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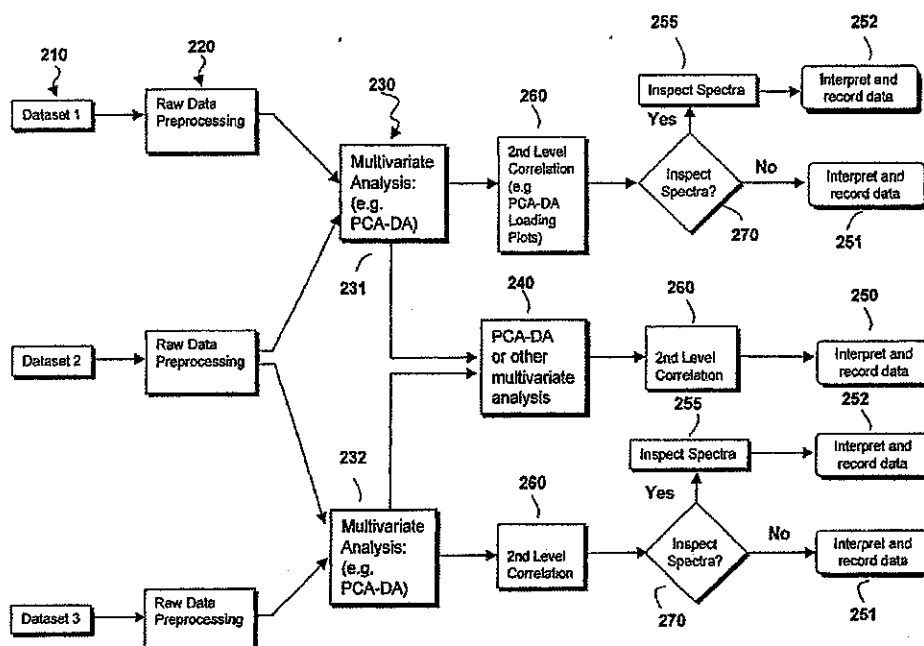
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(54) Title: METHOD AND SYSTEM FOR PROFILING BIOLOGICAL SYSTEMS



(57) Abstract: The present invention provides methods and systems for developing profiles of a biological system based on the discernment of similarities, differences, and/or correlations between biomolecular components, of a single biomolecular component type, of a plurality of biological samples. Preferably, the method comprises utilizing hierarchical multivariate analysis of spectroscopic data at one or more levels of correlation.

METHOD AND SYSTEM FOR PROFILING BIOLOGICAL SYSTEMS

CROSS REFERENCE TO RELATED APPLICATIONS

5 The present application claims the benefit of and priority to copending United States provisional application number 60/312,145, filed August 13, 2001, the entire disclosure
10 of which is herein incorporated by reference.

FIELD OF THE INVENTION

The invention relates to the field of data processing and evaluation. In particular, the invention relates to an analytical technology platform for separating and measuring multiple
15 components of a biological sample, and statistical data processing methods for identifying components and revealing patterns and relationships between and among the various measured components.

BACKGROUND

20 The characterization of complex mixtures has become important in a variety of research and application areas, including pharmaceuticals, biotechnological research, and nutraceutical (functional food) topics. One important area is the study of small molecules in pharmaceutical and biotechnology research, often referred to as metabolomics.

For example, an important challenge in the development of new drugs for complex
25 (multi-factorial) diseases is the tracing and validation of biomarkers/surrogate markers. Moreover, it appears that instead of single biomarkers, biomarker-patterns may be necessary to characterize and diagnose homeostasis or disease states for such diseases.

In the discipline of metabolomics, the current art in the field of biological sample profiling is based either on measurement by nuclear magnetic resonance ("NMR") or by mass
30 spectrometry ("MS") that focuses on a limited number of small molecule compounds. Both of these profiling approaches have limitations. The NMR approaches are limited in that they typically provide reliable profiles only of compounds present at high concentration. On the other hand, focused mass spectrometry based approaches do not require high concentrations but

can provide profiles of only limited portions of the metabolome. What is needed is an approach that can address limitations in current profiling techniques and that facilitates the discernment of correlations between components or patterns of component (such as biomarker patterns).

SUMMARY OF THE INVENTION

5 Embodiments of the present invention address limitations in current profiling techniques by providing a method and system (or collectively "technology platform") utilizing hierarchical multivariate analysis of spectrometric data on one or more levels. Embodiments of the present invention further provide a technology platform that facilitates the discernment of similarities, differences, and/or correlations not only between single biomolecular components
10 of a sample or biological system, but also between patterns of biomolecular components of a single biomolecular component type.

 As used herein, the term "biomolecule component type" refers to a class of biomolecules generally associated with a level of a biological system. For example, gene transcripts are one example of a biomolecule component type that are generally associated with
15 gene expression in a biological system, and the level of a biological system referred to as genomics or functional genomics. Proteins are another example of a biomolecule component type and generally associated with protein expression and modification, etc., and the level of a biological system referred to as proteomics. Further, another example of a biomolecule component type are metabolites, which are generally associated with the level of a biological
20 system referred to as metabolomics.

 According to a first aspect of the present invention, there is provided a method of profiling a state of a biological system in an animal through processing of a plurality of data sets, the method comprising the steps of: (a) optionally preprocessing a plurality of data sets comprising spectrometric measurements of a sample of a biological system, wherein the
25 spectrometric measurements are obtained using two different spectrometric techniques; and (b) generating a profile of one or more biomarkers for a state of the biological system by evaluating the plurality of data sets with a multivariate analysis and correlating spectrometric features among the plurality of data sets to determine one or more sets of differences among at least a portion of the plurality of data sets.

