data points based on a location of a citrate reference peak or peaks, then reducing the fitting region to about 30 data points centered about the calculated location of the defined TMAO peak region, and electronically curve fitting the defined TMAO peak region with a defined curve fitting function or functions to determine the level of TMAO.

5. The method of any preceding Claim, wherein the electronically determining the level of TMAO uses a linear calibration function which relates integral units from the NMR spectrum to micromolar concentration values.

6. The method of Claim 1, wherein the reference peak region is a glucose peak region, and optionally wherein the reference glucose peak region is associated with anomeric glucose at about 5.20 ppm.

7. The method of Claim 1, wherein the reference peak region is associated with anomeric glucose at about 5.20 ppm and wherein the calibration peak region is associated with one or more peaks of a citrate multiplet, e.g. a single peak of the citrate multiplet at about 3.7 ppm.

8. The method of Claim 1, wherein the biosample is a human blood plasma or serum sample, wherein the biosample comprises an acidic pH buffer so that the biosample has a pH between about 5.15 and 5.53, and wherein the electronic determination is carried out by applying curve fitting functions to NMR signal associated with the TMAO peak region to determine a first level of TMAO, then subtracting a known concentration of a TMAO standard that was added to the biosample to generate a patient-specific level of TMAO.

9. The method of Claim 1, further comprising:

   providing containers holding respective biosamples with a solution of citric acid and sodium dibasic phosphate in a defined ratio (buffer:serum) by volume, with the pH being between about 5.15 and 5.53;
   positioning a respective biosample in an NMR probe of an NMR spectrometer; and
   obtaining NMR signal to generate the NMR proton spectrum for determining the level of TMAO;
   optionally wherein the biosample is human serum and the obtaining step is carried out between 2-4 seconds on average of NMR acquisition time per scan with at least 16 scans per biosample.

10. The method of Claim 1, further comprising, before the electronic determination of the level of TMAO:

   providing containers holding small volumes of respective biosamples of about 50 μL or less with a buffer comprising citric acid, a TMAO standard concentration between about 1-100 μM, and sodium dibasic phosphate in a defined ratio, with the pH being between about 5.15 and 5.53;
   positioning respective biosamples from a respective container or the container with the biosample in an NMR probe of an NMR spectrometer; and
   obtaining NMR signal to generate the NMR proton spectrum for determining the level of TMAO,
   wherein the electronic determination is carried out by applying curve fitting functions to NMR signal associated with the TMAO peak to determine a first level of TMAO, then subtracting a known concentration of the TMAO standard that was added to the biosample to generate a patient-specific level of TMAO.

11. The method of any preceding Claim, further comprising generating an output of the determined level of TMAO with an indication of whether the determined level is considered high, and/or with visual indicia indicating a continuum of risk going from low to high or high to low and to indicate whether a subject is at risk of a complication of atherosclerotic cardiovascular disease, and wherein a subject whose TMAO is above a value associated with a level in a defined upper percentile of a reference population is at risk of experiencing a complication of atherosclerotic cardiovascular disease.

12. A computer program product for evaluating a proton NMR spectrum of an *in vitro* biosample, the computer program product comprising:

   a non-transitory computer readable storage medium having computer readable program code embodied in the medium, the computer-readable program code to cause a processor to perform the method of any one of the preceding claims.

13. An analysis system, comprising:

   at least one NMR spectrometer for acquiring at least one proton NMR spectrum of an *in vitro* biosample; and
   at least one processor in communication with the at least one NMR spectrometer, the at least one processor configured to perform the method of any one of claims 1 to 12.

Patentansprüche

1. Ein Verfahren zum Bestimmen eines Messwerts von Trimethylamin-N-oxid (TMAO) in einer biologischen In-vitro-Probe, das Folgendes beinhaltet:

elektronisches Ermitteln einer pH-stabilen Referenzpeakregion in einem Protonen-NMR-Spektrum der biologischen Probe;
elektronisches Ermitteln einer definierten Kalibrationspeakregion in dem NMR-Spektrum der biologischen Probe, wobei die Kalibrationspeakregion einen Ort aufweist, der sich auf der Grundlage des pH-Werts der biologischen Probe verändert;
elektronisches Berechnen einer Distanz zwischen der Referenzpeakregion und der Kalibrationspeakregion;
elektronisches Bestimmen eines Orts eines einzelnen TMAO-Peaks innerhalb einer definierten TMAO-Peakregion, der zwischen etwa 3,2 und etwa 3,4 ppm des Protonen-NMR-Spektrums liegt, wobei die Ermittlung des TMAO-Peaks auf der berechneten Distanz zwischen der Referenzpeakregion und der Kalibrationspeakregion basiert; dann
elektronisches Bestimmen eines TMAO-Levels in der biologischen Probe unter Verwendung des einzelnen TMAO-Peaks.

2. Verfahren gemäß Anspruch 1, das einen Messwert von TMAO in einem biologischen Bereich zwischen 1 und 50 μM bestimmt.

3. Verfahren gemäß Anspruch 1 oder Anspruch 2, wobei es sich bei der biologischen Probe um Folgendes handelt:

(i) ein Blutplasma oder Serum, wobei der definierte TMAO-Peak bei etwa 3,30 ppm liegt; oder
(ii) Speichel.

4. Verfahren gemäß einem der vorhergehenden Ansprüche, das vor dem Schritt des elektronischen Bestimmens des TMAO-Levels ferner Folgendes beinhaltet: elektronisches Berechnen eines Orts der definierten TMAO-Peakregion unter Verwendung einer Anpassungsregion mit einer Größe zwischen etwa 50 und 100 Datenpunkten auf der Grundlage eines Orts eines Citratreferenzpeaks oder mehrerer -peaks, dann Reduzieren der Anpassungsregion auf etwa 30 Datenpunkte, die um den berechneten Ort der definierten TMAO-Peakregion konzentriert sind, und elektronisches Kurvenanpassen der definierten TMAO-Peakregion mit einer definierten Kurvenanpassungsfunktion oder mehreren -funktionen, um den TMAO-Level zu bestimmen.

5. Verfahren gemäß einem der vorhergehenden Ansprüche, wobei das elektronische Bestimmen des TMAO-Levels eine lineare Kalibrationsfunktion verwendet, die ganzzahlige Einheiten von dem NMR-Spektrum in Beziehung mit mikromolaren Konzentrationswerten setzt.

6. Verfahren gemäß Anspruch 1, wobei die Referenzpeakregion eine Glucosepeakregion ist und wobei die Referenzglucosepeakregion optional mit anomerer Glucose bei etwa 5,20 ppm assoziiert ist.

7. Verfahren gemäß Anspruch 1, wobei die Referenzpeakregion mit anomerer Glucose bei etwa 5,20 ppm assoziiert ist und wobei die Kalibrationspeakregion mit einem oder mehreren Peaks eines Citrat-Multipletts assoziiert ist, z. B. einem einzelnen Peak des Citrat-Multipletts bei etwa 3,7 ppm.

8. Verfahren gemäß Anspruch 1, wobei die biologische Probe eine menschliche Blutplasma- oder Serumprobe ist, wobei die biologische Probe einen sauren pH-Puffer beinhaltet, so dass die biologische Probe einen pH-Wert zwischen etwa 5,15 und 5,53 aufweist, und wobei die elektronische Bestimmung ausgeführt wird, indem Kurvenanpassungsfunktionen auf ein NMR-Signal angewendet werden, das mit der TMAO-Peakregion assoziiert ist, um einen ersten TMAO-Level zu bestimmen, dann eine bekannte Konzentration eines TMAO-Standards, der zu der biologischen Probe zugegeben wurde, subtrahiert wird, um einen patientenspezifischen TMAO-Level zu erzeugen.

9. Verfahren gemäß Anspruch 1, das ferner Folgendes beinhaltet:

Versehen der Behälter, welche die jeweiligen biologischen Proben enthalten, mit einer Lösung von Zitronensäure und zweibasigem Natriumphosphat in einem definierten Verhältnis (Puffer zu Serum), bezogen auf das Volumen, wobei der pH-Wert zwischen etwa 5,15 und 5,53 beträgt;
Positionieren einer jeweiligen biologischen Probe in einer NMR-Sonde eines NMR-Spektrometers; und
Erhalten des NMR-Signals, um das NMR-Protonenspektrum zur Bestimmung des TMAO-Levels zu erzeugen;
wobei es sich bei der biologischen Probe optional um menschliches Serum handelt und der Schritt des Erhaltens bei einer durchschnittlichen NMR-Akquisitionszeit pro Scan von zwischen 2 bis 4 Sekunden mit mindestens 16 Scans pro biologischer Probe ausgeführt wird.

10. Verfahren gemäß Anspruch 1, das vor dem elektronischen Bestimmen des TMAO-Levels ferner Folgendes beinhaltet:

Versehen der Behälter, die kleine Volumen der jeweiligen biologischen Proben von etwa 50 μl oder weniger enthalten, mit einem Puffer, der Zitronensäure, eine TMAO-Standardkonzentration zwischen etwa 1 und 100 μM und zweibasiges Natriumphosphat in einem definierten Verhältnis enthält, wobei der pH-Wert zwischen etwa 5,15 und 5,53 beträgt;
Positionieren der jeweiligen biologischen Proben aus einem jeweiligen Behälter oder des Behälters mit der biologischen Probe in einer NMR-Sonde eines NMR-Spektrometers; und
Erhalten des NMR-Signals, um das NMR-Protonenspektrum zur Bestimmung des TMAO-Levels zu erzeugen, wobei die elektronische Bestimmung ausgeführt wird, indem Kurvenanpassungsfunktionen auf das NMR-Signal, das mit dem TMAO-Peak assoziiert ist, angewendet werden, um einen ersten TMAO-Level zu bestimmen, dann eine bekannte Konzentration des TMAO-Standards, der zu der biologischen Probe zugegeben wurde, subtrahiert wird, um einen patientenspezifischen TMAO-Level zu erzeugen.

11. Verfahren gemäß einem der vorhergehenden Ansprüche, das ferner das Erzeugen einer Ausgabe des bestimmten TMAO-Levels mit einer Angabe dazu, ob der bestimmte Level als hoch betrachtet wird, und/oder mit sichtbaren Anzeichen, die ein Risikokontinuum von niedrig zu hoch oder hoch zu niedrig anzeigen, beinhaltet, und um anzuzeigen, ob bei einem Individuum das Risiko einer Komplikation einer atherosklerotischen kardiovaskulären Erkrankung besteht, und wobei bei einem Individuum, dessen TMAO über einem Wert liegt, der mit einem Level in einem definierten oberen Perzentil einer Referenzpopulation assoziiert ist, das Risiko besteht, dass es zu einer Komplikation einer atherosklerotischen kardiovaskulären Erkrankung kommt.

12. Ein Computerprogramm zum Auswerten eines Protonen-NMR-Spektrums einer biologischen In-vitro-Probe, wobei das Computerprogrammprodukt Folgendes beinhaltet:

ein nichtflüchtiges computerlesbares Speichermedium mit computerlesbarem Programmcode, der in dem Medium verkörpert ist, wobei der computerlesbare Programmcode einen Prozessor veranlasst, das Verfahren gemäß einem der vorhergehenden Ansprüche durchzuführen.

13. Ein Analysesystem, das Folgendes beinhaltet:

mindestens ein NMR-Spektrometer zum Akquirieren mindestens eines Protonen-NMR-Spektrums einer biologischen In-vitro-Probe; und
mindestens einen Prozessor in Kommunikation mit dem mindestens einen NMR-Spektrometer, wobei der mindestens eine Prozessor dazu konfiguriert ist, das Verfahren gemäß einem der Ansprüche 1 bis 12 durchzuführen.

Revendications

1. Procédé de détermination d’une mesure de triméthylamine-N-oxyde (TMAO) dans un échantillon biologique *in vitro,* comprenant :

l’identification électronique d’une région du pic de référence à pH stable dans un spectre RMN du proton de l’échantillon biologique ;
l’identification électronique d’une région du pic d’étalonnage définie dans le spectre RMN de l’échantillon biologique, dans lequel la région du pic d’étalonnage présente un emplacement qui change en fonction du pH de l’échantillon biologique ;
le calcul électronique d’une distance entre la région du pic de référence et la région du pic d’étalonnage ;
la détermination électronique d’un emplacement d’un pic simple de TMAO à l’intérieur de la région de pic de TMAO définie résidant entre environ 3,2 et environ 3,4 ppm du spectre RMN du proton, l’identification du pic de TMAO étant basée sur la distance calculée entre la région du pic de référence et la région du pic d’étalonnage ; suivi de
la détermination électronique d’un niveau de TMAO dans l’échantillon biologique en utilisant le pic simple de TMAO.

2. Procédé selon la revendication 1 lequel détermine une mesure de TMAO dans une plage biologique de 1 à 50 μM.

3. Procédé selon la revendication 1 ou la revendication 2, dans lequel l'échantillon biologique est :

(i) un plasma ou un sérum sanguin dans lequel le pic de TMAO défini se trouve à environ 3,30 ppm ; ou
(ii) de la salive.

4. Procédé selon l'une quelconque des revendications précédentes, comprenant en outre, avant la détermination électronique du niveau de l'étape TMAO, le calcul électronique d'un emplacement de la région de pic de TMAO définie en utilisant une région de réglage ayant une taille entre 50 à 100 points de données basée sur un emplacement d'au moins un pic de référence de citrate, réduisant ainsi la région de réglage à environ 30 points de données centrés autour de l'emplacement calculé de la région de pic de TMAO définie, et l'ajustement de courbe électronique de la région de pic de TMAO définie, avec au moins une fonction d'ajustement de courbe définie pour déterminer le niveau de TMAO.

5. Procédé selon l'une quelconque des revendications précédentes, dans lequel la détermination électronique du niveau de TMAO utilise une fonction d'étalonnage linéaire qui rattache les unités intégrales du spectre RMN aux valeurs de concentration micromolaire.

6. Procédé selon la revendication 1, dans lequel la région du pic de référence est la région de pic du glucose et, éventuellement, dans lequel la région de pic de glucose de référence est associée au glucose anomérique à environ 5,20 ppm.

7. Procédé selon la revendication 1, dans lequel la région du pic de référence est associée au glucose anomérique à environ 5,20 ppm et dans lequel la région du pic d'étalonnage est associée à au moins un pic d'un multiplet de citrate, p. ex., un pic simple du multiplet de citrate à environ 3,7 ppm.

8. Procédé selon la revendication 1, dans lequel l'échantillon biologique est un échantillon de plasma ou de sérum sanguin humain, dans lequel l'échantillon biologique comprend un tampon à pH acide de sorte que l'échantillon biologique possède un pH situé entre environ 5,15 et 5,53, et dans lequel la détermination électronique est effectuée par l'application de fonctions d'ajustement de courbe à un signal RMN associé à la région du pic de TMAO pour déterminer un premier niveau de TMAO, en soustrayant ensuite une concentration connue de l'étalon de TMAO qui a été ajouté à l'échantillon biologique pour générer un niveau de TMAO particulier au patient.

9. Procédé selon la revendication 1, comprenant en outre :

la fourniture de récipients contenant des échantillons biologiques respectifs avec une solution d'acide citrique et de phosphate de sodium dibasique dans un rapport défini (tampon:sérum) par volume, avec un pH situé entre environ 5,15 et 5,53 ;

le positionnement d'un échantillon biologique respectif dans une sonde RMN d'un spectromètre RMN ; et
l'obtention d'un signal RMN pour générer le spectre RMN du proton pour déterminer le niveau de TMAO ;
éventuellement dans lequel l'échantillon biologique est un sérum humain et l'étape d'obtention est effectuée entre 2 à 4 secondes en moyenne du temps d'acquisition du spectre RMN par balayage avec au moins seize balayages par échantillon biologique.

10. Procédé selon la revendication 1, comprenant en outre, avant la détermination électronique du niveau de TMAO :

la fourniture de récipients contenant de faibles volumes d'échantillons biologiques respectifs d'environ 50 μl ou moins avec un tampon comprenant de l'acide citrique, une concentration d'étalon de TMAO entre environ 1 à 100 μM, et du phosphate de sodium dibasique dans un rapport défini, avec un pH situé entre environ 5,15 et 5,53 ;

le positionnement d'échantillons biologiques respectifs d'un récipient respectif ou le récipient avec l'échantillon biologique dans une sonde RMN d'un spectromètre RMN ; et
l'obtention d'un signal RMN pour générer le spectre RMN du proton pour déterminer le niveau de TMAO, dans lequel la détermination électronique est effectuée par l'application de fonctions d'ajustement de courbe au signal RMN associé au pic de TMAO pour déterminer un premier niveau de TMAO, en soustrayant ensuite une concentration connue de l'étalon de TMAO qui a été ajouté à l'échantillon biologique pour générer un niveau de TMAO particulier au patient.

