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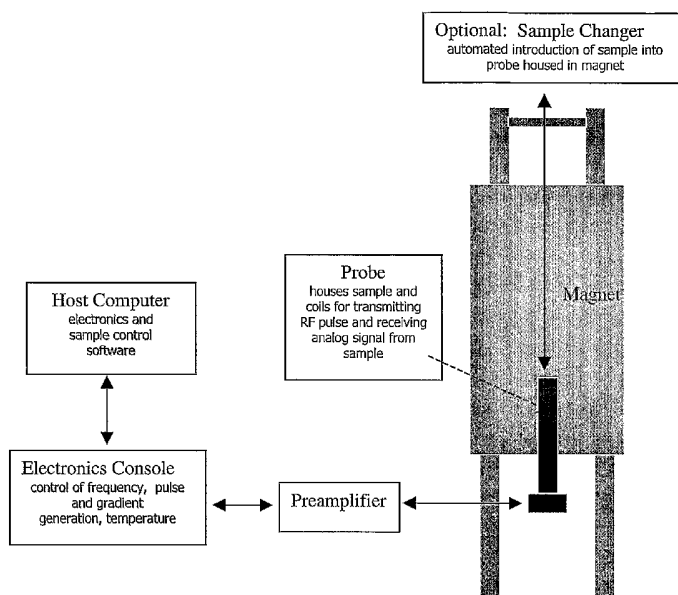
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(54) Title: ASSAY OF SEBUM LIPID COMPONENTS BY NUCLEAR MAGNETIC RESONANCE

Schematic of NMR spectrometer



(57) Abstract: The invention provides a method for identifying a compound that modulates sebum production or that modulates the amount of at least one individual lipid component of a sebum sample utilizing nuclear magnetic resonance (NMR) spectroscopy.

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ASSAY OF SEBUM LIPID COMPONENTS BY NUCLEAR MAGNETIC
RESONANCE

FIELD OF THE INVENTION

5 The present invention relates to an assay utilizing nuclear magnetic resonance spectroscopy for identifying compounds that modulate the lipid components of sebum.

BACKGROUND OF THE INVENTION

10 Human skin is composed of three primary layers, the stratum corneum, the epidermis, and the dermis. The outer layer of the skin, the stratum corneum, primarily functions as a barrier to the external environment preventing water loss and preventing the invasion of microorganisms. Lipids, secreted to the stratum corneum from the sebaceous glands, are the key components in maintaining this barrier. (Abramovits et al, Dermatologic Clinics, Vol. 18, Number 4, Oct. 2000).

15 Sebum, a complex mixture of proteins and lipids, is produced by the sebaceous glands. At maturation, the acinar cells of the sebaceous glands lyse and release sebum into the luminal duct, from which the sebum is secreted.

 Squalene, cholesterol, cholesterol esters, wax esters, and triglycerides are the primary lipids found in human sebum. Wax esters and squalene are
20 unique to sebum in that they are not synthesized by other cells in the body. During passage of sebum to the skin surface, bacterial enzymes hydrolyze some of the triglycerides, so that the lipid mixture reaching the skin surface also contains free fatty acids and small amounts of mono- and diglycerides.

 A number of methods to isolate, separate and qualitatively and/or
25 quantitatively analyze the lipid components of sebum and/or meibum have been reported (O'Neill and Gershbein, J. Chrom. Sci., 1976, (14) 28-36; Nordstrom et al., Journal of Investigative Dermatology, 1986; 86 (6), 700-5; Sullivan et al., Arch. Ophthalmol., 2002; 120:1689-1699). These methods are labor intensive, often require large amounts of sample and are not suitable for high throughput
30 screening of samples. What is needed is an accurate method for identifying compounds that modulate sebum production that is capable of qualitatively and/or quantitatively assessing changes in the lipid components of sebum.

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SUMMARY OF THE INVENTION

The invention provides a method for identifying a compound that modulates sebum production or that modulates the amount of at least one individual lipid component of a sebum sample, comprising subjecting a sample
5 from a treated subject to nuclear magnetic resonance (NMR) spectroscopy. In the method of the invention, a test compound is contacted with a source of sebum or meibum and, after an appropriate treatment period, the sebum, meibum or source of the sebum or meibum (collectively, hereinafter, "sebum") is collected from the test subject and submitted to NMR spectroscopy to determine the
10 amount of at least one lipid component of sebum. The amount of said lipid component is compared to the amount of lipid in a control sample. The amount of a lipid component in a test sample may be quantitated by NMR spectroscopy by acquiring proton NMR data from said test sample, processing the acquired NMR data to produce an NMR spectrum, and integrating the peak
15 area of a resonance assigned to the lipid component. The area can be related to a concentration by reference to NMR spectra of one or more reference lipids at multiple concentrations. The peak area of the spectral region for one or more sebum lipids of a treated sample can be compared to those of a control sample to determine whether a test compound inhibits the production of sebum or of one or
20 more individual lipids in sebum.

