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(54) Title: METHODS AND SYSTEMS FOR NON-DESTRUCTIVELY DETERMINING FERTILITY AND GENDER OF EGGS

(57) Abstract: This disclosure is directed to methods and systems for collecting, identifying and, in some instances quantifying, VOCs emitted from infertile, and fertile eggs. Determination of differences in VOC emissions by eggs by statistical analyses provide biological information regarding eggs such as fertilization-specific and gender-specific VOC biomarkers.

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METHODS AND SYSTEMS FOR NON-DESTRUCTIVELY DETERMINING FERTILITY AND GENDER OF EGGS

CROSS REFERENCE TO RELATED APPLICATION

[0001] The is application claims the benefit of U.S. Provisional Application No. 63/383,384 filed November 11, 2022.

FIELD OF THE INVENTION

[0002] The present disclosure generally relates to methods and systems for determining the fertility of an egg and the gender of a chick in-ovo.

BACKGROUND

[0003] Hatchability rates of incubated commercial broiler eggs generally range between 80-90%. The infertile or unhatched eggs represent a lost opportunity cost, as they take up valuable incubation space and energy that does not yield results. With the potential for achieving up to a 20% efficiency gain, technologies to non-destructively identify the fertility of an egg and determine the gender of a chick in-ovo could represent significant process and cost gains for the poultry industry. To date, this ability to do early detection in-ovo has proved elusive and very difficult.

SUMMARY

[0004] Various aspects of the present disclosure are directed to collecting, identifying and, in some instances quantifying, volatile organic compounds (VOCs) emitted from infertile, and fertile eggs to determine their differences by statistical analyses in order to explore potential biological information such as fertilization-specific and gender-specific VOC biomarkers. Methods of statistical analysis include, but are not limited to, Principal Component Analysis (PCA), Partial Least Squares (PLS), Linear Discriminant Analysis (LDA) and Canonical Discriminant Analysis (CDA). Current results indicate the ability to discriminate between fertilization status using headspace VOCs extracted. Compounds of particular significance in discriminating between fertilization status and/or gender may

span several chemical classes including, for example, aldehydes, alkanes, amines, ketones, and alcohols. In some instances, VOCs of chemical classes other than, or in addition to, aldehydes, alkanes, amines, ketones, and alcohols may also be of particular significance in discriminating between fertilization status and/or gender of eggs. Methods according to the disclosure exhibit the potential to non-destructively characterize and discriminate VOC biomarkers that differentiate fertilized and unfertilized and/or male and female eggs. Methods according to the disclosure can be used to develop fast, inline, and non-destructive tools to pre-screen eggs for fertility and, in some instances, gender identification before being passed on to incubation.

[0005] The present invention relates to a method for collecting VOCs and using gas chromatography (GC) coupled with mass spectrometry (MS) to determine egg properties such as fertility and gender. VOCs are extracted from poultry eggs using an absorbent material. In this work, stir base sorptive extraction (SBSE) was used. The preconcentrated VOCs are then analyzed using GC/MS or two-dimensional gas chromatography/mass spectrometry (GCxGC/MS). The resulting raw data is then subjected to statistical analysis allowing the differentiation of samples based on fertility status. In some instances, unique VOC profiles for male embryos can be used to isolate eggs that may not be suitable for certain purposes, such as raising, but may be suitable for other purposes such as table eggs or low-grade food stock.

[0006] In some instances, a first aspect of the disclosure can be described as a method of identifying infertile and fertile eggs, where the method comprises capturing volatile organic compounds (VOCs) emitted from each of a population of eggs; determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs; identifying infertile eggs from the population of eggs based upon a first VOC profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs; and identifying fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs.

[0007] In some instances, a second aspect of the disclosure can be described as a method according the first aspect, where the method further comprises determining amounts of one

or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

[0008] In some instances, a third aspect of the disclosure can be described as a method according the first or second aspect, where the first VOC profile and the second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

[0009] In some instances, a fourth aspect of the disclosure can be described as a method according any one of the first through third aspects, where the method further comprises determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs prior to incubation of said population of eggs.

[0010] In some instances, a fifth aspect of the disclosure can be described as a method of identifying fertile male eggs, where the method comprises capturing volatile organic compounds (VOCs) emitted from each of a population of eggs; determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs; identifying infertile eggs from the population of eggs based upon a first VOC profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs; identifying fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs; and identifying fertile male eggs from the fertile eggs based upon a third VOC profile, the third VOC profile corresponding to a set of the compositions of VOCs emitted by fertile male eggs.

[0011] In some instances, a sixth aspect of the disclosure can be described as a method according the fifth aspect, where the method further comprises determining amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

[0012] In some instances, a seventh aspect of the disclosure can be described as a method according the fifth or sixth aspect, where the first VOC profile and the second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

[0013] In some instances, an eighth aspect of the disclosure can be described as a method according any one of the fifth through seventh aspects, where the method further comprises

determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs prior to incubation of said population of eggs.

[0014] In some instances, a ninth aspect of the disclosure can be described as a method of identifying fertile female eggs, where the method comprises capturing volatile organic compounds (VOCs) emitted from each of a population of eggs; determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs; identifying infertile eggs from the population of eggs based upon a first VOC profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs; identifying fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs; and identifying fertile female eggs from the fertile eggs based upon a third VOC profile, the third VOC profile corresponding to a set of the compositions of VOCs emitted by fertile female eggs.

[0015] In some instances, a tenth aspect of the disclosure can be described as a method according the ninth aspect, where the method further comprises determining amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

[0016] In some instances, an eleventh aspect of the disclosure can be described as a method according the ninth or tenth aspect, where the first VOC profile and the second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

[0017] In some instances, a twelfth aspect of the disclosure can be described as a method according any one of the ninth through eleventh aspects, where the method further comprises determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs prior to incubation of said population of eggs.

[0018] In some instances, a thirteenth aspect of the disclosure can be described as a method of estimating fertility rate of a population of eggs, where the method comprises capturing volatile organic compounds (VOCs) emitted from each of a population of eggs during incubation of the eggs; determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs; identifying potentially infertile eggs from the population of eggs based upon a first VOC

profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs; and identifying potentially fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs.

[0019] In some instances, a fourteenth aspect of the disclosure can be described as a method according the thirteenth aspect, where the method further comprises determining amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

[0020] In some instances, a fifteenth aspect of the disclosure can be described as a method according the thirteenth or fourteenth aspect, where the first VOC profile and the second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

[0021] In some instances, a sixteenth aspect of the disclosure can be described as a method according any one of the thirteenth through fifteenth aspects, where the method further comprises determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs prior to incubation of said population of eggs.

[0022] In some instances, a seventeenth aspect of the disclosure can be described as a method according any one of the first through sixteenth aspects, where the method steps are performed at one or more timepoints during incubation.

[0023] In some instances, an eighteenth aspect of the disclosure can be described as a method according to the seventeenth aspect, where the he one or more timepoints include one or more of day 0, day 1, day 3, day 6, day 7, day 9, day 10, day 14 and day 15 of incubation.

[0024] In some instances, a nineteenth aspect of the disclosure can be described as a method according any one of the first through eighteenth aspects, where the method steps are performed at one or more time points prior to incubation and one or more timepoints during incubation.

[0025] In some instances, a twentieth aspect of the disclosure can be described as a method according any one of the first through nineteenth aspects, where the method is performed

non-destructively, without destroying or otherwise compromising the eggs in the population of eggs.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] In order that the present disclosure may be readily understood, aspects of the vial adapter are illustrated by way of examples in the accompanying drawings, in which like parts are referred to with like reference numerals throughout.

[0027] FIG. 1 is a schematic illustration of an exemplary volatile organic compound (VOC) collection apparatus according to various aspects of the present disclosure.

[0028] FIG. 2 is a graphical display showing the results of Principle Component Analysis for eggs' VOCs of different classes at day 0 of an incubation period.

[0029] FIG. 3 is a graphical display showing the results of Principle Component Analysis for eggs' VOCs of different classes at day 3 of an incubation period.

[0030] FIG. 4 is a graphical display showing the results of Principle Component Analysis for eggs' VOCs of different classes at day 9 of an incubation period.

[0031] FIG. 5 is a graphical display showing the results of Principle Component Analysis for eggs' VOCs of different classes at day 15 of an incubation period.

[0032] FIG. 6 is a graphical display showing abundance of hexanal, 4-methyl-octane and phenol for fertile, infertile and early dead eggs after 3 days of incubation.

[0033] FIG. 7 is a graphical display showing abundance of hexanal, methenamine and 3-methyl-butanol for fertile, infertile and early dead eggs after 15 days of incubation.

[0034] FIG. 8 is a color map showing VOCs emitted by infertile eggs or fertile eggs. The color map displays relative amounts of individual compounds, with the amounts of individual VOCs emitted increasing from blue to red color representations.

[0035] FIG. 9 is a graphical display showing the results of Principle Component Analysis for fertile eggs' VOCs of different classes during cold storage and at days 0, 3, 7, 10 and 14 of an incubation period.

[0036] FIG. 10 is a graphical display showing the results of Principle Component Analysis for infertile eggs' VOCs of different classes during cold storage and at days 0, 3, 7, 10 and 14 of an incubation period.

[0037] FIG. 11 is a graphical display showing the results of Principle Component Analysis of VOCs emission for fertile and infertile eggs at day 0 of an incubation period.

[0038] FIG. 12 is a graphical display showing the results of Principle Component Analysis of VOCs emission for fertile and infertile eggs at day 3 of an incubation period.

[0039] FIG. 13 is a graphical display showing the results of Principle Component Analysis of VOCs emission for fertile and infertile eggs at day 7 of an incubation period.

[0040] FIG. 14 is a graphical display showing the results of Principle Component Analysis of VOCs emission for fertile and infertile eggs at day 10 of an incubation period.

[0041] FIG. 15 is a graphical display comparing the amount of hexanal emitted from fertile and infertile eggs during cold storage and at days 0, 3, 7, 10 and 14 of an incubation period.

[0042] FIG. 16 is a graphical display comparing the amount of 1-octen-3-ol emitted from fertile and infertile eggs during cold storage and at days 0, 3, 7, 10 and 14 of an incubation period.

[0043] FIG. 17 is an illustration of classification results of a Random Forrest Test analysis for fertile and infertile eggs at day 0 of an incubation period.

[0044] FIG. 18 is an illustration of classification results of a Random Forrest Test analysis for fertile and infertile eggs at day 7 of an incubation period.

[0045] FIG. 19 is an illustration of classification results of a Random Forrest Test analysis for fertile and infertile eggs at day 14 of an incubation period.

DETAILED DESCRIPTION

[0046] The following description of the embodiments is merely exemplary in nature and is in no way intended to limit the subject matter of the present disclosure, their application, or uses.