11. Procédé selon l'une quelconque des revendications précédentes, comprenant en outre la génération d'une sortie du niveau de TMAO déterminé avec une indication à savoir si le niveau déterminé est considéré comme élevé, et/ou avec un indice visuel indiquant un continuum de risque allant de faible à élevé ou d'élevé à faible et pour indiquer si un sujet est à risque d'une complication de maladie cardiovasculaire athérosclèreuse, et dans lequel un sujet dont le TMAO est au-dessus d'une valeur associée à un niveau d'un percentile supérieur défini d'une population de référence est à risque de subir une complication de maladie cardiovasculaire athérosclèreuse.

12. Programme informatique pour l'évaluation d'un spectre RMN du proton d'un échantillon biologique *in vitro*, le programme informatique comprenant :

un support de stockage non transitoire lisible par un ordinateur avec un code programme lisible par un ordinateur incorporé dans le support, le code de programme lisible par un ordinateur amenant un processeur à réaliser le procédé selon l'une quelconque des revendications précédentes.

13. Système d'analyse comprenant :

au moins un spectromètre RMN pour l'acquisition d'au moins un spectre RMN du proton d'un échantillon biologique *in vitro* ; et
au moins un processeur en communication avec le au moins un spectromètre RMN, le au moins un processeur étant configuré pour réaliser le procédé selon l'une quelconque des revendications 1 à 12.

SERUM NMR SPECTRUM WITH EXPANSION FROM 3.2 TO 3.4 ppm TO SHOW TMAO PEAK

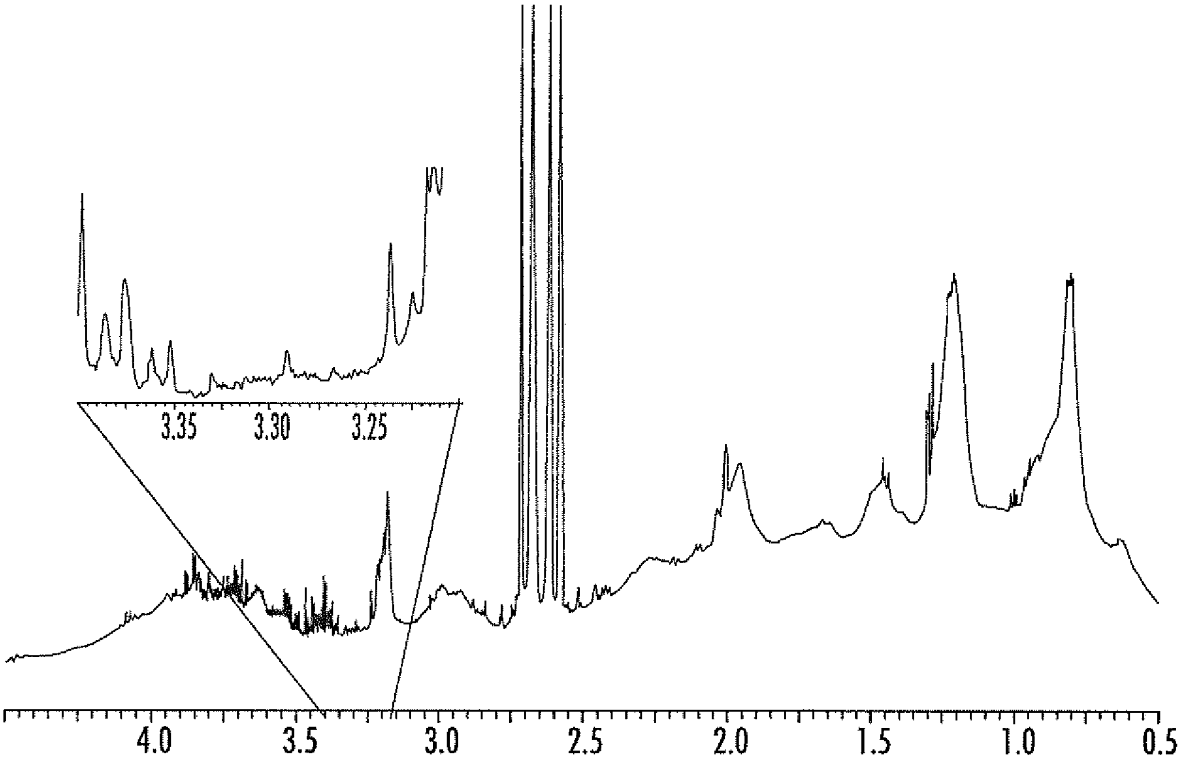


FIG. 1A

URINE NMR SPECTRUM WITH EXPANSION FROM 3.1 TO 3.4 ppm TO SHOW TMAO PEAK

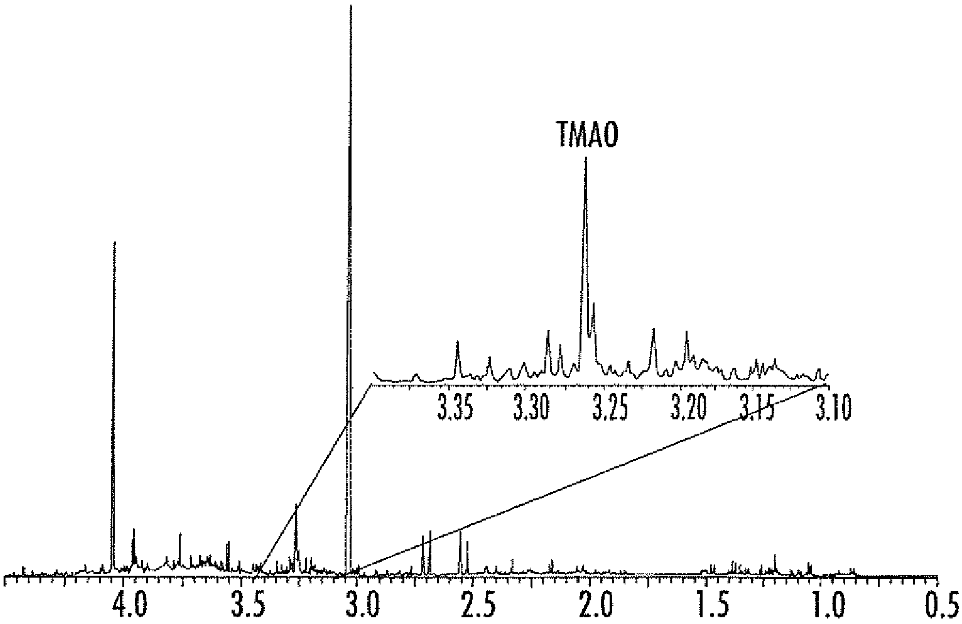


FIG. 1B

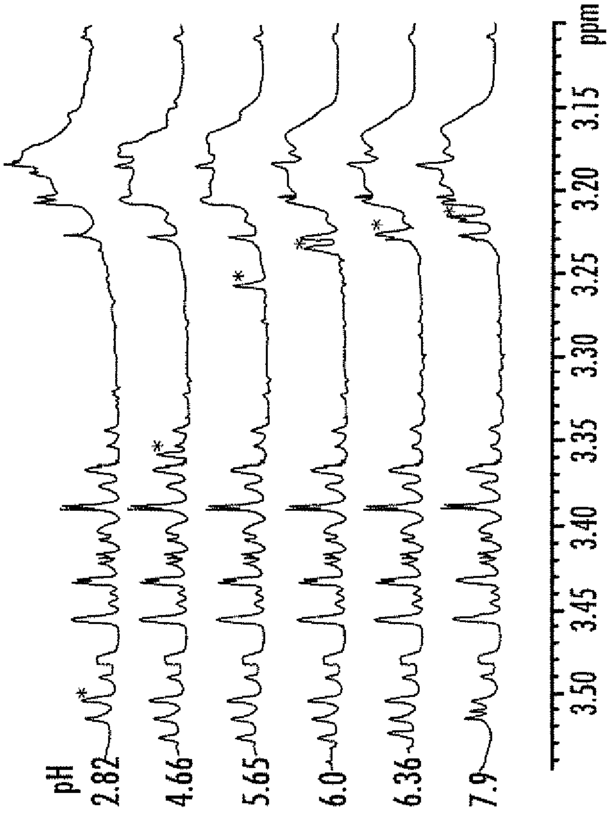
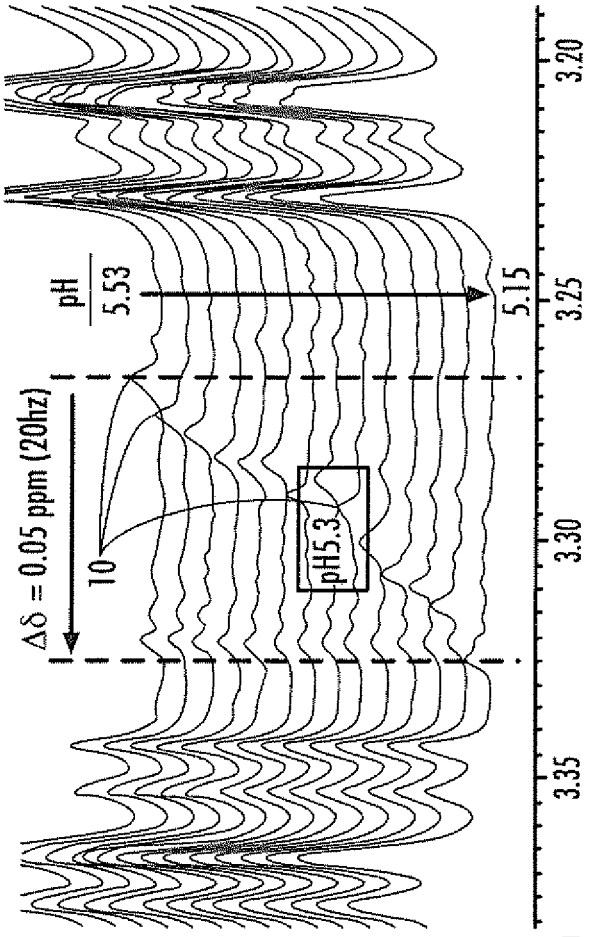


FIG. 2A



pH ASSAY FROM 2.82 TO 7.9 AND 5.15 TO 5.53

FIG. 2B

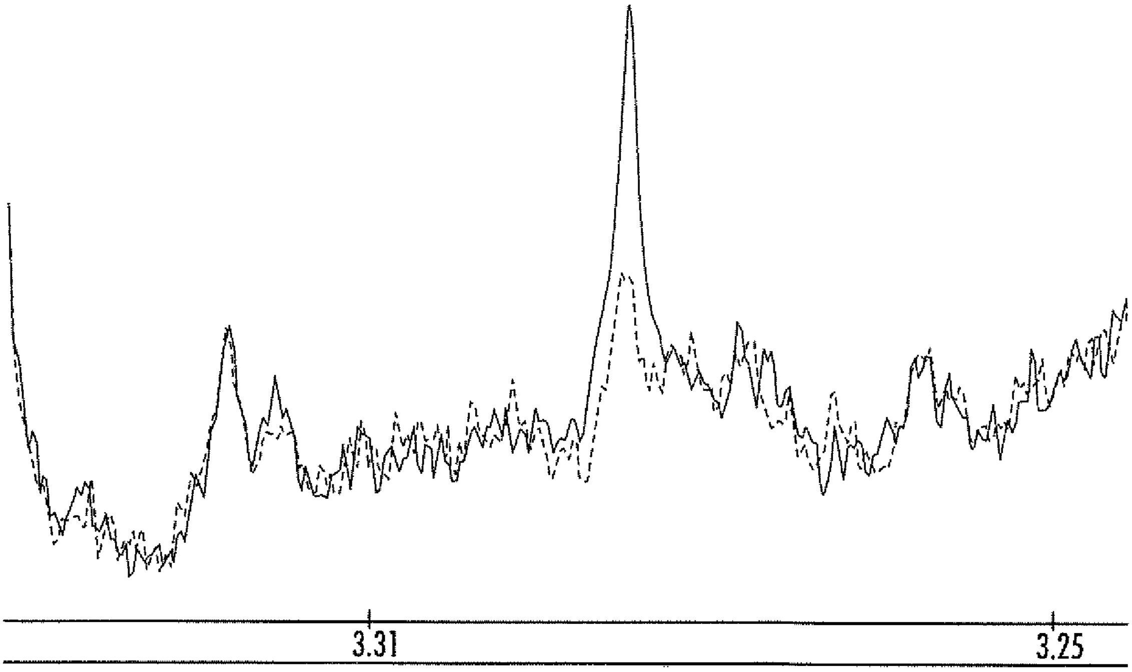

3.31
3.25

FIG. 2C

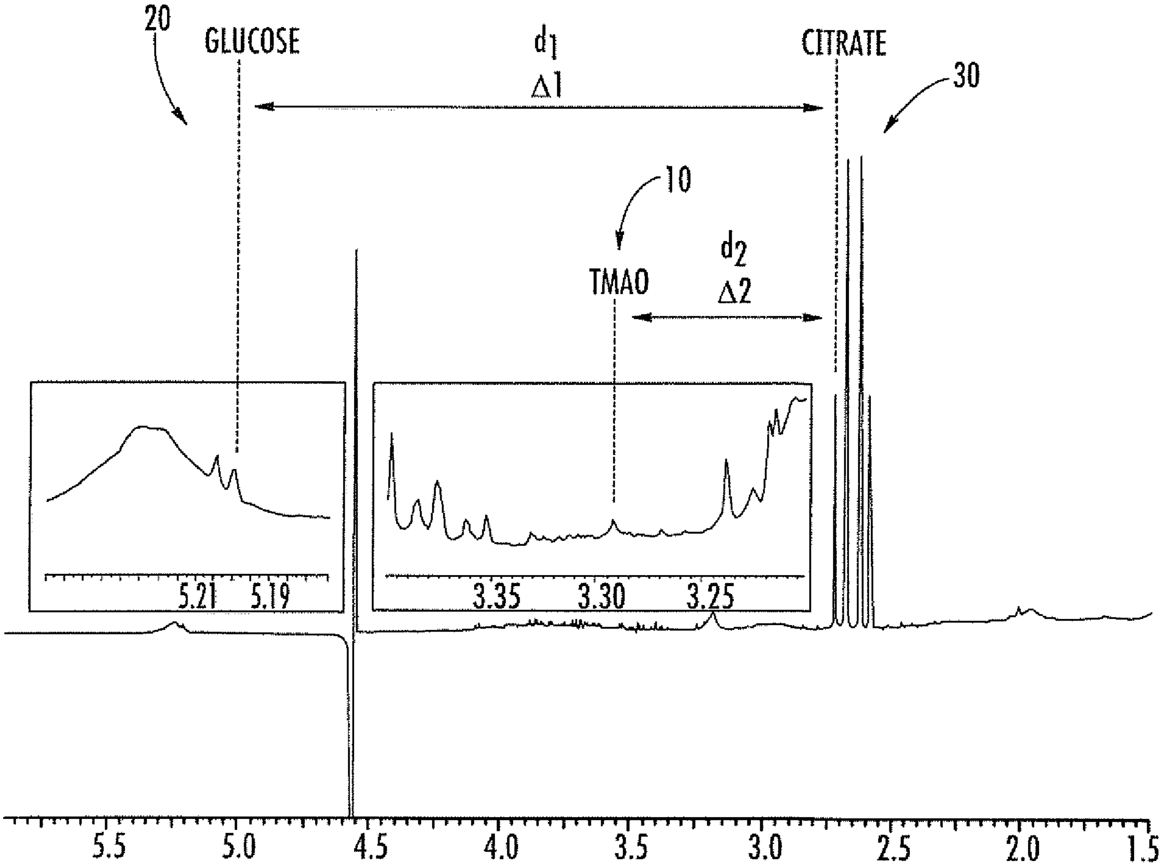

20
GLUCOSE
d1
Δ1
CITRATE
30
10
TMAO
d2
Δ2
5.21
5.19
3.35
3.30
3.25
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5