Embodiments of the present invention provide a method and system for profiling a biological system utilizing a hierarchical multivariate analysis of spectrometric data to generate a profile of a state of a biological system. The states of a biological system that may be profiled by the invention include, but are not limited to, disease state, pharmacological agent response, toxicological state, biochemical regulation (e.g., apoptosis), age response, environmental response, and stress response. Embodiments of the present invention may use data on a biomolecule component type (e.g., metabolites, proteins, gene transcripts, etc.) from multiple biological sample types (e.g., body fluids, tissue, cells) obtained from multiple sources (such as, for example, blood, urine, cerebrospinal fluid, epithelial cells, endothelial cells, different subjects, the same subject at different times, etc.). In addition, the present invention may use spectrometric data obtained on one or more platforms including, but not limited to, MS, NMR, liquid chromatography ("LC"), gas-chromatography ("GC"), high performance liquid chromatography ("HPLC"), capillary electrophoresis ("CE"), and any known form of hyphenated mass spectrometry in low or high resolution mode, such as LC-MS, GC-MS, CE-MS, LC-UV, MS-MS, MSⁿ, etc.

As used herein, the term "spectrometric data" includes data from any spectrometric or chromatographic technique and the term "spectrometric measurement" includes measurements made by any spectrometric or chromatographic technique. Spectrometric techniques include, but are not limited to, resonance spectroscopy, mass spectroscopy, and optical spectroscopy. Chromatographic techniques include, but are not limited to, liquid phase chromatography, gas phase chromatography, and electrophoresis.

As used herein, the terms "small molecule" and "metabolite" are used interchangeably. Small molecules and metabolites include, but are not limited to, lipids, steroids, amino acids, organic acids, bile acids, eicosanoids, peptides, trace elements, and pharmacophore and drug breakdown products.

In one embodiment, the present invention provides a method of spectrometric data processing utilizing multiple steps of a multivariate analysis to process data in a hierarchical procedure. In one embodiment, the method uses a first multivariate analysis on a plurality of data sets to discern one or more sets of differences and/or similarities between them and then uses a second multivariate analysis to determine a correlation (and/or anti-correlation, i.e., negative correlation) between at least one of these sets of differences (or similarities) and one or

more of the plurality of data sets. The method may further comprise developing a profile for a state of a biological system based on the correlation.

As used herein, the term "data sets" refers to the spectrometric data associated with one or more spectrometric measurements. For example, where the spectrometric technique is NMR, a data set may comprise one or more NMR spectra. Where the spectrometric technique is UV spectroscopy, a data set may comprise one or more UV emission or absorption spectra. Similarly, where the spectrometric technique is MS, a data set may comprise one or more mass spectra. Where the spectrometric technique is a chromatographic-MS technique (e.g., LC-MS, GC-MS, etc), a data set may comprise one or more mass chromatograms. Alternatively, a data set of a chromatographic-MS technique may comprise one or more a total ion current ("TIC") chromatograms or reconstructed TIC chromatograms. In addition, it should be realized that the term "data set" includes both raw spectrometric data and data that has been preprocessed (e.g., to remove noise, baseline, detect peaks, to normalize, etc.).

Moreover, as used herein, the term "data sets" may refer to substantially all or a sub-set of the spectrometric data associated with one or more spectrometric measurements. For example, the data associated with the spectrometric measurements of different sample sources (e.g., experimental group samples v. control group samples) may be grouped into different data sets. As a result, a first data set may refer to experimental group sample measurements and a second data set may refer to control group sample measurements. In addition, data sets may refer to data grouped based on any other classification considered relevant. For example, data associated with the spectrometric measurements of a single sample source (e.g., experimental group) may be grouped into different data sets based, for example, on the instrument used to perform the measurement, the time a sample was taken, the appearance of the sample, etc. Accordingly, one data set (e.g., grouping of experimental group samples based on appearance) may comprise a sub-set of another data set (e.g., the experimental group data set).

In another embodiment, the present invention provides a method of spectrometric data processing utilizing multivariate analysis to process data at two or more hierarchical levels of correlation. In one embodiment, the method uses a multivariate analysis on a plurality of data sets to discern correlations (and/or anti-correlations) between data sets at a first level of correlation, and then uses the multivariate analysis to discern correlations (and/or anti-

correlations) between data sets at a second level of correlation. The method may further comprise developing a profile for a state of a biological system based on the correlations discerned at one or more levels of correlation.

In yet another embodiment, the present invention provides a method of
5 spectrometric data processing utilizing multiple steps of a multivariate analysis to process data sets in a hierarchal procedure, wherein one or more of the multivariate analysis steps further comprises processing data at two or more hierarchal levels of correlation. For example, in one embodiment, the method comprises: (1) using a first multivariate analysis on a plurality of data
10 sets to discern one or more sets of differences and/or similarities between them; (2) using a second multivariate analysis to determine a first level of correlation (and/or anti-correlation) between a first sets of differences (or similarities) and one or more of the data sets; and (3) using the second multivariate analysis to determine a second level of correlation (and/or anti-correlation) between the first sets of differences (or similarities) and one or more of the data sets. The method of this aspect may also comprise developing a profile for a state of a
15 biological system based on the correlations discerned at one or more levels of correlation.

In other embodiments, the present invention provide systems adapted to practice the methods of the invention set forth above. In one embodiment, the system comprises a spectrometric instrument and a data processing device. In another embodiment, the system further comprises a database accessible by the data processing device. The data processing
20 device may comprise an analog and/or digital circuit adapted to implement the functionality of one or more of the methods of the present invention.

In some embodiments, the data processing device may implement the functionality of the methods of the present invention as software on a general purpose computer. In addition, such a program may set aside portions of a computer's random access memory to provide
25 control logic that affects the hierarchical multivariate analysis, data preprocessing and the operations with and on the measured interference signals. In such an embodiment, the program may be written in any one of a number of high-level languages, such as FORTRAN, PASCAL, C, C++, or BASIC. Further, the program may be written in a script, macro, or functionality embedded in commercially available software, such as EXCEL or VISUAL BASIC.

Additionally, the software could be implemented in an assembly language directed to a microprocessor resident on a computer. For example, the software could be implemented in Intel 80x86 assembly language if it were configured to run on an IBM PC or PC clone. The software may be embedded on an article of manufacture including, but not limited to,

- 5 “computer-readable program means” such as a floppy disk, a hard disk, an optical disk, a magnetic tape, a PROM, an EPROM, or CD-ROM.

