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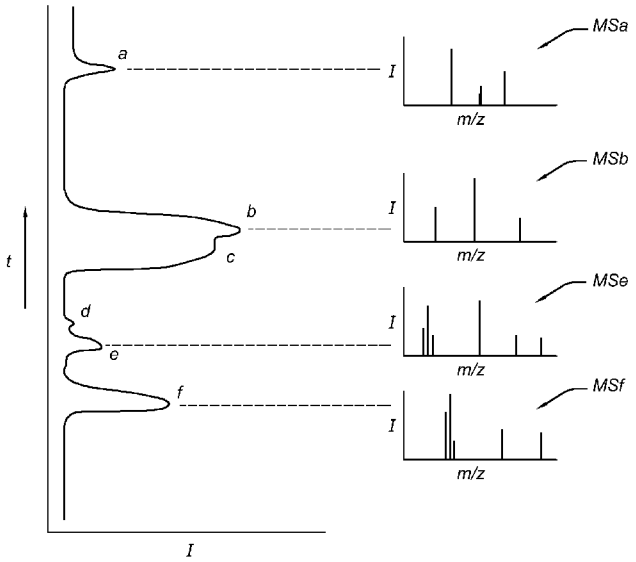
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(54) **Title:** METHOD FOR THE DETECTION OF MASTITIS PATHOGENS IN A MILK SAMPLE

Fig 3



(57) **Abstract:** The invention provides a method for the detection of mastitis pathogens in a milk sample comprising analyzing the milk sample by headspace analysis with a detector, thereby providing an analysis result, then comparing the analysis result with reference analysis data of metabolites of mastitis pathogens, and further determining the presence of the mastitis pathogens in the milk sample.

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Method for the detection of mastitis pathogens in a milk sample

Field of the invention

[0001] The invention relates to a method for the detection of mastitis pathogens in a milk sample and to a computer program product for use in or for such method.

Background of the invention

[0002] A major cause of loss in dairy farming is an infection, known as mastitis, which occurs in an animal's udder. Mastitis is the inflammation of the mammalian breast. It is one of the mostly costly diseases of dairy cows. In the Netherlands, the average costs of a clinical mastitis case of a cow are estimated between €168 and €277, depending mainly on the stage of lactation. Mastitis is caused by contagious pathogens invading the udder and producing toxins that are harmful to the mammary glands. Generally, mastitis starts in one udder quarter.

[0003] Somatic cells, predominantly white blood cells and epithelial cells, enter the mammary gland as a result of damage to the alveolar lining by infection or chemical irritation. The counting of somatic cells excreted in the milk has become a widely used measure of mammary gland inflammation and mastitis. The somatic cell s can be counted by a laborious direct microscopic method on stained milk smears, or by fluoro-opto-electronic counting, or the cell numbers can also be estimated by direct chemical tests.

[0004] Other methods measure milk somatic cells indirectly or by determining the concentration of various by-products of the inflammatory response. One of these methods is based on measuring changes in the electrical conductivity of milk, as generally ion concentration, and thus electrical conductivity, in mastitis milk is higher than in normal milk. Electrical conductivity is generally measured with a DC or AC circuit having a probe positioned in the flow of milk. WO2005006849, for instance, describes a method for indicating a health condition of a milking animal on-line during milking of said milking animal by a milking machine, characterized by the steps of measuring the concentration of a specified ion in the milk as drawn from an udder quarter or udder of said milking animal during said milking by means of an ion-selective sensor, comparing the measured concentration of said specified ion with a reference concentration value, and indicating a health condition of said milking animal depending on the outcome of said comparison.

[0005] Another method is described in WO2001035728, wherein a method is disclosed for testing a milk flow for mastitis, wherein a diverted milk flow is automatically tested by measuring DNA of any somatic cells in at least part of the milk of that sample. In an

embodiment of WO2001035728, measuring is by reference to a gelling characteristic of the milk under the action of an anionic detergent.

Summary of the invention

[0006] According to an aspect of the invention, an alternative method for the detection of mastitis, especially of mastitis pathogens , is provided, which method is further preferably quicker and/or more reliable than state of the art methods. Fast diagnosis of the pathogen causing mastitis is important, for instance in view of treatment of the animal hosting the mastitis pathogen or for instance for measures to be taken on the farm where mastitis hosting animals are (were) present.

[0007] According to a first aspect, the invention provides a method for the detection of mastitis pathogens in a milk sample comprising:

a analyzing the milk sample by headspace analysis with a detector, thereby providing an analysis result;

b comparing the analysis result with reference analysis data of n metabolites of m individual mastitis pathogens, wherein n is at least 5 and m is at least 1; and

c determining the presence of the m individual mastitis pathogens in the milk sample.

[0008] According to another aspect of the invention, the invention provides a computer program product comprising executable instructions, which instructions, when executed on a computer, allow the computer to compare the analysis result with reference analysis data of n metabolites of m individual mastitis pathogens and to determine the presence of the m individual mastitis pathogens in the milk sample according to the method of the invention. In this embodiment, n is especially at least 9, and m is especially at least 2.

[0009] According to yet another aspect of the invention, a computer program product comprising reference analysis data of n metabolites of m individual mastitis pathogens is provided, which when read by a computer, allows the computer to compare the analysis result with the reference analysis data of n metabolites and to determine the presence of the m individual mastitis pathogens in the milk sample according to the method of the invention. In this embodiment, n is also especially at least 9, and m is also especially at least 2.

[0010] Advantageously, the method of the invention and the computer program products according to the invention allow a fast and reliable analysis of milk samples of animals, especially lactating animals, such as cows. Elaborate sample preparation is not required and no difficult method has to be applied. Further, the detection method can be performed within hours and without further time consuming analysis of the results. This speeds up the process

of detection of the possible presence of mastitis pathogens and thereby it also allows a quick response, such as a treatment (including curative and/or preventive treatment) of animals in farms that produced samples wherein mastitis pathogens could be detected.

Detailed description of the invention

[0011] Mastitis with or without clinical symptoms is most often caused by bacteria. Although many bacteria can cause mastitis, a limited group of about 5 bacteria (Staphylococcus aureus, coagulase-negative staphylococci (CNS), Streptococcus uberis, Streptococcus dysgalactiae, and Escherichia coli) is responsible for about 80% of the (Dutch) mastitis cases in which bacteria could be cultured from the milk.

[0012] When animals suffer from mastitis, they host pathogens. Due to the presence of pathogens, metabolites are formed. It appeared that a number of pathogens have their own characterizing metabolite pattern ("fingerprint") . In general, each pathogen generates a plurality of metabolites. Some pathogens may generate the same metabolites, but each pathogen has its own specific pattern of metabolites (see also below). Hence, when detecting metabolites, depending upon the type (and relative amounts) of these metabolites, it can be determined which pathogen(s) were hosted by the animal. More precisely, when detecting metabolites in a milk sample by head space analysis, depending upon the type (and relative amounts) of the metabolites detected (if any), it can be determined which pathogen(s) (if any) were hosted by the lactating animal. Knowing the mastitis pathogens, a dedicated treatment can be selected. Hence, the invention has further the advantage that not only mastitis per se is detected, but also the specific pathogen(s) causing the mastitis can be determined (in case the milk sample is of an animal having mastitis).

[0013] The invention provides a fast and reliable method for the detection of mastitis pathogens. Herein, the term "method of the invention" shortly refers to the method for the detection of mastitis pathogens in a milk sample.

[0014] The milk sample herein can be any milk sample, such as milk of a lactating animal, especially a cow. Especially, the milk sample is non-human animal milk.

[0015] In general, a farmer with milk producing animals sends milk samples on a regular base to laboratories (or control stations). Such milk samples can be analyzed with the method according to the invention.

[0016] In the method of the invention, the milk sample is analyzed on the presence of a plurality of metabolites by headspace analysis with a detector. Headspace analysis is known in the art, see for instance Marsili, J. Agric. Food Chem. 1999, 47, 648-654. Headspace

analysis refers to the method wherein part of the gaseous compounds present above or escaping from a sample or part of a sample are exhausted or removed by suction or absorption and analyzed. In this way, only gaseous compounds are removed and analyzed.

[0017] In general, the milk directly after milking, such as by hand or by a mechanical device, has no or a relatively low amount of metabolites. This amount may be too low to be detected by a detector, such as a GC-MS or GC-FID apparatus. Hence, in a specific embodiment, the milk sample is incubated prior to analyzing the sample. Incubating in general includes heating the milk sample to a temperature above about 30ºC. In a specific embodiment, incubating comprises heating the milk sample to a temperature of at least 37ºC during at least 1 minute. Especially, incubation is at least 15 minutes, more especially at least 30 minutes, even more especially at least 60 minutes, yet even more especially at least 4 hours. In general incubation will not be longer than about 24 hours, especially not longer than about 12 hours. The incubation temperature is especially in the range of about 5-50 ºC, more especially in the range of about 10-50 ºC, such as about 37ºC, even more especially in the range of about 30-40 ºC. An advantage of the method of the invention is that the pathogen(s) have not to be isolated from the milk sample, for instance with classical microbiological methods, which are time consuming. Hence, in the method of the invention, the presence of pathogen(s) in a milk sample is determined without prior isolation from the pathogen(s) from the milk sample. This may advantageously save time and costs.

[0018] The detector herein can be any detector, but is preferably a detector comprising a GC-MS apparatus (gas chromatograph – mass spectrometer). In another embodiment, the detector preferably comprises a GC-FID apparatus (gas chromatograph with flame ionization detector). Hence, in a specific embodiment, the detector comprises a gas chromatograph coupled to a mass spectrometer. An apparatus comprising a gas chromatograph coupled to a mass spectrometer is herein indicated as GC-MS or GC-MS apparatus. Hence, in another specific embodiment, the detector comprises a gas chromatograph coupled with a flame ionization detector. An apparatus comprising a gas chromatograph which such flame ionization detector is herein indicated as GC-FID or GC-FID apparatus. Such apparatus(s) may be designed for head space analysis, i.e. it may comprise a suction or absorption element for removing or absorbing gaseous compounds present over the milk sample. Those gaseous compounds are then subsequently fed to the detector. The sample measured by the detector is herein also indicated as “headspace sampled sample”. Hence, in an embodiment, a method may be applied wherein the detector comprises a GC-FID apparatus designed for headspace

analysis, and in another embodiment, a method may be applied wherein the detector comprises a GC-MS apparatus designed for headspace analysis.