FIG. 3A

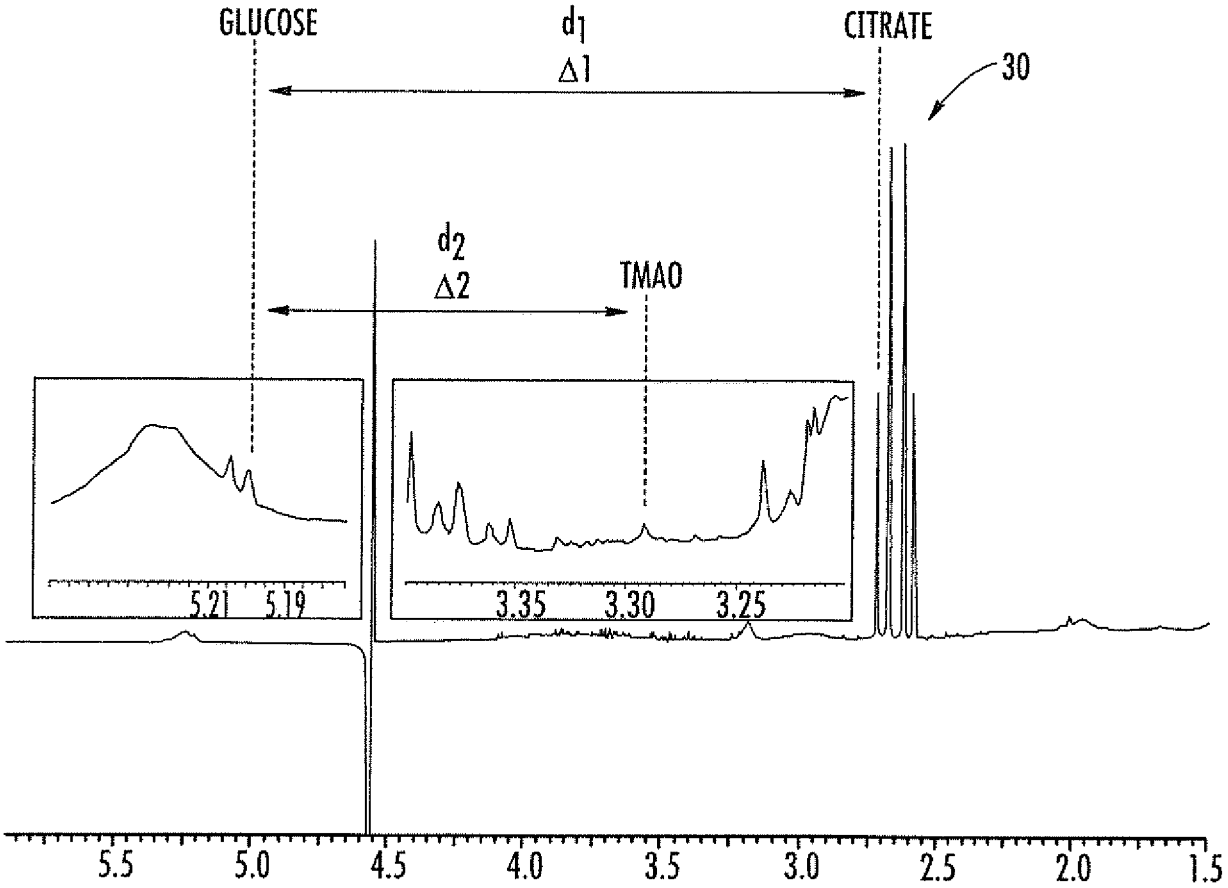

GLUCOSE
d1
Δ1
CITRATE
30
d2
Δ2
TMAO
5.21
5.19
3.35
3.30
3.25
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5

FIG. 3B

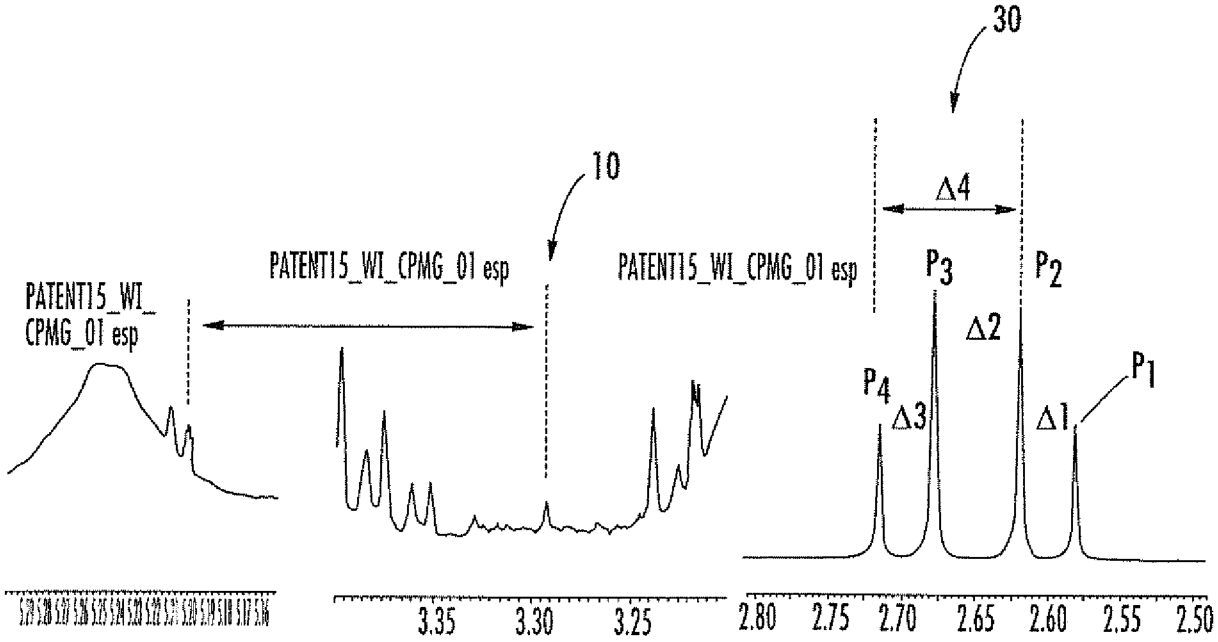

30
10
Δ4
PATENT15_WI_CPMG_01 esp
PATENT15_WI_CPMG_01 esp
PATENT15_WI_
CPMG_01 esp
P3
P2
P4
Δ3
Δ2
Δ1
P1
3.35
3.30
3.25
2.80
2.75
2.70
2.65
2.60
2.55
2.50

FIG. 3C

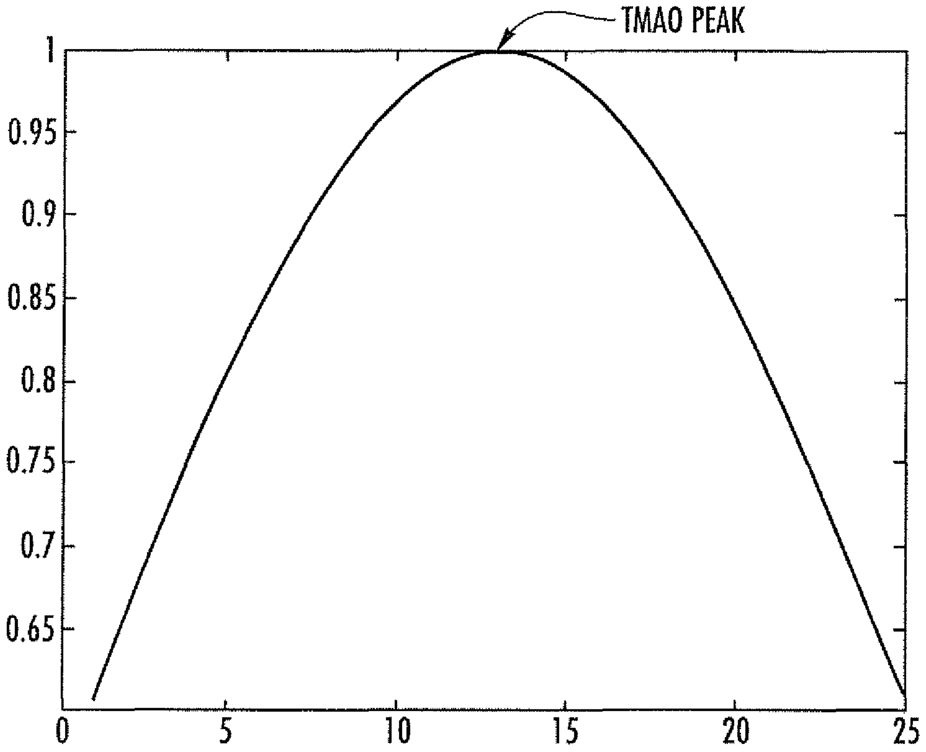

TMAO PEAK
1
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0
5
10
15
20
25

FIG. 3D

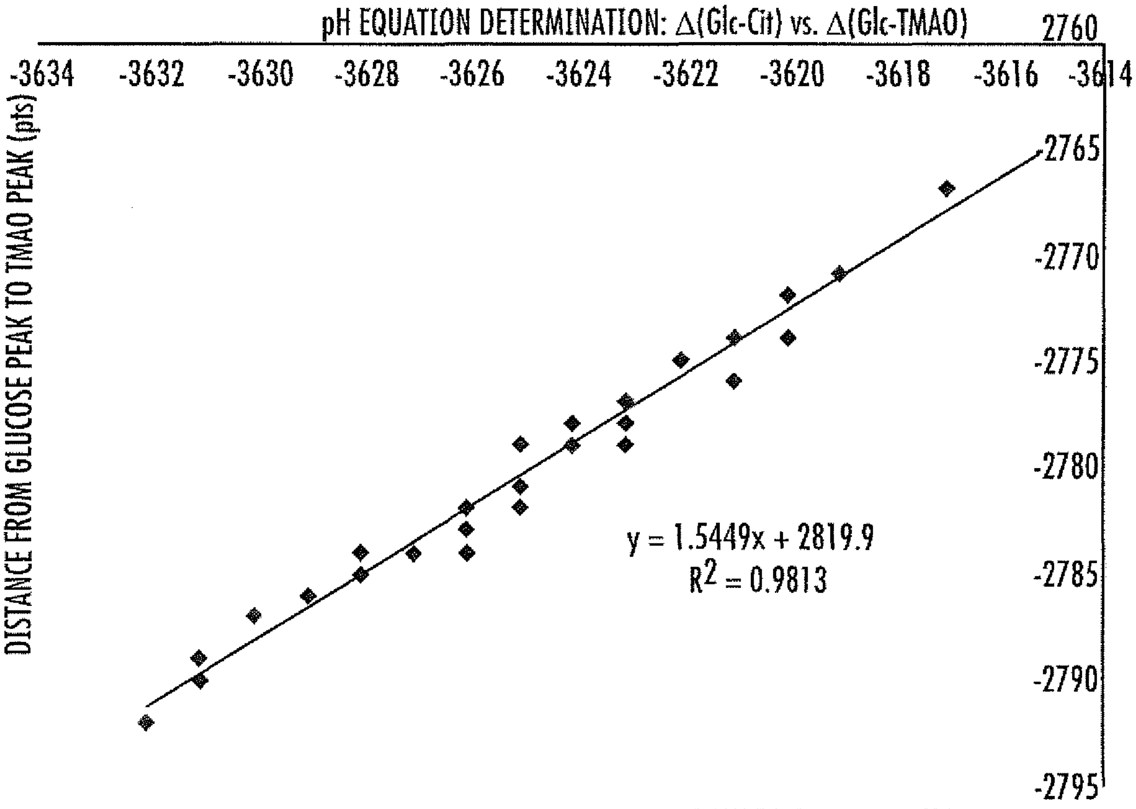

pH EQUATION DETERMINATION: Δ(Glc-Cit) vs. Δ(Glc-TMAO)
-3634
-3632
-3630
-3628
-3626
-3624
-3622
-3620
-3618
-3616
-3614
2760
-2765
-2770
-2775
-2780
-2785
-2790
-2795
DISTANCE FROM GLUCOSE PEAK TO TMAO PEAK (pts)
y = 1.5449x + 2819.9
R2 = 0.9813
DISTANCE FROM ANOMERIC GLUCOSE PEAK TO CITRATE PEAK (pts)

FIG. 4A

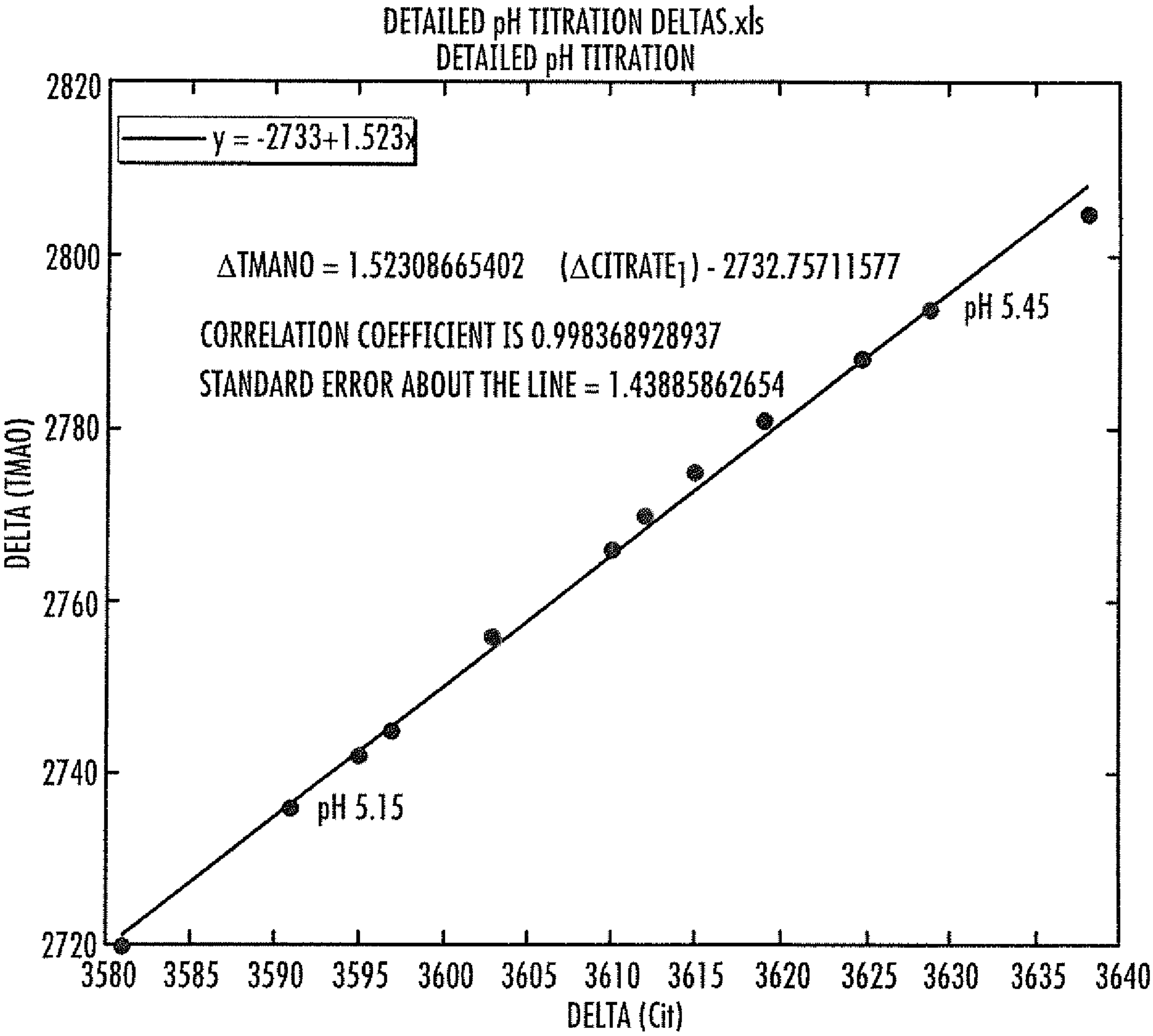
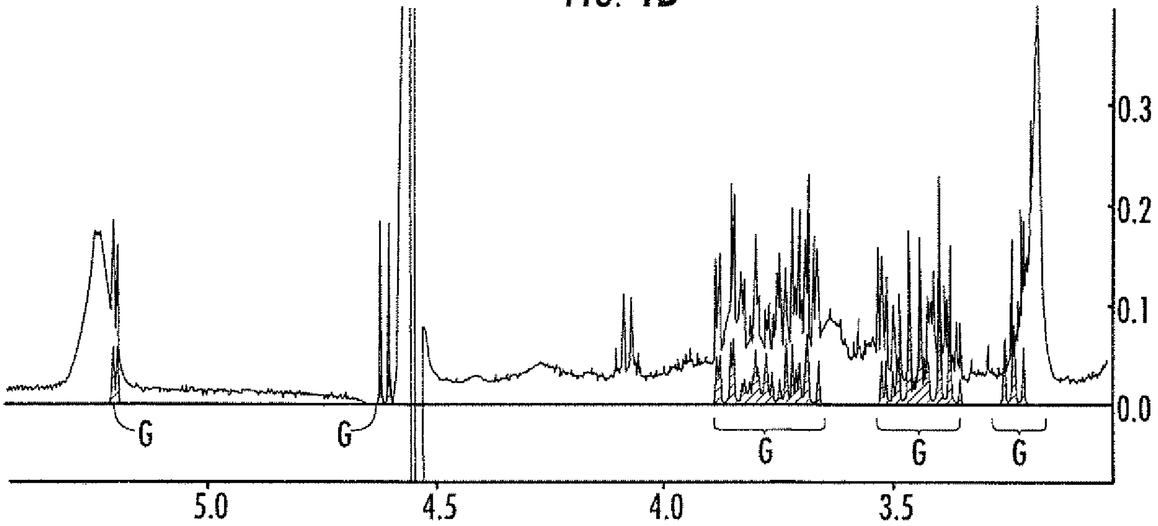