The sebum lipid component may be selected from the group consisting of triglycerides, cholesterol, cholesterol esters, wax esters, and hydrocarbons, such as squalene.

The NMR spectrometer is selected from a continuous-wave (CW) and a
25 pulsed or Fourier-transform (FT) spectrometer. Typically, a FT-NMR spectrometer is used in the present invention. FT-NMR spectrometers have a host computer, an electronics console, a preamplifier, and a magnet into which a probe is inserted. NMR spectrometers may also operate with an automated sample changer.

30 Typically, the NMR spectrometer includes a superconducting magnet. The NMR spectrometer useful in the present invention typically operates at proton frequencies above 400 MHz. Superconducting magnets with strengths above 11.7 Tesla are typically used in the assay of the present invention. A NMR spectrometer having a magnet with a strength of 11.7 Tesla operates at a proton

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frequency of about 500 MHz or higher. Typically, the method of the present invention utilizes a NMR spectrometer operating at proton frequencies of about 500 to 600 MHz.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1. Schematic of NMR Spectrometer
- Figure 2. Proton NMR Spectrum of Hamster Ear Extract
- 5 Figure 3. Proton NMR Spectrum of Sebum Extracted into Deuterated Cyclohexane
- Figure 4. Proton NMR Spectrum of Calibration Mixtures at lipid concentrations ranging from 5 μ M to 1000 μ M.
- 10 Figure 5. Distribution of Components in Sebum From Scalp of Male Subjects
- Figure 6. Proton NMR Spectrum of Meibum Extracted into Deuterated Cyclohexane

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DETAILED DESCRIPTION OF THE INVENTION

5 The present invention provides a method for identifying compounds that modulate the production of sebum by quantitatively detecting one or more sebum lipids in a sample by proton NMR spectroscopy.

10 The assay of the present invention may be utilized to assess whether a test compound modulates the production, composition, and/or secretion of sebum. It may be used to quantitate the amount of sebum produced, to determine if a test compound modulates (increases or decreases) the amount of at least one individual sebum lipid component and/or to quantitate the relative amounts of the sebum lipid components.

The method of the present invention can rapidly and accurately quantitate the amount of sebum components and compare the effectiveness of a treatment to modulate the production and/or secretion of sebum.

15 Briefly, a test agent that potentially modulates sebum production is applied to a subject or to cells. After an appropriate treatment period, a test sample from a treated subject (a subject exposed to a test compound) and a sample from an untreated subject (control sample) are -collected, the lipids are extracted from the test samples and at least one or more of the lipids are
20 analyzed by proton NMR spectroscopy. A compound inhibits the production of sebum or the amount of a sebum lipid component if the peak area under the NMR resonance (referred to as peak area) for said lipid component in said treated sample is less than the peak area under the NMR resonance in a control sample. The test compound increases the amount of a lipid if the peak area
25 under NMR resonance for said lipid component in a treated sample is higher than the peak area under the NMR resonance in a control sample. A control sample may also refer to reference standards prepared as in Examples 2 or 3.

The term "subject" refers to a human, an animal, or cells from a human or animal source that can produce a sample containing sebum lipids.

30 The term "sample" refers to any biological sample that may be a source of sebum or meibum (hereinafter, collectively "sebum"), including, but not limited to, sebaceous glands, meibomian glands, normal cells, tumors, skin, hair, specific layers of skin, i.e. the dermis, epidermis, stratum corneum or tear film or fluid. The tissue may be of animal origin. Thus, it may be derived from fur, feathers,
35 animal skin or other tissues. For instance, the "sample" may be a skin surface sample collected from the skin surface by, for instance blotting, wiping the

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surface of the skin or by absorbing skin surface materials on to absorbent papers or absorbent tapes. One such absorbent tape is described in US Patent No. 4,532,937, which is incorporated herein by reference. Skin surface samples may include samples from which all of part of the stratum corneum has been removed such as that described by Cotterill et al. (Br. J. Derm. (1972) Vol. 86, pp 356-360) or may include cells of the stratum corneum.