[0019] The term "analyzing" herein, refers in a specific embodiment to analyzing by GC-MS. Hence, in an embodiment, the phrase "analyzing the milk sample by headspace analysis with a detector" refers to taking a headspace sample, subjecting this headspace sample to gas chromatography and subjecting the separated components in the effluent of the GC to the MS. The term "analyzing" herein, refers in another specific embodiment to analyzing by GC-FID. Hence, in an embodiment, the phrase "analyzing the milk sample by headspace analysis with a detector" refers to taking a headspace sample, subjecting this headspace sample to gas chromatography and subjecting the separated components in the effluent of the GC to the FID. As described above, the milk sample is analyzed on the presence of a plurality of n metabolites. The number n especially indicates the number of metabolites of which reference analysis data, especially GC and/or MS reference analysis data, are available. These MS reference data may especially include MS spectral data, i.e. m/z data and optionally also relative intensities of the m/z data. Hence, the MS reference data may especially be mass spectra, i.e. mass spectra of metabolites. The GC reference analysis data may especially include retention times and/or retention indices and optionally also relative intensities (i.e. relative abundances). See further below.

[0020] For a useful analysis, the method is preferably able to detect (i.e. recognize) a plurality of metabolites. This does not imply that the metabolites must be present in the milk sample, i.e. taken from the head space, but that the method must in principle be able to ascribe a gaseous component as a specific metabolite, i.e. recognize such gaseous component as metabolite. To this end, a plurality of reference analysis data (such as MS spectra and retention times) may be available. The plurality of metabolites that is known (for instance by analysis software) is indicated with the number n. Especially, n is larger than 1, more especially equal to or larger than 2, even more especially at least 5, yet even more especially at least 9.

[0021] In a specific embodiment, the n metabolites comprise one or more compounds selected from the group consisting of ketones, aldehydes and branched free fatty acids, and more especially comprises one or more compounds selected from the group consisting of ketone alcohols, branched aldehydes, branched free fatty acids and ethyl esters of free fatty acids. Herein the terms "ketone", "ketone alcohol", "aldehyde", "branched aldehyde", "branched free fatty acid" and ethyl esters of free fatty acids refer to a ketone, to a ketone alcohol, to an aldehyde, to a branched aldehyde, to a branched free fatty acid, or ethyl esters of free fatty

acid, respectively, but may in an embodiment also refer to a plurality of different ketones, a plurality of different ketone alcohols, a plurality of different aldehydes, a plurality of different branched aldehydes, a plurality of different branched free fatty acids, and a plurality of different ethyl esters of free fatty acids, respectively. Herein, the terms "ketone(s)" and "a ketone", indicate that at least one predetermined ketone (such as for instance indicated in table 1, see below) is analysable. Likewise, the terms "aldehyde(s)" and "an aldehyde" imply at least one predetermined aldehyde (such as for instance indicated in table 1, see below) is analysable. Likewise, the terms "branched free fatty acid(s)" and "a branched free fatty acid(s)" imply at least one predetermined branched free fatty acid (such as for instance indicated in table 1, see below) is analysable. Likewise this applies for the other mentioned metabolite classes. In this context, predetermined means that at least reference analysis MS-data and/or GC data of such metabolite (s) are available, more especially MS and GC reference analysis data are available. It surprisingly appears that especially ketones, branched aldehydes and free fatty acids are distinguishing metabolites, more especially ketone alcohols, branched aldehydes, branched free fatty acids and ethyl esters of free fatty acids are distinguishing metabolites. This is also indicated in table 2, see below.

[0022] In yet another embodiment, the n metabolites belong to at least k different chemical classes, wherein k is at least 3, and wherein the k chemical classes are selected from the group comprising ketones, branched aldehydes, free fatty acids, ethyl esters of free fatty acids, branched free fatty acids, ethyl esters of branched free fatty acids, alcohols, and ketone alcohols. As mentioned above, the n metabolites especially comprise one or more ketone alcohols, one or more branched aldehydes and one or more branched free fatty acids and one or more ethyl esters of free fatty acids. In a specific embodiment, k is at least 4. This implies that with the method of the invention at least one predetermined member out of at least 4 of such classes can be recognized. As mentioned above, this implies that reference analysis data of n metabolites of k different classes are available. Especially, the n metabolites comprise ketone alcohols, branched aldehydes, branched free fatty acids and ethyl esters of free fatty acids (i.e. at least one compound from each of these classes, respectively); and reference analysis data of these metabolites are available.

[0023] In yet a more specific embodiment, the at least n metabolites are selected from the group comprising acetaldehyde, ethanol, acetone, dimethylsulfide, 2,3-butadione, 2-butanone, ethyl acetate, 3-methylbutanal, 2-methylbutanal, 2-pentanone, acetic acid, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl butanoate, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, 2-methylbutanoate, 2-heptanone, and ethyl hexanoate.

[0024] In a specific embodiment, the at least n metabolites comprise ethyl acetate, 3-methylbutanal, 2-methylbutanal, 3-hydroxy-2-butanone, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, and 2-methylbutanoate (here, n = 9). Especially these compounds are distinguishing compounds (see also table 2), and reference analysis data of these metabolites can be applied to detect metabolites in the milk sample.

[0025] More especially, the at least n metabolites comprise acetaldehyde, ethanol, 2,3-butadione, 2-butanone, ethyl acetate, 3-methylbutanal, 2-methylbutanal, 2-pentanone, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl butanoate, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, 2-methylbutanoate, 2-heptanone, and ethyl hexanoate (i.e. n = 17), more especially acetaldehyde, ethanol, acetone, dimethylsulfide, 2,3-butadione, 2-butanone, ethyl acetate, 3-methylbutanal, 2-methylbutanal, 2-pentanone, acetic acid, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl butanoate, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, 2-methylbutanoate, 2-heptanone, and ethyl hexanoate (i.e. n = 21). This implies that with the method of the invention, all these compounds can be recognized (but are not necessarily all present in the sample).

[0026] As will be clear to the person skilled in the art, the phrase "can be recognized" especially means one or more of such metabolites is within the detection limits of the detector, and, by comparing the analysis results with the available reference analysis data of the metabolites, the presence (and type) of the one or more metabolites in the milk sample can be determined. When present within the detection limits of the detector, these compounds will be detected, and the analysis of the milk sample on the presence of the plurality of metabolites leads to a result indicating that the specific metabolite(s) is(are) present.

[0027] Having performed the analysis, an analysis result is obtained (i.e. analysis data are obtained). Herein, the terms "analysis result" or "GC data" or "MS data" refer to analysis data, GC analysis data, and MS analysis data, respectively.

[0028] This may mean in an embodiment that a GC chromatogram has been produced, and from the separated components in the effluent of the gas chromatograph, MS spectra have been measured. Hence, in an embodiment, wherein a GC-MS analysis is performed, the analysis result may include gas chromatographic data of the headspace sampled milk sample and MS data from the separated components in the effluent of the gas chromatograph measured by MS. Chromatographic data (GC data) may especially include retention times and or retention indices, but may further also include relative peak heights and/or relative peak

areas. MS data may especially include m/z values of a plurality of fragments, but may further also include (relative) peak heights ((relative) abundances). Further, the MS data may be used to quantify the amount of metabolite present in the sample. This may for instance be done by integrating the signal of the most abundant m/z fragment in the MS spectrum.

[0029] This may mean in another embodiment that a GC chromatogram has been produced. Hence, in an embodiment, wherein a GC-FID analysis is performed, the analysis result may include gas chromatographic data of the headspace sampled milk sample, such as retention times, but may further also include relative peak heights and/or relative peak areas. The peak height may be used to quantify the amount of metabolite present in the sample. This may for instance be done by integrating the FID signal.

[0030] Hence, for identification, when applying GC-MS, retention times and MS data may be used, while when applying GC-FID, for identification only retention times may be used. When using retention times, especially is referred to the relative retention time, more especially the retention index. As known in the art, retention indices can be obtained from capillary GC to provide additional criteria for identification according to the method of Kovats. Hence, GC (reference) (analysis) data herein may refer to GC data obtainable with a GC-FID apparatus or with a GC from a GC-MS apparatus.

[0031] With this analysis result, it can be determined whether or not mastitis pathogens are (were) present in the milk sample, and if any, which mastitis pathogen(s). To that end, the analysis results are compared with reference analysis data of the metabolites of the individual mastitis pathogens. Comparison can be done by hand, but in general this will be done by dedicated software, known to the person skilled in the art (such as NeuralTools from Palisade).

[0032] The analysis results will in general comprise:

GC analysis results may comprise:

a.1. one or more of retention times, relative retention times and retention indices of the components separated in the GC;

a.2. optionally the relative peak heights and/or relative peak areas of the separated components (i.e. the relative abundances of the components); and

MS analysis results may comprise:

a.3. the m/z values of ionized fragments of the separated components in the effluent of the GC; and

a.4. optionally their relative peak heights (i.e. the relative abundances of the components).

[0033] Similarly, also the reference analysis data will in general comprise:

GC reference analysis data may comprise:

a.1. one or more of retention times, relative retention times and retention indices of metabolites of a specific pathogen;

a.2. optionally the relative peak heights and/or relative peak areas of the separated metabolites of the specific pathogen (i.e. the relative abundances of the metabolites of a specific pathogen); and

MS reference analysis data may comprise:

a.3. the m/z values of ionized fragments of the separated metabolites in the effluent of the GC; and

a.4. optionally their relative peak heights (i.e. the relative abundances of the metabolites of a specific pathogen).

[0034] Relative peak heights, especially relative peak heights related to different metabolites can be used to determine the relative abundance of the metabolites. The relative abundance of metabolites for a specific mastitis pathogen especially characterizes the specific mastitis pathogen, and can be used as reference analysis data in the correlation with analysis results. Hence, with GC (i.e. GC-FID) or GC-MS the presence of specific metabolites can be determined and by determining the relative abundances with GC or GC-MS and by comparing and correlating the obtained metabolites patterns with reference analysis data (such as the reference metabolite pattern), the presence of specific mastitis pathogens can be determined. Thus, with (GC-)MS reference analysis data such as m/z values of metabolites and relative abundances of the metabolites for a specific pathogen, milk samples can be analysed with GC-MS on the presence of the specific pathogen. Optionally, also GC reference analysis data may be applied (such as one or more of retention times, relative retention times and retention indices; especially retention indices). Likewise, with GC reference analysis data such as one or more of retention times, relative retention times and retention indices; especially retention indices, of metabolites and relative abundances of the metabolites for a specific pathogen, milk samples can be analysed with GC-FID on the presence of the specific pathogen. In a specific embodiment, the reference analysis data comprise relative abundances of metabolites of m individual mastitis pathogens, respectively, i.e. for each of the m individual mastitis pathogens, the relative abundances (i.e. pattern or fingerprint) is available as reference analysis data.

[0035] The term reference analysis data relate to data which are used as reference data to compare with the analysis results and determine from the comparison whether a specific compound is a metabolite and/or whether a specific metabolite pattern can be ascribed to the

metabolite pattern of a pathogen, i.e. whether the presence of the pathogen can be determined. The term reference analysis data refers to a library of data. In general, the reference analysis data comprise analysis data obtained with a similar or preferably identical analysis method as per which the milk sample is measured. The term "metabolite pattern" especially refers to the type and relative abundance of metabolites for a specific pathogen. Metabolite patterns can be derived form GC-MS and/or GC-FID data of pathogen containing milk samples. The phrase "determining the presence of the m individual mastitis pathogens" especially refers to a determination based on the comparison made. The analysis results are compared with the reference analysis data of n metabolites of m individual mastitis pathogens and this comparison or correlation may lead to a determination whether a specific mastitis pathogen is present or not. Hence, the phrases "method for the detection of mastitis pathogens" and "determining the presence of mastitis pathogens" does not necessarily imply that indeed mastitis pathogens were present in the milk sample. The terms "detection" and "determining" may include a determination that no pathogens were present.