FIG. 4B



SERUM SAMPLE WITH GLUCOSE REFERENCE SPECTRUM BELOW GLUCOSE MULTIPLET AT 5.2 ppm, THERE ARE MULTIPLETS CENTERED AT 4.6, 3.9, 3.8, 3.7, 3.5, 3.4 AND 3.2 ppm

FIG. 5

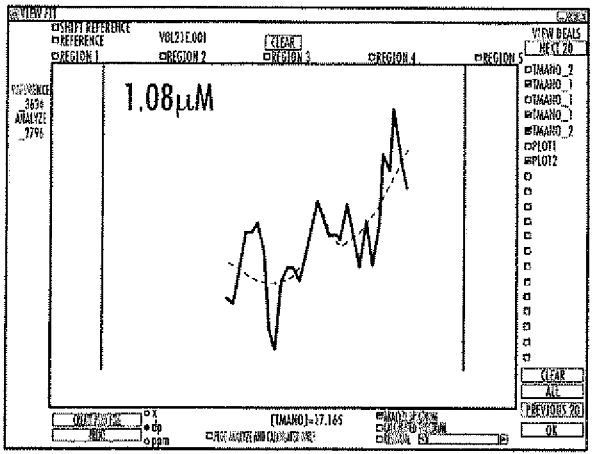


FIG. 6A

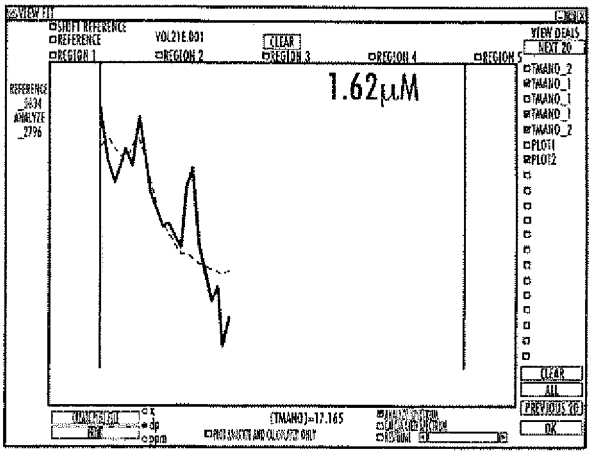


FIG. 6B

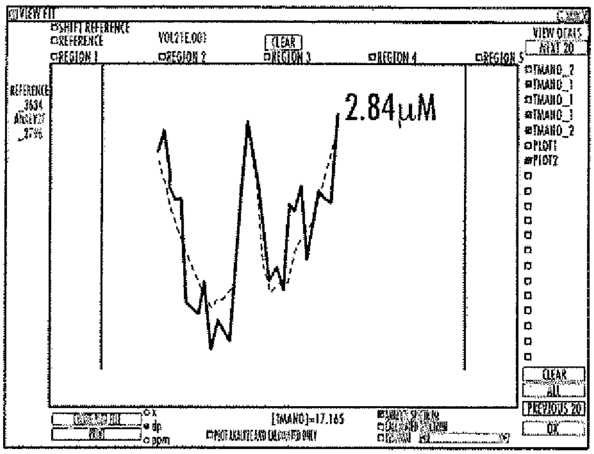


FIG. 6C

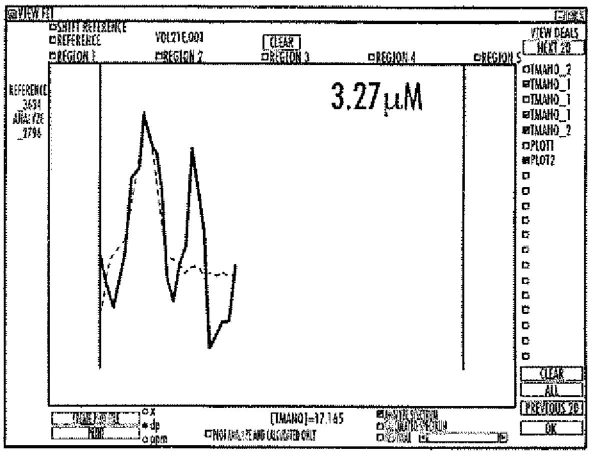


FIG. 6D

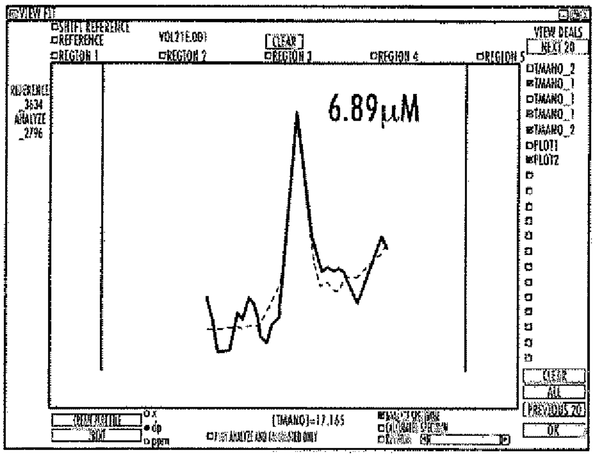


FIG. 6E

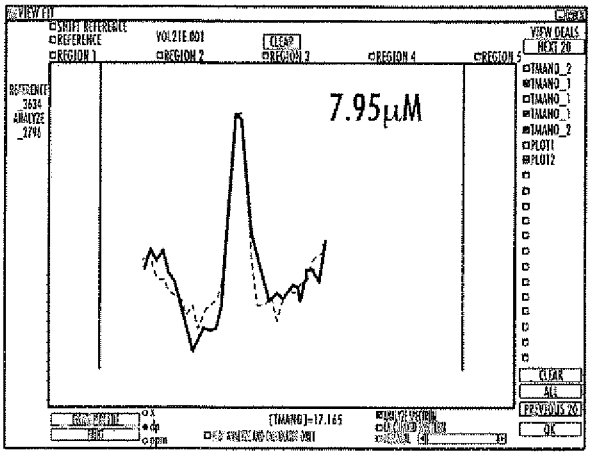
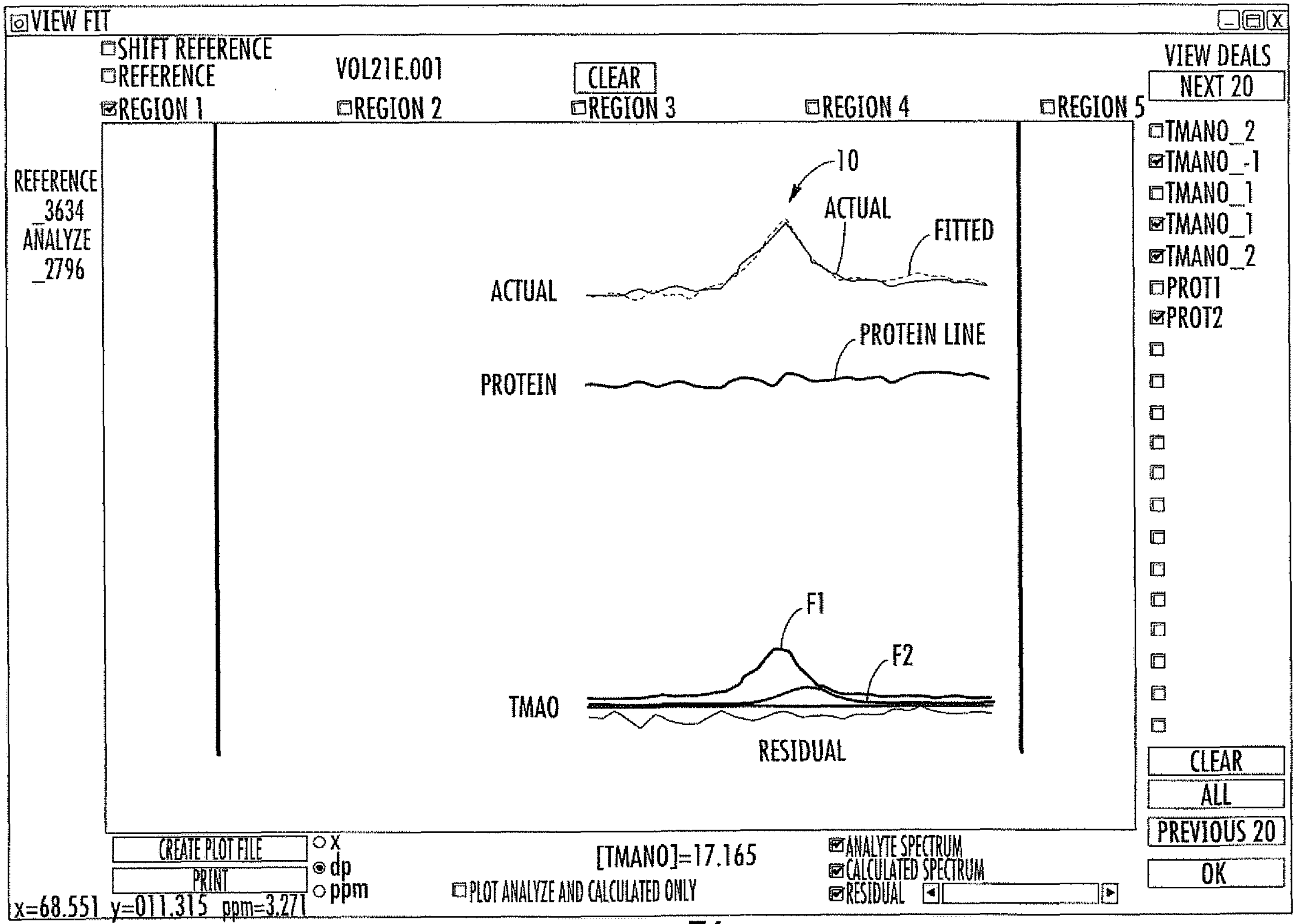


FIG. 6F

FITTING OF PATIENT TMAO PEAKS WITH CONCENTRATIONS IN THE 1ST (A-B), 2ND (C-D) AND 3RD (E-F) QUARTILES.



☐ VIEW FIT

☐ SHIFT REFERENCE
☐ REFERENCE
☑ REGION 1
☐ REGION 2
☐ REGION 3
☐ REGION 4
☐ REGION 5

VOL21E.001

REFERENCE
_3634
ANALYZE
_2796

VIEW DEALS

☐ TMANO_2
☑ TMANO_-1
☐ TMANO_1
☑ TMANO_1
☑ TMANO_2
☐ PROT1
☑ PROT2

○ x
● dp
○ ppm

[TMANO]=17.165

☐ PLOT ANALYZE AND CALCULATED ONLY

☑ ANALYTE SPECTRUM
☑ CALCULATED SPECTRUM
☑ RESIDUAL

x=68.551 y=011.315 ppm=3.271

FIG. 7A

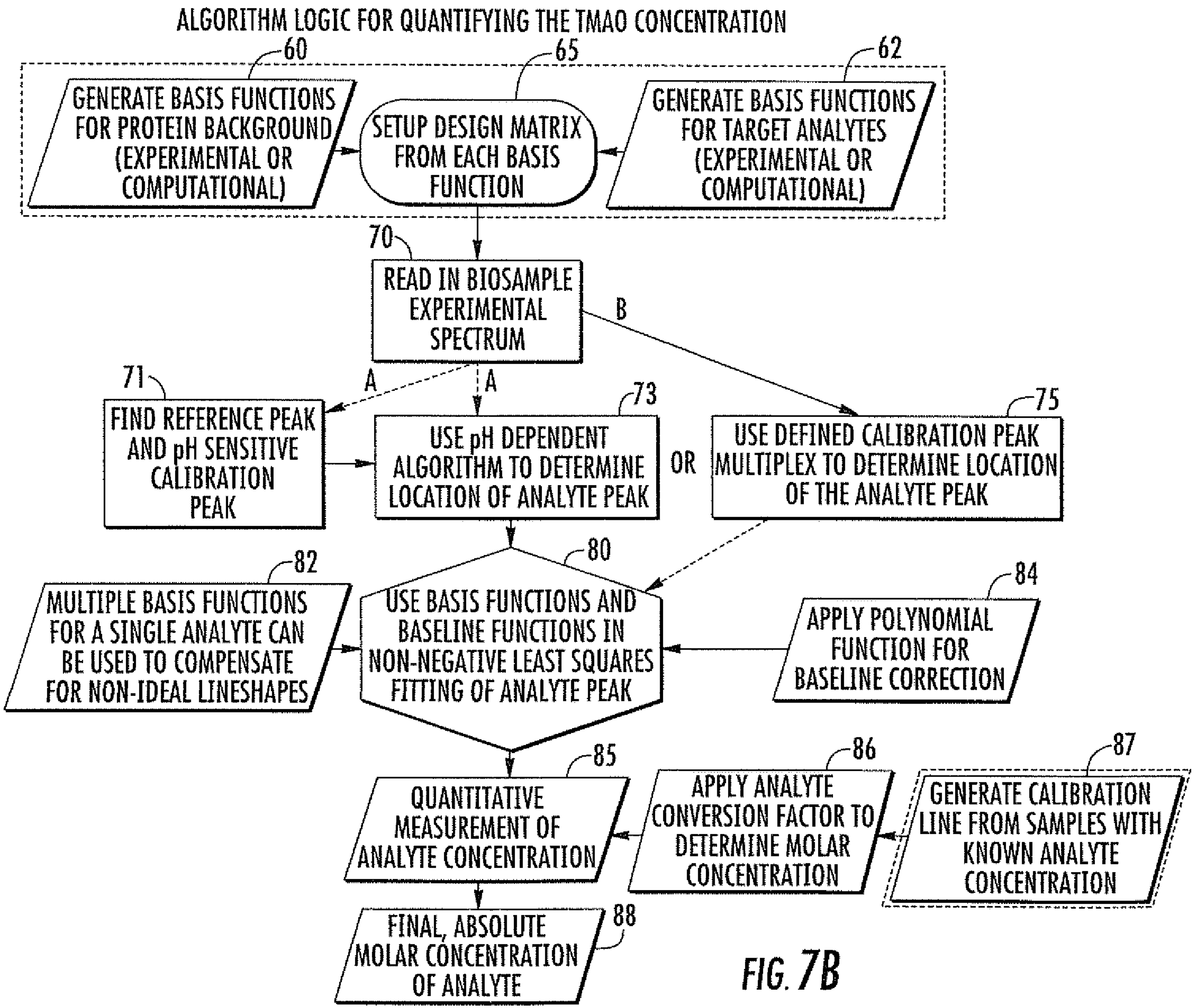

ALGORITHM LOGIC FOR QUANTIFYING THE TMAO CONCENTRATION
60
GENERATE BASIS FUNCTIONS FOR PROTEIN BACKGROUND (EXPERIMENTAL OR COMPUTATIONAL)
65
SETUP DESIGN MATRIX FROM EACH BASIS FUNCTION
62
GENERATE BASIS FUNCTIONS FOR TARGET ANALYTES (EXPERIMENTAL OR COMPUTATIONAL)
70
READ IN BIOSAMPLE EXPERIMENTAL SPECTRUM
B
A
A
71
FIND REFERENCE PEAK AND pH SENSITIVE CALIBRATION PEAK
73
USE pH DEPENDENT ALGORITHM TO DETERMINE LOCATION OF ANALYTE PEAK
OR
75
USE DEFINED CALIBRATION PEAK MULTIPLEX TO DETERMINE LOCATION OF THE ANALYTE PEAK
82
MULTIPLE BASIS FUNCTIONS FOR A SINGLE ANALYTE CAN BE USED TO COMPENSATE FOR NON-IDEAL LINESHAPES
80
USE BASIS FUNCTIONS AND BASELINE FUNCTIONS IN NON-NEGATIVE LEAST SQUARES FITTING OF ANALYTE PEAK
84
APPLY POLYNOMIAL FUNCTION FOR BASELINE CORRECTION
85
QUANTITATIVE MEASUREMENT OF ANALYTE CONCENTRATION
86
APPLY ANALYTE CONVERSION FACTOR TO DETERMINE MOLAR CONCENTRATION
87
GENERATE CALIBRATION LINE FROM SAMPLES WITH KNOWN ANALYTE CONCENTRATION
88
FINAL, ABSOLUTE MOLAR CONCENTRATION OF ANALYTE