[0047] It is noted that, as used in this specification and the appended claims, the singular forms “a,” “an,” and “the,” include plural references unless expressly and unequivocally limited to one referent. As used herein, the term “include” and its grammatical variants are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that can be substituted or added to the listed items. For example, as used in this specification and the following claims, the terms “comprise” (as well as forms, derivatives, or variations thereof, such as “comprising” and “comprises”), “include” (as

well as forms, derivatives, or variations thereof, such as "including" and "includes") and "has" (as well as forms, derivatives, or variations thereof, such as "having" and "have") are inclusive (i.e., open-ended) and do not exclude additional elements or steps. Accordingly, these terms are intended to not only cover the recited element(s) or step(s), but may also include other elements or steps not expressly recited. Furthermore, as used herein, the use of the terms "a" or "an" when used in conjunction with an element may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." Therefore, an element preceded by "a" or "an" does not, without more constraints, preclude the existence of additional identical elements.

[0048] Various aspects of the present disclosure are directed to collecting, identifying and optionally quantifying the amount of individual volatile organic compounds (VOCs) emitted from a population of eggs prior to and/or at various timepoints during incubation of said population of eggs. VOC data, specifically the identity and quantity one or more VOCs emitted from a population of eggs, can be subjected to various forms of statistical analysis to determine fertilization-specific and gender-specific VOC biomarkers and/or profiles. Individual eggs within the population of eggs can then be identified as fertile or infertile and male or female based upon VOC biomarkers exhibited by said individual eggs. Methods of statistical analysis include, but are not limited to Principal Component Analysis (PCA), Partial Least Squares (PLS), Linear Discriminant Analysis (LDA), Canonical Discriminant Analysis (CDA), Hierarchical Clustering and Random Forrest analysis. Current results indicate the ability to discriminate between fertilization status of eggs, specifically poultry eggs, using headspace VOCs extracted and analyzed by various techniques. Compounds of particular significance in discriminating between fertilization status and gender of eggs may include various chemical classes such as, but not limited to, aldehydes, alkanes, amines, ketones, and alcohols. In some instances, VOCs of chemical classes other than, or in addition to, aldehydes, alkanes, amines, ketones, and alcohols may also be of particular significance in discriminating between fertilization status and/or gender of eggs.

[0049] Methods according to the disclosure have the ability to non-destructively characterize and discriminate VOC biomarkers that differentiate between fertilized and unfertilized eggs and between male and female eggs. Methods according to the disclosure

can be used to develop fast, in-line, and non-destructive tools to pre-screen eggs for fertility status and optionally gender identification before being passed on to incubation and at various stages during incubation.

[0050] In some instances, populations of eggs according to various aspects of the disclosure can be eggs of a single breed of chicken or of multiple breeds of chickens. In some instances, populations of eggs according to various aspects of the disclosure can be eggs from single poultry farm or hatchery. In some instances, populations of eggs according to various aspects of the disclosure can be eggs from a plurality of poultry farms or hatcheries in a common region, such as a city, county or state. In some instances, populations of eggs according to various aspects of the disclosure can be eggs laid from a specified set of laying hens. In some instances, populations of eggs according to various aspects of the disclosure can be eggs laid from a specified set of laying hens having a common rooster. In some instances, populations of eggs according to various aspects of the disclosure can be eggs laid from a specified set of laying hens having a common diet. In some instances, populations of eggs according to various aspects of the disclosure can be eggs laid from a specified set of laying hens that are housed under common environmental conditions (e.g., temperature, humidity, caged, cage-free, and so on). In some instances, populations of eggs according to various aspects of the disclosure can be eggs stored under common environmental conditions (e.g., temperature, humidity, light and so on) pre-incubation. In some instances, populations of eggs according to various aspects of the disclosure can be eggs stored under common environmental conditions (e.g., temperature, humidity, light and so on) during incubation. In some instances, populations of eggs according to various aspects of the disclosure can be eggs stored under a first set of common environmental conditions (e.g., temperature, humidity, light and so on) pre-incubation and under a second set of common environmental conditions during incubation. Generally, one or ordinary skill in the art may choose to evaluate any population of eggs based upon any one or more traits specific to an egg population.

[0051] In some instances, unique VOC profiles for eggs can be used to isolate eggs that may not be suitable for certain purposes, such as raising, but may be suitable for other purposes such as table eggs or low-grade food stock. In some instances, unique VOC profiles for eggs (and by extension the amount of fertile or infertile and male or female

eggs) in a tested population of eggs can be used by poultry farmers or hatcheries as a guide for making changes to their egg laying process, their pre-incubation process, their incubation process, or any combination thereof. For example, poultry farmers or hatcheries may be guided to change one or more of the diet, surrounding environment or rooster(s) of a population of laying hens, or discontinue the use of certain laying hens, based upon an obtained set of VOC biomarkers or a VOC profile of a tested population of eggs indicating an unacceptable percentage of fertile or infertile eggs. Generally, one or ordinary skill in the art may choose to make numerous decisions as to how to modify their poultry farming procedures based upon any particular result from subjecting populations of their eggs to VOC testing and statistical analysis procedures as described herein.

[0052] According to various aspects of the disclosure, methods for collecting volatile organic compounds (VOCs) use GC/MS to determine egg properties such as fertility and gender. VOCs can be extracted or emitted from poultry eggs and collected using an absorbent material. The preconcentrated VOCs are then analyzed using GC/MS or GCxGC/MS. The resulting raw data of the analyzed VOCs can then be subjected to statistical analysis to generate a VOC profile or VOC biomarkers specific to or indicating the statistical likelihood of the fertility status and optionally gender identification of each individual egg.

[0053] To measure the VOC's emitted from an egg, any non-destructive methodology may be utilized. In some instances, VOCs emitted from an eggs can be measured by sealing an egg in a sample jar and collecting the VOCs present in the headspace of sample jar for subsequent analyses such as, but not limited to characterization and quantification. In some instances, VOCs can be collected from the headspace using, for example a Thin Films Solid Phase Micro Extraction (TF-SPME) device or a Twister® (Stir Bar Sorptive Extraction (SBSE), made by Gerstel) device located within the sealed sample jar. In some instances, VOCs can be collected from the headspace using, for example a Solid Phase Micro Extraction (SPME) device that is insertable into the headspace from a port located on a surface, preferably a septum in the lid, of the sealed sample jar. FIG. 1 is a schematic illustration of sealed VOCs collection sample jar having 1) an egg located therein and 2) one or more of a TF-SPME, an SBSE and an SPME incorporated therewith.

[0054] According to various aspects of the disclosure, a method of identifying infertile and fertile eggs includes capturing volatile organic compounds (VOCs) emitted from each of a population of eggs; determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during, and optionally prior to, incubation of said population of eggs; identifying infertile eggs from the population of eggs based upon a first VOC profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs; and identifying fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs. In some instances, the method further incudes determining amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs prior to and during incubation of said population of eggs. In some instances, the first VOC profile and the second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during prior to and during incubation of said population of eggs.

[0055] According to various aspects of the disclosure, a method of identifying fertile male eggs includes capturing volatile organic compounds (VOCs) emitted from each of a population of eggs; determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during, and optionally prior to, incubation of said population of eggs; identifying infertile eggs from the population of eggs based upon a first VOC profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs; identifying fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs; and identifying fertile male eggs from the fertile eggs based upon a third VOC profile, the third VOC profile corresponding to a set of the compositions of VOCs emitted by fertile male eggs. In some instances, the method further includes determining amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during, and optionally prior to, incubation of said population of eggs. In some instances, the first VOC profile and the second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs

from the population of eggs during, and optionally prior to, incubation of said population of eggs.

[0056] According to various aspects of the disclosure, a method of identifying fertile female eggs includes capturing volatile organic compounds (VOCs) emitted from each of a population of eggs; determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during, and optionally prior to, incubation of said population of eggs; identifying infertile eggs from the population of eggs based upon a first VOC profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs; identifying fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs; and identifying fertile female eggs from the fertile eggs based upon a third VOC profile, the third VOC profile corresponding to a set of the compositions of VOCs emitted by fertile female eggs. In some instances, the method further includes determining amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during, and optionally prior to, incubation of said population of eggs. In some instances, the first VOC profile and the second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during, and optionally prior to, incubation of said population of eggs.

[0057] According to various aspects of the disclosure, a method of estimating fertility rate of a population of eggs comprises capturing volatile organic compounds (VOCs) emitted from each of a population of eggs during incubation of the eggs; determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs; identifying potentially infertile eggs from the population of eggs based upon a first VOC profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs; and identifying potentially fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs. In some instances, the method further incudes determining amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs prior to and during incubation of said population of eggs. In some instances, the first VOC profile and the

second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during prior to and during incubation of said population of eggs.

[0058] As discussed above, compounds of particular significance in discriminating between fertilization status and gender of eggs include various chemical classes such as, but not limited to, aldehydes, alkanes, amines, ketones, and alcohols. Even within these general chemical classes, different breeds of chicken eggs may exhibit unique VOC profiles due to the emissions of specific VOCS prior to and/or during incubation. As such, a first breed of chicken eggs may be found to emit a first set of one or more specific VOCs which provides said first breed of chicken eggs with a first set of VOC biomarker profiles for fertile, infertile, male and/or female eggs. A second breed of chicken eggs, on the other hand, may be found to emit a second set of one or more specific VOCs which provides said second breed of chicken eggs with a second set of VOC biomarker profiles for fertile, infertile, male and/or female eggs. Generally, as may be appreciated by one of ordinary skill in the art, the present invention is capable of determining a first breed of chicken eggs may be found to emit a first set of one or more specific VOCs which provides said first breed of chicken eggs with a first set of VOC biomarker profiles for fertile, infertile, male and/or female eggs within any particular population of eggs.

[0059] In some instances, specific VOCs we have observed to be useful to generate VOC biomarker profiles for fertile, infertile, male and/or female eggs include, but are not limited to, hexanal, octanal, decanal, nonanal, hexadecanal, 3-methyl-butanal, 1-octen-3-ol, phenol, 4-methyloctane, and methenamine.

[0060] According to various aspects of the disclosure, the methods disclosed herein can be performed non-destructively, without destroying or otherwise compromising the analyzed eggs.

[0061] Example 1

[0062] One object of the invention is to identify and characterize the volatile organic compounds (VOCs) emitted from infertile and fertile eggs to determine their differences using Principal Component and Linear Discriminant analyses in order to explore potential biological information such as fertilization-specific and gender-specific VOC biomarkers.

[0063] Fifteen infertile eggs and fifteen fertile eggs from non-inseminated and inseminated hens respectively were collected from the University of Georgia Poultry Research Center. Each egg was independently placed inside 250mL glass jars where VOCs emitted from the eggs were collected for one hour using two Stir Bar Sorptive Extraction (SBSE) devices (Twister®) that were magnetically attached to the lid. VOCs sampling was conducted prior to incubation as well as on incubation days 0, 3, 6, 9, 12 and 15 respectively.