[0036] In an embodiment wherein GC-MS analysis is applied, the peaks in the gas chromatogram of the GC analysis result of the milk sample can be ascribed to specific compounds, based on prior art data bases, and when metabolites are present in the milk sample, also to metabolites, based on the MS reference analysis data (and/or GC reference analysis data, such as retention time data). Then, the metabolite peaks in the GC can be deconvoluted into the separate contribution(s) to the spectrum of metabolites of the m individual mastitis pathogens. In this way, the presence and optionally the amount of (specific) mastitis pathogens can be detected. Hence, in an embodiment the reference analysis data comprise MS reference analysis data (such as m/z values of one or more specific metabolites and optionally the relative abundances of the one or more metabolites of the one or more specific pathogens, respectively), more especially GC-MS reference analysis data (such as m/z values of one or more specific metabolites and optionally the relative abundances of the one or more specific metabolites of the one or more specific pathogens, respectively, and one or more of retention times, relative retention times and retention indices of the one or more specific metabolites).

[0037] In yet another embodiment wherein GC-FID analysis is applied, the peaks in the gas chromatogram of the GC analysis result of the milk sample can be ascribed to specific compounds, based on prior art data bases, and when metabolites are present in the milk sample, also to metabolites, based on the GC reference analysis data (such as one or more of retention times, relative retention times and retention indices of the one or more specific

metabolites, and optionally the relative abundances of the metabolites of the one or more specific pathogens, respectively). Then, the metabolite peaks in the GC can be deconvoluted into the separate contribution(s) to the spectrum of metabolites of the m individual mastitis pathogens. In this way, the presence and optionally the amount of (specific) mastitis pathogens can be detected.

[0038] The invention in an aspect further provides a computer program product (such as software) comprising executable instructions, which instructions, when executed on a computer, allow the computer to compare the analysis result with reference analysis data of n metabolites of m individual mastitis pathogens and to determine the presence of the m individual mastitis pathogens in the milk sample, according to the method of the invention. In this embodiment, n is especially at least 9, and m is especially at least 2. Not only may dedicated software be applied for the method of the invention, also commercially available software may be applied, which has access to the reference analysis data (the library). Hence, the invention further provides, a computer program product (such as a non executable file with (only) the reference analysis data) comprising m reference analysis data of n metabolites of m individual mastitis pathogens, which when read by a computer, allows the computer to compare the analysis result with the reference analysis data of n metabolites of m individual mastitis pathogens and to determine the presence of the m individual mastitis pathogens in the milk sample, according to the method of the invention. As mentioned above, the phrase "to determine the presence" may include the determination that a specific mastitis pathogen is not present (in detectable amounts).

[0039] The GC analysis data for a specific pathogen may not always be the same, due to genetical variation within a type of pathogens. Due to natural variations, the GC analysis data and thus to a certain extent also the MS data when using GC-MS may differ from sample to sample. Advantageously, when using a neural network, the analysis data are compared or correlated with the reference analysis data, and when the GC or GC-MS analysis data best fit with one of the metabolite patterns of the specific mastitis pathogen, then the sample will be considered to contain the specific mastitis pathogen. Advantageously, the neural network can be trained. Hence, in a specific embodiment, the method for the detection further comprises using a neural network for determining the presence of the m individual mastitis pathogens in the milk sample. Therefore, the herein mentioned analysis software may include a neural network model.

[0040] Hence, the invention further provides the use of reference analysis data (such as in the form of a computer program product) of n metabolites selected from the group comprising

acetaldehyde, ethanol, acetone, dimethylsulfide, 2,3-butadione, 2-butanone, ethyl acetate, 3-methylbutanal, 2-methylbutanal, 2-pentanone, acetic acid, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl butanoate, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, 2-methylbutanoate, 2-heptanone, and ethyl hexanoate, wherein n is at least 5, for determining with a detection method the presence of the n metabolites, and especially the presence of m individual mastitis pathogens in a milk sample, wherein the m individual mastitis pathogens are selected from the group comprising Staph. aureus, Coagulase Negative Stapylococci, Strep. uberis, Strep. dysgalactiae and E. coli, wherein m is at least m 1, more preferably 5.

[0041] As mentioned above, no elaborate sample preparation or difficult methods have to be applied with the method of the invention. Further, analysis can be performed within hours and without further time consuming analysis of the results. This speeds up the process of detection of the possible presence of mastitis pathogens and thereby it also allows a quick response, such as a treatment (including curative and/or preventive treatment) of animals in farms that produced samples wherein mastitis pathogens could be detected. Since in some case, especially in the case of gram-negative bacteria, such as E. coli, treatment is of no or limited use, the method of the invention can further also be used to evaluate the whether or not to treat animals suffering from mastitis. Hence, in a specific embodiment, the invention further comprises:

d. under the condition that a pathogen is determined present in the milk sample: treating the animal (and/or herd) from which the milk sample originates, optionally unless all pathogen(s) detected in the milk sample are gram negative bacteria.

[0042] Herein, the term “treating” especially refers to a treatment of the animal(s) with antibiotics or other curative and/or anti inflammatory substances.

[0043] When one or more pathogens can be detected in the milk sample and all these one or more pathogens are gram negative bacteria, a treatment with antibiotics may not be useful. However, when one or more gram-positive bacteria (such as Staph. aureus, Coagulase Negative Stapylococci, Strep. uberis, and Strep. dysgalactiae) are detected in the milk sample, the animal (and/or its herd) from which the milk originates can advantageously be treated with (specific) antibiotics. The type of antibiotics might advantageously be selected depending upon the type of pathogen(s) detected. Herein, the phrase “treating the animal from which the milk sample originates” in an embodiment also refers to “treating the animals from the same farm or stable (herd) from which the animal that produced the milk sample that

contained the pathogen (during analysis) originates. This is also indicated by the phrase "the animal (and/or herd)". Hence, when a milk sample is determined to contain a pathogen, the whole stable or herd may be treated with (specific) antibiotics or other (specific) curative and/or anti inflammatory substances.

Brief description of the drawings

[0044] Embodiments of the invention will now be described, by way of example only, with reference to the accompanying schematic drawings and graphs in which corresponding reference symbols indicate corresponding parts.

[0045] Figure 1 schematically depicts an embodiment of the mastitis pathogen detection method, within a production chain of milk and milk analysis (and optional treatment of animals infected with mastitis pathogens). In a first stage 10, milk is produced, for instance in a farmhouse. Then samples are taken (for instance from each cows milk) and incubated in a sample preparation step 20. This may be a simple heating of the milk of the cow, and does not imply isolation of mastitis pathogens, if any. Then, headspace analysis takes place in a head space analysis stage 30. Based on the analysis results from the headspace analysis, in a comparison stage 40, the analysis results of the (individual) milk samples are compared with the reference analysis results of individual mastitis pathogens. Based on this comparison in the comparison stage 40, it can be determined in a determination stage 50 whether and/or which mastitis pathogens were present in the milk sample.

[0046] Based on this determination, optionally, a treatment in a treatment decision stage 60 can be decided, for treating the animals infected with mastitis or for treating animals in a herd or cowshed of which one or more animals are (were) infected with mastitis pathogens. In the treatment decision stage 60, it may also be decided not to treat the animal(s), for instance when the animals were infected with gram-negative bacteria only. Hence, based on the decision in the treatment decision stage 60, the animal from which the milk sample originates, which appeared to contain one or more mastitis pathogens, may be treated (especially with antibiotics) unless all pathogen(s) detected in the milk sample are gram negative bacteria. This optional treatment is indicated with the arrow from decision stage 60 to the milk production stage 10.

[0047] Figures 2a and 2b schematically depict an apparatus that can be used in the method of the invention. Figure 2a schematically depicts an incubator 22, such as a stove or furnace, for incubating the milk sample 21. Figure 2b schematically shows the detector 35, here a GC-MS apparatus for analysing the milk sample 21 (especially after incubation). Here, the detector 35

includes a suction opening 31 for headspace analysis of samples (here sample 21). The gas layer over the sample 21 is at least partially introduced via suction opening 31 into a GC 32 and the headspace sampled sample is separated over a GC column. Especially suitable GC columns are thick film apolar columns or normal film polar columns. For the GC analysis a thick film apolar column (e.g. a column with a film based on 100% Dimethylpolysiloxane or 95% Dimethylpolysiloxane combined with 5% Phenyl-methylpolysiloxane with a film thickness of at least 0.5μm) or a normal thickness film polar column (e.g. a column with a film based on poly ethylene glycol with a film thickness of 0.1-0.53 μm) may for instance be applied. Examples of the apolar columns are the DB-1/DB-5 and examples of the polar columns are DB-WAX/DB-FFAP (supplier Agilent J&W, Santa Clara, CA, US).

[0048] The effluent of the GC may at least partially fed into a MS 32, which analyses the separate components of the effluent, respectively. The analysis results of the GC 32 and MS 33 may be fed to a computer 45, for comparing the analysis results with the reference analysis results of metabolites of individual mastitis pathogens and for determining whether or not mastitis pathogens were present in sample 21; and if present, which mastitis pathogen(s). The sample 21 is no part of the detector 35. The detector 35 may comprise the computer 45, but optionally, the analysis results of the detector 35 may also be evaluated (i.e. comparing and/or determining) on another computer, not in connection with the detector 35.

[0049] Figure 3 schematically depicts the relation between GC and MS data. On the left hand side of the figure, schematically a GC chromatogram is depicted, with on the horizontal axis the GC-signal (Relative abundance, RA), and on the vertical axis the time. A number of peaks are displayed, indicated with a-f. These peaks correspond to GC separated components of the headspace sampled milk sample. In the effluent of the GC (not depicted), these components appear with time, respectively.

[0050] The MS coupled to the GC produces MS spectra of the separated components of the effluent of the GC. Schematically, on the left hand side of figure 3, MS spectra are shown, wherein schematic MS spectra MSa corresponds to peak a in the GC, etc. For the sake of clarity, not all schematic MS spectra are drawn. The MS spectra have in this drawing on the horizontal axis m/z and on the vertical axis the MS signal (relative abundance, RA).

[0051] GC-MS is a powerful tool. Having available the reference analysis data such as retention time and/or relative retention time and/or retention index and m/z values per metabolite, when measuring a milk sample, GC-MS signals of compounds in a headspace sampled milk sample corresponding to the reference analysis data lead to a positive determination of the presence of the specific metabolite. Moreover, referring to the GC part in

figure 3, for instance, it could be the case that signals a and e only occur and/or only occur in this ratio, when a specific mastitis pathogen is in the milk sample, whereas signals e and f only occur, and/or only occur in this ratio, when another mastitis pathogen is present in the milk sample. Hence, armed with the reference analysis data, the GC spectrum can be deconvoluted and the presence of specific mastitis pathogens can positively be determined. This is further elucidated in the next figures.