FIG. 7B

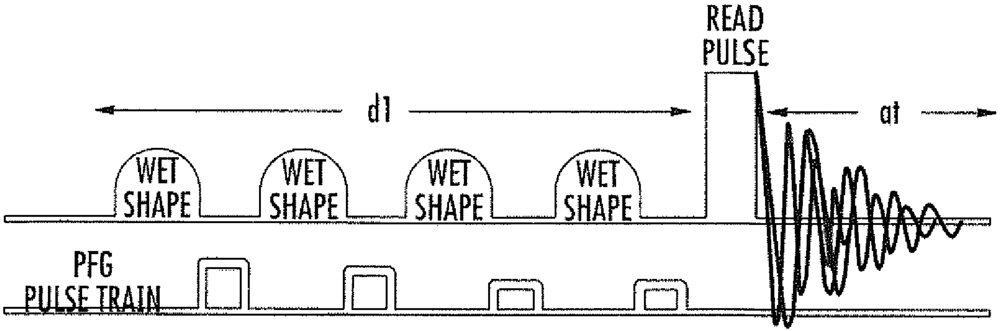


FIG. 8

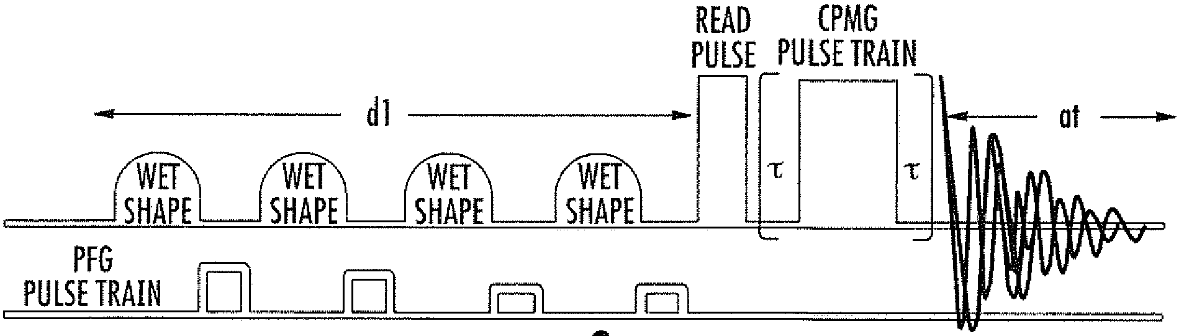


FIG. 9

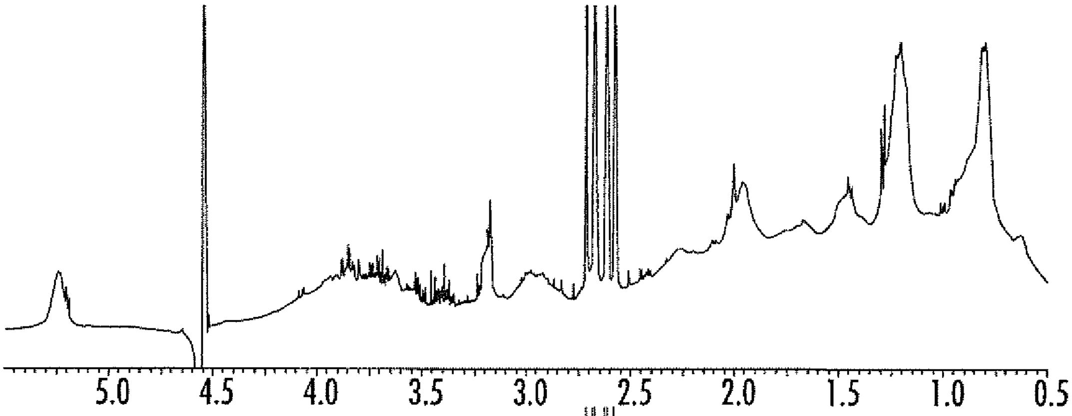


FIG. 10A

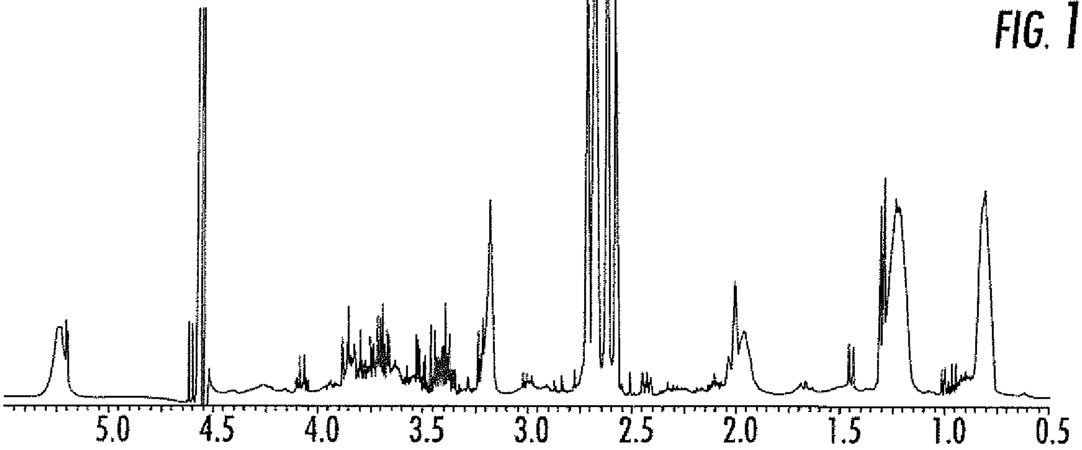
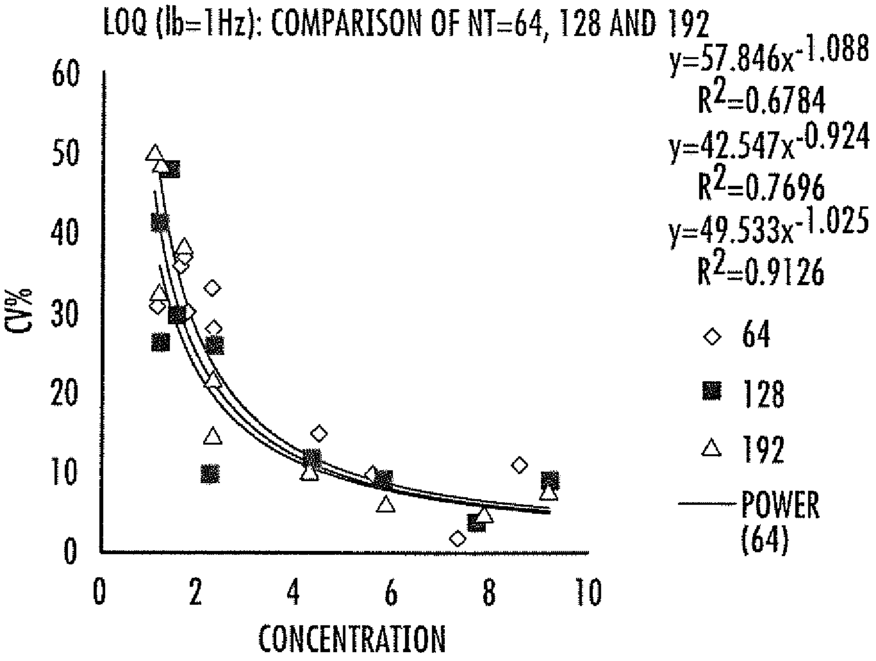


FIG. 10B



ASSAY PERFORMANCE (%CV) WITH SPIKED DIALYZED SERUM WITH 64, 128 AND 192 SCANS.

FIG. 11

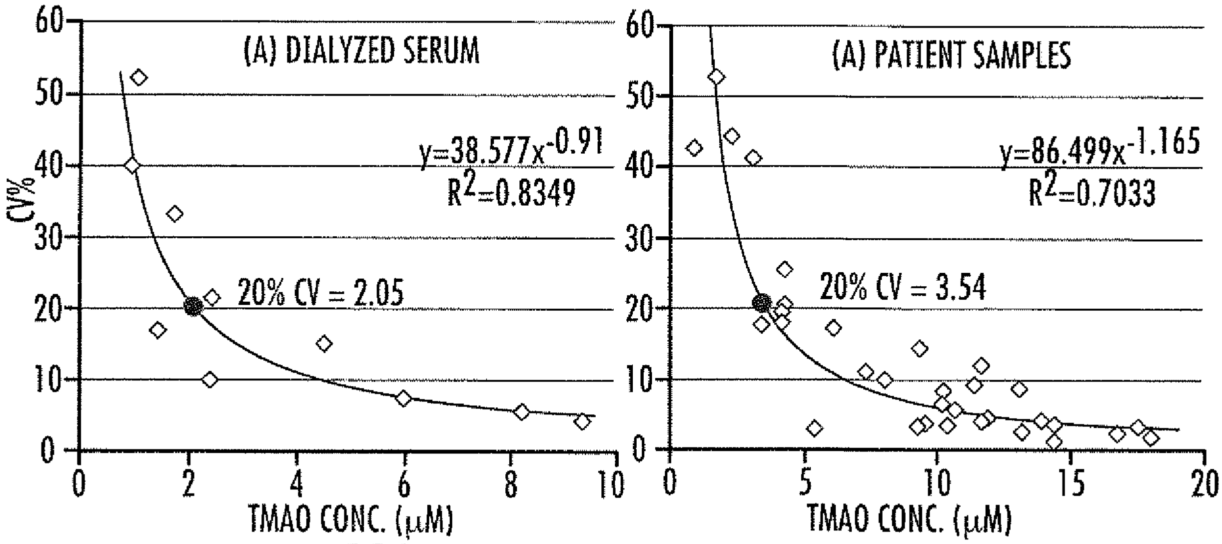


FIG. 12A

FIG. 12B

FIGURE 12. ASSAY PERFORMANCE (%CV) WITH (A) SPIKED DIALYZED SERUM AND (B) PATIENT SAMPLES.

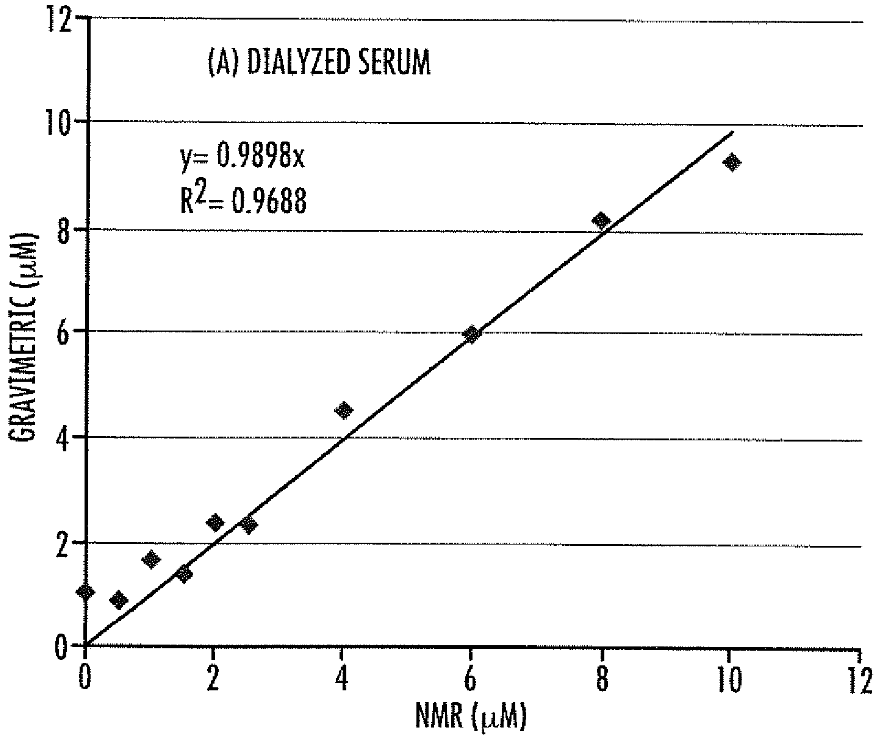


FIG. 13A

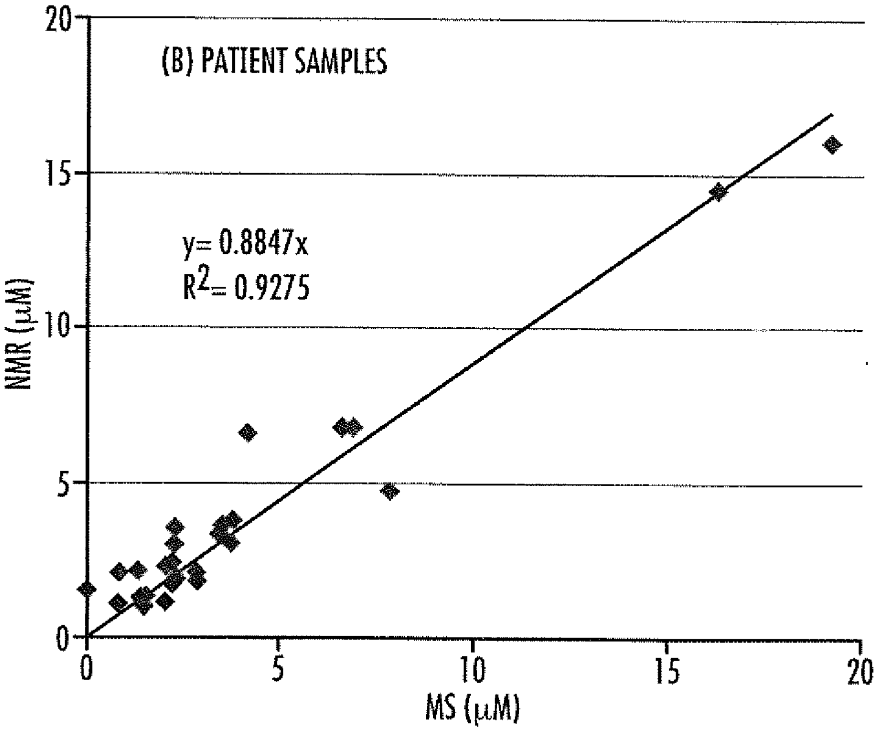


FIG. 13B

FIGURE 13. COMPARISON OF NMR DETERMINATION OF TMAO (A) IN DIALYZED SERUM WITH GRAVIMENTRALLY DETERMINED TMA AND (B) IN PATIENT SAMPLES WITH MS DETERMINED TMAO