[0064] VOCs were desorbed from the Twisters® using a thermal desorption unit (TDU) coupled to a cooled injection system (CIS) which serves both as a cryo-focusing trap as well as a temperature programmable GC inlet. Desorbed VOCs were then analyzed using a two-dimensional gas chromatograph coupled to a time-of-flight mass spectrometer (GCxGC/TOF-MS) in a non-targeted approach. In some instances, Agilent 7250 Accurate-Mass Quadrupole Time-of-flight GC/MS system (Agilent Technologies, Palo Alto, USA), coupled with Gerstel Thermal Desorption Unit (TDU 2)/CIS system (Gerstel, Inc., Linthicum, MD, USA)were employed to carry out the VOC sample analysis.

[0065] LECO® ChromaTOF® software was used for data acquisition. Compounds were identified using the NIST 2017 mass spectral database within LECO® ChromaTOF TILE®. Additionally, class comparison and Principle Component Analysis (PCA) were also conducted using the same software package. PCA results indicated the ability to discriminate between fertilization statuses using headspace VOCs extracted with this method. Compounds of particular significance (or Most Influential Compounds) in discriminating between fertilization statuses spanned several chemical classes including aldehydes, alkanes, ketones, and alcohols. As illustrated herein, the combination of SBSE and GCxGC/TOF-MS has the ability to non-destructively characterize and discriminate VOC.

[0066] *Fertility Analyses*. Principle Component Analysis (PCA), Hierarchical Clustering and Linear Discriminant Analysis (LDA) were used to separate eggs into two classes: Fertile and Infertile based on respective VOC signatures. FIGS. 2-5 show the results of PCA for eggs' VOCs of different classes throughout the at Days 0, 3, 9 and 15, respectively, of the incubation period. Increasing separation between VOC signatures of Fertile and Infertile eggs correlates with increase in incubation days. Other classification techniques (Hierarchical Clustering and LDA) show similar outcomes. Inspection of PCA loading

plots allowed for identification of compounds that are most influential for classification models that assign a sample VOC to an appropriate class.

[0067] FIGS. 6 and 7 are graphical displays showing identified Most Influential Compounds at days 3 and 15 of incubation, respectively, of fertile, infertile and early dead eggs. FIG. 6 shows the abundance of hexanal, 4-methyl-octane and phenol for fertile, infertile and early dead eggs after 3 days of incubation. FIG. 7 shows the abundance of hexanal, methenamine and 3-methyl-butanol for fertile, infertile and early dead eggs after 15 days of incubation.

[0068] Based upon the identification and quantification of VOCs emitted by infertile and fertile eggs, color maps illustrating VOCs emitted by infertile eggs, fertile eggs, or neither infertile nor fertile eggs can be generated. Such color maps can display relative amounts of individual VOC compounds, with blue representing a low amount of a particular VOC, while the sample colors are indicative of fertility status with red and yellow representing fertile and infertile respectively. FIG. 8 is an illustration of exemplary color map.

[0069] **Example 2**

[0070] Sixty eggs of unknown fertility (fertile or infertile) status were independently placed inside a corresponding 185mL glass jar (without lid) and stored at 18.3°C (i.e., cold storage) for seven days prior to incubation. VOCs emitted from each egg were collected during this storing period by magnetically attaching a Twister® to the inside of a lid and sealing the jar for 1 hour. Prior to incubation, the eggs were allowed to warm to room temperature for two hours, during which VOCs were collected in the same manner.

[0071] During incubation, VOC sampling was conducted on incubation days 3, 7, 10, and 14 in the same manner for each egg. Overall, three VOC collection temperatures were used, refrigerated, room temperature, and incubation temperature to cover all aspects of the incubation process. VOCs were desorbed from the Twisters® using a thermal desorption unit (TDU) coupled to a cooled injection system (CIS). Desorbed VOCs were then analyzed using two-dimensional gas chromatography-mass spectrometry (GCxGC/TOFMS) in a non-targeted approach. On day 15 of incubation, the fertility status of each egg was determined by visual examination.

[0072] LECO® ChromaTOF® software was used for data acquisition. Compounds were identified using the NIST 2017 mass spectral database within LECO® ChromaTOF

TILE®. Linear Discriminant Analysis (LDA) was utilized to classify eggs into "Fertile" and "Infertile" categories. Preliminary results have shown a good performance on a training dataset (0% misclassified on day 7 of incubation) and promising results on a validation set (20% misclassified) in Random Forrest tests. Additionally, a trend among fertile and infertile groups is visible in PCA results as early as day 0. Several chemical classes including aldehydes, alkanes, ketones, and alcohols were again shown to be of significant importance in assessing the fertility status of eggs.

[0073] *Fertility Analyses*. Principle Component Analysis (PCA), Hierarchical Clustering, Linear Discriminant Analysis (LDA) and Random Forrest Analysis were used to separate eggs into fertile and infertile classes based on respective VOC signatures. FIGS. 9 and 10 show the combined results of PCA for eggs' VOCs of different classes during cold storage and throughout incubation at days 0, 3, 7, 10 and 14 for the fertile (FIG. 9) and infertile (FIG. 10) eggs. FIGS. 11-14 are plots showing PCA results for fertile and infertile eggs (combined in each graph) at days 0, 3, 7, 10 of incubation. As can be seen in FIGS. 9-14, increasing separation between VOC signatures of fertile and infertile eggs correlates with increase in incubation days. Hierarchical Clustering and LDA analyses showed similar outcomes. Inspection of PCA loading plots allowed for identification of compounds that are most influential for classification models that assign a sample VOC to an appropriate class.

[0074] *Infertile/Fertile Eggs Comparison by VOC Emission Analysis*

[0075] In Example 2, among the VOCs emitted by the eggs during cold storage and the incubation period, hexanal and 1-octen-3-ol abundances were found to be surprisingly telling as to the fertility status of eggs. FIG. 15 is a graphical display comparing the amount of hexanal emitted from fertile and infertile eggs during cold storage and at days 0, 3, 7, 10 and 14 of an incubation period. As shown in FIG. 15, both fertile and infertile eggs emitted similar amounts of hexanal during cold storage and through day 3 of incubation. On days 7-14 of incubation, however, it became clear that infertile eggs emitted approximately 3-9-times more hexanal than fertile eggs. FIG. 16 is a graphical display comparing the amount of 1-octen-3-ol emitted from fertile and infertile eggs during cold storage and at days 0, 3, 7, 10 and 14 of an incubation period. As shown in FIG. 16, infertile eggs emitted

increasing amounts of 1-octen-3-ol during incubation while fertile eggs, on the other hand did not emit 1-octen-3-ol at any stage of the incubation period.

[0076] FIGS. 17-19 and Table 1 illustrate classification results of a Random Forrest Test analysis for fertile and infertile eggs at days 0, 7 and 14 of the incubation period, respectively, using a subset of the eggs tested in Example 2. The Random Forest analysis showed 86% accuracy rate with 1 fertile egg was misclassified for the day 7 incubation data, and 100% accuracy rate for the day 14 incubation data.

Day	Source	Cross Validation Score (Train)	Accuracy (Test)
0	Training (90%)/Test (10%)	82%	85%
7		87%	86%
14		91%	100%

Table 1.

[0077] From the results of Example, multiple conclusions can be drawn. First, PCA (as well as Hierarchical Clustering and LDA) can effectively separate eggs into fertile and infertile classes, with increasing separation correlating with increase in incubation days, based on VOC signature. Second, Random Forest analysis can be used to predict egg fertility status with high accuracy and increasing accuracy with increasing incubation time. Third, while the emission of various VOCs can be monitored, accurate discrimination between fertile and infertile eggs can, in some instances, be effective by analysis of a limited number of VOCs (for example, 1 or 2 VOCs).

[0078] The following references, which have been cited in this application, may be pertinent background:

[0079] 1) Adegbenjo, A.O. et al., "Non-Destructive Assessment of Chicken Egg Fertility", *Sensors*, 2020, 20, 5546; doi:10.3390/s20195546;

[0080] 2) Xiang, X. et al., "Nondestructive characterization gender of chicken eggs by odor using SPME/GC-MS coupled with Chemometrics", *Poultry Science*, March 2022, Vols 101, Issue 3, 101619, doi: 10.1016/j.psj.2021.101619; and

[0081] 3) Gabbai, N., "A system and method for non-invasively determining egg properties", WO 2019/021275 A1.

[0082] While certain implementations have been described in terms of what may be considered to be specific aspects, the present disclosure is not limited to the disclosed aspects. Additional modifications and improvements to the aforementioned vial adapter may be apparent to those skilled in the art. Moreover, the many features and advantages of the disclosure are apparent from the detailed specification, and thus, it is intended by the appended claims to cover all such features and advantages of the present disclosure which fall within the spirit and scope of the disclosure.

CLAIMS

What is claimed is:

1. A method of identifying infertile and fertile eggs, the method comprising:

capturing volatile organic compounds (VOCs) emitted from each of a population of eggs;

determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs;

identifying infertile eggs from the population of eggs based upon a first VOC profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs; and

identifying fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs.

2. The method of claim 1, further comprising determining amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

3. The method of claim 1, wherein the first VOC profile and the second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

4. The method of claim 1, further comprising determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs prior to incubation of said population of eggs.

5. A method of identifying fertile male eggs, the method comprising:

capturing volatile organic compounds (VOCs) emitted from each of a population of eggs;

determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs;

identifying infertile eggs from the population of eggs based upon a first VOC profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs;

identifying fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs; and

identifying fertile male eggs from the fertile eggs based upon a third VOC profile, the third VOC profile corresponding to a set of the compositions of VOCs emitted by fertile male eggs.

6. The method of claim 5, further comprising determining amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

7. The method of claim 5, wherein the first VOC profile and the second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

8. The method of claim 5, further comprising determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs prior to incubation of said population of eggs.

9. A method of identifying fertile female eggs, the method comprising:

capturing volatile organic compounds (VOCs) emitted from each of a population of eggs;

determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs;

identifying infertile eggs from the population of eggs based upon a first VOC profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs;

identifying fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs; and

identifying fertile female eggs from the fertile eggs based upon a third VOC profile, the third VOC profile corresponding to a set of the compositions of VOCs emitted by fertile female eggs.

10. The method of claim 9, further comprising determining amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

11. The method of claim 9, wherein the first VOC profile and the second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

12. The method of claim 9, further comprising determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs prior to incubation of said population of eggs.

13. A method of estimating fertility rate of a population of eggs, the method comprising:

capturing volatile organic compounds (VOCs) emitted from each of a population of eggs during incubation of the eggs;

determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs;

identifying potentially infertile eggs from the population of eggs based upon a first VOC profile, the first VOC profile corresponding to a set of the compositions of VOCs emitted by infertile eggs; and

identifying potentially fertile eggs from the population of eggs based upon a second VOC profile, the second VOC profile corresponding to a set of the compositions of VOCs emitted by fertile eggs.