[0052] Figures 4a-e show GC chromatograms of a plurality of metabolites of 5 metabolites. Below, a summary is given of the peaks detected and their assignment. These results were obtained with a DB-5 column (95% Dimethylpolysiloxane combined with 5% Phenyl-methylpolysiloxane).

Table 1: retention times and retention indices of metabolites (see also figures 4a-e)

Peak number (in figure 4a)	Retention time (min)	Retention index	Identity of component	CAS number
1	5.21	443	Acetaldehyde	75-07-0
2	5.47	465	Ethanol	64-17-5
3	5.72	486	Acetone	67-64-1
4	6.68	568	2,3-Butadione	431-03-8
5	6.72	571	2-Butanone	78-93-3
6	6.88	585	Ethyl acetate	141-78-6
7	7.34	624	3-Methylbutanal	590-86-3
8	7.44	632	2-Methylbutanal	69-17-3
9	7.71	655	2-Pentanone	107-87-9
10	8.17	694	Acetic acid	64-19-7
11	8.27	703	3-Methyl-1-butanol	123-51-3
12	8.63	734	3-Hydroxy-2-butanone	513-86-0
13	8.82	750	Ethyl butanoate	105-54-4
14	9.04	768	Butanoic acid	107-92-6
15	9.27	788	Ethyl 2-methylbutanoate	53956-13-1
16	9.32	792	Ethyl 3-methylbutanoate	108-64-5
17	9.43	802	3-Methylbutanoate	503-74-2
18	9.53	810	2-methylbutanoate	116-53-0
19	9.77	830	2-Heptanone	110-43-0
20	10.63	904	Ethyl hexanoate	123-66-0
	5.81	498	Dimethylsulfide	75-18-3

[0053] Below, in table 2, the metabolite patterns of the 5 investigate pathogens are displayed and related to a headspace sampled and analyzed milk sample of milk not containing mastitis pathogens (reference sample).

Table 2: mastitis pathogen metabolite patterns and their relative abundances

	Mastitis Pathogen					
Metabolite	*Staph. aureus*	CNS[1]	*Strep. uberis*	*Strep. dysgalactiae*	*E. coli*	No mastitis[2]
Acetaldehyde	$7{,}4\cdot10^{6}$	$6{,}4\cdot10^{4}$	$2{,}8\cdot10^{5}$	$1{,}4\cdot10^{5}$	$2{,}7\cdot10^{5}$	$1{,}2\cdot10^{5}$
Ethanol	$1{,}1\cdot10^{8}$	$1{,}6\cdot10^{5}$	$1{,}6\cdot10^{7}$	$5{,}8\cdot10^{6}$	$1{,}3\cdot10^{8}$	0
Acetone	$4{,}3\cdot10^{7}$	$3{,}7\cdot10^{7}$	$3{,}6\cdot10^{7}$	$7{,}1\cdot10^{7}$	$1{,}7\cdot10^{7}$	$1{,}0\cdot10^{8}$
Dimethylsulfide	$9{,}5\cdot10^{4}$	$3{,}5\cdot10^{4}$	$1{,}7\cdot10^{5}$	$1{,}2\cdot10^{5}$	$1{,}2\cdot10^{6}$	$3{,}9\cdot10^{5}$
2,3-Butadione	$1{,}1\cdot10^{6}$	$5{,}0\cdot10^{4}$	$5{,}6\cdot10^{5}$	$1{,}4\cdot10^{5}$	$1{,}5\cdot10^{4}$	0
2-Butanone	$2{,}1\cdot10^{6}$	$3{,}2\cdot10^{5}$	$1{,}4\cdot10^{6}$	$7{,}0\cdot10^{5}$	$5{,}0\cdot10^{5}$	$3{,}0\cdot10^{6}$
Ethyl acetate	$1{,}1\cdot10^{6}$	0	$5{,}0\cdot10^{4}$	$2{,}0\cdot10^{4}$	$6{,}9\cdot10^{5}$	0
3-Methylbutanal	$2{,}8\cdot10^{7}$	$2{,}7\cdot10^{6}$	$8{,}8\cdot10^{4}$	$4{,}5\cdot10^{5}$	$4{,}6\cdot10^{4}$	$6{,}4\cdot10^{3}$
2-Methylbutanal	$3{,}7\cdot10^{5}$	$5{,}9\cdot10^{4}$	$2{,}7\cdot10^{3}$	$2{,}7\cdot10^{3}$	0	0
2-Pentanone	$8{,}1\cdot10^{4}$	$8{,}3\cdot10^{3}$	$3{,}6\cdot10^{4}$	$7{,}1\cdot10^{4}$	$9{,}6\cdot10^{5}$	0
Acetic acid	$6{,}0\cdot10^{6}$	$5{,}1\cdot10^{5}$	$2{,}7\cdot10^{6}$	$3{,}4\cdot10^{6}$	$9{,}8\cdot10^{6}$	$3{,}7\cdot10^{6}$
3-Methyl-1-butanol	$7{,}3\cdot10^{6}$	$1{,}1\cdot10^{6}$	0	$1{,}3\cdot10^{5}$	$3{,}9\cdot10^{6}$	0
3-Hydroxy-2-butanone	$8{,}2\cdot10^{5}$	$3{,}2\cdot10^{3}$	$3{,}6\cdot10^{3}$	0	0	0
Ethyl butanoate	$7{,}2\cdot10^{5}$	0	$7{,}2\cdot10^{4}$	$1{,}0\cdot10^{5}$	$3{,}3\cdot10^{5}$	0
Butanoic acid	$2{,}9\cdot10^{6}$	$2{,}0\cdot10^{5}$	$7{,}8\cdot10^{5}$	$1{,}0\cdot10^{6}$	$1{,}0\cdot10^{6}$	$4{,}15\cdot10^{6}$
Ethyl 2-methylbutanoate	$2{,}2\cdot10^{4}$	$3{,}6\cdot10^{2}$	0	0	0	0
Ethyl 3-methylbutanoate	$9{,}6\cdot10^{4}$	$4{,}6\cdot10^{2}$	$3{,}2\cdot10^{3}$	$2{,}8\cdot10^{3}$	0	0
3-Methylbutanoate	$1{,}4\cdot10^{2}$	0	0	$7{,}2\cdot10^{2}$	0	0
2-Methylbutanoate	$6{,}0\cdot10^{4}$	0	0	0	0	0
2-Heptanone	$1{,}7\cdot10^{5}$	$1{,}9\cdot10^{4}$	$1{,}0\cdot10^{5}$	$1{,}3\cdot10^{5}$	$3{,}2\cdot10^{5}$	$2{,}0\cdot10^{4}$
Ethyl hexanoate	$3{,}7\cdot10^{5}$	$7{,}6\cdot10^{2}$	$6{,}4\cdot10^{4}$	$9{,}2\cdot10^{4}$	$2{,}2\cdot10^{5}$	0
	Fig. 4a	Fig. 4b	Fig. 4c	Fig. 4d	Fig. 4e	

1: CNS: Coagulase negative staphylococci;
2: Milk from cows without clinical mastitis and low somatic cell count

[0054] From this table can be concluded that especially the metabolites ethyl acetate, 3-methylbutanal, 2-methylbutanal, 3-hydroxy-2-butanone, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, and 2-methylbutanoate, more especially, ethanol, 2,3-butadione, 2-butanone, ethyl acetate, 3-methylbutanal, 2-methylbutanal, 2-pentanone, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl butanoate, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, 2-methylbutanoate, 2-heptanone, and ethyl hexanoate are characterizing metabolites, since these compounds are not substantially present in milk from cows without clinical mastitis and low

somatic cell count. Hence, in a specific embodiment, the reference analysis data comprise reference analysis data of z metabolites selected from the group consisting of acetaldehyde, ethanol, acetone, dimethylsulfide, 2,3-butadione, 2-butanone, ethyl acetate, 3-methylbutanal, 2-methylbutanal, 2-pentanone, acetic acid, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl butanoate, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, 2-methylbutanoate, 2-heptanone, and ethyl hexanoate, wherein z is at least 1, more especially at least 5, and even more especially in the range of 9-21. In a specific embodiment, the reference analysis data comprise reference analysis data of all 21 herein defined metabolites. Hence, in a specific embodiment, a method is provided wherein the at least n metabolites comprise ethyl acetate, 3-methylbutanal, 2-methylbutanal, 3-hydroxy-2-butanone, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, and 2-methylbutanoate, more especially, ethanol, 2,3-butadione, 2-butanone, ethyl acetate, 3-methylbutanal, 2-methylbutanal, 2-pentanone, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl butanoate, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, 2-methylbutanoate, 2-heptanone, and ethyl hexanoate.

[0055] Referring to tables 1 and 2, GC reference analysis data may for instance comprise the relative retention times, and especially the retention indices. By determining the relative retention times and especially the retention indices of the metabolites in the milk sample (if present), the presence of the metabolites can be determined. GC can, as mentioned above, especially be used as GC-FID or GC-MS. Hence, the reference analysis data may comprise relative retention times, and especially the retention indices, and in a further embodiment also MS reference analysis data (i.e. M/z values for one or more fragments of the metabolites). Hence, the reference analysis data may in an embodiment comprise the relative retention times, and especially the retention indices according to table 1 for one or more of the metabolites mentioned in this table. Further, the reference analysis data may especially further comprise the relative abundances of the metabolites for a specific pathogen. Therefore, in a specific embodiment, the reference analysis data further comprise the relative abundances according to table 2 of m specific mastitis pathogens. Further, the reference analysis data may also comprise the relative abundances of compounds (also known as metabolites) for milk not containing, or substantially not containing one or more of the m mastitis pathogens, also according to table 2 (last column). Table 2 in fact gives the (metabolite) spectral data or (metabolite) reference pattern for the in table 2 indicated mastitis pathogens and milk substantially free of mastitis pathogens.

[0056] Tables 3a-3e gives MS data of some specific metabolites.