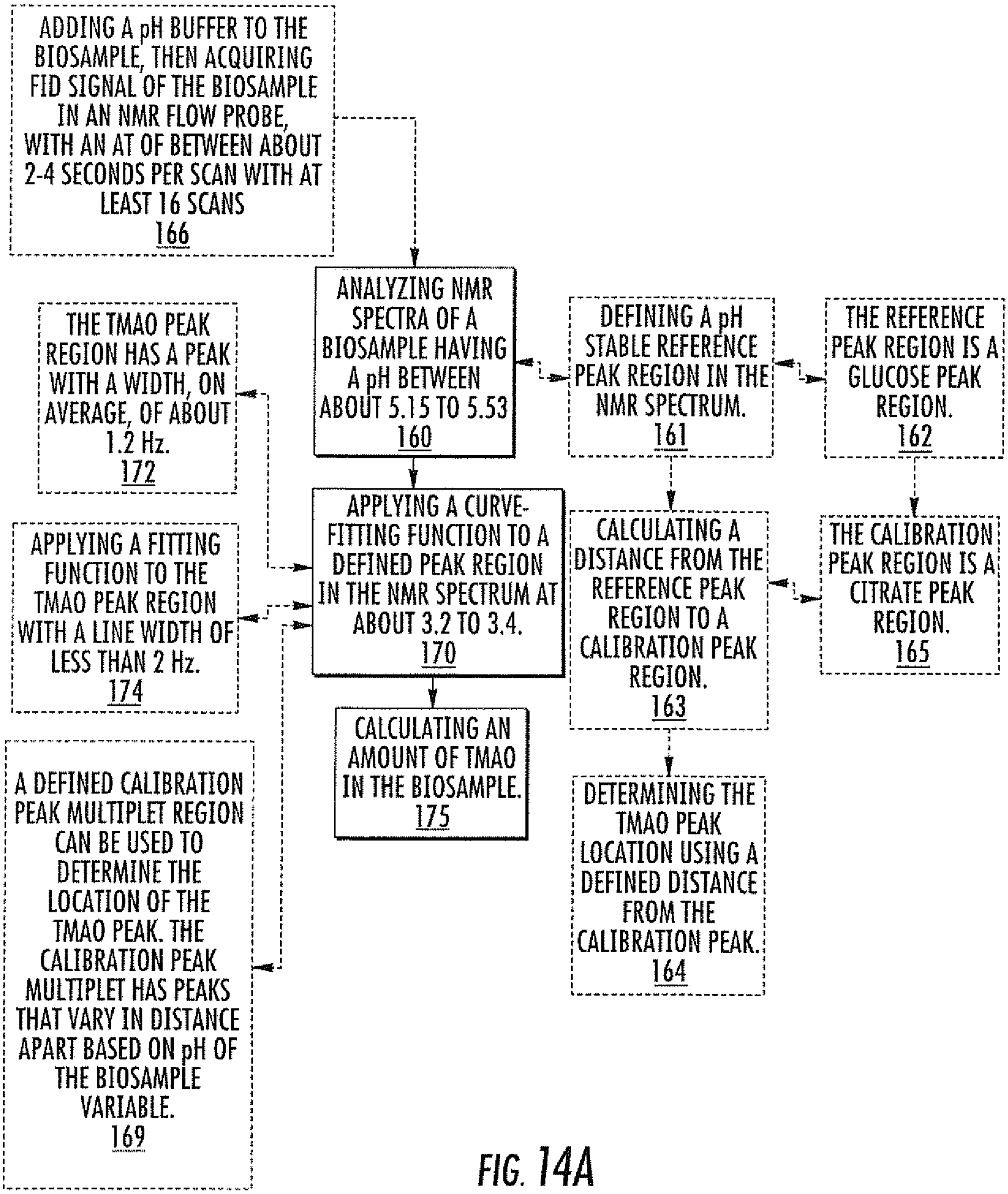

ADDING A pH BUFFER TO THE BIOSAMPLE, THEN ACQUIRING FID SIGNAL OF THE BIOSAMPLE IN AN NMR FLOW PROBE, WITH AN AT OF BETWEEN ABOUT 2-4 SECONDS PER SCAN WITH AT LEAST 16 SCANS
166
ANALYZING NMR SPECTRA OF A BIOSAMPLE HAVING A pH BETWEEN ABOUT 5.15 TO 5.53
160
DEFINING A pH STABLE REFERENCE PEAK REGION IN THE NMR SPECTRUM.
161
THE REFERENCE PEAK REGION IS A GLUCOSE PEAK REGION.
162
THE TMAO PEAK REGION HAS A PEAK WITH A WIDTH, ON AVERAGE, OF ABOUT 1.2 Hz.
172
APPLYING A CURVE-FITTING FUNCTION TO A DEFINED PEAK REGION IN THE NMR SPECTRUM AT ABOUT 3.2 TO 3.4.
170
CALCULATING A DISTANCE FROM THE REFERENCE PEAK REGION TO A CALIBRATION PEAK REGION.
163
THE CALIBRATION PEAK REGION IS A CITRATE PEAK REGION.
165
APPLYING A FITTING FUNCTION TO THE TMAO PEAK REGION WITH A LINE WIDTH OF LESS THAN 2 Hz.
174
CALCULATING AN AMOUNT OF TMAO IN THE BIOSAMPLE.
175
DETERMINING THE TMAO PEAK LOCATION USING A DEFINED DISTANCE FROM THE CALIBRATION PEAK.
164
A DEFINED CALIBRATION PEAK MULTIPLET REGION CAN BE USED TO DETERMINE THE LOCATION OF THE TMAO PEAK. THE CALIBRATION PEAK MULTIPLET HAS PEAKS THAT VARY IN DISTANCE APART BASED ON pH OF THE BIOSAMPLE VARIABLE.
169

FIG. 14A

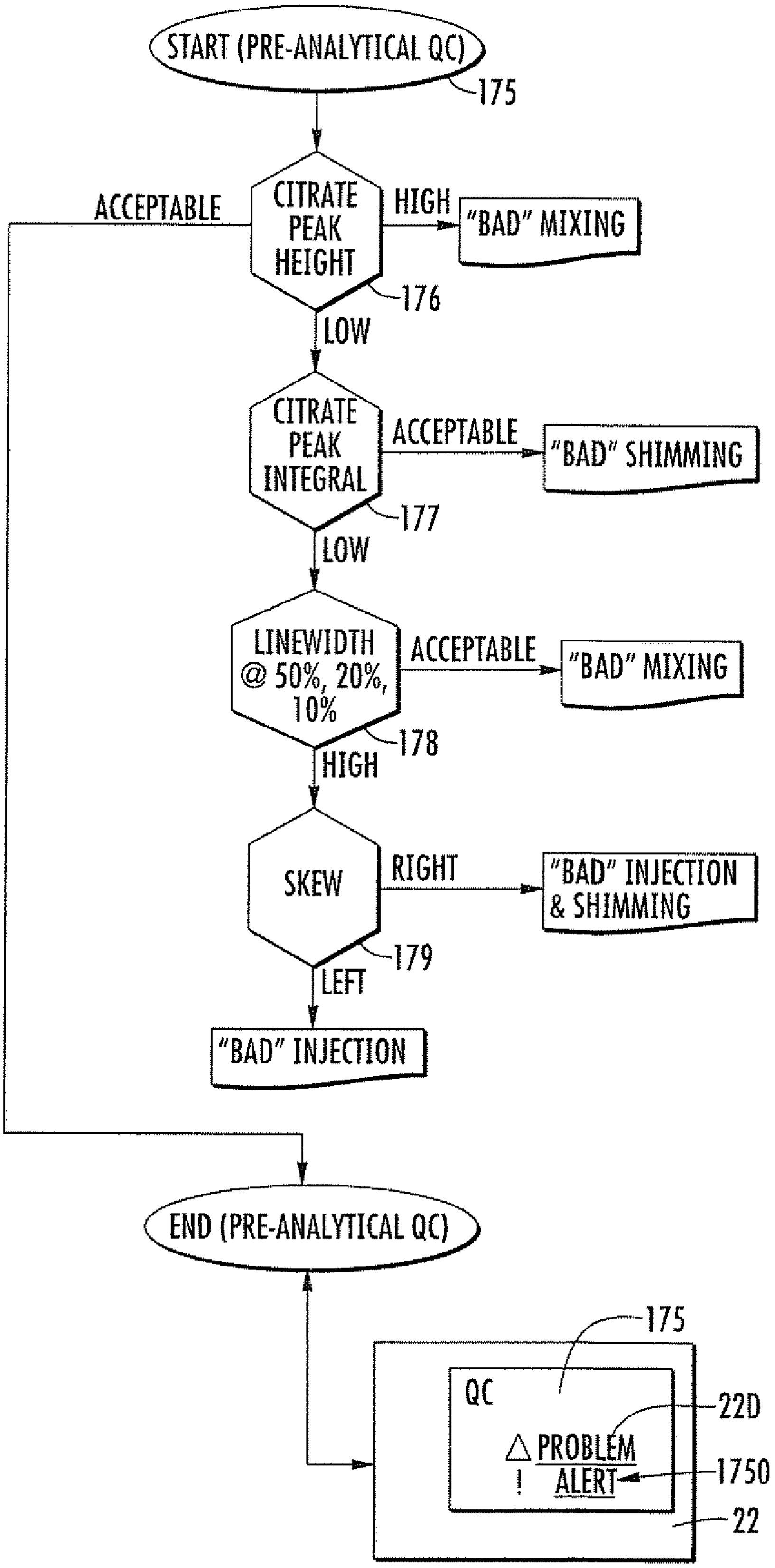

START (PRE-ANALYTICAL QC)
175
CITRATE PEAK HEIGHT
176
ACCEPTABLE
HIGH
"BAD" MIXING
LOW
CITRATE PEAK INTEGRAL
177
ACCEPTABLE
"BAD" SHIMMING
LOW
LINEWIDTH @ 50%, 20%, 10%
178
ACCEPTABLE
"BAD" MIXING
HIGH
SKEW
179
RIGHT
"BAD" INJECTION & SHIMMING
LEFT
"BAD" INJECTION
END (PRE-ANALYTICAL QC)
175
QC
22D
PROBLEM ALERT
1750
22