14. The method of claim 13, further comprising determining amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

15. The method of claim 13, wherein the first VOC profile and the second VOC profile further incorporate amounts of one or more of the individual VOCs emitted by each of the eggs from the population of eggs during incubation of said population of eggs.

16. The method of claim 13, further comprising determining the compositions of individual VOCs emitted by each of the eggs from the population of eggs prior to incubation of said population of eggs.

17. The method of any one of claims 1 to 16, wherein the method steps are performed at one or more timepoints during incubation.

18. The method of claim 17, wherein the one or more timepoints include one or more of day 0, day 1, day 3, day 6, day 7, day 9, day 10, day 14 and day 15 of incubation.

19. The method of any one of claims 1 to 18, wherein the method steps are performed at one or more time points prior to incubation and one or more timepoints during incubation.

20. A method according to any one of claims 1 to 19, wherein the method is performed non-destructively, without destroying or otherwise compromising the eggs in the population of eggs.

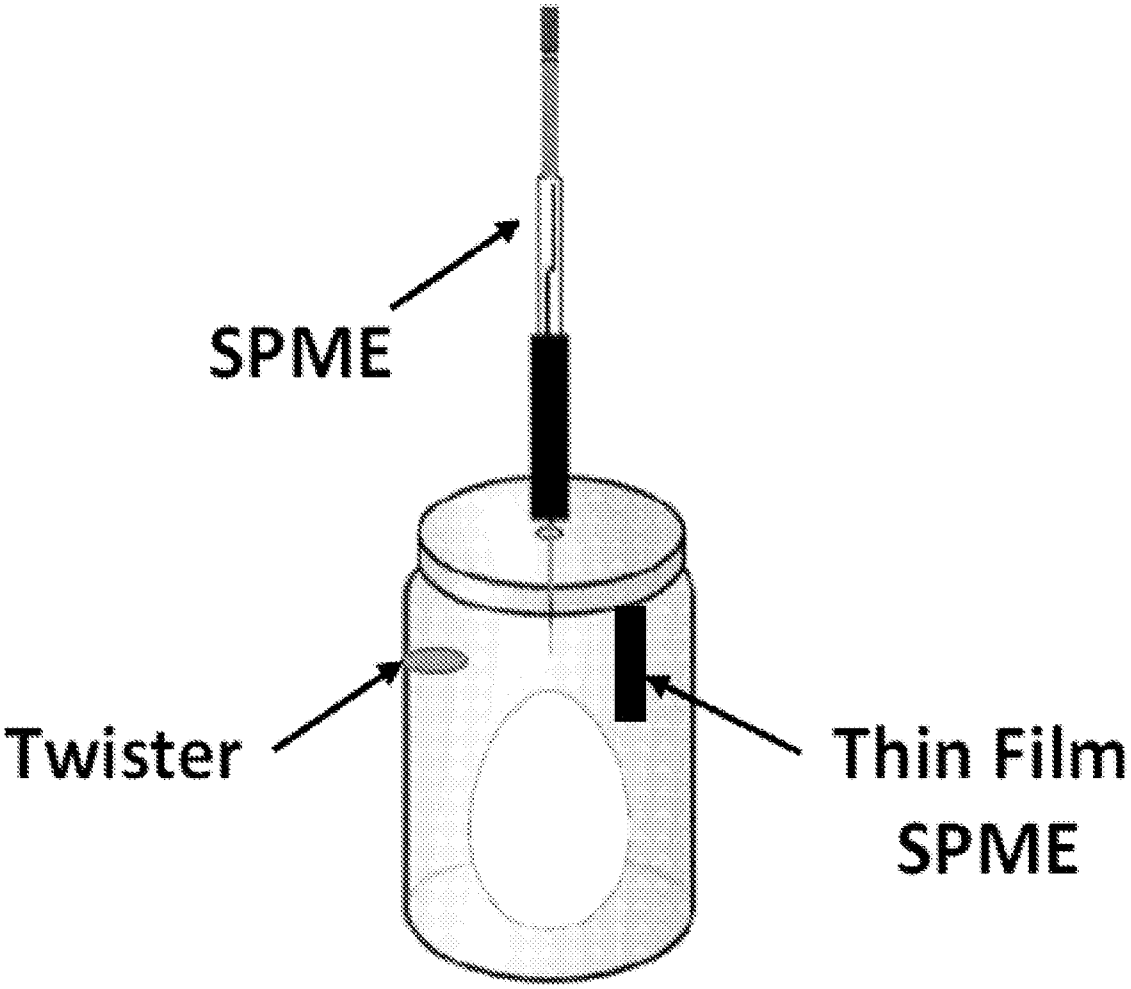

FIG. 1

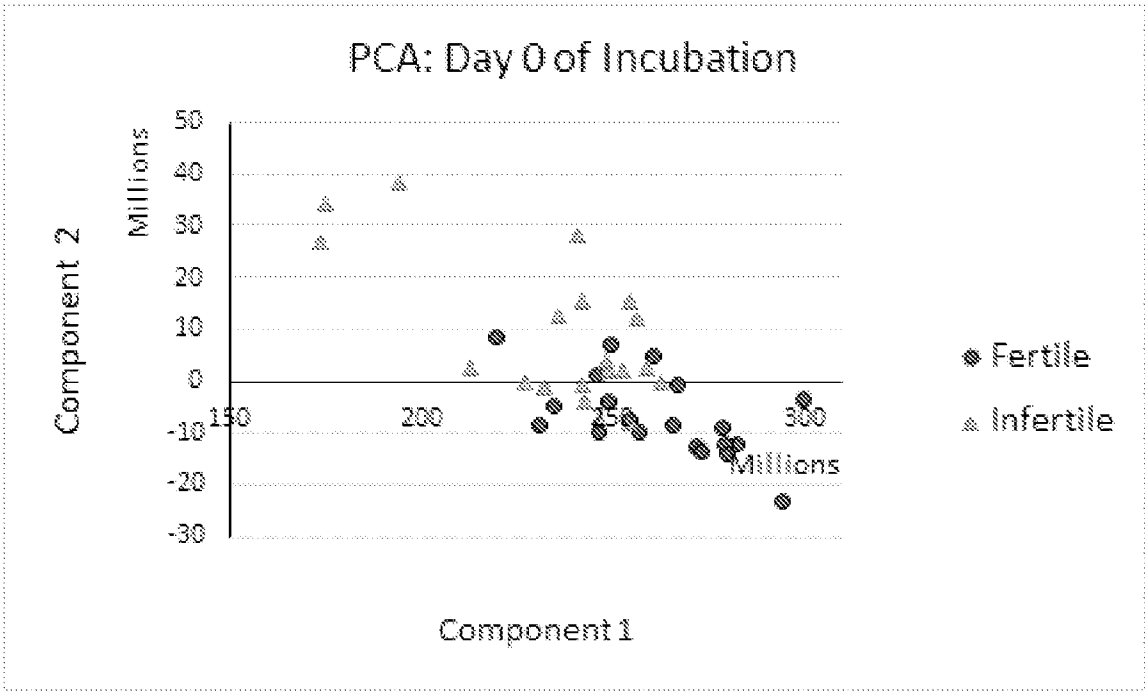
PCA: Day 0 of Incubation
Component 2
Millions
50
40
30
20
10
0
-10
-20
-30
150
200
300
Millions
Component 1
Fertile
Infertile

FIG. 2

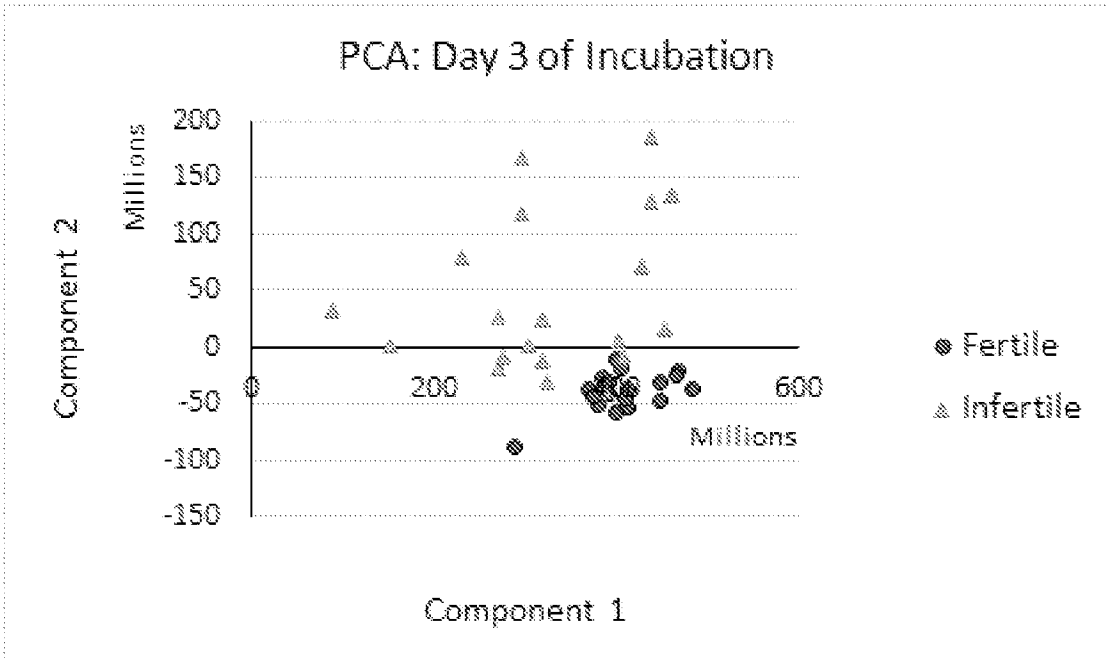
PCA: Day 3 of Incubation
Component 2
Millions
200
150
100
50
0
-50
-100
-150
0
200
600
Millions
Component 1
Fertile
Infertile