In a further embodiment, the present invention provides an article of manufacture where the functionality of a method of the present invention is embedded on a computer-readable medium, such as, but not limited to, a floppy disk, a hard disk, an optical disk, a
10 magnetic tape, a PROM, an EPROM, CD-ROM, or DVD-ROM.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other features and advantages of the invention, as well as the invention itself, will be more fully understood from the description, drawings, and claims that follow. The drawings are not necessarily drawn to scale, and like reference numerals refer to the same parts throughout the different views.

5 Figure 1A is a flow diagram of analyzing a plurality of data sets according to various embodiments of the present invention.

Figure 1B is a flow diagram of analyzing a plurality of data sets according to various other embodiments of the present invention.

10 Figures 2A and 2B are flow diagrams of the analysis performed according to various embodiments of the present invention on a plurality of data sets of multiple biological sample types obtained from wildtype mice and APO E3 Leiden mice.

Figures 3A and 3B are examples of partial 400 MHz ^1H -NMR spectra for urine samples of wildtype mouse samples, Figure 3A and APO E3 mouse samples, Figure 3B.

15 Figures 4A and 4B are examples of partial 400 MHz ^1H -NMR spectra for urine samples of wildtype mouse samples, Figure 4A and APO E3 mouse samples, Figure 4B.

Figures 5A and 5B are examples of partial 400 MHz ^1H -NMR spectra for blood plasma samples of wildtype mouse samples, Figure 5A, and APO E3 mouse samples, Figure 5B.

20 Figures 6A and 6B are examples of partial 400 MHz ^1H -NMR spectra for blood plasma samples of wildtype mouse samples, Figure 6A, and APO E3 mouse samples, Figure 6B.

Figures 7A and 7B are examples of a blood plasma lipid profile obtained by a LC-MS spectrometric technique using ESI on APO E3 mouse blood plasma samples, Figure 7A, and wildtype mouse samples, Figure 7B.

25 Figure 8 is an example of a PCA-DA score plot of the NMR data for the urine samples of data sets 1 and 2 of Figures 2A and 2B.

Figure 9 is an example of a PCA-DA score plot of the NMR data for the urine samples of data set 1 (wildtype mouse) of Figures 2A and 2B.

Figure 10 is an example of a PCA-DA score plot of the NMR data for the urine samples of data set 2 (APO E3 mouse) of Figures 2A and 2B.

Figure 11 is an example of a PCA-DA score plot of the NMR data for the urine samples of both wildtype and APO E3 mice.

5 Figure 12 is an example of a PCA-DA score plot of the NMR data for the blood plasma samples of data sets 3 and 4 of Figures 2A and 2B.

Figure 13 is an example of a PCA-DA score plot of the LC-MS data on the blood plasma samples of data sets 5, 6 of Figures 2A and 2B and human samples.

Figure 14 is an example of a loading plot for axis D2 of Figure 13.

10 Figure 15 is an example of the comparison of normalized blood plasma lipid profiles obtained by an LC-MS spectrometric technique for wildtype mouse samples (thin sold line) and APO E3 mouse samples (thick sold line).

Figure 16 is an example of the comparison of normalized blood plasma lipid profiles obtained by an LC-MS spectrometric technique for wildtype mouse samples (thin sold line) and APO E3 mouse samples (thick sold line).
15

Figure 17 is an example of a canonical correlation score plot for spectrometric data for one biological sample type (blood plasma) from two different spectrometric techniques (NMR and LC-MS).

Figure 18 is an example of a canonical correlation score plot for spectrometric data for one biological sample type (blood plasma) from the same general spectrometric technique but different instrument configurations.
20

Figure 19 is a schematic representation of one embodiment of a system adapted to practice the methods of the invention.

25 DETAILED DESCRIPTION

Referring to Figure 1A, a flow chart of one embodiment of a method according to the present invention is shown. One or more of a plurality of data sets **110** are preferably subjected to a preprocessing step **120** prior to multivariate analysis. Suitable forms of

preprocessing include, but are not limited to, data smoothing, noise reduction, baseline correction, normalization and peak detection. Preferable forms of data preprocessing include entropy-based peak detection (such as disclosed in pending U.S. Patent Application, Serial No. 09/920,993, filed August 2, 2001, the entire contents of which are hereby incorporated by
5 reference) and partial linear fit techniques (such as found in J.T.W.E. Vogels *et al.*, "Partial Linear Fit: A New NMR Spectroscopy Processing Tool for Pattern Recognition Applications," Journal of Chemometrics, vol. 10, pp. 425-38 (1996)). A multivariate analysis is then performed at a first level of correlation 130 to discern differences (and/or similarities) between the data sets. Suitable forms of multivariate analysis include, for example, principal component
10 analysis ("PCA"), discriminant analysis ("DA"), PCA-DA, canonical correlation ("CC"), partial least squares ("PLS"), predictive linear discriminant analysis ("PLDA"), neural networks, and pattern recognition techniques. In one embodiment, PCA-DA is performed at a first level of correlation that produces a score plot (i.e., a plot of the data in terms of two principal components; see, e.g. Figures 8-12 which are described further below). Subsequently,
15 the same or a different multivariate analysis is performed on the data sets at a second level of correlation 140 based on the differences (and/or similarities) discerned from the first level of correlation.

For example, in one embodiment, where the first level comprises a PCA-DA score plot, the second level of correlation comprises a loading plot produced by a PCA-DA analysis.
20 This second level of correlation bears a hierarchical relationship to the first level in that loading plots provide information on the contributions of individual input vectors to the PCA-DA that in turn are used to produce a score plot. For example, where each data set comprises a plurality of mass chromatograms, a point on a score plot represents mass chromatograms originating from one sample source. In comparison, a point on a loading plot represents the contribution of
25 a particular mass (or range of masses) to the correlations between data sets. Similarly, where each data set comprises a plurality of NMR spectra, a point on a score plot represents one NMR spectrum. In comparison, a point on the corresponding loading plot represents the contribution of a particular NMR chemical shift value (or range of values) to the correlations between data sets.