FIG. 14B

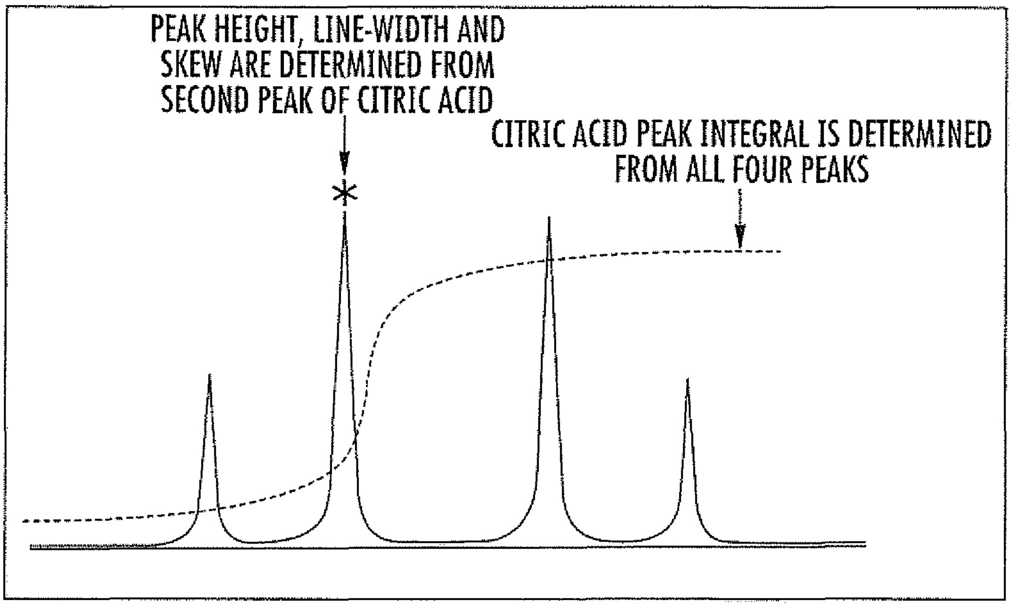


FIG. 14C

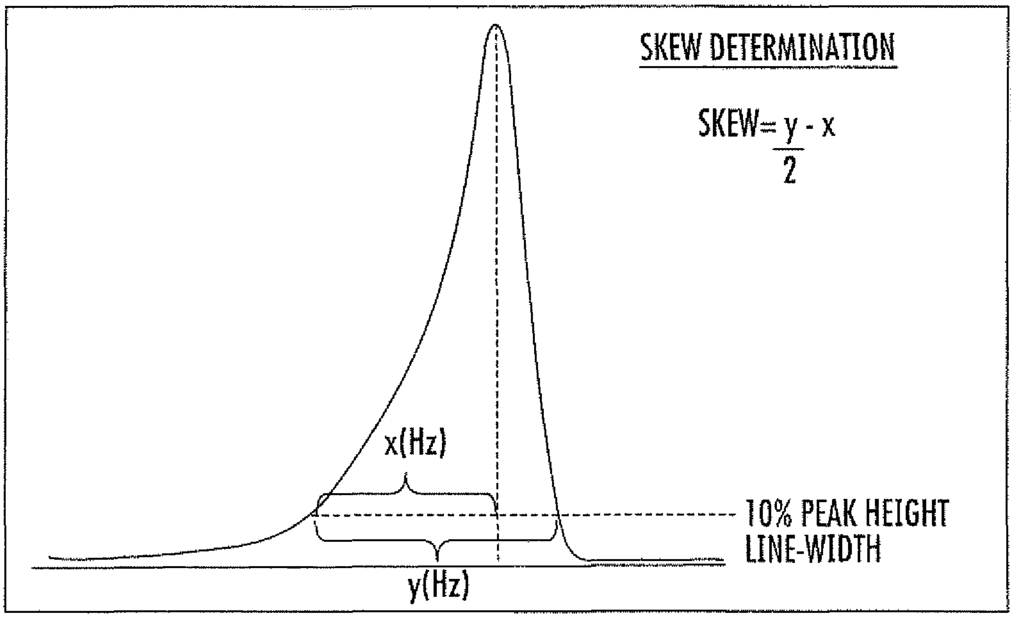


FIG. 14D

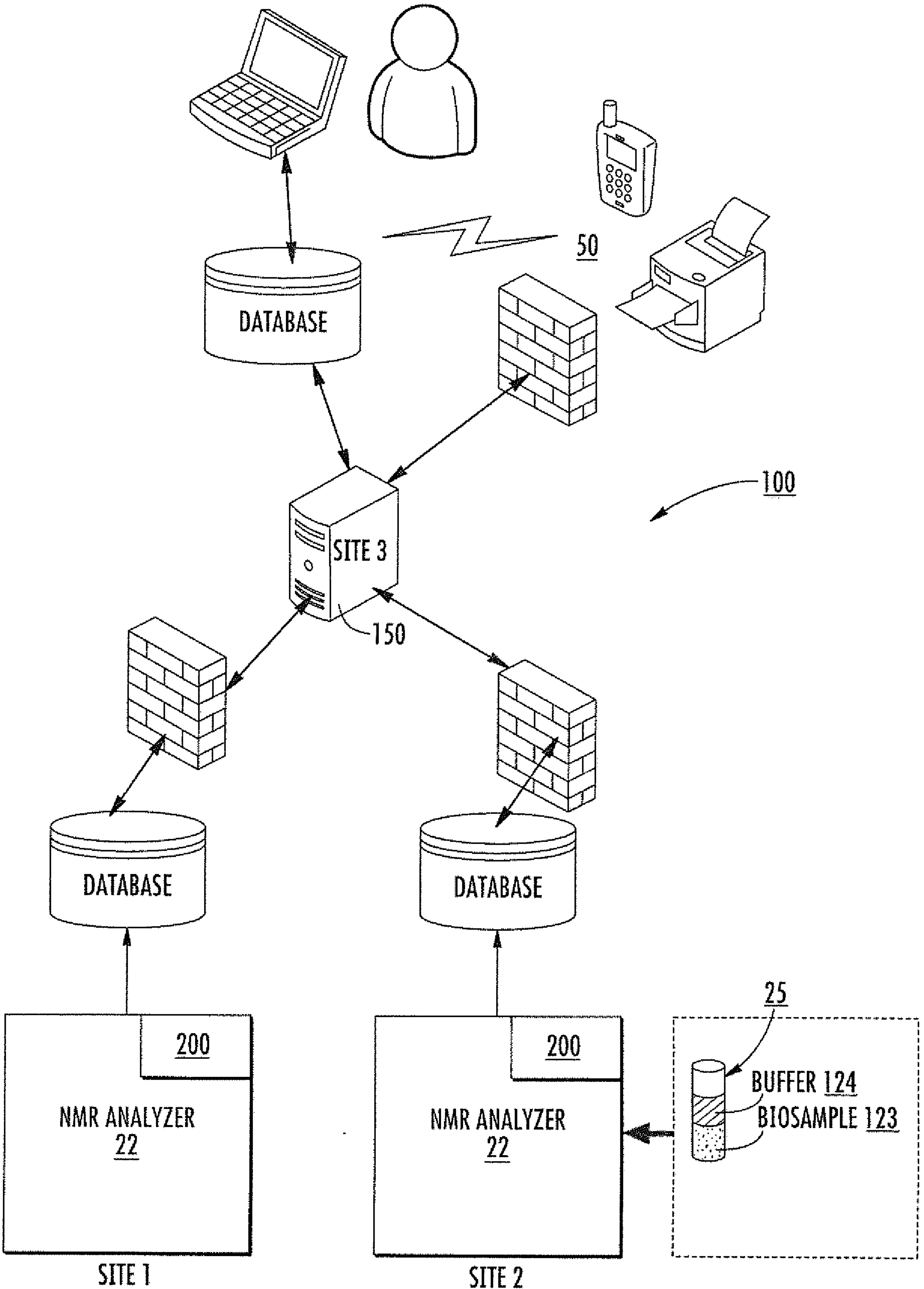


FIG. 15A

FIG. 15B

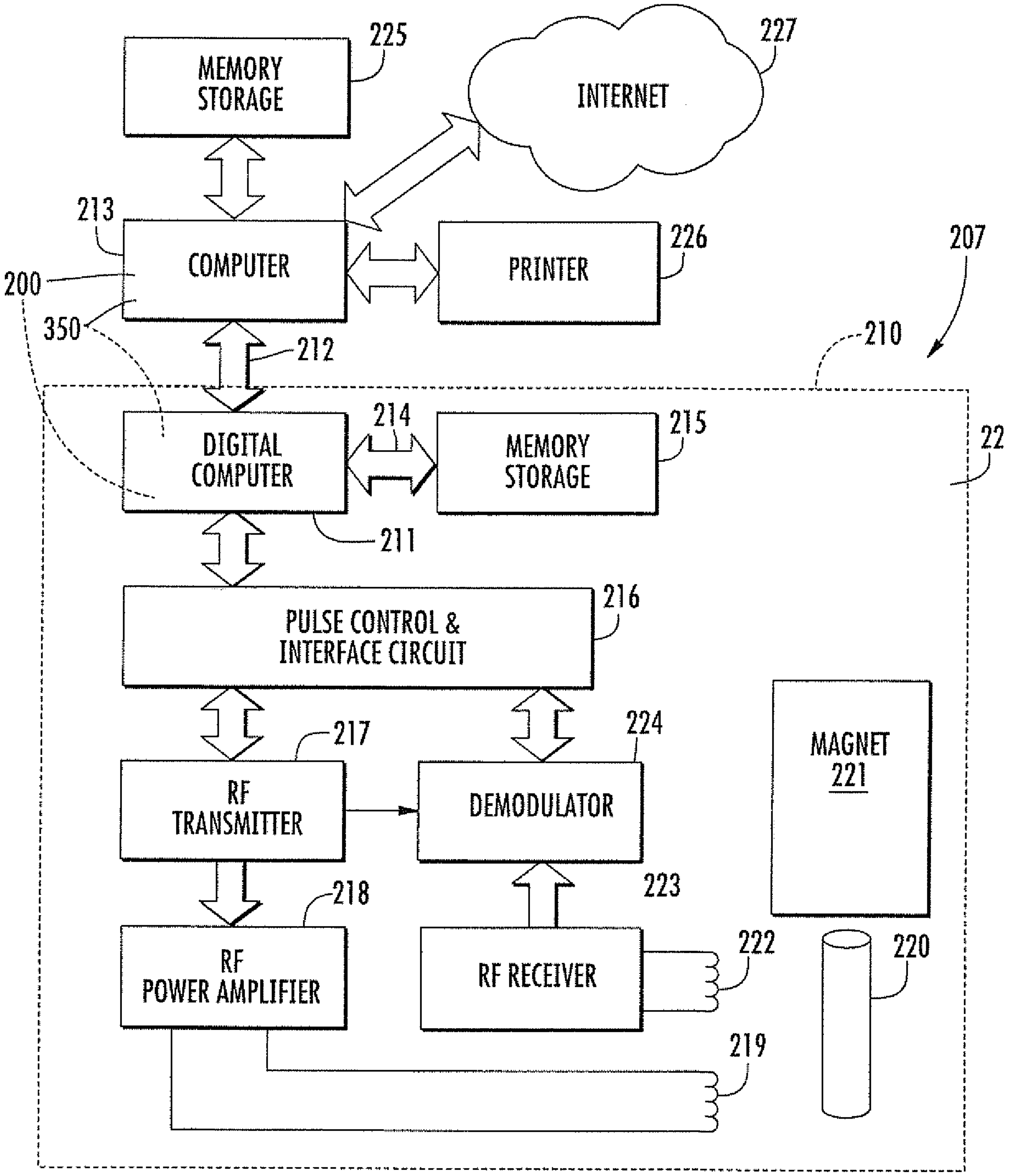

MEMORY STORAGE
225
INTERNET
227
213
COMPUTER
PRINTER
226
207
200
350
212
210
DIGITAL COMPUTER
214
MEMORY STORAGE
215
22
211
PULSE CONTROL & INTERFACE CIRCUIT
216
217
224
MAGNET
221
RF TRANSMITTER
DEMODULATOR
223
218
RF POWER AMPLIFIER
RF RECEIVER
222
220
219

FIG. 16

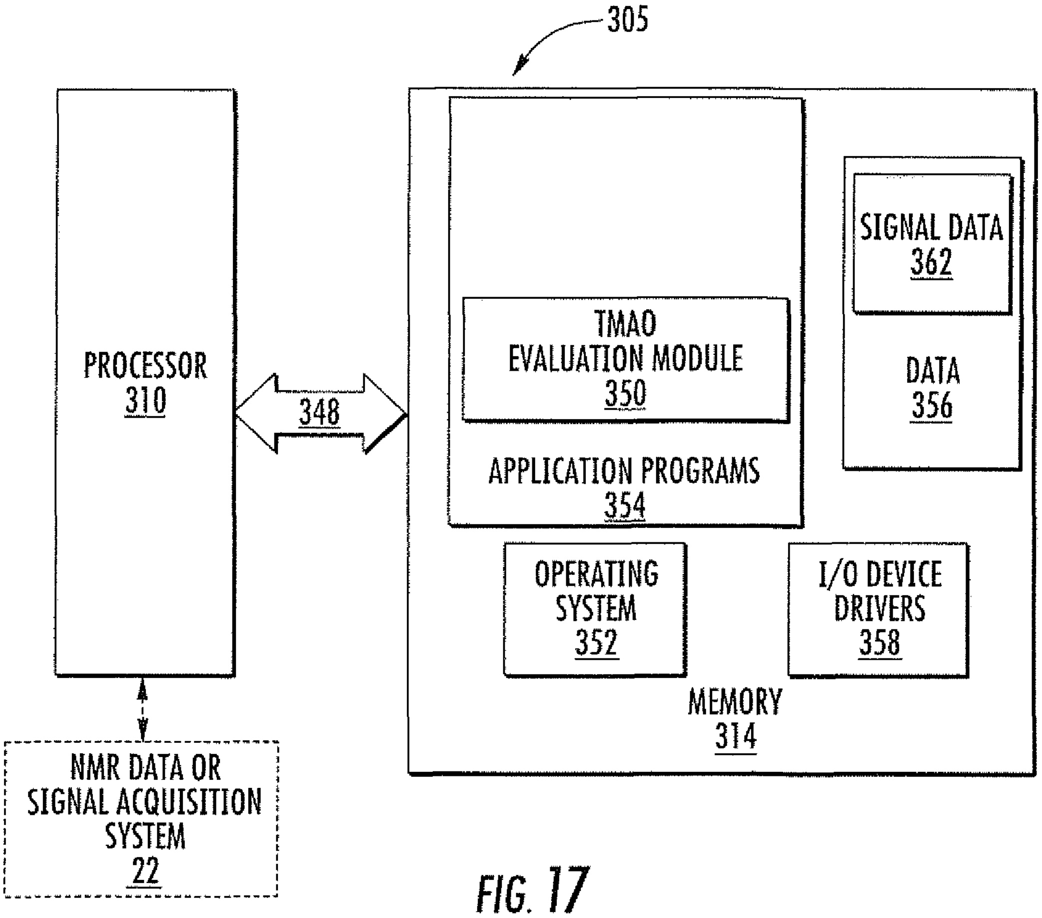

305
PROCESSOR
310
348
TMAO
EVALUATION MODULE
350
APPLICATION PROGRAMS
354
SIGNAL DATA
362
DATA
356
OPERATING
SYSTEM
352
I/O DEVICE
DRIVERS
358
MEMORY
314
NMR DATA OR
SIGNAL ACQUISITION
SYSTEM
22

FIG. 17

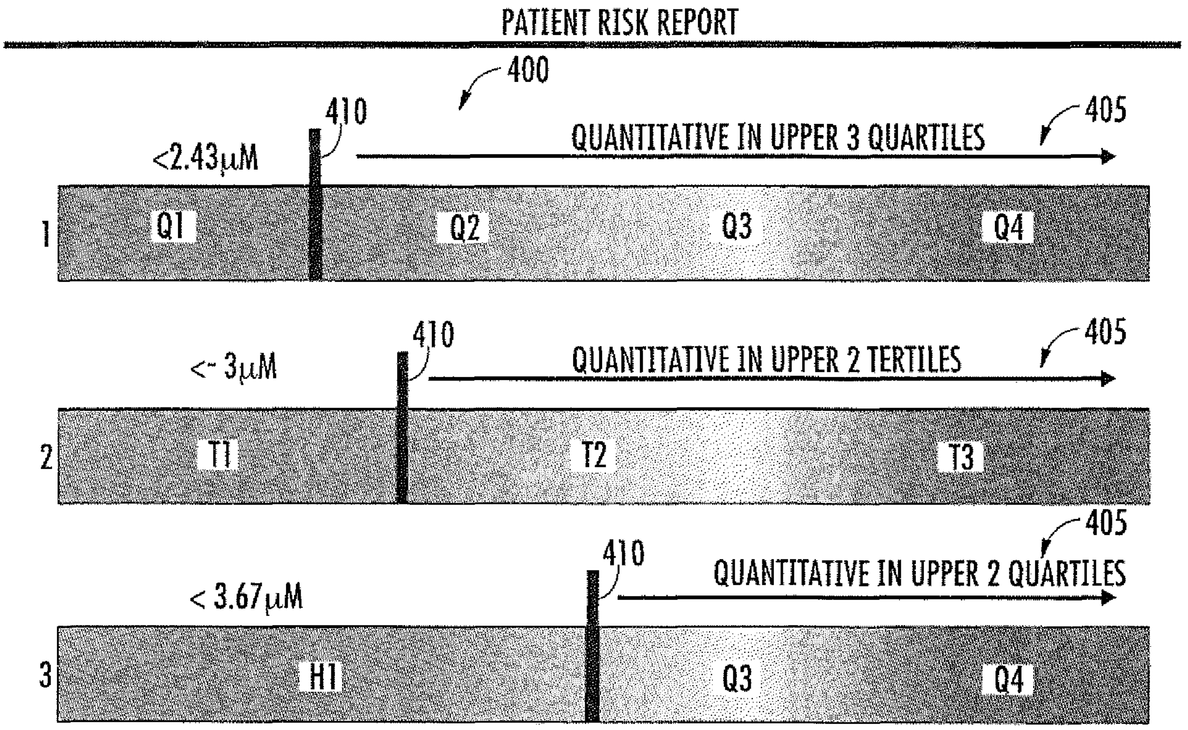

PATIENT RISK REPORT
400
410
405
<2.43μM
QUANTITATIVE IN UPPER 3 QUARTILES
1
Q1
Q2
Q3
Q4
410
405
<~ 3μM
QUANTITATIVE IN UPPER 2 TERTILES
2
T1
T2
T3
410
405
< 3.67μM
QUANTITATIVE IN UPPER 2 QUARTILES
3
H1
Q3
Q4

FIG. 18

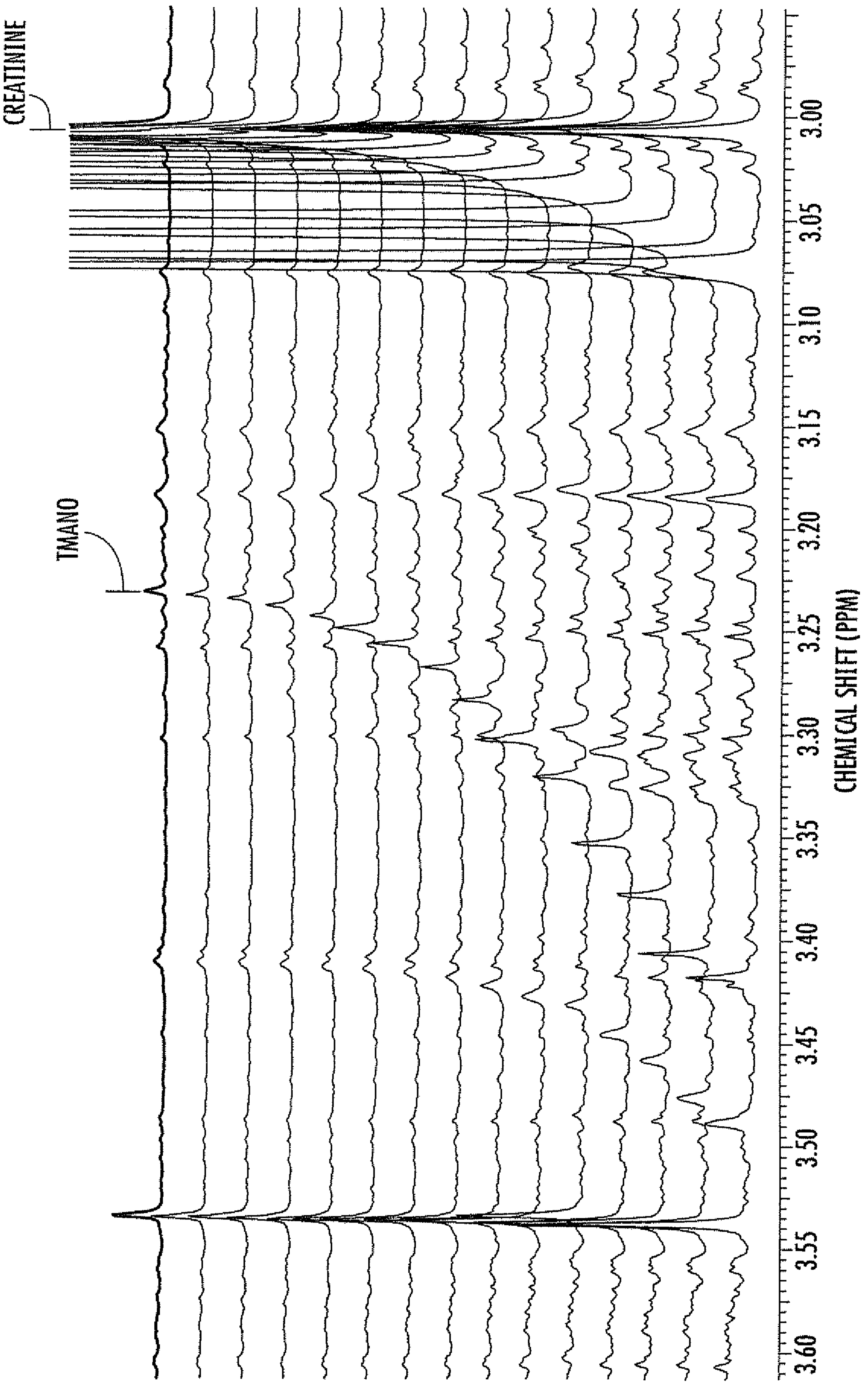

CREATININE
TMANO
3.00
3.05
3.10
3.15
3.20
3.25
3.30
3.35
3.40
3.45
3.50
3.55
3.60
CHEMICAL SHIFT (PPM)