FIG. 3

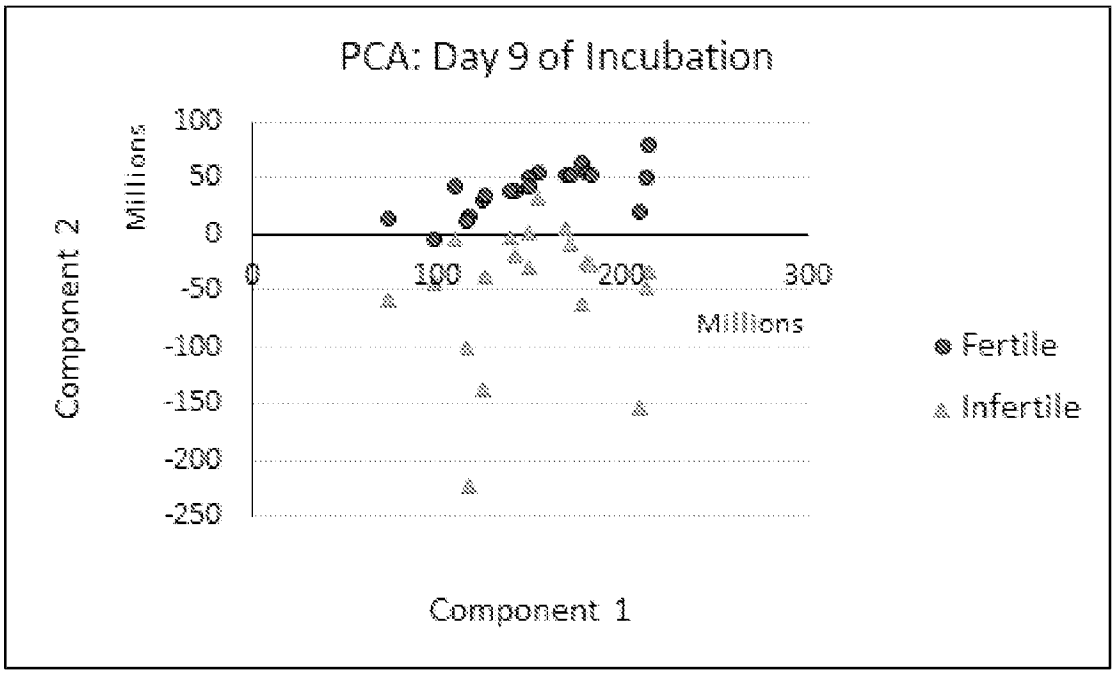
PCA: Day 9 of Incubation
Component 2
Millions
100
50
0
-50
-100
-150
-200
-250
0
100
200
300
Millions
Component 1
Fertile
Infertile

FIG. 4

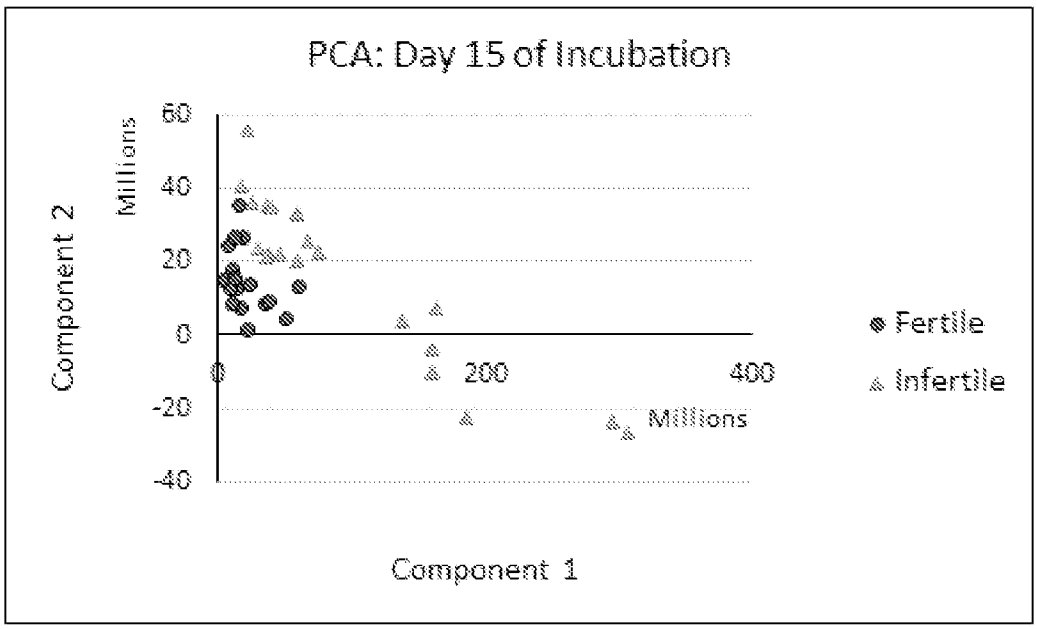
PCA: Day 15 of Incubation
Component 2
Millions
60
40
20
0
-20
-40
0
200
400
Millions
Component 1
Fertile
Infertile

FIG. 5

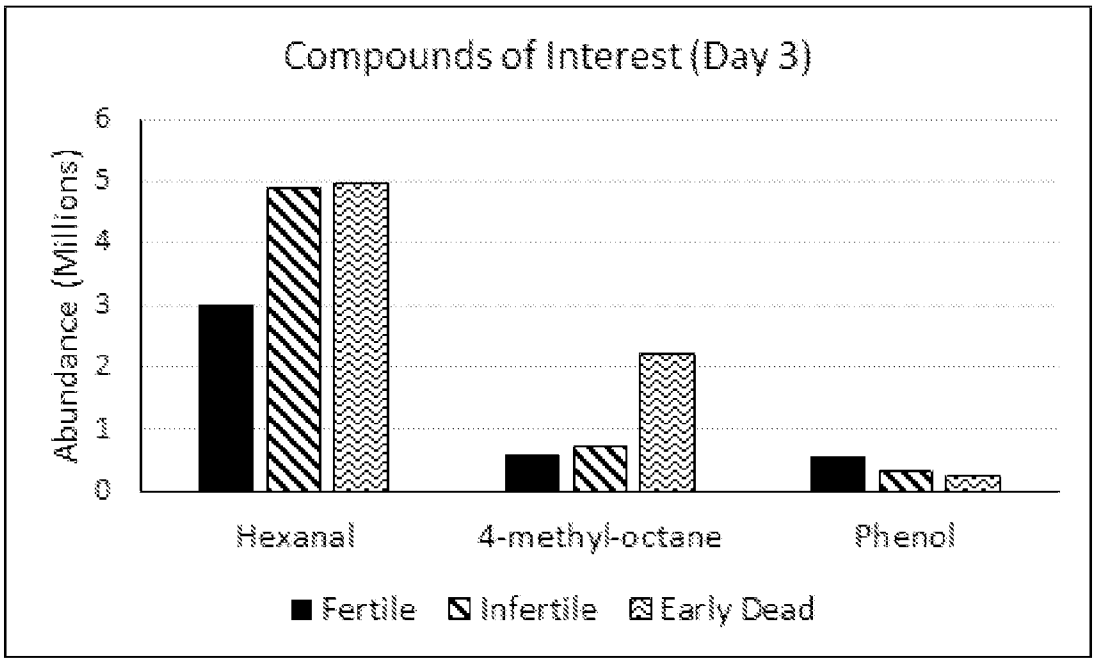
Compounds of Interest (Day 3)
Abundance (Millions)
0
1
2
3
4
5
6
Hexanal
4-methyl-octane
Phenol
Fertile
Infertile
Early Dead

FIG. 6

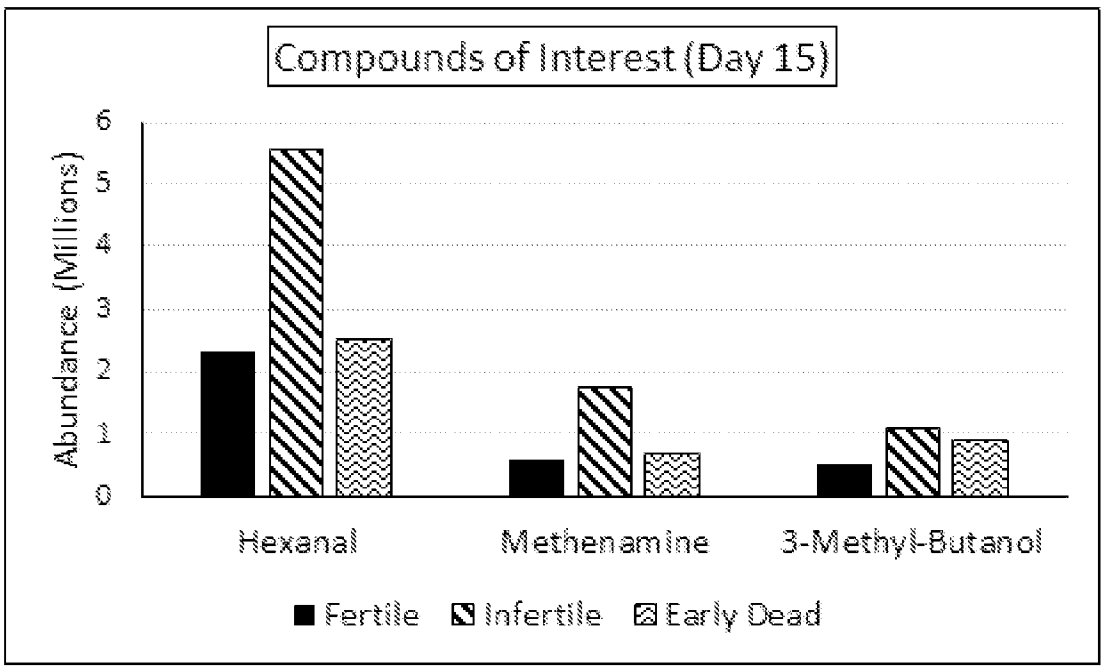
Compounds of Interest (Day 15)
Abundance (Millions)
0
1
2
3
4
5
6
Hexanal
Methenamine
3-Methyl-Butanol
Fertile
Infertile
Early Dead