30 Referring again to Figure 1A, based on the correlations discerned in the analysis at the first level of correlation 130 and/or that at the second level of correlation 140 a profile may

be developed 151 ("NO" to inspect spectra query 160). For example, the region in a score plot where the data points fall for a certain group of data sets may comprise a profile for the state of a biological system associated with that group. Further, the profile may comprise both the above region in a score plot and a specific level of contribution from one or more points in an associated loading plot. For example, where the data sets comprise mass chromatograms and/or mass spectra, a biological system may only fit into the profile of a state if spectrometric data sets from appropriate samples fall in a certain region of the score plot and if the mass chromatograms for a particular range of masses provide a significant contribution to the correlation observed in the score plot. Similarly, where the data sets comprise NMR spectra, a biological system may only fit into the profile of a state if spectrometric data sets from appropriate samples fall in a certain region of the score plot and if a particular range of chemical shift values in the NMR spectra provide a significant contribution to the correlation observed in the score plot.

In addition, the method may further include a step of inspection 155 of one or more specific spectra of the data sets ("YES" to inspect spectra query 160) based on the correlations discerned in the analysis at the first level of correlation 130 and/or that at the second level of correlation 140. A profile based on this inspection is then developed 152. For example, where the spectra of the data sets comprise mass chromatograms, the method inspects the mass chromatograms of those mass ranges showing a significant contribution to the correlation based on the loading plot. Inspection of these mass chromatograms, for example, may reveal what species of chemical compounds are associated with the profile. Such information may be of particular importance for biomarker identification and drug target identification.

Referring to Figure 1B, a flow chart of another embodiment of a method according to the present invention is shown. One or more of a plurality of data sets 210 are preferably subjected to a preprocessing step 220 prior to multivariate analysis. A first multivariate analysis is then performed 230 on a plurality of data sets to discern one or more sets of differences and/or similarities between them. The first multivariate analysis may be performed between sub-sets of the data sets. For example, the first multivariate analysis may be performed between data set 1 and data set 2, 231 and the first multivariate analysis may be performed separately between data set 2 and data set 3, 232. The method then uses a second multivariate analysis 240 to determine a correlation between at least one of the sets of

differences (or similarities) discerned in the first multivariate analysis and one or more of the data sets. This second multivariate analysis 240 bears a hierarchal relationship to the first 230 in that the differences between data sets are discerned in a hierarchal fashion. For example, the differences between data sets 1 and 2 (and data sets 2 and 3) are first discerned 231, 232 and then those differences are subjected to further multivariate analysis 240. In one embodiment, a profile based on the correlations discerned in the second multivariate analysis 240 is developed 250.

In addition, any of the multivariate analysis steps 231, 232, 240 may further comprise a step of performing the same or a different multivariate analysis at another level of correlation 260 (for example, such as described with respect to Figure 1A) based on the differences (and/or similarities) discerned from the level of correlation used in a prior multivariate analysis step 231, 232, 240. A profile based on the information from one or more of these levels of correlation may then be developed 250, 251 ("NO" to inspect spectra query 270). Alternatively, the method may further include a step of inspection 255 of one or more specific spectra of the data sets ("YES" to inspect spectra query 270) based on the correlations discerned in the analysis at one or more levels of correlation and/or one or more multivariate analysis steps. A profile based on this inspection then may be developed 252.

The methods of the present invention may be used to develop profiles on any biomolecular component type. Such profiles facilitate the development of comprehensive profiles of different levels of a biological system, such as, for example, genome profiles, transcriptomic profiles, proteome profiles, and metabolome profiles. Further, such methods may be used for data analysis of spectrometric measurements (of, for example, plasma samples from a control and patient group), may be used to evaluate any differences in single components or patterns of components between the two groups exist in order to obtain a better insight into underlying biological mechanisms, to detect novel biomarkers/surrogate markers, and/or develop intervention routes.

In various embodiments, the present invention provides methods for developing profiles of metabolites and small molecules. Such profiles facilitate the development of comprehensive metabolome profiles. In other various embodiments, the present invention provides methods for developing profiles of proteins, protein-complexes and the like. Such profiles facilitate the development of comprehensive proteome profiles. In yet other various

embodiments, the present invention provides methods for developing profiles of gene transcripts, mRNA and the like. Such profiles facilitate the development of comprehensive genome profiles.

In one version of these embodiments, the method is generally based on the following steps: (1) selection of biological samples, for example body fluids (plasma, urine, cerebral spinal fluid, saliva, synovial fluid etc.); (2) sample preparation based on the biochemical components to be investigated and the spectrometric techniques to be employed (e.g., investigation of lipids, proteins, trace elements, gene expression, etc.); (3) measurement of the high concentration components in the biological samples using methods mass spectrometry and NMR; (4) measurement of selected molecule subclasses using NMR-profiles and preferred MS-approaches to study compounds such as, for example, lipids, steroids, bile acids, eicosanoids, (neuro)peptides, vitamins, organic acids, neurotransmitters, amino acids, carbohydrates, ionic organics, nucleotides, inorganics, xenobiotics etc.; (5) raw data preprocessing; (6) data analysis using multivariate analysis according to any of the methods of the present invention (e.g., to identify patterns in measurements of single subclasses of molecules or in measurements of high concentration components using NMR or mass spectrometry); and (7) using of multivariate analysis to combine data sets from distinct experiments and find patterns of interest in the data. In addition, the method may further comprise a step of (8) acquiring data sets at a number of points in time to facilitate the monitoring of temporal changes in the multivariate patterns of interest.

The methods of the present invention may be used to develop profiles on a biomolecular component type obtained from a wide variety of biological sample types including, but not limited to, blood, blood plasma, blood serum, cerebrospinal fluid, bile acid, saliva, synovial fluid, pleural fluid, pericardial fluid, peritoneal fluid, feces, nasal fluid, ocular fluid, intracellular fluid, intercellular fluid, lymph urine, tissue, liver cells, epithelial cells, endothelial cells, kidney cells, prostate cells, blood cells, lung cells, brain cells, adipose cells, tumor cells and mammary cells.