Table 3a: M/z values and relative intensities (I(rel)) for a number of metabolites

Acetaldehyde		Ethanol		Acetone		2,3-butadione		acetic acid	
m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)
40	10	41	13	37	17	41	21	41	35
41	50	42	47	38	21	42	88	42	130
42	127	43	114	39	41	43	999	43	999
43	474	45	514	41	19	44	28	44	24
44	825	46	216	42	90	86	186	45	903
45	27			43	999			46	10
				44	33			60	747
				57	16			61	19
				58	637				
				59	30				

Table 3b: M/z values and relative intensities (I(rel)) for a number of metabolites

Dimethyl-sulfide		2-butanone		ethyl acetate		3-methyl-butanal		2-methyl-butanal	
m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)
34	10	39	20	42	46	38	26	37	21
35	322	41	10	43	999	39	237	38	47
37	13	42	30	44	29	40	55	39	312
43	16	43	999	45	146	41	903	41	999
44	40	44	30	61	153	42	223	42	67
45	408	57	80	70	118	43	821	43	127
46	361	71	10	73	49	44	1000	44	10
47	954	72	250	88	70	45	118	50	29
48	33	73	10			50	16	51	31
49	42					51	16	53	48
57	22					53	39	55	97
58	29					55	42	56	92
59	28					56	29	57	888
61	333					57	278	58	580
62	999					58	619	59	21
63	43					59	22	71	44
64	43					67	17	86	113
						68	16		
						69	14		
						71	201		
						85	19		
						86	72		

Table 3c: M/z values and relative intensities (I(rel)) for a number of metabolites

2-Pentanone		3-methyl-1-butanol		3-hydroxy-2-butanone		ethyl butanoate		butanoic acid	
m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)
39	44	37	25	41	17	38	23	38	10

2-Pentanone		3-methyl-1-butanol		3-hydroxy-2-butanone		ethyl butanoate		butanoic acid	
m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)
41	91	38	53	42	62	39	165	39	65
42	39	39	436	43	642	40	31	40	11
43	999	40	68	44	46	41	356	41	162
44	26	41	805	45	1000	42	233	42	139
57	49	42	896	46	23	43	937	43	140
58	81	43	823	55	17	44	58	45	98
71	91	44	67	73	18	45	185	55	66
86	193	45	199	88	139	46	10	60	999
87	18	46	78			47	11	61	26
		47	15			55	48	69	12
		50	22			57	13	70	14
		51	25			60	232	71	28
		53	28			61	100	73	324
		54	17			69	17	74	11
		55	999			70	176	87	18
		56	128			71	999	88	25
		57	307			72	56	89	16
		58	16			73	210		
		60	10			88	568		
		69	53			89	159		
		70	705			90	11		
		71	49			101	90		
						116	34		

Table 3d: M/z values and relative intensities (I(rel)) for a number of metabolites

Ethyl 2-methyl-butanoate		Ethyl 3-methyl-butanoate		3-Methyl-butanoate		2-Methyl-butanoate		2-Heptanone	
m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)
39	85	39	85	39	63	38	19	38	11
40	14	40	14	41	294	39	163	39	101
41	477	41	477	42	154	40	18	40	17
42	37	42	37	43	270	41	528	41	116
43	71	43	71	45	109	42	41	42	69
44	12	44	12	55	51	43	54	43	999
45	101	45	101	56	95	45	167	44	27
47	12	47	12	57	584	50	13	53	12
53	16	53	16	58	32	51	14	55	45
55	75	55	75	59	107	53	24	56	10
56	142	56	142	60	275	55	107	57	18
57	999	57	999	61	183	56	133	58	549
58	52	58	52	70	199	57	638	59	86
59	25	59	25	73	66	58	28	71	133
60	36	60	36	84	30	59	18	72	18
61	23	61	23	85	716	60	14	85	17

Ethyl 2-methyl-butanoate		Ethyl 3-methyl-butanoate		3-Methyl-butanoate		2-Methyl-butanoate		2-Heptanone	
m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)	m/z	I(rel)
69	25	69	25	86	64	69	41	99	25
70	29	70	29	87	148	73	148	114	52
73	67	73	67	88	1000	74	999		
74	216	74	216	89	36	75	35		
75	25	75	25	103	34	87	238		
85	404	85	404	115	99	88	10		
86	18	86	18						
87	117	87	117						
88	96	88	96						
102	644	102	644						
115	100	115	100						
130	33	130	33						

Table 3e: M/z values and relative intensities (I(rel)) for a number of metabolites

Ethyl hexanoate	
m/z	I(rel)
39	232
40	46
41	430
42	350
43	859
44	41
45	230
51	14
53	30
54	13
55	234
56	57
57	52
59	16
60	450
61	247
69	85
70	222
71	268
72	18
73	274
74	48
75	10
83	15
87	73
88	999
89	58
97	19
98	14
99	539

Ethyl hexanoate	
m/z	I(rel)
100	39
101	262
102	35
115	84
117	48
144	15

[0057] The reference analysis data may in an embodiment also comprise the m/z values and I(rel) values (i.e. relative abundances of the respective fragments) of one or more of the n metabolites according to tables 3a-e.

[0058] Hence, in a specific embodiment, the GC reference analysis data comprise the retention times, and more especially the retention indices of n metabolites, as indicated in table 1. Such GC reference analysis data may especially be applied for analysis of samples with a detector comprising an apolar GC column. Hence, in a further specific embodiment, the GC reference analysis data (especially for GC-FID) or the MS reference analysis data (especially for GC-MS) comprise the relative abundances data as indicated in table 2, for n metabolites of m mastitis pathogens. As will be clear to the person skilled in the art, the relative abundances according to table 2 or multiples of these values indicated in this table may be used. Hence, in a further specific embodiment, the MS reference analysis data comprise the relative intensities of n metabolites, as indicated in one or more of tables 3a-3e. The value of n is as indicated herein. When referring to n, it indicates that n arbitrary or n non-arbitrary metabolites may be selected as "reference" metabolites. Likewise, the value of m is as indicated herein. When referring to m, it indicates that m arbitrary or m non-arbitrary pathogens may be selected as pathogens to be detected (if present). As mentioned herein, n is especially 5, and more especially 9, and m is especially 1, and more especially 5. Further, the reference analysis data may especially comprise GC or MS reference analysis data, respectively, of ethyl acetate, 3-methylbutanal, 2-methylbutanal, 3-hydroxy-2-butanone, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, and 2-methylbutanoate (here, n = 9).

[0059] Below, examples are described how the reference analysis data were obtained.

Examples

Milk Samples

[0060] 50 milk samples from cows with clinical mastitis were selected from the bacteriological diagnostic lab of the Dutch Animal Health Service. Samples had first been

screened for the presence of bacteria. If one of the 5 pathogens of interest (*Staph. aureus*, coagulase-negative *staphylococci*, *Strep. uberis*, *Strep. dysgalactiae*, or *E. coli*) was cultured, the remainder of the sample (approx. 5 mL) was frozen at -20°C before analysis of their volatile metabolites.

[0061] 10 milk samples of cows, without clinical mastitis and low somatic cell count, were supplied by the "De Ossekampen", the university farm of Wageningen University and Research Centre. These samples were kept frozen at -20°C before analysis of their volatile metabolites.

Bacteriological Screening

[0062] Bacteriological culturing was carried out according to NMC protocols (NMC, 1999; NMC, 2004). Briefly, the milk sample was incubated overnight at 37°C. Next, an inoculum of 0.01 mL of milk was spread on a sheep blood agar plate and on Edward's medium. Edward's medium was used for selective isolation of streptococci.

[0063] All plates were incubated at 37°C and examined after 24 and 48 h. Isolates of staphylococci showing α, α and β, or β haemolysis were presumptively identified as *Staph. aureus*. A colony was characterized as CNS when the tube coagulase test was negative. All coagulase positive colonies were assumed to be *Staph. aureus*. Only milk samples from which one bacterial species was cultured, were included in the study.

Analysis of Volatile Metabolites

[0064] 5 mL milk samples were preheated in 20 mL vials sealed with silicon/Teflon septa and magnetic caps for 1 min at 60°C. Volatile metabolites were extracted from the headspace for 5 min with a 75 μm PDMS-carboxen SPME fiber (Supelco, Bellefonte, PA) using the combiPAL autosampler (CTC Analytics AG, Switzerland). The volatile metabolites were thermally desorbed from the fiber by heating it in a Best PTV injector with an empty liner for 5 min at 250°C. The fiber was subsequently cleaned for 10 min at 290°C. A vial with 5 mL demineralised water was used as blank. Because analysis of volatile metabolites occurred only after bacteriological screening, the milk samples had thus been incubated overnight at 37°C.

[0065] GC separation of the volatile components was performed on a Finnigan Trace GC gas chromatograph coupled to a Finnigan DSQ mass spectrometer. Volatiles were separated on an apolar BPX-5 column of 30 m length, 0.15 mm i.d. (internal diameter), and 0.25 μm film thickness (SGE, Austin, TX). Oven temperature was held at -30°C for 3 min, raised to 230°C

at 20°C/min, followed by 1 min holding. Helium was used as the carrier gas at a flow rate of 0.6 mL/min. The MS interface and the ion source were kept at 250°C. Acquisition was performed in electron impact mode (70 eV) with 2 scans/s; the mass range used was m/z 33-250.

[0066] The resulting chromatograms were analyzed using the AMDIS software (NIST, Gaithersburg, MD); data was deconvoluted to obtain pure mass spectra, to improve peak identification. Identification of volatile metabolites was based on matching mass spectra and retention time with pure standard, if possible. Otherwise, spectra were compared to the NIST/EPA/NIH mass spectral database and the Kovats index was compared to data from literature (Acree and Arn, 2004). Peak integration was subsequently performed using the XCalibur software package. Peak area was corrected for the blank sample.

Statistical Analysis

[0067] SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL) was used for comparisons between groups. Because data was not normally distributed, first the Kruskal-Wallis test was performed to test for differences between groups. If significant differences between groups were observed, the Nemenyi test (Zar, 1999) was used for subsequent pair wise multiple comparisons.

[0068] NeuralTools (Palisade, Ithaca, NY) was used to develop artificial neural networks (ANN) for this study. Probabilistic neural networks (PNN) were the type of ANN used for this study. The conjugate gradient descent method was used for training the PNN. Training of the neural networks was carried out using cross validation, with 70% of the samples used for training and 30% for validation. Samples were distributed randomly between the training and validation group. This randomization of samples was repeated 5 times, to check for consistency of results. The reported correct classification rates are always based on the validation group.

Analysis of Volatile Metabolites

[0069] After successful microbiological analysis of the milk samples, their volatiles were analyzed using GC/MS. Table 1 (see above) contains an overview of the results of the GC/MS analysis, grouped by pathogen. The samples from healthy cows contained a lower number and lower amount of volatile components, compared to the mastitis milk samples.

[0070] The statistical differences between the groups of samples did give a clear distinction between the samples from healthy cows compared to cows with mastitis, see table 2.

[0071] Four groups can thus be identified based on the statistical analysis of the volatile bacterial metabolites: *Staph. aureus*, coagulase negative staphylococci, *streptococcus* (both *streptococcus* species as one group), and *E. coli*. However, classification based on this statistical analysis alone is difficult, because in every group, some samples have more similarity with samples of another group compared to the other samples in the group; e.g. a specific metabolite was not detected, or the concentration (i.e. relative abundance) of a specific metabolite was different.

[0072] Because a clear classification of the mastitis pathogens based on this statistical analysis alone could not be established, the use of a PNN was tested. A neural network enables to determine a weighing factor for each volatile pathogen metabolite. The neural network uses the weighing factors to determine the similarity of the analysis results of the detector, such as GC-FID or GC-MS with the metabolite patterns of the m specific pathogens (and optionally of a milk sample substantially free of pathogens)(see also especially table 2).