FIG. 7

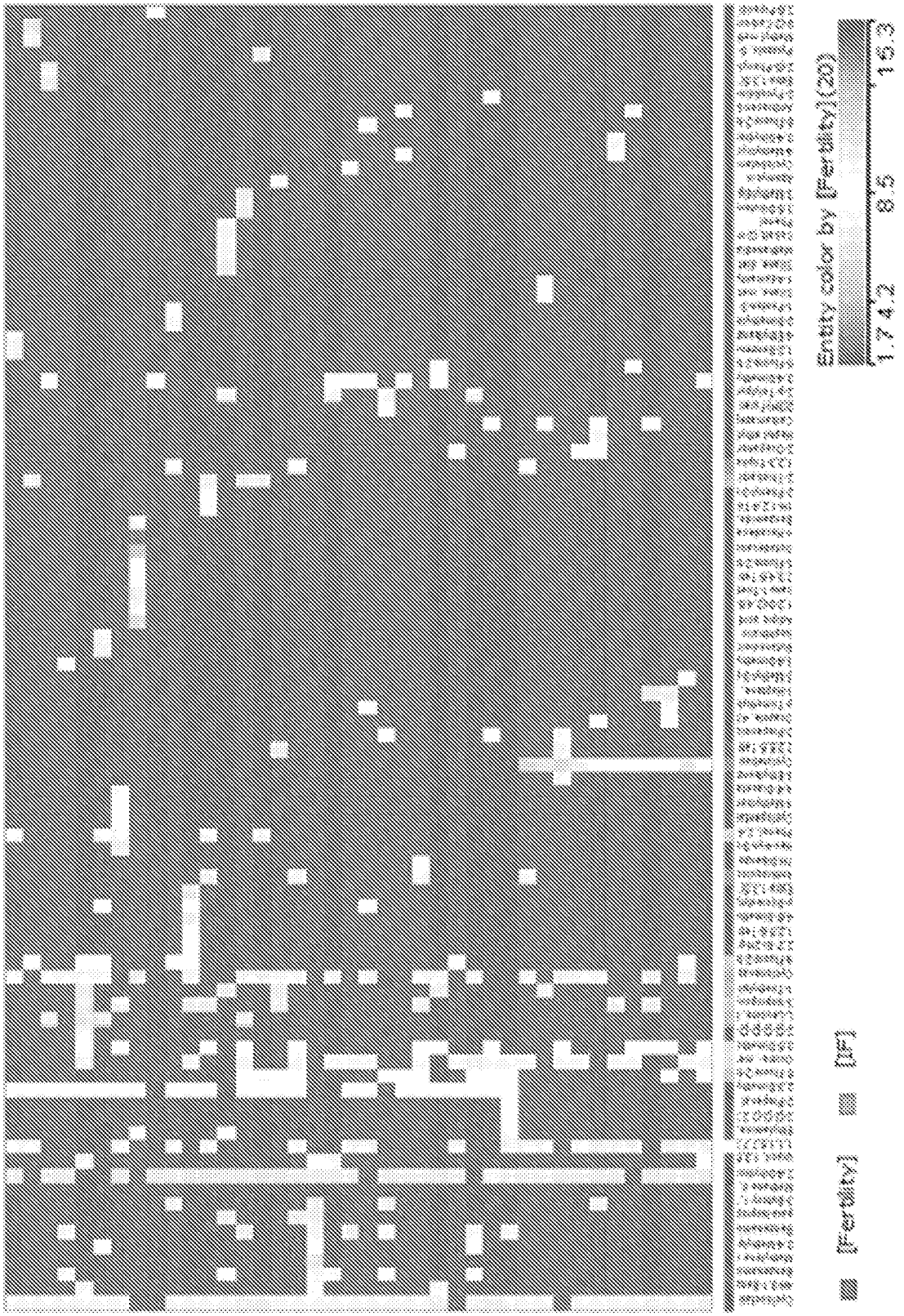

FIG. 8

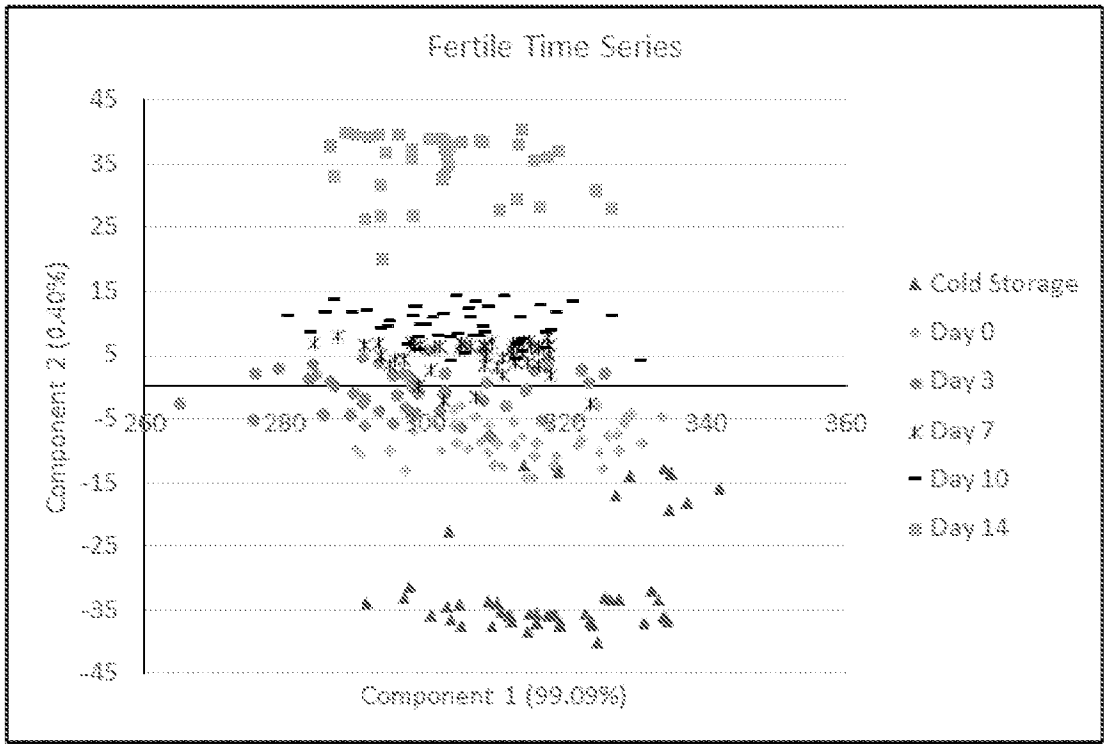
Fertile Time Series
Component 2 (0.40%)
Component 1 (99.09%)
45
35
25
15
5
-5
-15
-25
-35
-45
260
280
300
320
340
360
Cold Storage
Day 0
Day 3
Day 7
Day 10
Day 14

FIG. 9

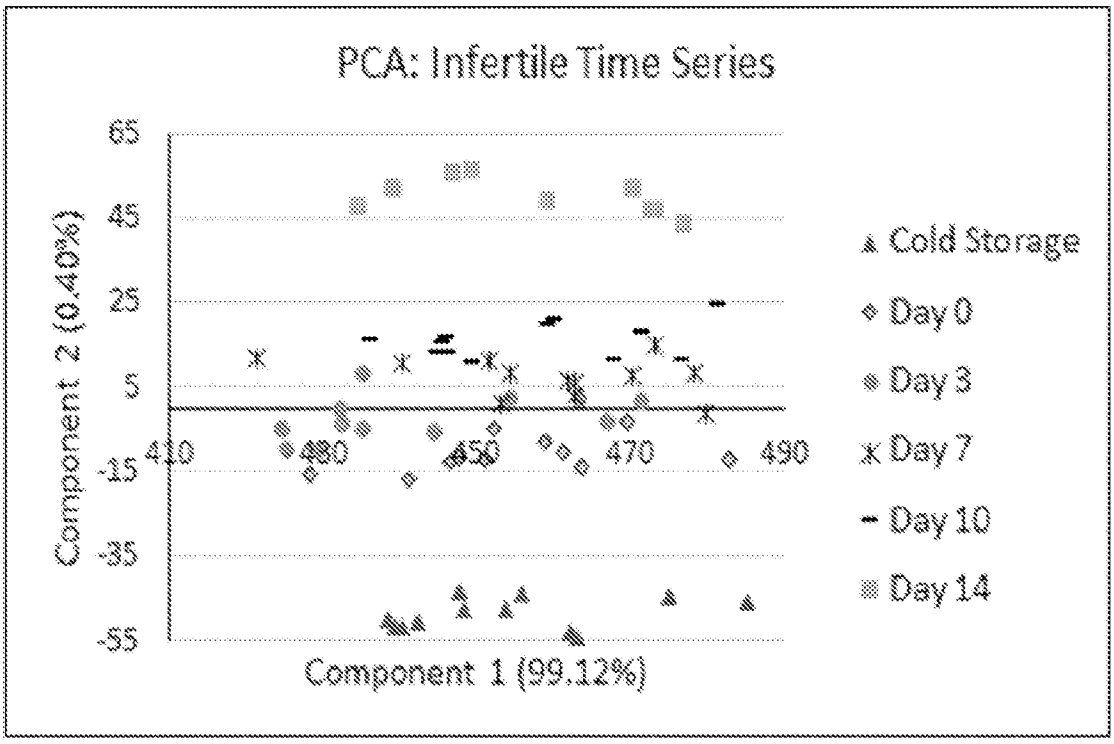
PCA: Infertile Time Series
Component 2 (0.40%)
Component 1 (99.12%)
65
45
25
5
-15
-35
-55
410
430
450
470
490
Cold Storage
Day 0
Day 3
Day 7
Day 10
Day 14

FIG. 10

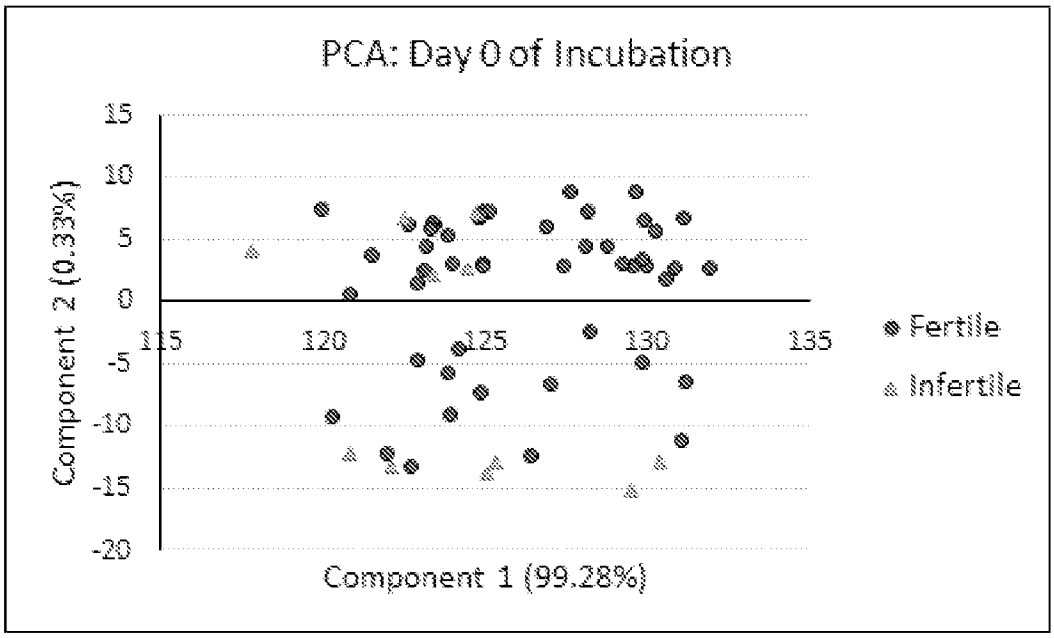
PCA: Day 0 of Incubation
Component 2 (0.33%)
15
10
5
0
-5
-10
-15
-20
115
120
125
130
135
Component 1 (99.28%)
Fertile
Infertile