The method of the present invention may be used to identify compounds that modulate the amount of one or more sebum lipids selected from the group consisting of wax esters, cholesterol esters, triglycerides, and hydrocarbons.

Proton Nuclear Magnetic Resonance Spectroscopy (proton NMR) possesses a number of advantages for the analysis and quantitation of sebum components compared to previously described assays. It allows for the simultaneous quantification of multiple lipid classes to be conducted with significantly smaller quantities of test samples, a significant advantage when the assay is being conducted on human subjects.

Nuclear magnetic resonance (NMR) spectroscopy uses radiofrequency radiation to induce transitions between different nuclear spin states of samples in an applied magnetic field. NMR spectroscopy can be used for quantitative measurements, as well as determining the structure of molecules.

Nuclei with an odd number of protons, neutrons, or both, will have an intrinsic nuclear angular momentum or "nuclear spin". When a nucleus with a non-zero spin is placed in a magnetic field, the nuclear spin can align in either the same direction or in the opposite direction as the external magnetic field. Nuclei that are commonly observed in NMR spectroscopy include ^1H , ^{13}C , ^{19}F , ^{15}N , and ^{31}P . A nucleus that has its spin aligned with the external field will have a lower energy than when it has its spin aligned in the opposite direction to the field. Thus, these two nuclear spin alignments have different energies in the presence of an applied magnetic field.

The magnitude of the energy splitting between these levels for nuclei in a strong magnetic field is in the range of radiofrequency (RF) radiation. Absorption of the RF radiation causes nuclear spins to realign or flip in the higher-energy direction. After absorbing energy, nuclei will reemit RF radiation and return to the lower-energy state.

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The energy (and thus frequency) of an NMR transition depends on the magnetic-field strength and a proportionality factor for each nucleus called the magnetogyric ratio, γ . The frequency of a transition is given by:

$$\nu = \frac{\gamma}{2\pi} H$$

5 where ν is the frequency of the resonant radiation and H is the strength of the magnetic field.

The utility of NMR spectroscopy for structural characterization arises because different atoms in a molecule experience slightly different magnetic fields and therefore have transitions at slightly different resonance frequencies in
10 an NMR spectrum. Furthermore, multiplicity of the spectra lines arises due to interactions between different nuclei, which provide information about the proximity of different atoms in a molecule.

The utility of NMR spectroscopy for quantitative analysis arises from its inherent linear response over concentrations spanning multiple orders of
15 magnitude. The area of an NMR resonance is directly proportional to the molar quantity of that proton present in the sample. Selecting NMR data acquisition parameters for quantitative analysis are generally understood by those skilled in the art (Traficante, Daniel D. "Optimum Tip Angle and Relaxation Delay for Quantitative Analysis," Concepts in Magnetic Resonance, 1992, 4, 153-160).

20 The resonance frequencies of different nuclei in an atom are described by a relative shift compared to the frequency of a standard. This relative shift is called the chemical shift, δ , and is given by:

$$\delta = \left(\frac{\nu_{sample} - \nu_{ref}}{\nu_{ref}} \right) 1 \times 10^6$$

25 where δ has units of ppm (parts per million). For proton NMR spectroscopy, a common reference compound in organic solvents is tetramethylsilane, $\text{Si}(\text{CH}_3)_4$, or TMS. The chemical shift of proton resonances from the solvent may also be used to reference spectra. This is commonly referred to as external referencing.

30 There are two main NMR spectrometer designs, continuous-wave (CW) and pulsed or Fourier-transform (FT). The most commonly used design in modern spectrometers is Fourier-transform NMR spectrometers (FT-NMR).

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Fourier-transform NMR spectrometers use a pulse of radiofrequency (RF) radiation to cause nuclei in a magnetic field to flip into the higher-energy alignment. The frequency width of the RF pulse (typically 1-10 μ s) is wide enough to simultaneously excite nuclei in all local environments. All of the nuclei will re-emit RF radiation at their respective resonance frequencies, creating an interference pattern in the resulting RF emission versus time, known as a free-induction decay (FID). The frequencies are extracted from the FID by a Fourier transform of the time-based data, resulting in a NMR spectrum where the x-axis is chemical shift (in ppm) and the y-axis is the signal intensity.