Training the PNN to Classify Milk Samples

[0073] A PNN was first trained to categorize milk as either milk from healthy cows, or cows with mastitis. The correct classification rate for this categorization was 100%.

[0074] The PNN was then trained for categorizing all 5 pathogens. The correct classification rate for this PNN was 66%.

[0075] The PNN was subsequently trained to classify the samples in 4 groups. The correct classification rate was 93%.

[0076] It has been widely discussed whether *E. coli* mastitis should be treated with antibiotics at all, because cows with this type of mastitis often don't respond well to antibiotics (Pyorala, 2003). Fast classification of samples between *E. coli* and non-*E. coli* is thus very useful. A new PNN was trained to classify samples to the groups *E. coli* and non-*E. coli*. The correct classification rate was 100%.

[0077] These results show that mastitis pathogen classification using volatile bacterial metabolites looks very promising.

[0078] It should be noted that the above-mentioned embodiments and examples illustrate rather than limit the invention, and that those skilled in the art will be able to design many alternative embodiments without departing from the scope of the appended claims. In the claims, any reference signs placed between parentheses shall not be construed as limiting the

claim. Use of the verb "to comprise" and its conjugations does not exclude the presence of elements or steps other than those stated in a claim. The article "a" or "an" preceding an element does not exclude the presence of a plurality of such elements. The invention may be implemented by means of hardware comprising several distinct elements, and by means of a suitably programmed computer. In the device claim enumerating several means, several of these means may be embodied by one and the same item of hardware. The mere fact that certain measures are recited in mutually different dependent claims does not indicate that a combination of these measures cannot be used to advantage.

Claims

1. A method for the detection of mastitis pathogens in a milk sample comprising:
   a analyzing the milk sample by headspace analysis with a detector, thereby providing an analysis result;
   b comparing the analysis result with reference analysis data of n metabolites of m individual mastitis pathogens, wherein n is at least 5 and m is at least 1; and
   c determining the presence of the m individual mastitis pathogens in the milk sample.
2. The method according to claim 1, wherein the milk sample is incubated prior to analyzing the sample, and wherein incubating comprises heating the sample to a temperature of at least 37 °C during at least 1 minute.
3. The method according to any one of the preceding claims, wherein the detector comprises a GC-MS apparatus designed for headspace analysis.
4. The method according to claim 3, where the reference analysis data comprise MS reference analysis data, more especially GC-MS reference analysis data.
5. The method according to any one of the preceding claims, wherein the detector comprises a GC-FID apparatus designed for headspace analysis.
6. The method according to any one of the preceding claims, wherein the reference analysis data comprise relative abundances of metabolites of m individual mastitis pathogens, respectively.
7. The method according to any one of the preceding claims, wherein the n metabolites comprise ketone alcohols, branched aldehydes, branched free fatty acids and ethyl esters of free fatty acids.
8. The method according to any one of the claims 1-6, wherein the n metabolites belong to at least k different chemical classes, wherein k is at least 3, and wherein the k chemical classes are selected from the group comprising ketones, ketone alcohols, branched aldehydes, free fatty acids, branched free fatty acids, ethyl esters of free fatty acids, ethyl esters of branched free fatty acids, and alcohols.
9. The method according to claim 8, wherein k is at least 4.
10. The method according to any one of the preceding claims, wherein at least n metabolites are selected from the group comprising acetaldehyde, ethanol, acetone, dimethylsulfide, 2,3-butadione, 2-butanone, ethyl acetate, 3-methylbutanal, 2-methylbutanal, 2-pentanone, acetic acid, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl butanoate, butanoic acid,

ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, 2-methylbutanoate, 2-heptanone, and ethyl hexanoate.

11. The method according to claim 10, wherein n is at least 9.
12. The method according to any one claims 1-11, wherein the at least n metabolites comprise ethyl acetate, 3-methylbutanal, 2-methylbutanal, 3-hydroxy-2-butanone, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, and 2-methylbutanoate, more especially, ethanol, 2,3-butadione, 2-butanone, ethyl acetate, 3-methylbutanal, 2-methylbutanal, 2-pentanone, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl butanoate, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, 2-methylbutanoate, 2-heptanone, and ethyl hexanoate.
13. The method according to any one of the preceding claims, wherein the m mastitis pathogens are selected from the group comprising Staph. aureus, Coagulase Negative Stapylococci, Strep. uberis, Strep. dysgalactiae and E. coli.
14. The method according to claim 13, wherein m is 5 and wherein the m mastitis pathogens comprise Staph. aureus, Coagulase Negative Stapylococci, Strep. uberis, Strep. dysgalactiae and E. coli.
15. The method according to any one of the preceding claims, wherein the milk sample is milk from a lactating animal.
16. The method according to any one of the preceding claims, wherein the method further comprises

    d. under the condition that a pathogen is determined present in the milk sample, treating the animal from which the milk sample originates, unless all pathogen(s) detected in the milk sample are gram negative bacteria.
17. A computer program product comprising executable instructions, which instructions, when executed on a computer, allow the computer to compare the analysis result with reference analysis data of n metabolites of m individual mastitis pathogens and to determine the presence of the m individual mastitis pathogens in the milk sample, according to the method according to any one of claims 1-16.
18. A computer program product comprising reference analysis data of n metabolites of m individual mastitis pathogens, which when read by a computer, allow the computer to compare the analysis result with the reference analysis data of n metabolites of m individual mastitis pathogens and to determine the presence of the m individual mastitis pathogens in the milk sample, according to the method according to any one of claims 1-16.

19. Use of reference analysis data of n metabolites selected from the group comprising acetaldehyde, ethanol, acetone, dimethylsulfide, 2,3-butadione, 2-butanone, ethyl acetate, 3-methylbutanal, 2-methylbutanal, 2-pentanone, acetic acid, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl butanoate, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, 2-methylbutanoate, 2-heptanone, and ethyl hexanoate, wherein n is at least 5, for determining with a detection method the presence of m individual mastitis pathogens in a milk sample, wherein the m individual mastitis pathogens are selected from the group comprising Staph. aureus, Coagulase Negative Stapylococci, Strep. uberis, Strep. dysgalactiae and E. coli, wherein m is at least 1, more preferably 5.
20. Use according to claim 19, where the reference analysis data comprise MS reference analysis data, more especially GC-MS reference analysis data and wherein the detection method is GC-MS.
21. Use according to any one of claims 19-20, wherein the n metabolites comprise ketone alcohols, branched aldehydes, branched free fatty acids and ethyl esters of free fatty acids.
22. Use according to any one of claims 19-21, wherein n is at least 9.
23. Use according to any one of claims 19-22, wherein n metabolites comprise acetaldehyde, ethanol, acetone, dimethylsulfide, 2,3-butadione, 2-butanone, ethyl acetate, 3-methylbutanal, 2-methylbutanal, 2-pentanone, acetic acid, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl butanoate, butanoic acid, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 3-methylbutanoate, 2-methylbutanoate, 2-heptanone, and ethyl hexanoate.