FIG. 11

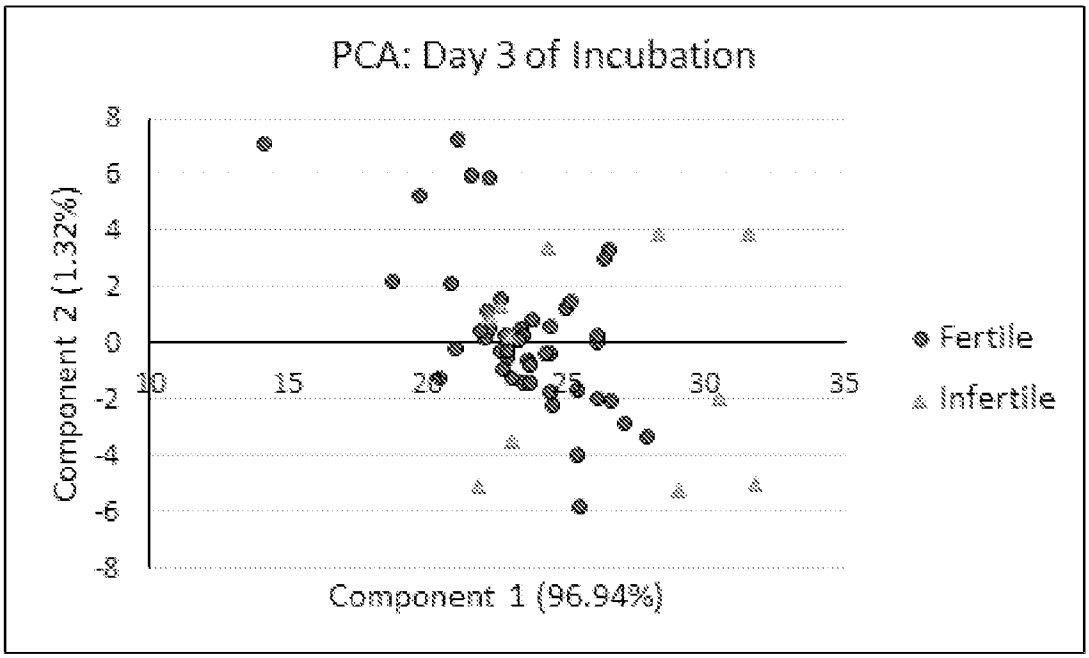
PCA: Day 3 of Incubation
Component 2 (1.32%)
8
6
4
2
0
-2
-4
-6
-8
10
15
20
25
30
35
Component 1 (96.94%)
Fertile
Infertile

FIG. 12

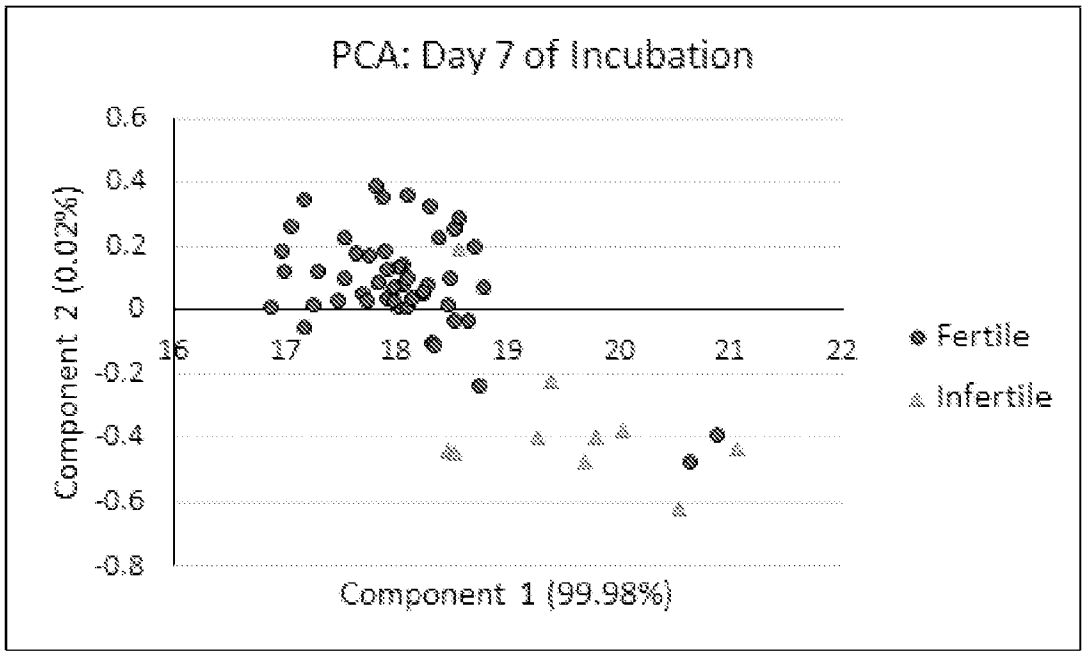
PCA: Day 7 of Incubation
Component 2 (0.02%)
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
16
17
18
19
20
21
22
Component 1 (99.98%)
Fertile
Infertile

FIG. 13

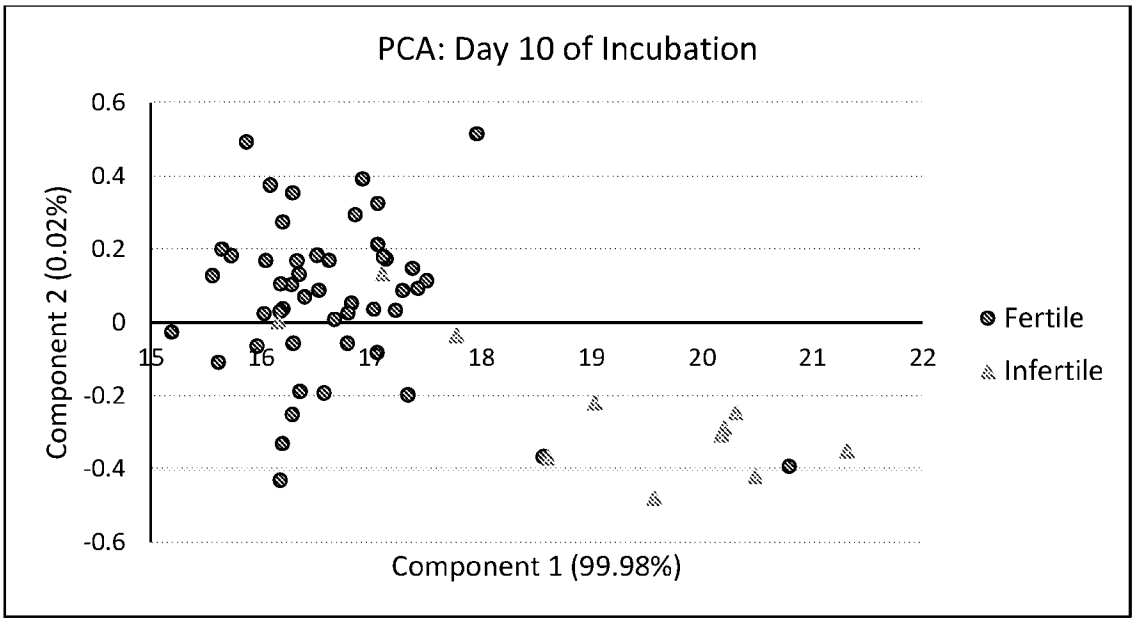
PCA: Day 10 of Incubation
Component 2 (0.02%)
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
15
16
17
18
19
20
21
22
Component 1 (99.98%)
Fertile
Infertile

FIG. 14

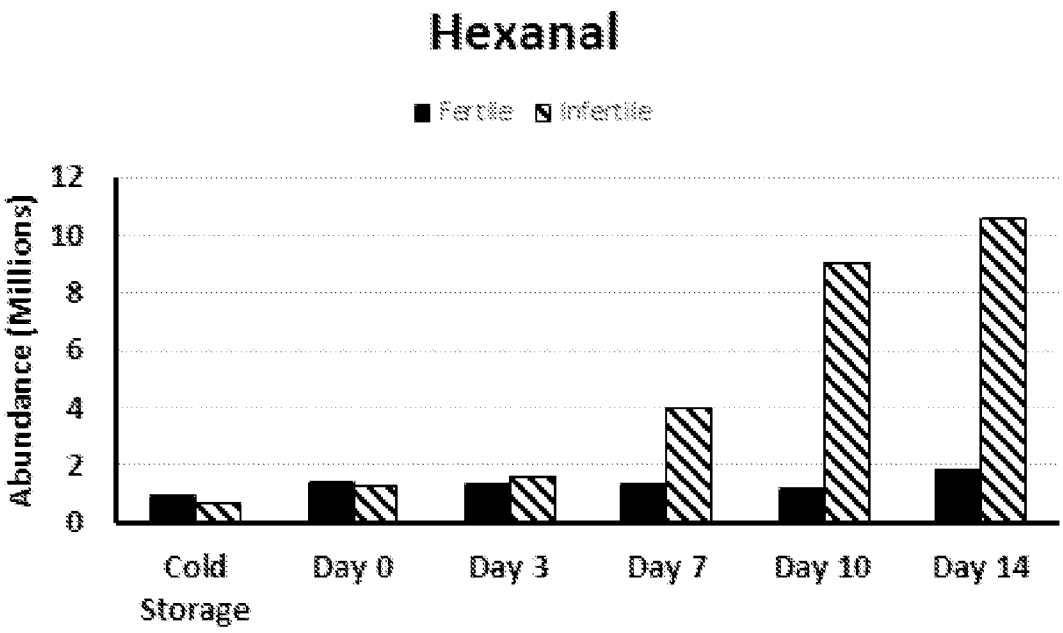
Hexanal
Fertile
Infertile
Abundance (Millions)
12
10
8
6
4
2
0
Cold Storage
Day 0
Day 3
Day 7
Day 10
Day 14