Figure 1 depicts the main components of a typical modern NMR spectrometer. As shown in Figure 1, NMR spectrometers have a host computer, an electronics console, a preamplifier, and a magnet into which a probe is inserted. NMR spectrometers may also operate with an automated sample changer.

The host computer runs software to control data acquisition and processing as well as to control the sample changer.

The electronics console contains hardware to generate precisely-timed RF pulses at specific frequencies and a system to receive and process the NMR signal. The console also contains a separate acquisition computer that communicates with the host computer to execute the NMR experiment. The console may also have electronics to control the temperature of the sample in the probe.

During an NMR experiment, an RF pulse is generated in the console and applied to the sample via a RF coil in the probe. The receiver coil in the probe detects the NMR signal as an analog voltage. The signal is amplified by the preamplifier and then converted to a digital output by the ADC (analog-digital converter) board in the electronics console. After digitizing the data, the data is transferred to the acquisition computer and ultimately to the host computer.

Electromagnets or superconducting magnets are available.

Superconducting magnets with strengths above 11.7 Tesla are most useful in the assay of the present invention.

A test sample is inserted into the probe. The probe contains the RF transmitter and receiver coils. The probe may also contain a heating element to maintain samples at a given temperature, as well as a gradient coil for applying magnetic field gradients. Many probes are commercially available to observe a

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wide range of nuclei. A probe designed to observe three nuclei is referred to as a triple resonance probe.

Probes may accept test samples directly or in tubes that are pneumatically lowered into the probe. Probes that accept test samples contained in tubes are commonly referred to as conventional probes. Typically, tubes from about 1 to about 10 mm in diameter may be utilized. Probes that accept test samples directly are commonly referred to as flow probes. Most common flow probes are designed to accommodate anywhere from 1 μL to 250 μL . Flow probes containing less than 10 μL are usually referred to as capillary flow probes.

Electronics within the probe may operate at about room temperature or temperatures well below room temperature (approximately 25 Kelvin). The combination of a preamplifier and probe with an RF coil operating at cryogenic temperature are referred to as cryoprobes, cold probes, or cryogenically cooled probes by those skilled in the art. Cryoprobes generally have significantly less noise resulting in up to a four-fold increase in sensitivity. In one embodiment, the assay is performed using a 5mm triple resonance cryogenically cooled probe equipped with a z-axis gradient.

Introduction of the sample into a probe may be automated through the use of robotic samples changers or robotic liquid handlers, depending on the probe configuration. These are not required, but are useful in operating NMR spectrometers without manual intervention. In one embodiment, the assay is performed using a Bruker Avance NMR spectrometer equipped with a Bruker automatic sample changer with a capacity for 120 samples (B-ACS 120) (Billerica, Massachusetts).

NMR spectrometers are available from a number of commercial sources. These include, but are not limited to, Varian (Palo Alto, California), Bruker, (Billerica, Massachusetts), and JEOL-USA (Peabody, Massachusetts). Any commercially available or home built NMR spectrometer may be used in the assay of the present invention.

Typically, the assay used in the present invention is carried out in the following manner. A sample from a subject that has been treated with a test compound is collected and placed in a 5mm glass NMR tube and inserted into the NMR probe. Typically, the sample is solubilized in a volume of solvent that is from about 5 to about 100 times the volume of the lipid of the sample. However, smaller or larger amounts of solvent may be used. One of skill in the art will

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readily be able to determine the amount of solvent suitable for their particular sample. Proton NMR spectra may be acquired with a Bruker Avance NMR spectrometer operating at 599.8 MHz for proton, using a 5 mm triple resonance cryoprobe equipped with a z-axis gradient. The acquisition parameters included
5 a 15 ppm spectral width, 90 degree pulse, a 1.8 second acquisition time, a 6 second relaxation delay, 32K data points, and scanned 64 times. All data were acquired at 25 °C using an automated sample changer.