In another aspect, the present invention provides an article of manufacture where the functionality of a method of the present invention is embedded on a computer-readable medium, such as, but not limited to, a floppy disk, a hard disk, an optical disk, a magnetic tape, a PROM, an EPROM, CD-ROM, or DVD-ROM. The functionality of the method may be

embedded on the computer-readable medium in any number of computer-readable instructions, or languages such as, for example, FORTRAN, PASCAL, C, C++, BASIC and assembly language. Further, the computer-readable instructions can, for example, be written in a script, macro, or functionally embedded in commercially available software (such as, e.g., EXCEL or
5 VISUAL BASIC).

In other aspects, the present invention provides systems adapted to practice the methods of the present invention. Referring to Figure 19, in one embodiment, the system comprises one or more spectrometric instruments 1910 and a data processing device 1920 in electrical communication, wireless communication, or both. The spectrometric instrument may
10 comprise any instrument capable of generating spectrometric measurements useful in practicing the methods of the present invention. Suitable spectrometric instruments include, but are not limited to, mass spectrometers, liquid phase chromatographers, gas phase chromatographer, and electrophoresis instruments, and combinations thereof. In another embodiment, the system further comprises an external database 1930 storing data accessible by the data processing
15 device, wherein the data processing device implement the functionality of one or more of the methods of the present invention using at least in part data stored in the external database.

The data processing device may comprise an analog and/or digital circuit adapted to implement the functionality of one or more of the methods of the present invention using at least in part information provided by the spectrometric instrument. In some embodiments, the
20 data processing device may implement the functionality of the methods of the present invention as software on a general purpose computer. In addition, such a program may set aside portions of a computer's random access memory to provide control logic that affects the spectrometric measurement acquisition, multivariate analysis of data sets, and/or profile development for a biological system. In such an embodiment, the program may be written in any one of a number
25 of high-level languages, such as FORTRAN, PASCAL, C, C++, or BASIC. Further, the program can be written in a script, macro, or functionality embedded in proprietary software or commercially available software, such as EXCEL or VISUAL BASIC. Additionally, the software could be implemented in an assembly language directed to a microprocessor resident on a computer. For example, the software can be implemented in Intel 80x86 assembly
30 language if it is configured to run on an IBM PC or PC clone. The software may be embedded on an article of manufacture including, but not limited to, a computer-readable program

medium such as a floppy disk, a hard disk, an optical disk, a magnetic tape, a PROM, an EPROM, or CD-ROM.

EXAMPLE : SMALL MOLECULE STUDY OF THE
APO E3 MOUSE MODEL FOR ATHEROSCLEROSIS

An example of the practice of various embodiments of the present invention is illustrated below in the context of a small molecule study of the APO E3 Leiden transgenic mouse model.

A. The APO E3 Leiden Mouse

The APO E3 Leiden mouse model is a transgenic animal model described in "The Use of Transgenic Mice in Drug Discovery and Drug Development," by P.L.B. Bruijnzeel, TNO Pharma, October 24, 2000. Briefly, the APO E3-Leiden allele is identical to the APO E4 (Cys112 → Arg) allele, but includes an in frame repeat of 21 nucleotides in exon 4, resulting in tandem repeat of codon 120-126 or 121-127. Transgenic mice expressing APO E3-Leiden mutation are known to have hyperlipidemic phenotypes that under specific conditions lead to the development of atherosclerotic plaques. The model has a high predicted success rate in finding differences at the small molecule (metabolite) and protein levels, while the gene level is very well characterized.

In the present example, 10 wildtype and 10 APO E3 male mice were sacrificed after collection of urine in metabolic cages. The APO E3 mice were created by insertion of a well-defined human gene cluster (APO E3 – APC1), and a very homogeneous population was generated by at least 20 inbred generations.

The following samples were available for analysis: (1) 10 wildtype and 10 APO E3 urine samples (about 0.5 ml/animal or more); (2) 10 wildtype and 10 APO E3 (heparin) plasma samples (about 350 µl/animal); (3) 10 wildtype and 10 APO E3 liver samples. From the plasma samples 100 microliters were used for NMR and the same samples were used for LC-MS, about 250 µl is available for protein work and duplicates. All samples were stored at -20°C. In total, 19 plasma samples were received. One sample, animal #6 (APO-E3 Leiden group) was not present. After cleanup, (described below) the portions reserved for proteomics research were transferred to -70°C.

B. Experimental Details, Plasma and Urine Samples

Plasma sample extraction was accomplished with isopropanol (protein precipitation). LC-MS lipid profile measurements of the plasma samples were obtained with on an electrospray ionization ("ESI") and atmospheric pressure chemical ionization ("APCI") LC-MS system. The resultant raw data was preprocessed with an entropy-based peak detection technique substantially similar to that disclosed in pending U.S. Patent Application Serial No. 09/920,993, filed August 2, 2001. The preprocessed data was then subjected to principal component analysis ("PCA") and/or discriminant analysis ("DA") according to the methods of the present invention. The raw data from the NMR measurements of the plasma samples was subjected to a pattern recognition analysis ("PARC"), which included preprocessing (such as a partial linear fit), peak detection and multivariate statistical analysis.

Urine samples were prepared and NMR measurements of the urine samples were obtained. The raw NMR data on the urine samples was also subjected to a PARC analysis, which included preprocessing, peak detection and multivariate statistical analysis.

B.1. Mouse Blood Plasma Preparation and Cleanup

The mouse plasma samples were thawed at room temperature. Aliquots of 100 μ l were transferred to a clean eppendorf vials and stored at -70°C . The sample volume for sample #12 was low and only 50 μ l was transferred. For NMR and LC-MS lipid analysis 150 μ l aliquots were transferred to clean eppendorf vials.

Plasma samples were cleaned up and handled substantially according to the following protocol: (1) add 0.6 ml of isopropanol; (2) vortex; (3) centrifuge at 10,000 rpm for 5 min.; (4) transfer 500 μ l to clean tube for NMR analysis; (5) transfer 100 μ l to clean eppendorf vial; (6) add 400 μ l water and mix; and (7) transfer 200 μ l to autosampler vial insert. The remaining extract and pellet (precipitated protein) were stored at -20°C .

B.2. Human Blood Plasma Preparation and Cleanup

Human heparin plasma was obtained from a blood bank. In a glass tube, 1 ml of human plasma and 4 ml of isopropanol were mixed (vortexed). After centrifugation, 1 ml of extract was transferred to a tube and 4 ml of water was added. The resulting solution was transferred to 4 autosampler vials (1 ml).