FIG. 15

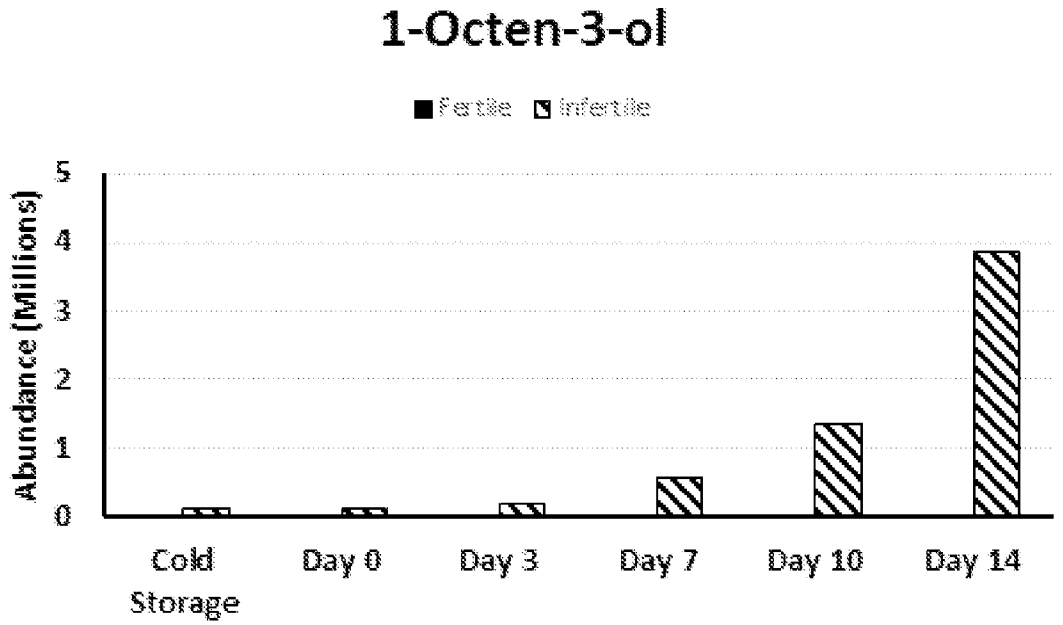
1-Octen-3-ol
Fertile
Infertile
Abundance (Millions)
5
4
3
2
1
0
Cold Storage
Day 0
Day 3
Day 7
Day 10
Day 14

FIG. 16

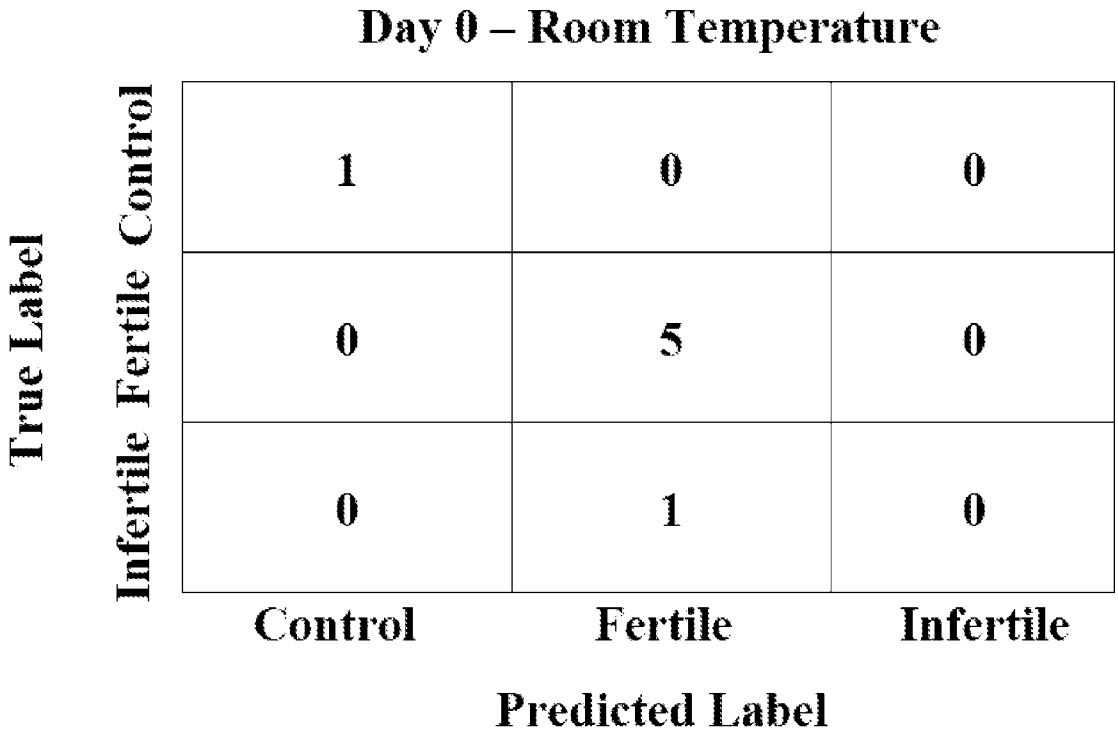
Day 0 – Room Temperature
True Label
Infertile Fertile Control
1
0
0
0
5
0
0
1
0
Control
Fertile
Infertile
Predicted Label

FIG. 17

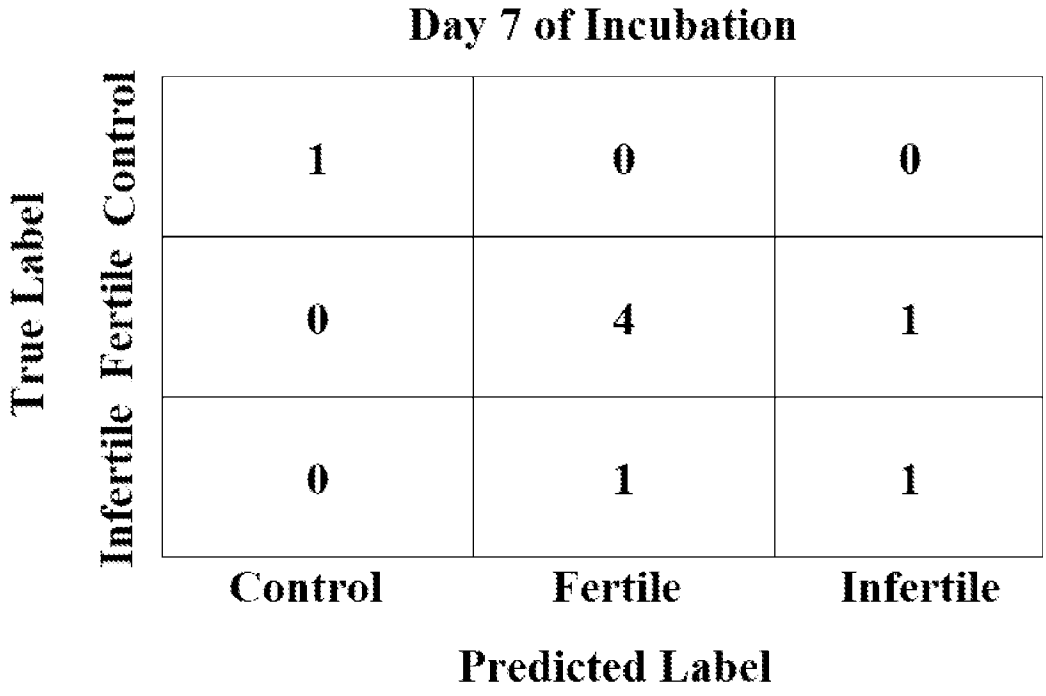
Day 7 of Incubation
True Label
Infertile Fertile Control
1
0
0
0
4
1
0
1
1
Control
Fertile
Infertile
Predicted Label

FIG. 18

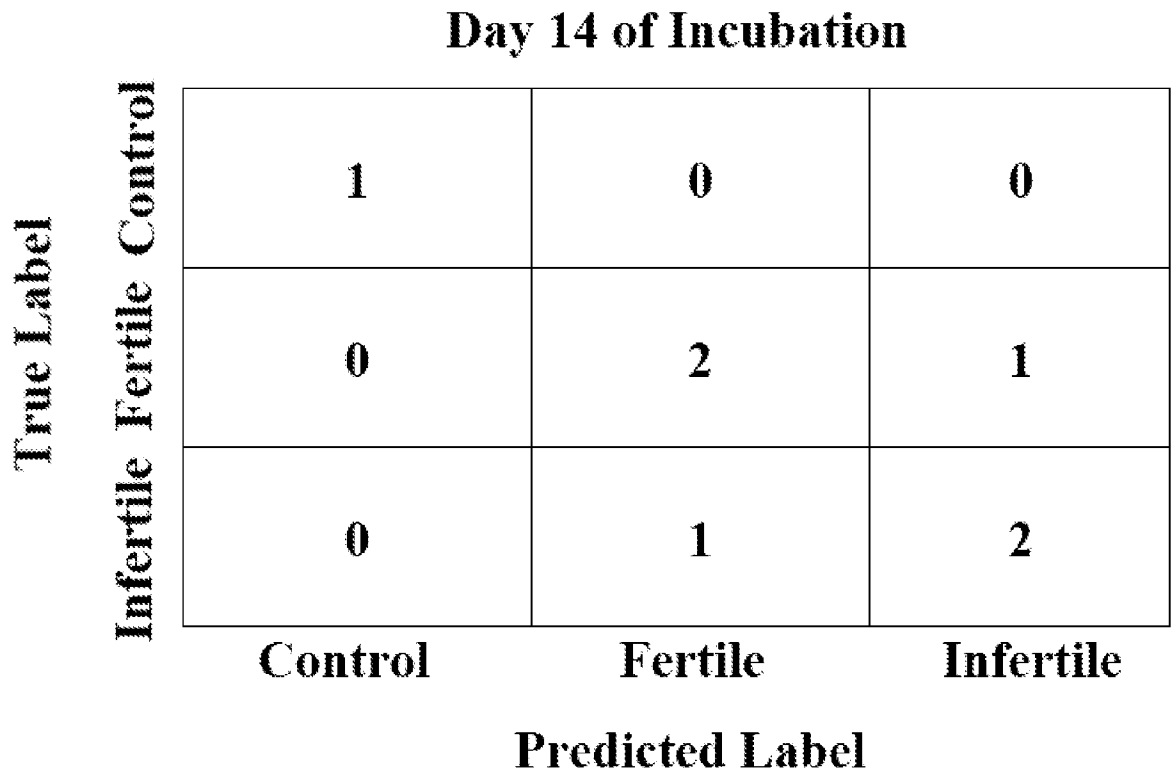
Day 14 of Incubation
True Label
Infertile Fertile Control
1
0
0
0
2
1
0
1
2
Control
Fertile
Infertile
Predicted Label

FIG. 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US23/37161

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. G01N 33/08 (2023.01)
ADD. A01K 43/00; B01D 53/02; H01J 49/26 (2023.01)

CPC - INV. G01N 33/08
ADD. A01K 43/00; B01D 53/02; H01J 49/26

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

Electronic database consulted during the international search (name of database and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WEBSTER, B. ET AL. "Avian Egg Odour Encodes Information on Embryo Sex, Fertility and Development" 10(1): 0116345. PLOS ONE. Online. 28 January 2015; Entire Document; DOI: https://doi.org/10.1371/journal.pone.0116345	1-18
A	US 2021/0212297 A1 (NOVATRANS GROUP S.A.) 15 July 2021; entire document	1-18
A	US 2019/0174726 A1 (NOVATRANS GROUP S.A.) 13 June 2019; entire document	1-18
A	XIANG, X ET AL. "Nondestructive characterization gender of chicken eggs by odor using SPME/GC-MS coupled with chemometrics" 101:101619. Poultry Science. Online. 29 November 2021; Entire Document; DOI: https://doi.org/10.1016/j.psj.2021.101619	1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
31 January 2024 (31.01.2024)	MAR 04 2024
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US23/37161

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 19-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.