The amount or modulation of sebum lipids in a sample from a subject treated with a compound (treated subject) is determined by measuring the peak
10 area of an NMR resonance assigned to one or more lipids and comparing the peak area of said lipids to those obtained for standard curves for selected lipids either alone or in a mixture and comparing said peak area with that produced by samples from subjects that were not exposed to the test compound (control subjects). Alternately, the modulation of sebum lipids in a sample from a treated
15 subject may be determined by comparing the intensity of an NMR resonance assigned to one or more lipids to the intensity of an NMR resonance of said lipids in sample from a control subject. The method of the invention allows the determination of changes in the amount of at least one of the lipids selected from the group consisting of squalene, cholesterol esters, triglycerides, and wax esters
20 in a test sample by comparison of the NMR peak area of the lipids in the test sample with the NMR peak area of said lipids in a sample from untreated subjects. The peak area may also be converted to a concentration using a calibration curve. The modulation of sebum lipids in a sample from a treated subject is then determined by comparing the concentration of one more lipids to the concentration of said lipids in a sample from a control subject. For purposes
25 of this invention, a calibration curve for at least one sebum lipid may be utilized as a control sample. The calibration curve may be performed at the same time as the sample analysis or may be one that had been previously carried out under the same conditions as those used for the test sample. Similarly, for the purpose
30 of this invention, historical data may be used to determine whether a compound modulates the amount of sebum produced or the amount of at least one sebum lipid component.

One skilled in the art, based upon the teachings of this application, could carry out the assay with a wide variety of NMR spectrometers.

SAMPLE PREPARATION

Prior to contacting the samples with the NMR spectrometer, the sebum lipids may be extracted from the sample by contacting the sample with an extraction medium. The extraction can be carried out in an extraction medium in any manner that extracts the lipid components of sebum. The term "extraction medium" is meant to include any solution with which the test sample is contacted prior to subjecting said test sample to NMR analysis. The extraction medium may comprise a single solvent or a combination of solvents in which the lipids of interest are soluble. Typically, the extraction media may be composed of polar and/or non-polar organic solvents such as chloroform, methanol, propanol, isopropanol, di-chloromethane, tri-methyl-pentene, hexane, cyclohexane, toluene, benzene, or heptane or their combinations and may contain an aqueous phase with or without modifiers (such as acids or bases). The extraction media may also be composed the above mentioned solvents with protons replaced with deuterons, commonly referred to as deuterated solvents. The type of extraction media used depends on the lipids of interest. As a general rule of thumb, polar lipids require polar extraction media, while non-polar lipids are better extracted in non-polar solvents.

In one embodiment, the sebum is extracted from the test sample by contacting the test sample with an extraction medium comprising at least one volatile organic solvent for a sufficient period of time to sufficiently release the lipid components, so that they may be detected by NMR spectroscopy. The extraction medium may also comprise an aqueous phase in addition to the organic solvents. Any of these methods may be used in conjunction with the NMR analysis of the present invention. The extraction can be carried out in any manner that extracts the lipid components of sebum. Several methods for extracting the sebum components have been reported. Sebum lipids are most commonly extracted using a mixture of chloroform:methanol (2:1 v/v; "Folch solvent") or ether [J. of Dermatological Science, 1 (1990) 269-276, Invest. Ophthalmol. Vis. Sci. 20, 4, 1981, 522-536]. The volume of extraction medium used to extract the lipids from a sebum sample is not critical. It is only required that the volume of the extraction medium exceed the volume of the lipid in the sample. Typically, the extraction medium is used at a ratio of from about 2 to about ten times the sample volume. Typically the ratio of the volume of extraction medium to the volume of sample lipid is about from about 5 to 1 to about 10 to 1.

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The extracted test sample may be analyzed directly by NMR spectroscopy. Alternately, the extraction medium may be removed by evaporation and the sample comprising the lipids stored for later analysis. The test sample may then be reconstituted in a solvent compatible with NMR spectroscopy.

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SEBACEOUS GLANDS

A test compound is applied to the sebaceous gland of a subject (treated subject). After a suitable time, the sebaceous glands are isolated and the lipids extracted by contacting the glands with an extraction medium. Typically, such samples are homogenized and extracted in an extraction medium. The homogenates are centrifuged and the lipid-containing layer can be used directly for NMR analysis. Alternatively, the lipid containing extract can also be dried down and reconstituted in a suitable solvent mixture for NMR spectroscopy. The sebaceous glands from any species may be utilized. Sebaceous glands from the ears of hamsters are used as a model to study the effect of compounds on the modulation of sebum production.