B.3. LC-MS of blood plasma samples:

Spectrometric measurements of plasma samples were made with a combination HPLC-time-of-flight MS instrument. Effluent emerging from the chromatograph was ionized by electrospray ionization ("ESI") and atmospheric pressure chemical ionization ("APCI").

- 5 Typical instrument parameters used with HPLC instrument are given in Table 1 and details of the gradient in Table 2; typical parameters for the ESI source are given in Table 3, and those for the APCI source are given in Table 4.

Table 1: HPLC Parameters

Column:	Inertsil ODS3 5 μ m, 100 x 3 mm i.d. (Chrompack); R ₂ guard column (Chrompack)
Mobile phase A:	5% acetonitrile, 50 ml MeCN, water <i>ad</i> 1000 ml, 10 ml ammonium acetate solution (1 mol/l), 1 ml formic acid
Mobile phase B:	30% isopropanol in acetonitrile, 300 ml isopropanol, acetonitrile <i>ad</i> 1000 ml, 10 ml ammonium acetate solution (1 mol/l), 1 ml formic acid
Mobile phase C:	50% dichloromethane in isopropanol, 500 ml isopropanol, dichloromethane <i>ad</i> 1000 ml, 10 ml ammonium acetate solution (1 mol/l), 1 ml formic acid
Temperature:	ca. 20 °C (conditioned laboratory)
Injection volume:	75 μ l

Table 2: HPLC Gradient

Time (min)	Flow (ml/min)	%A	%B	%C
0	0.7	70	30	
2	0.7	70	30	
15	0.7	5	95	
35	0.7	5	35	60
40	0.7	5	35	60
41	0.7	5	95	
45	0.7	70	30	

Table 3: Electrospray (ESI) Parameters

Mode:	positive (+)
Cap. Heater:	250 °C
Spray voltage:	4 kV
Sheath gas:	70 units
Aux. Gas:	15 units
Scan:	200 to 1750, 1 s/scan

Table 4: Atmospheric Pressure Chemical Ionization (APCI) Parameters

Mode:	positive (+)
Cap. Heater:	175 °C
Vaporizer:	450 °C
Corona:	5 μ A
Sheath gas:	70 units
Aux. Gas:	0 units
Scan:	200 to 1750, 1 s/scan

5 The injection sequence for samples was as follows. The mouse plasma extracts were injected twice in a random order. The human plasma extract was injected twice at the start of the sequence and after every 5 injections of the mouse plasma extracts to monitor the stability of the LC-MS conditions. The random sequence was applied to prevent the detrimental effects of possible drift on the multivariate statistics.

10 B.4. NMR of plasma and urine samples:

 NMR spectrometric measurements of plasma samples were made with a 400 MHz ^1H -NMR. Samples for the NMR were prepared and handled substantially in accord with the following protocol. Isopropanol plasma extracts (500 μl from 2.3.1) were dried under nitrogen, whereafter the residues were dissolved in deuterated methanol (MeOD). Deuterated methanol
15 was selected because it gave the best NMR spectra when chloroform, water, methanol and dimethylsulfoxide (all deuterated) were compared.

 NMR spectrometric measurements of urine samples were also made with a 400 MHz ^1H -NMR.

C. Spectrometric Measurements and Analysis

20 The following spectrometric measurements were made at metabolite/ small molecule level:

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- NMR-measurements of urine, multiple measurements (preferably triplicate measurements) on a total of 40 samples;
 - NMR- measurement of plasma, multiple measurements (preferably triplicate measurements) on a total of 40 samples; and
- 5 • LC/MS- measurement of plasma (plasmalipid profile), multiple measurements (preferably triplicate measurements) on a total of 40 samples.

A flow chart illustrating the analysis of the spectrometric data of this example according to one embodiment of the present invention is shown in Figures 2A and 2B.

Referring to Figure 2A, the spectrometric data obtained was grouped into eight data
10 sets **301-308**. The data sets were as follows: (1) data set 1 comprised 400 MHz ¹H-NMR spectra of wildtype mouse urine samples **301**; (2) data set 2 comprised 400 MHz ¹H-NMR spectra of APO E3 mouse urine samples **302**; (3) data set 3 comprised 400 MHz ¹H-NMR spectra of APO E3 mouse blood plasma samples **303**; (4) data set 4 comprised 400 MHz ¹H-NMR spectra of wildtype mouse blood plasma samples **304**; (5) data set 5 comprised LC-MS
15 spectra (using ESI) of wildtype mouse blood plasma lipid samples **305**; (6) data set 6 comprised LC-MS spectra (using ESI) of APO E3 mouse blood plasma lipid samples **306**; (7) data set 7 comprised LC-MS spectra (using APCI) of APO E3 mouse blood plasma lipid samples **307**; and (8) data set 8 comprised LC-MS spectra (using APCI) of wildtype mouse blood plasma lipid samples **308**. Examples of the spectrometric measurements obtained for each of these data
20 sets is as follows: Figures 3A and 4A for data set 1; Figures 3B and 4B for data set 2; Figures 5B and 6B for data set 3; Figures 5A and 6A for data set 4; Figure 7B for data set 5; and Figure 7A for data set 6. Various features were noted in the data of Figures 3A-7B.

Referring to Figures 3A and 3B, it was noted that peaks associated with hippuric acid **410** were observed in the wildtype mouse urine sample ¹H-NMR spectra, while such peaks
25 were substantially absent from the APO E3 mouse urine sample ¹H-NMR spectra, indicating a possible biochemical process unique to the APO E3 mouse. Referring to Figures 4A and 4B, in addition, peaks associated with an unidentified component **420** were observed in the wildtype mouse urine sample ¹H-NMR spectra, which were also substantially absent from corresponding ¹H-NMR spectra of the APO E3 mouse urine samples.