Fig 1

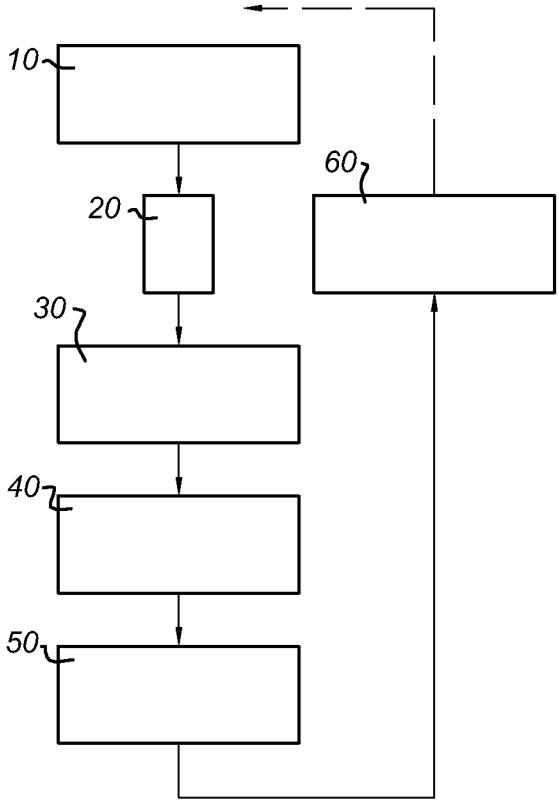


Fig 2a

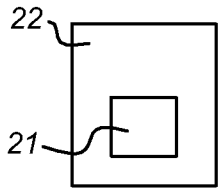


Fig 2b

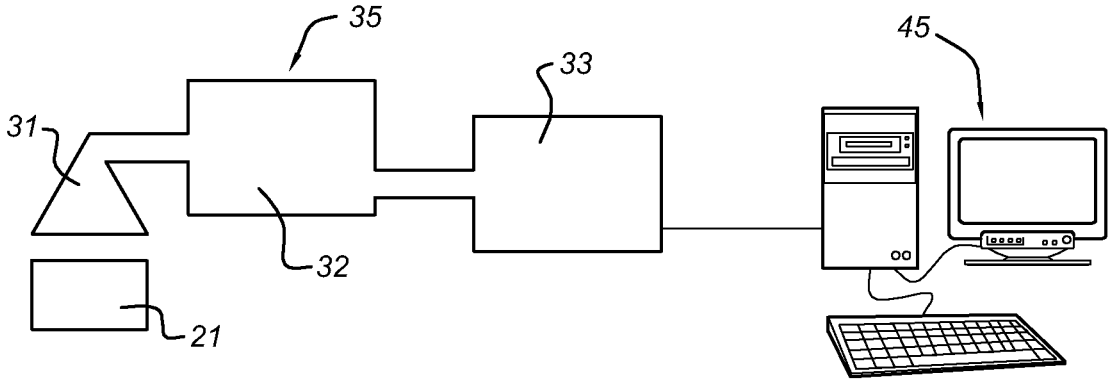
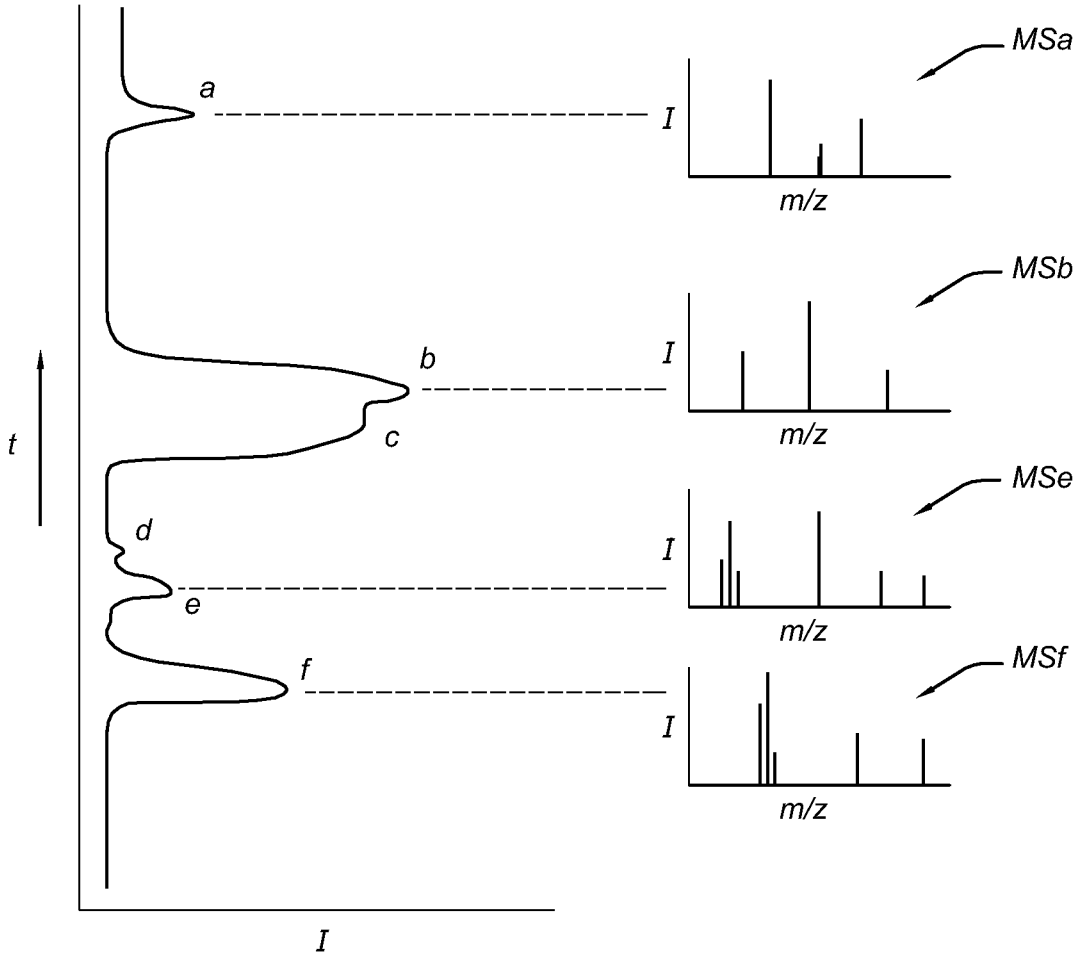


Fig 3



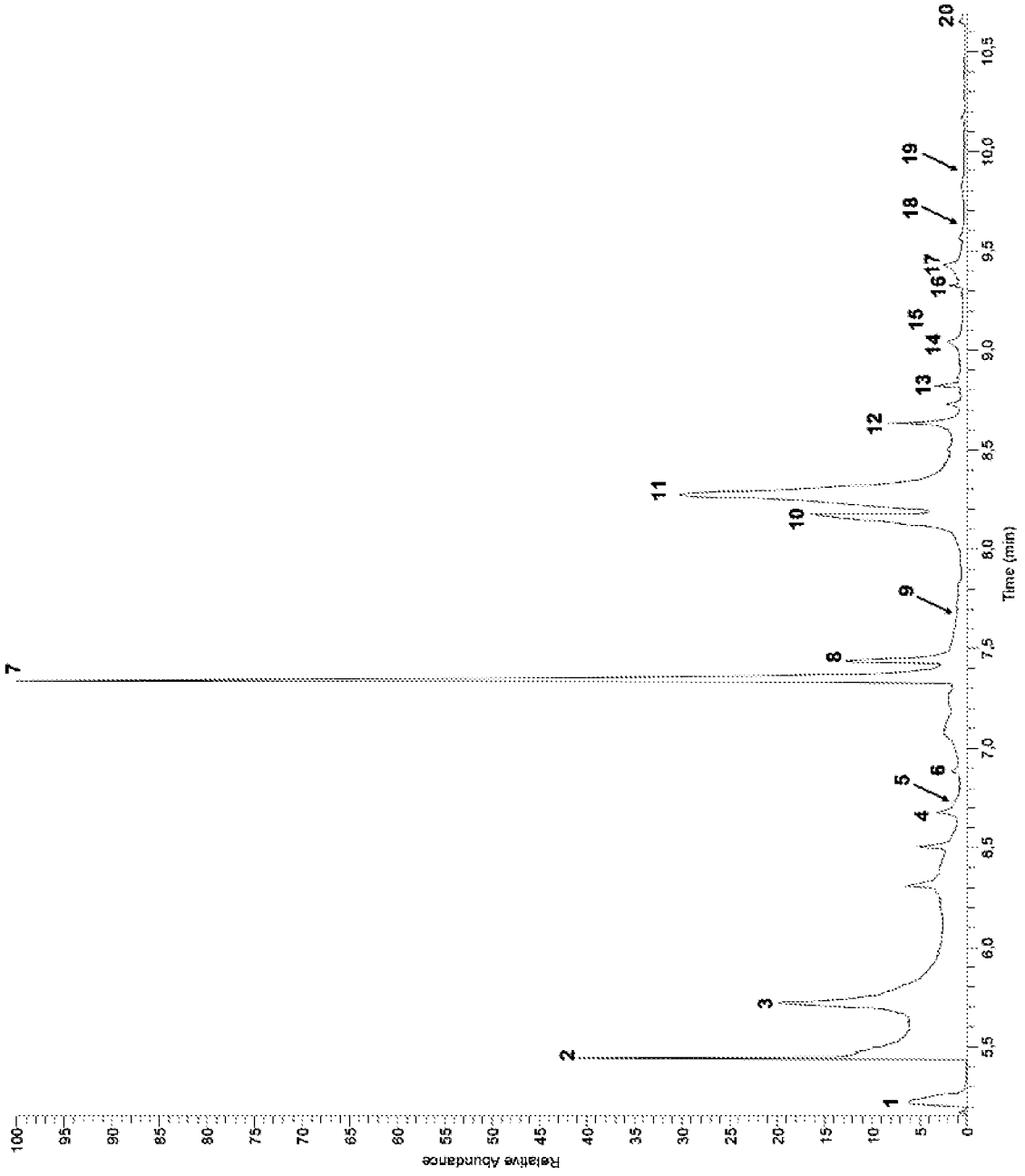

Relative Abundance
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17
18
19
20

Fig 4a

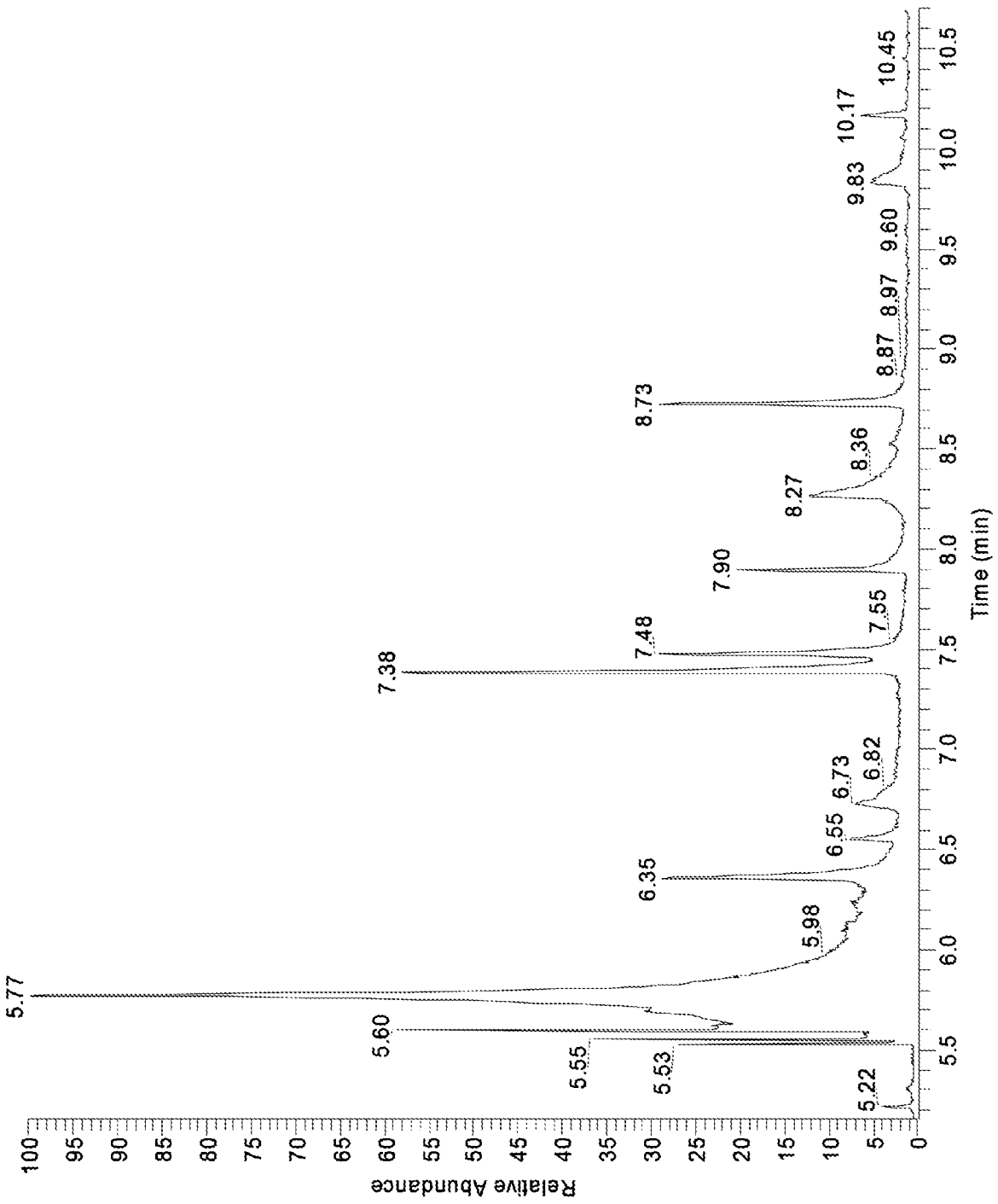

Relative Abundance
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7.48
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8.97
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10.45