15

PREPARATION OF SEBUM SAMPLES

In one embodiment of the invention, the test sample is selected from human sebum. The sebum samples are collected, placed in a suitable solvent and subjected to analysis by proton NMR spectroscopy. The samples may be collected by any means known in the art. For instance, samples can be collected using absorbents, such as Sebutape® (CuDerm Corporation, Dallas, TX) (lipid absorbing polymeric film), cigarette paper, clean tissue or filter paper that can absorb lipid components from tissue surfaces. Absorbents can be soaked in organic solvents (e.g. ether) prior to application to aid the absorption of lipids from the surface of the tissues. Bentonite clay patches have also used to collect surface lipids. (Clarys et al. in Clinics in Dermatology 1995, 13, 307-321) Meibum samples can also be collected by touching microcapillaries or cotton tipped applicators to the outer surface of the eye and collecting the expelled fluid with a chalazion curette (surgical stainless steel) (Sullivan et al. The Journal of Clinical and Endocrinology & Metabolism Vol. 85, No. 12. p. 4866-4872).

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In order to provide greater speed and higher sample throughput, the extracted sample can be directly analyzed by NMR spectroscopy without further processing. In one embodiment, the present invention allows for the direct analysis of extracted sample by proton NMR spectroscopy by using a lipid-

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solubilizing NMR-compatible extracting medium that allows for dissolution of the sebum components into the extraction medium.

5 The sebum may be collected on Sebutape®. Typically, 0.1-10 mg of sebum will be analyzed. The amount of extraction medium used for each sample may vary from, for instance, about 0.5 mL to about 5mL. Typically, 0.5 mL of the extraction medium will be used per Sebutape® test sample.

10 In one embodiment, the samples are extracted in an extraction medium of deuterated cyclohexane. The extraction is carried out for a sufficient period of time at a temperature sufficient to extract the lipids in the test sample. Typically, this will range from about 5 min to 0.5 hrs. Typically, the sample is shaken or vortexed during the extraction period. The extraction may be carried out at any temperature that is compatible with the integrity of the lipids to be analyzed. Typically, the extraction is carried out at a temperature ranging from about 20° C to about 60°C. In one embodiment, the extraction is carried out at approximately
15 20°C to about 25°C. After a sufficient period of time, the extracted lipids may be directly analyzed by NMR spectroscopy. Alternately, the extraction medium may be removed, for instance, by evaporation, and the lipid sample may be stored until subjected to NMR analysis.

20 Prior to NMR analysis, the test sample is reconstituted in a medium compatible with proton NMR spectroscopy.

QUANTIFICATION OF SEBUM COMPONENTS

25 In order to identify compounds that modulate the production of sebum or the amount of one or more sebum lipids, the present invention enables one to quantitate the amount of selected lipids of a test sample, for instance, the cholesterol ester or triglyceride content of a sebum sample. The amount or modulation of sebum lipids in a sample from a subject treated with a compound (treated subject) is determined by measuring the
30 peak area of an NMR resonance assigned to one or more lipids or measuring the signal intensity of the NMR resonance assigned to one or more lipids. Modulation of lipids is determined by comparing the measurement of peak area or signal intensity of said lipids to those measured in samples from subjects that were not exposed to the test
35 compound (control subjects). The peak area may also be converted to

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concentration unit using a calibration curve. The empirical relationship between the response detected by the NMR spectrometer from selected lipid components of a test sample is determined by comparison to a calibration curve of known lipids using standard solutions. The calibration curve is typically set up by preparing at least five standard solutions in deuterated cyclohexane of the cholesterol ester or triglyceride of interest ranging from 5 μ M to 1000 μ M. For example see Figure 4.

The test samples may be extracted and analyzed in parallel with the standards of the calibration curve using identical conditions. Alternatively, a calibration curve may have been previously run and the test sample data compared to the calibration curve for the specific lipid component of interest. The test sample content of a specific lipid component of interest, for instance a cholesterol ester or triglyceride, is calculated using linear regression analysis of the peak area responses from the cholesterol ester or triglyceride content of the test samples and correlating it to the cholesterol ester or triglyceride concentrations from the calibration curve. For purposes of this invention a calibration curve may be utilized as a control sample. The calibration curve may be performed at the same time as the sample analysis or may be one that had been previously carried out under the same conditions as those used for the test sample. Similarly, for the purpose of this invention, historical data may be used to determine whether a compound modulates the amount of sebum produced or the amount of at least one sebum lipid component.

Quantification may be performed, for instance, by proton NMR spectroscopy on a Bruker Avance (Billerica, Mass.) NMR spectrometer operating at a proton frequency of 599.98 MHz. The NMR spectrometer may be equipped with a cryogenically-cooled triple-resonance probe and an automatic sample changer.