Referring to Figures 5A and 5B, a two series of peaks 510, 520 were observed in the APO E3 mouse blood plasma sample ¹H-NMR spectra, which were either substantially absent from the wildtype spectra 510 or substantially reduced 520. As shown in Figures 6A and 6B, the peaks associated with the first series of peaks 510 are substantially absent from the resonance shift region in wildtype spectra 610, while the second series of peaks 520 are present but reduced in the wildtype spectra 620.

Referring to Figures 7A and 7B, it was noted that peaks associated with lyso-phosphatidylcholines ("lyso-PC") 710 were slightly reduced in intensity in the APO E3 mouse spectra relative to those for the wildtype, that peaks associated with phospholipids 720 were substantially equal in intensity between the APO E3 and wildtype spectra, and that peaks associated with triglycerides 730 were substantially increased in intensity in the APO E3 mouse spectra relative to those for the wildtype.

The raw data from data sets 1 to 8 was preprocessed 320 and a first multivariate analysis was performed between data sets 1 and 2, 3 and 4, 5 and 6, and 7 and 8, respectively, each at a first level of correlation 330, i.e., PCA-DA score plots. Examples of the results of the first multivariate analysis at a first level of correlation are illustrated in Figures 8-11 for data sets 1 and 2; Figure 12 for data sets 3 and 4; and Figure 13 for data sets 5 and 6 (which includes data from human samples). Data from the first multivariate analysis was then used to produce an analysis at a second level of correlation 340, i.e., PCA-DA loading plots. An example of one such PCA-DA loading plot is shown in Figure 14.

Referring to Figure 8, a PCA-DA score plot of the NMR data for the urine samples of data sets 1 and 2 is shown. As illustrated, the analysis groups NMR data for APO E3 and wildtype group into two substantially distinct regions in the score plot, an APO E3 region 810 and a wildtype region 820, indicating that urine samples alone may suffice to develop a profile that reflects the transgenic nature of the APO E3 mice and serve as a bodyfluid biomarker profile for distinguishing APO E3 mice from other types of mice.

Referring to Figure 9, a score plot of the NMR data for the urine samples of data set 1 is shown. As illustrated, the analysis indicates that there are similarities and differences within the urine samples of data set 1 that correlate with urine color. Specifically, the analysis illustrates three distinct regions in the score plot correlated to deep brown urine 910, brown

urine 920, and yellow urine 930. Figure 9 illustrates that there are three distinct subgroups of mouse urine profiles in the wildtype mouse cohort.

Similarly in Figure 10, a score plot of the NMR data for the urine samples of data set 2 is shown. As illustrated, the analysis indicates that there are similarities and differences within the urine samples of data set 2 that correlate with urine color. Specifically, the analysis illustrates three regions in the score plot, one correlated to brown urine 1010, and another to pale brown urine 1020, that slightly overlaps with a yellow urine correlated region 1030. Figure 10 illustrates that there are three subgroups of mouse urine profiles in the APO E3 mouse cohort.

Referring to Figure 11, a PCA-DA score plot of the NMR data for the urine samples of both wildtype and APO E3 mice is shown. As illustrated, the analysis indicates that there are similarities and differences within the urine samples of data sets 1 and 2 even for urine with the same color. Specifically, the analysis illustrates three regions in the score plot, one correlated to yellow APO E3 mouse urine 1110, one to pale brown APO E3 mouse urine 1120, and another to yellow wildtype mouse urine 1130. Figure 11 illustrates that there are three distinct subgroups of mouse urine profiles which can be used as profiles to distinguish between APO E3 animals from wildtype animals, and to distinguish animals producing yellow urine from pale brown urine.

Referring to Figure 12, a PCA-DA score plot of the NMR data for the blood plasma samples of data sets 3 and 4 is shown. As illustrated, the analysis groups NMR data for APO E3 and wildtype group into two substantially distinct regions in the score plot, a wildtype region 1210 and an APO E3 region 1220, indicating that blood samples alone may be suffice to develop a profile that distinguishes APO E3 mice from wildtype mice.

Referring to Figure 13, a PCA-DA score plot of the NMR data for the blood plasma samples of data sets 5, 6 and the human samples is shown. As illustrated, the analysis groups NMR data regions corresponding to each organism type, a human region 1310, a wildtype region 1320 and an APO E3 region 1330. Figure 13 indicates that blood plasma samples may suffice to develop a profile that distinguishes organisms and genotypes. In one embodiment, information at a second level of correlation is obtained from the analysis illustrated in Figure 13 to investigate, for example, the contribution of each metabolite measured by the NMR technique to the segregation of the data into three regions. In one version a loading plot is used

to determine a second level of correlation. An example of a loading plot for axis D2 of Figure 13 is shown in Figure 14.

Referring to Figure 14 and 2A, four ranges of numbers are circled 1401-1404. The abscissa corresponds to masses (or mass-to-charge ranges). Points with positive values along the ordinate indicate component masses that are lower in abundance in the APO E3 mouse versus wildtype, and negative values indicate the reverse. As can be seen in Figure 14, the circled ranges are a significant contribution to the correlations of, for example, Figure 13. The mass chromatograms associated these regions were investigated 350 and the upper circled ranges 1401, 1403 found to be associated with lyso-phosphatidylcholines ("lyso-PC"), and the lower ranges 1402, 1404 with triglycerides. An example of the phosphatidylcholine mass chromatograms for both wildtype and APO E3 mouse are shown in Figure 15, and an example of the lyso-phosphatidylcholine mass chromatograms for both wildtype and APO E3 mouse are shown in Figure 16.

Referring to Figure 15, a series of peaks corresponding phosphatidylcholines, where n refers to the number of residues, is shown for both wildtype (thin solid line) and APO E3 (thick solid line) plasma samples. The chromatograms in Figure 15 are each normalized such that the maximum intensity of the n=3 peak 1510 is equal for all the spectra and it should be noted that although some n=1 is present, the majority of the signal corresponding to this peak location 1540 is not believed to arise from a phosphatidylcholine. As illustrated, it was observed that the peaks corresponding to n=5 1520, 1530 were substantially reduced in the APO E3 mouse spectra relative to wildtype.