FIG. 19

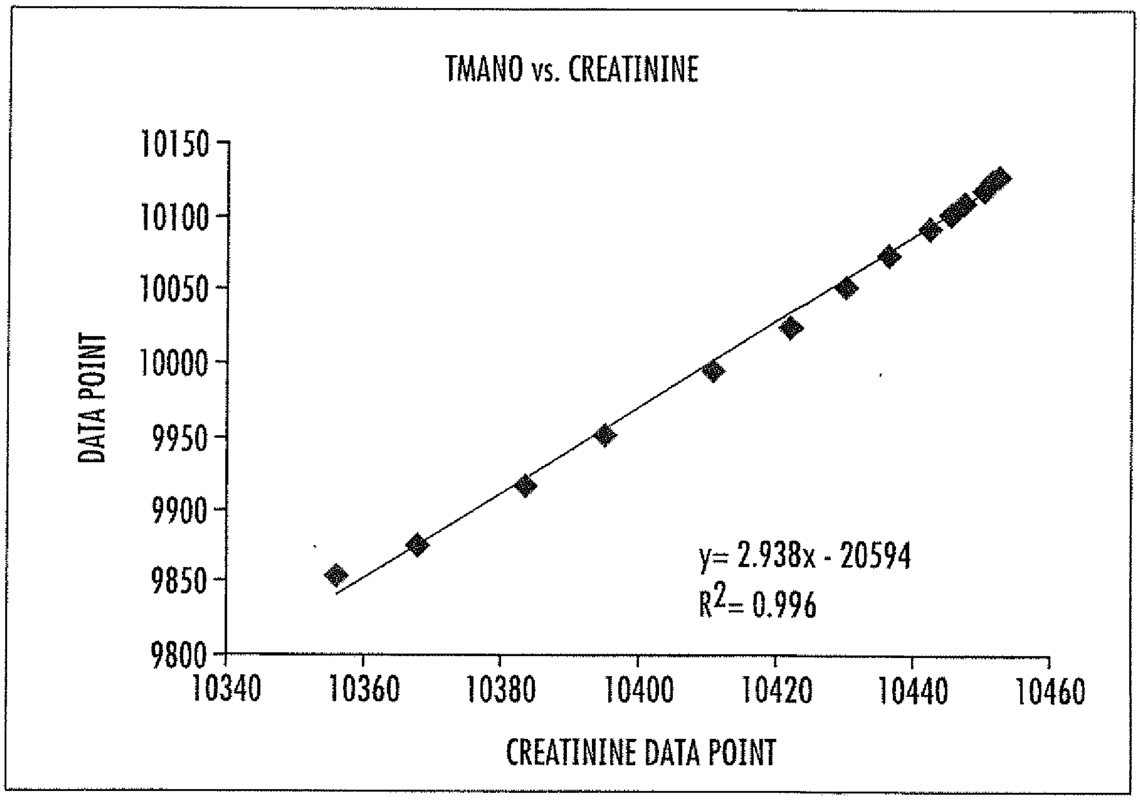


FIG. 20A

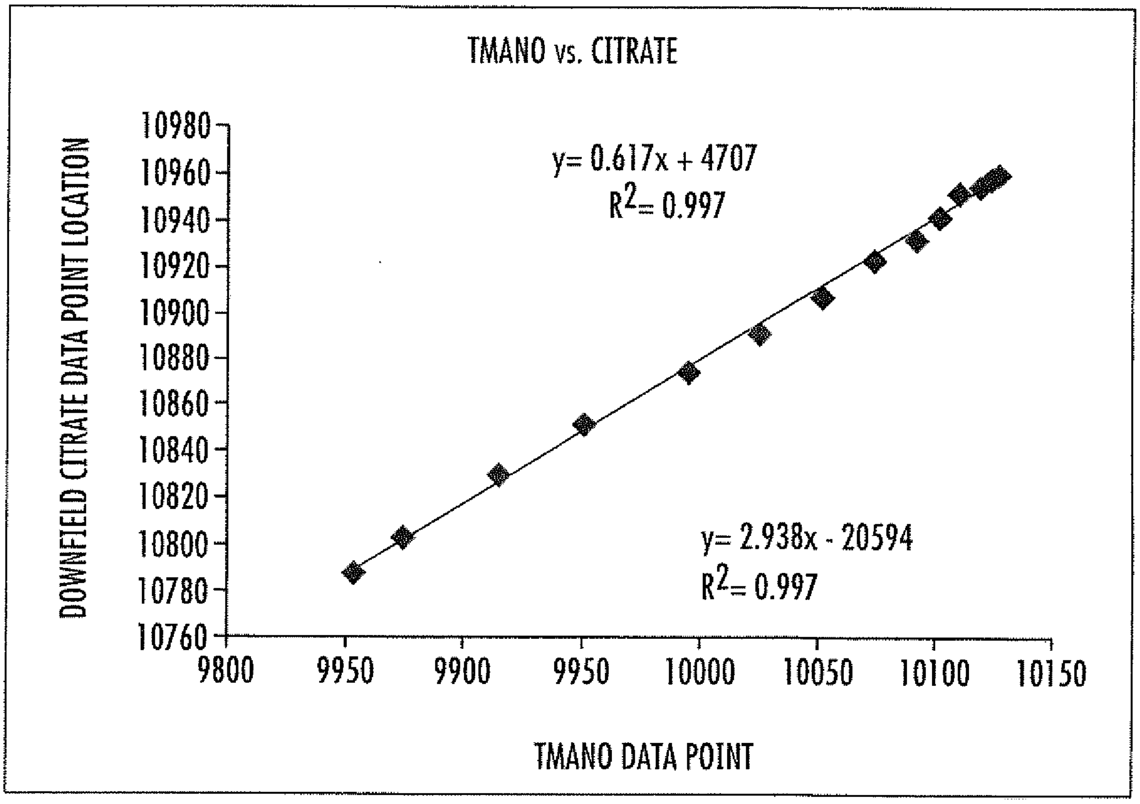


FIG. 20B

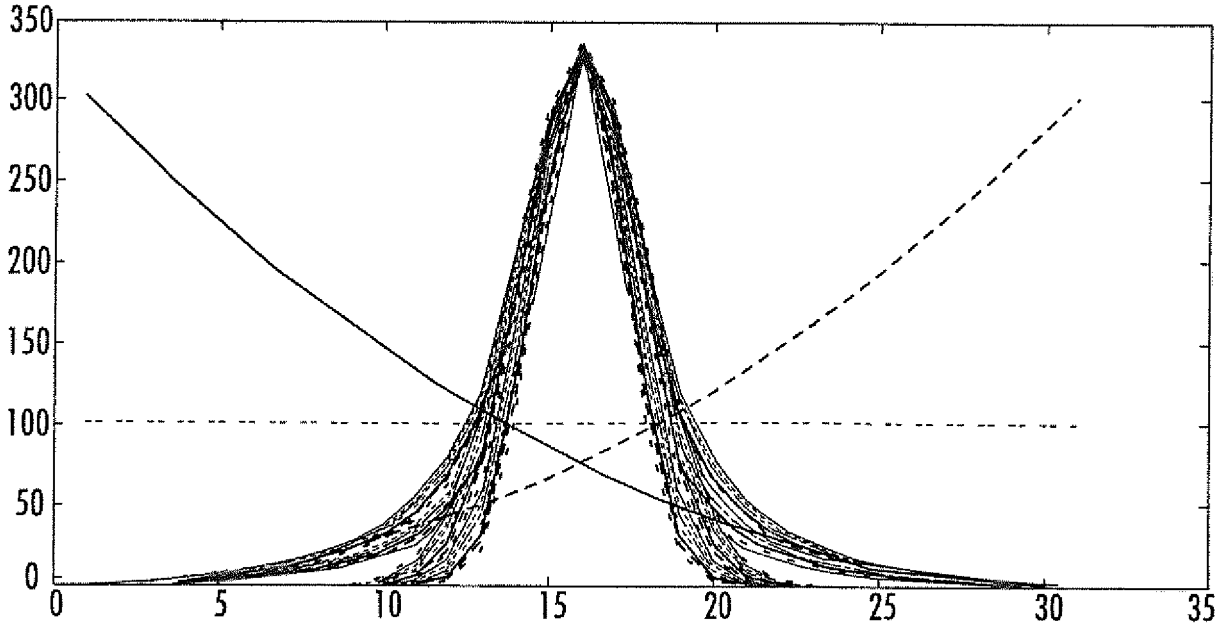

350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35

FIG. 21

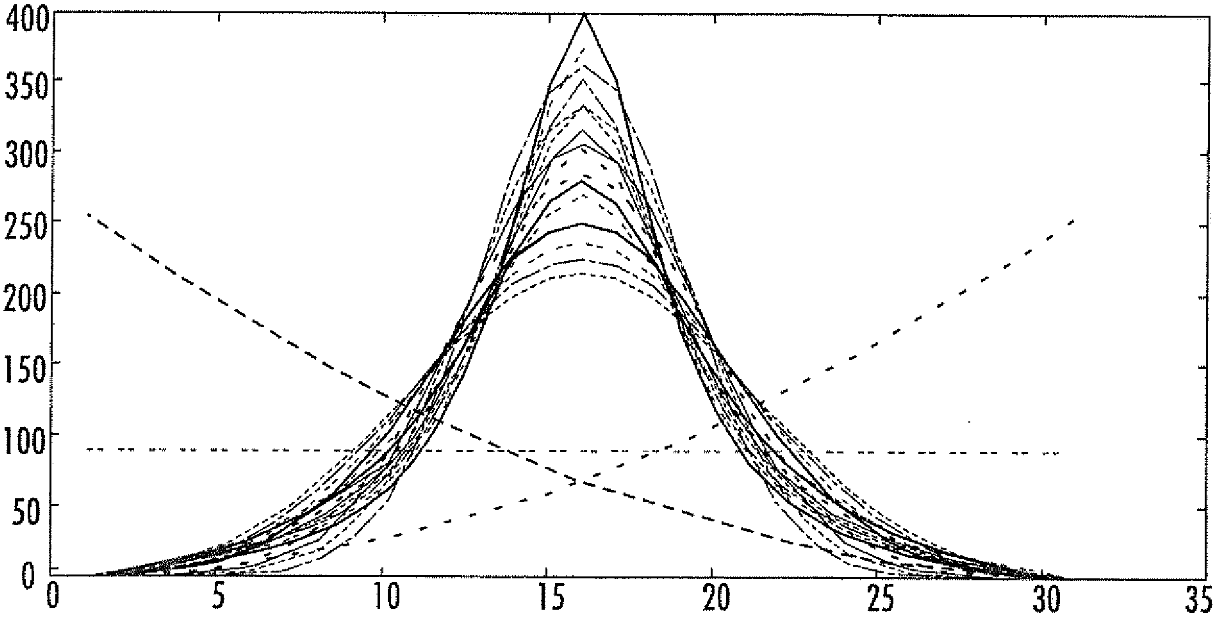

400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35

FIG. 22

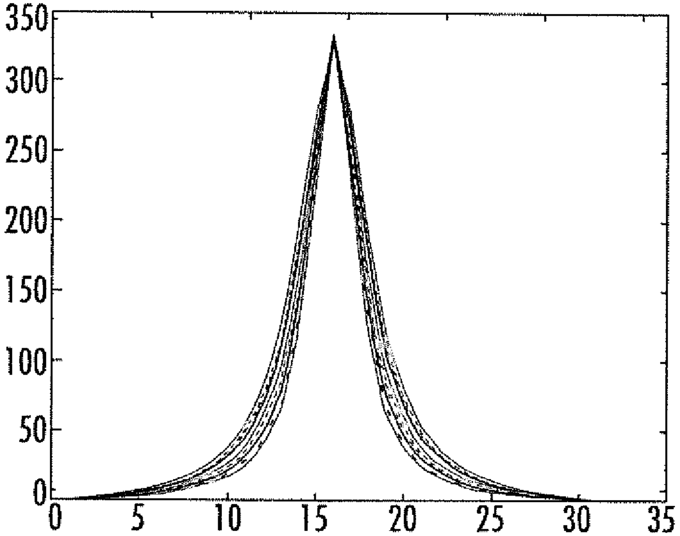


FIG. 23

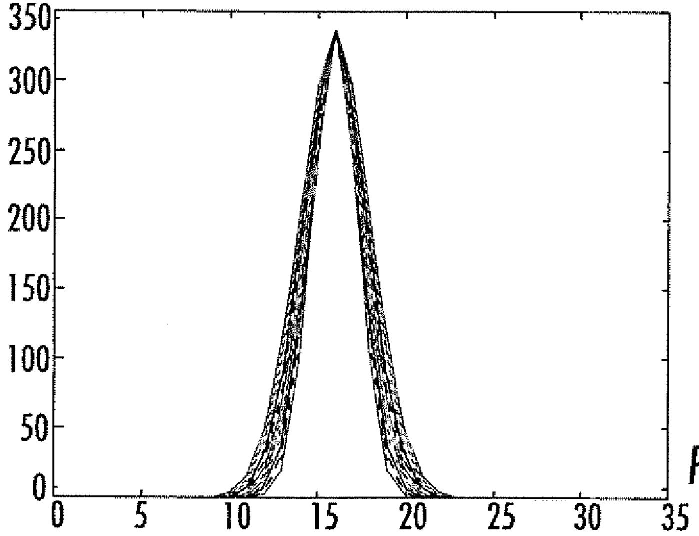


FIG. 24

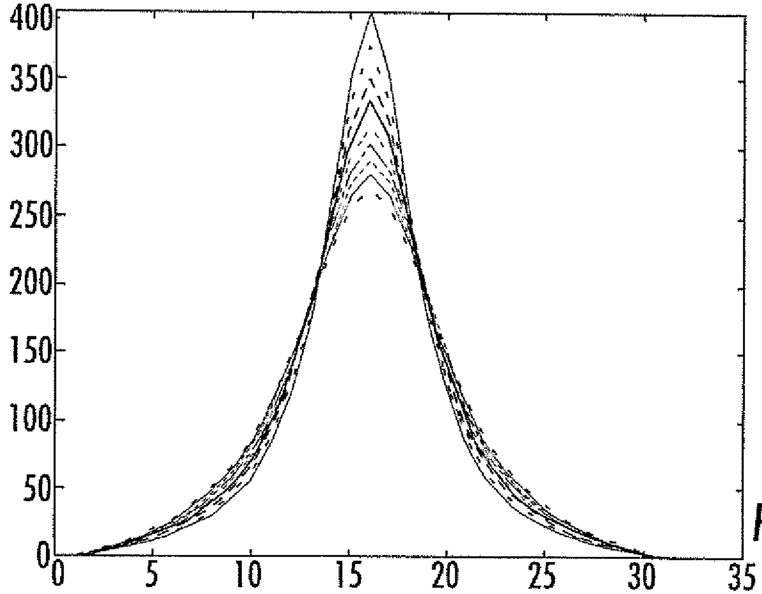


FIG. 25

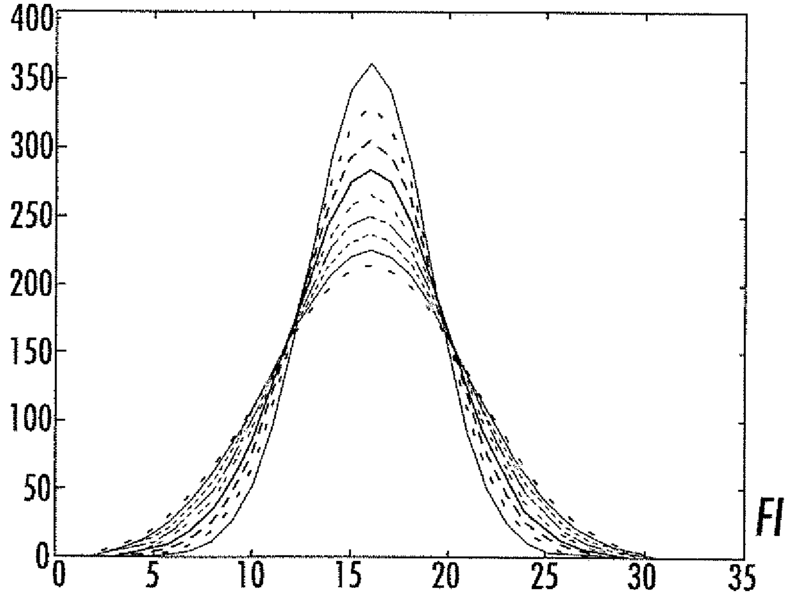


FIG. 26

REFERENCES CITED IN THE DESCRIPTION

Patent documents cited in the description

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- US 8013602 B **[0055] [0125]**
- US 7243030 B **[0077]**

Non-patent literature cited in the description

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- **BELL J D et al.** Nuclear magnetic resonance studies of blood plasma and urine from subjects with chronic renal failure: identification of trimethylamine-N-oxide. *Biochimica et Biophysica Acta,* 1991, vol. 1096, 101-107 **[0003]**
- **IJARE O. B.** The Effect of pH on the Analysis of 1H MRS Data of Urine. *Biomedical Applications* **[0004]**
- **OGG, R. J. ; KINGSLEY, R. B. ; TAYLOR, J. S. J.** *Magn. Reson., Ser. B,* 1994, vol. 104, 1-10 **[0044]**
- **SMALLCOMBE, S. H. ; PATT, S. L. ; KEIFER, P. A. J.** *Magn. Reson., Ser. A,* 1995, vol. 117, 295-303 **[0044]**
- **WANG et al.** Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature,* 07 April 2011, vol. 472 (58 **[0071]**
- **LAWSON, CL ; HANSON RJ.** Solving Least Squares Problems. Prentice-Hall, 1974 **[0077]**
- **OTVOS, JD ; JEYARAJAH, EJ ; BENNETT, DW.** *Clin Chem,* 1991, vol. 37, 377 **[0077]**
- **BECKONERT, O. ; KEUN, H. C. ; EBBELS, T. M.** *Nat. Protoc.,* 2007, vol. 2, 2692-2703 **[0088]**
- **SIMPSON, A. J. ; BROWN, S. A.** *J. Magn. Reson.,* 2005, vol. 175, 340-346 **[0088]**
- **SMALLCOMBE, S. H. ; PATT, S. L. ; KEIFER, P. A.** *J. Magn. Reson.,* 1995, vol. 117, 295-303 **[0092]**