EXAMPLES

EXAMPLE 1

5 Determination of Effect of Flutamide and Accutane on Hamster ear Sebum by
10 Proton NMR Spectroscopy

Male Syrian Hamsters approximately 8-10 weeks old were housed in individual cages. The animals were acclimated to 16-hour light cycles for 2 weeks prior to dosing. For each treatment, test animals are anesthetized using isoflurane gas. Each treatment group consisted of 9 animals. In one experiment, twenty microliters of a test compound was applied topically to the ventral side of each ear using a positive displacement pipette. The subjects were treated twice a day, 7 days a week, for four weeks with at least 6 hours between treatments. The test compound A, 3% flutamide, was prepared in 50:50 (v/v) ethanol:propylene glycol (EtOH:PG). Control animals were treated with 50:50 (v/v) EtOH:PG. In a separate experiment, animals were treated with the test compound at a dose of 20 mg/kg, given orally to animals. The subjects were treated twice a day, 7 days a week, for four weeks with at least 6 hours between treatments. The test compound B, Accutane (Isotretinoin), was prepared as a suspension in PG. Control animals were treated with PG alone.

At the end of the treatment period, one 8mm distal biopsy punch was taken, just above the anatomical "V" mark in the ear to normalize the sample area. The punch was pulled apart. The ventral biopsy surface (the area where the topical dose was directly applied to the sebaceous glands) was retained for testing and the dorsal surface of the biopsy punch was discarded. Tissue samples were dried using nitrogen gas and stored at -80°C under nitrogen until analysis.

Prior to NMR analysis, the tissue samples were removed from the freezer and allowed to come to room temperature in capped vials. Fifty microliters of a 2 mg/mL archidonic alcohol (AA) was added to each sample to serve as the internal standard for the analysis. Tissue samples were contacted with 3ml of solvent (a 4:1 v/v mixture of 2,2,4-trimethylpentane and isopropyl alcohol). The mixture was shaken for 15 minutes and stored overnight at room temperature, protected from light. One milliliter of water was added to the sample and the sample was shaken for 15 minutes. The sample was then centrifuged at

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approximately 1500rpm for 15 minutes. One and one-third ml of the organic phase (top layer) was transferred to a glass vial, dried at 37°C, under nitrogen, for approximately 1 hour, and then further dried under vacuum for approximately 48 hours. The samples were then removed from the lyophilizer and each vial was reconstituted with 600 µL of deuterated chloroform. The vials were capped and manually shaken. The entire sample was transferred to a 5 mm NMR tube.

Within 24 hours of sample preparation, the NMR tube was inserted into a spinner, and placed in an autosampler. The autosampler was interfaced with a Bruker Avance NMR spectrometer operating at 599.98 MHz for proton. The spectrometer was outfitted with a triple resonance cryoprobe equipped with a z-axis gradient. The acquisition parameters included a pulse width of 90 degrees, acquisition time of 1.8 seconds, a relaxation delay of 6 seconds, a spectral width of 15 ppm, 32K data points, and scanned 64 times. All data were acquired at temperature of 25 °C. Spectra were processed using Metabonomi software (see International Patent Application WO2004038602) in which NMR data are Fourier transformed. Within the software, the resulting spectra were phased, baseline corrected, referenced to the low levels of protonated chloroform present in the solvent at 7.26 ppm, and standardized to 32K data points in the region from 10 to -0.5 ppm. Using Metabonomi, the area of spectral regions were measured including regions assigned to cholesterol esters (CE) (4.66 to 4.76 ppm), wax esters (WE) (3.98 to 4.07 ppm), and arachidonic alcohol (AA) (3.60 to 3.70 ppm). The peak area for wax esters and cholesterol esters were normalized using the area of the internal standard, arachidonic alcohol.

A representative NMR spectrum from a control hamster ear extract is shown in Figure 2.

The NMR peak areas of cholesterol esters and wax esters in the hamster ear sebum sample extracts (600 µL volume each) of untreated subjects and subjects (n=9 subjects/group) treated with 3 % Flutamide are summarized in Table 1 below. Table 1 shows that subjects treated with 3% Flutamide had 18.7 % less cholesterol esters and 32.9 % less wax esters than the untreated subjects.