Referring to Figure 16, a series of peaks corresponding lyso-phosphatidylcholines, where the designation x:y refers to x number of carbon atoms on the fatty acids and y carbon bonds, is shown for both wildtype (thin solid line) and APO E3 (thick solid line) plasma samples. The chromatograms in Figure 16 are each normalized such that the maximum intensity of peak 1610 is equal for all the spectra. As illustrated, it was observed that the peaks corresponding to arachidonic acid 1620, and linoleic acid 1630 were substantially reduced in the APO E3 mouse spectra relative to wildtype.

Referring again to Figures 2A and 2B, a second multivariate analysis was also performed ("YES" to query 360) comprising a canonical correlation. This second multivariate analysis was performed on data sets 3, 4, 5, and 6, 371, to produce a canonical correlation score

plot 381. An example of the results of this second multivariate analysis is shown in Figure 17. It should be noted that analysis 371 correlates data from two very different spectrometric techniques: data sets 3 and 4 from NMR, and 5 and 6 from LC-MS. Such an analysis, for example, may discern whether different information is being provided by such different techniques.

As illustrated in Figure 17, the canonical correlation groups both NMR and LC-MS results for the APO E3 mouse and wildtype mouse into two substantially distinct regions in the plot, a wildtype region 1710 and an APO E3 region 1720, indicating that both NMR and LC-MS techniques result in segregation into distinct regions, however the LC-MS method yielded a more pronounced separation.

A second multivariate analysis was performed on data sets 5, 6, 7 and 8, 372, to produce a canonical correlation score plot 382. An example of the results of this second multivariate analysis is shown in Figure 18. It should be noted that analysis 372 correlates data from in many respects the same spectrometric technique LC-MS, but different instrument configurations: data sets 5 and 6 using ESI, and 7 and 8 using APCI. Such an analysis, for example, may discern whether different information is being provided by such different instrument configurations. In addition, such a multivariate analysis may be used to discern whether different machines (that use the exact same instrumentation) provide different information. In cases where different machines provide significantly different information (on the same sample, using the same technique, parameters, and instrumentation) user or machine errors may be detected.

As illustrated in Figure 18, the canonical correlation groups both ESI LC-MS results (crosses +) and APCI LC-MS results (asterisks *) for the APO E3 mouse and wildtype mouse into two substantially distinct regions in the plot, a wildtype region 1810 and an APO E3 region 1820, indicating that both ESI LC-MS and APCI LC-MS techniques result in segregation into distinct regions.

While the invention has been particularly shown and described with reference to specific embodiments, it should be understood by those skilled in the art that various changes in form and detail may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. The scope of the invention is thus indicated by

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the appended claims and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced.

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CLAIMS

1. A method of profiling a state of a biological system in an animal through processing of a plurality of data sets, the method comprising the steps of:
 - (a) optionally preprocessing a plurality of data sets comprising spectrometric measurements of a sample of a biological system, wherein the spectrometric measurements are obtained using two different spectrometric techniques; and
 - (b) generating a profile of one or more biomarkers for a state of the biological system by evaluating the plurality of data sets with a multivariate analysis and correlating spectrometric features among the plurality of data sets to determine one or more sets of differences among at least a portion of the plurality of data sets.
2. The method of claim 1 wherein the plurality of data sets are derived from samples taken at different times for the same organism.
3. The method of claim 1 wherein the plurality of data sets are derived from one biological sample type.
4. The method of claim 1 wherein the plurality of data sets are derived from a single sample source that is treated differently.
5. The method of claim 1 wherein the two different spectrometric techniques comprise different instrument configurations of the same spectrometric technique.
6. The method of claim 1 wherein the spectrometric measurements comprise data from a spectrometric technique comprising at least one of liquid chromatography, gas chromatography, high performance liquid chromatography, capillary electrophoresis, nuclear magnetic resonance, ultraviolet spectrometry, liquid chromatography-ultraviolet spectrometry, mass spectrometry, liquid chromatography-mass spectrometry, gas chromatography-mass spectrometry, high performance liquid chromatography-mass spectrometry, capillary electrophoresis-mass spectrometry, mass spectrometry-mass spectrometry, and third order and higher mass spectrometry.

7. The method of claim 1 wherein the plurality of data sets comprise spectrometric measurements of a biomolecular component type.
8. The method of claim 7 wherein the biomolecular component type is a gene transcript, a protein, or a metabolite.
9. The method of claim 1, wherein the multivariate analysis comprises at least one of principal component analysis, discriminant analysis, principal component analysis with discriminant analysis, canonical correlation, kernel principal component analysis, non-linear principal component analysis, factor analysis, multidimensional scaling, and cluster analysis.
10. The method of claim 1, wherein the multivariate analysis comprises a hierarchical cascade of two or more multivariate analyses.
11. The method of claim 1, wherein the plurality of data sets are derived from one or more biological sample types comprising at least one of blood, blood plasma, blood serum, cerebrospinal fluid, bile acid, saliva, synovial fluid, pleural fluid, pericardial fluid, peritoneal fluid, feces, nasal fluid, ocular fluid, intracellular fluid, intercellular fluid, lymph fluid, and urine.
12. The method of claim 1, wherein the plurality of data sets are derived from one or more biological sample types comprising at least one of liver cells, epithelial cells, endothelial cells, kidney cells, prostate cells, blood cells, lung cells, brain cells, skin cells, adipose cells, tumor cells, and mammary cells.
13. The method of claim 1, wherein the profile comprises a biomarker.
14. The method of claim 1, comprising the step of comparing the profile to a database of profiles.
15. The method of claim 1, wherein step (b) comprises evaluating the plurality of data sets for differences arising from spectrometric measurement technique based on a quality factor for the data sets of two or more spectrometric measurement techniques.

16. The method of claim 1, wherein the state of the biological system comprises a disease state.

17. The method of claim 1, wherein the state of the biological system comprises a response to a pharmacological agent.

18. The method of claim 1, wherein the state of the biological system comprises a response to at least one of age, environment, and stress.

19. The method of claim 1, comprising measuring components in the sample to generate the spectrometric measurements.

20. The method of claim 1, comprising showing at least a portion of data relevant to the profile.

21. An article of manufacture having a computer-readable medium with computer-readable instructions embodied thereon for performing the method of claim 1.