Fig 4b

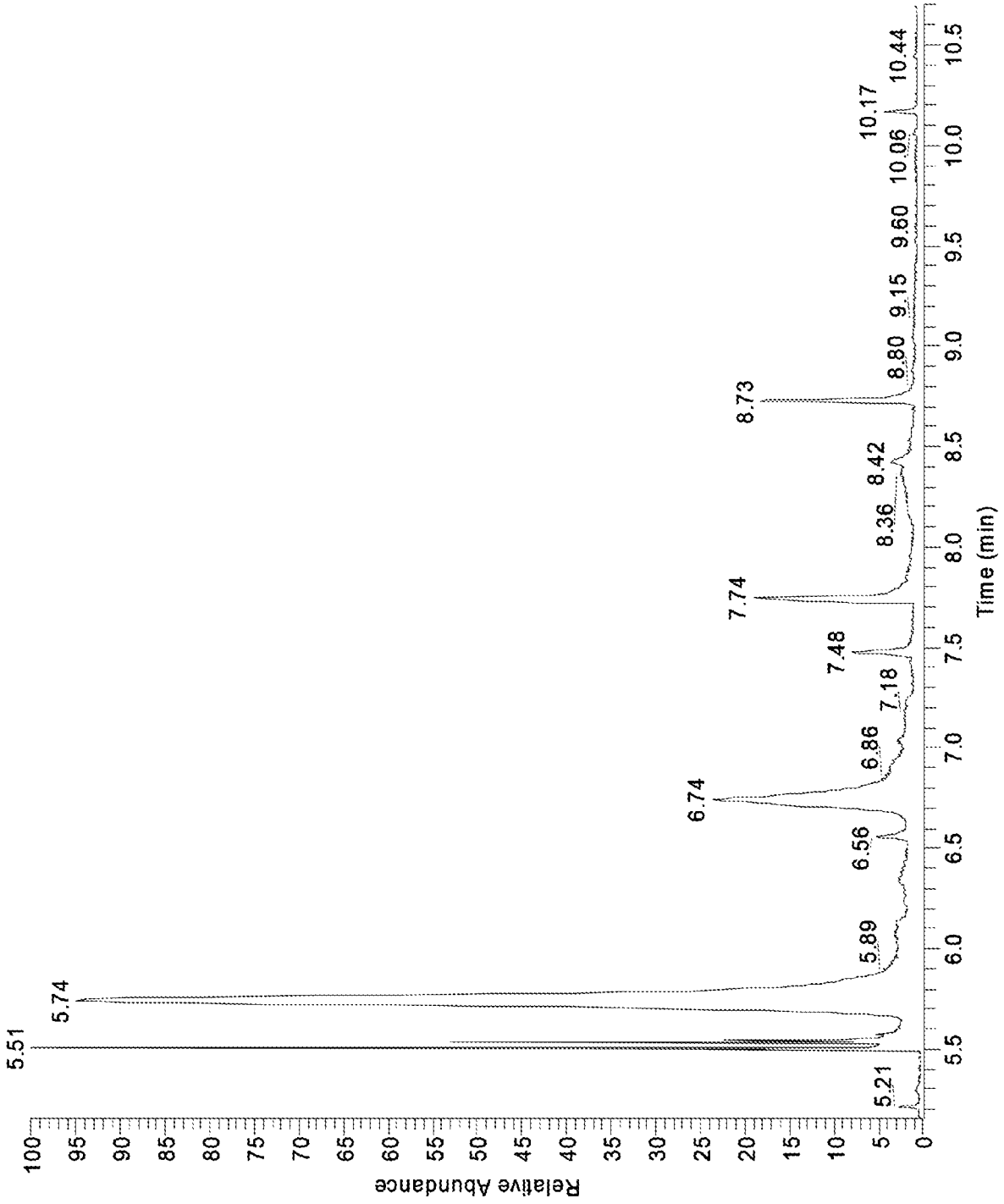

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10.17
10.44

Fig 4c

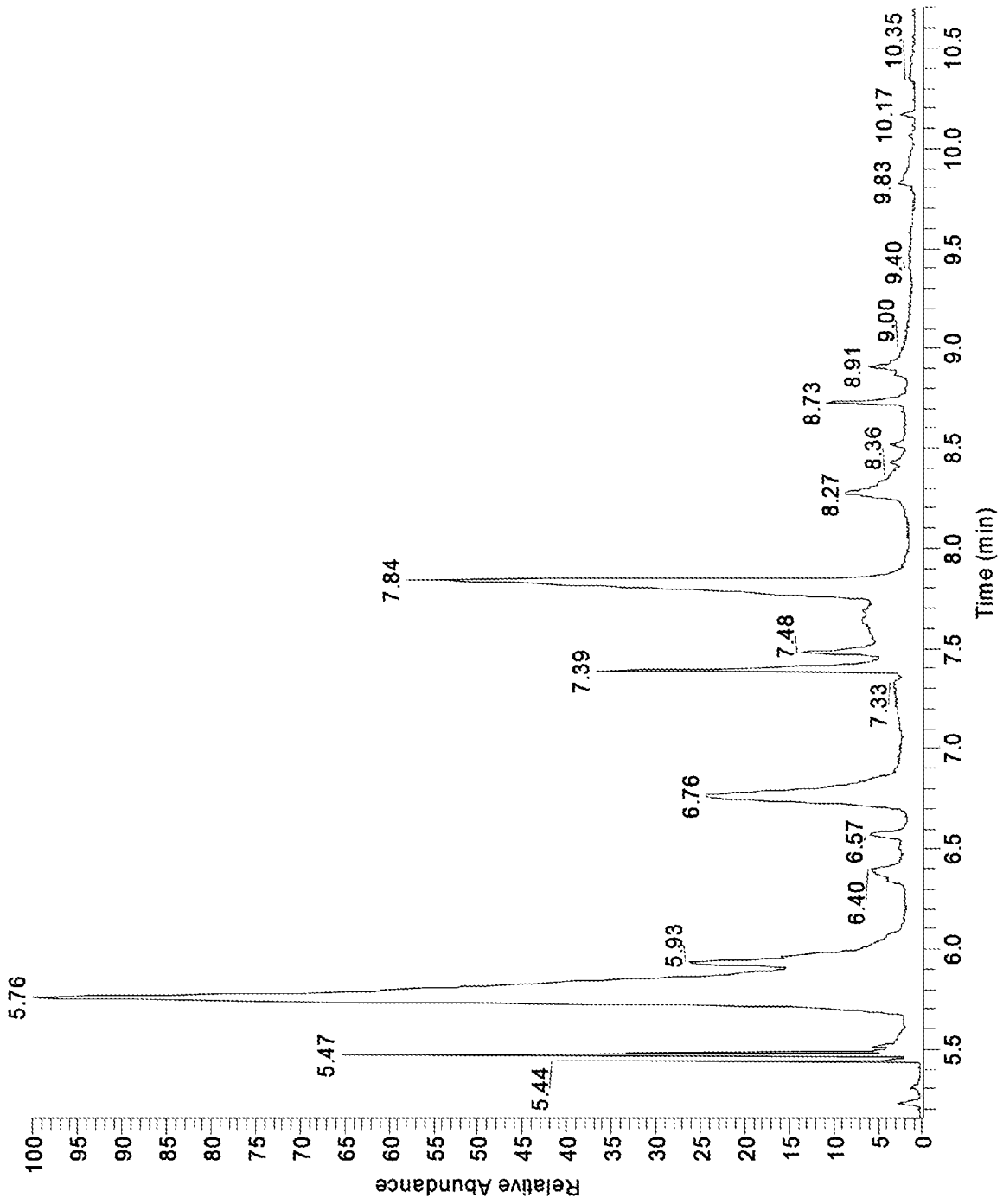

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10.17
10.35

Fig 4d

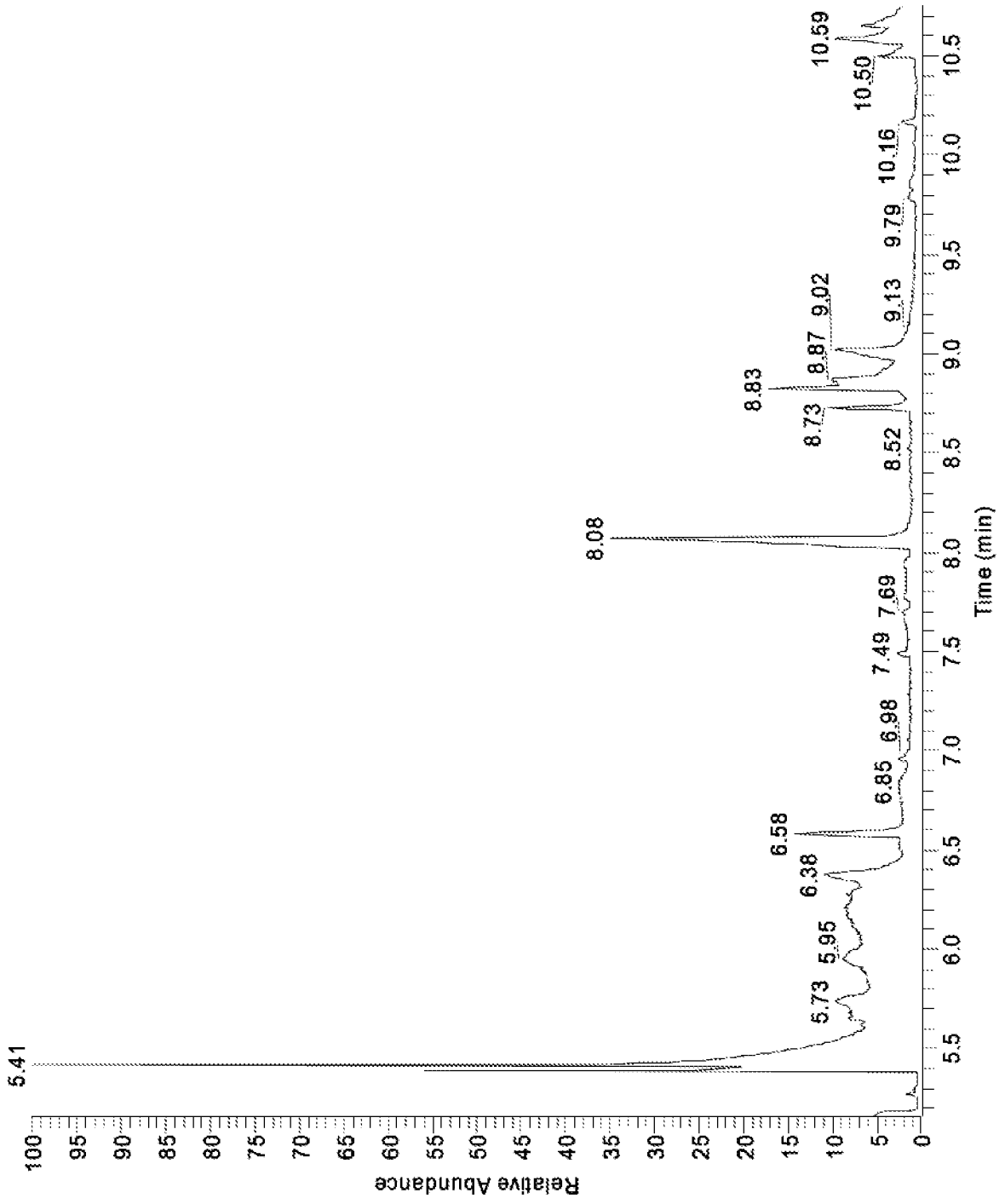

Relative Abundance
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8.73
8.83
8.87
9.02
9.13
9.79
10.16
10.50
10.59

Fig 4e