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Table 1

Sebum Component	Measured NMR Peak Areas		% Change in Mean Peak Area
	Untreated (Mean $\pm$ SE*)	Treated 3% Flutamide (Mean $\pm$ SE*)	
Cholesterol Esters	0.71 $\pm$ 0.03	0.58 $\pm$ 0.02	-18.7
Wax Esters	0.85 $\pm$ 0.03	0.57 $\pm$ 0.01	-32.9

* SE – standard error calculated by dividing standard deviation by square root of n (number of measurements)

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The NMR peak areas of cholesterol esters and wax esters in the hamster ear sebum sample extracts (600 μ L volume each) of untreated subjects and subjects (n=10 subjects/group) treated with 20 mg/kg Accutane are summarized in Table 2 below. Table 2 shows that subjects treated with 20 mg/kg Accutane had 30 % less the cholesterol esters and 38.1 % less wax esters than the untreated subjects.

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Table 2

Sebum Component	Measured NMR Peak Areas		% Change in Mean Peak Area
	Untreated Mean $\pm$ SE*	Treated 20 mg/kg Accutane Mean $\pm$ SE*	
Cholesterol Esters	0.59 $\pm$ 0.06	0.41 $\pm$ 0.01	-30.0
Wax Esters	0.75 $\pm$ 0.09	0.47 $\pm$ 0.02	-38.1

* SE – standard error calculated by dividing standard deviation by square root of n (number of measurements)

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Tables 1 and 2 further demonstrated that the method of the present invention is suitable for the detection of changes in the concentration of the selected sebum lipids as a response to drug treatment.

EXAMPLE 2

Determination of sebum components in human sebum collected on Sebutape®
by proton NMR spectroscopy

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The proton NMR spectroscopy method of the current invention was used to determine the concentration profile of sebum lipids in human subjects.

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Sebum was collected from human volunteers on three separate visits. Samples were taken from the right and left sides of the malar and forehead regions of female volunteers and the right and left sides of the malar, forehead, and scalp regions from male volunteers. Prior to sample collection, the region was cleansed with an alcohol wipe. For the male volunteers, samples from the scalp region were collected 5 minutes after cleansing. Samples were collected by applying Sebutape® to the skin for 30 seconds. The Sebutape® was removed from the cardboard backing using tweezers and placed into a 1 dram glass vial. The samples were stored at – 70 °C until sample extraction and analysis. Additional samples from the malar, scalp, and forehead regions were collected three hours after cleansing. Samples were collected by applying Sebutape® to the skin for 30 seconds. The Sebutape® was removed from the cardboard

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backing using tweezers and placed into a 1 dram glass vial. The samples were stored at – 70 °C until sample extraction and analysis.

The samples were removed from the freezer and allowed to come to room temperature in capped vials without assistance. Vials were uncapped and 600 uL of deuterated cyclohexane was added to each vial. Samples were capped and agitated for 15 minutes. The entire solution was transferred to a 5 mm NMR tube while the Sebutape® remained in the vial.

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Within 24 hours of sample preparation, the NMR tube was inserted into a spinner, and placed in an autosampler. The autosampler was interfaced with a Bruker Avance NMR spectrometer operating at 599.98 MHz for proton. The spectrometer was outfitted with a triple resonance cryogenically-cooled probe equipped with a z-axis gradient. The acquisition parameters included a pulse width of 85 degrees, acquisition time of 1.8 seconds, a relaxation delay of 1 second, a spectral width of 15 ppm, 32K data points, and scanned 512 times. All data were acquired at temperature of 25 °C. Spectra were processed using a software package, Metabonomi, (see patent WO2004038602) in which NMR data

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are Fourier transformed. Within the software, the resulting spectra were phased, baseline corrected, referenced to the low levels of protonated cyclohexane present in the solvent at 1.38 ppm, and standardized to 32K data points in the region from 10 to -0.5 ppm. Using Metabonomi, the area of spectral regions were measured including regions assigned to cholesterol esters (CE) (4.52 to 4.60 ppm), wax esters (WE) (3.96 to 3.99 ppm), triglycerides (TG) (4.21 to 4.30 ppm), and squalene (5.06 to 5.13 ppm). A representative NMR spectrum of human sebum is shown in Figure 3.

Peak areas measured in the NMR spectra were converted to concentration values using a calibration function relating NMR peak areas to concentration. The calibration function was constructed from NMR data on six concentrations of a mixture of calibration standards. The calibration mixture was prepared from an equimolar, four-component stock solution prepared from commercially available reference compounds dissolved in deuterated cyclohexane. The reference compounds were chosen to include at least one component from each class of compounds being measured with the NMR method. The reference compounds included squalene, triolein (a triglyceride), cholesteryl palmitate (a cholesterol ester), and palmitic acid stearyl ester (a wax ester). The concentrations included 5, 50, 100, 500, 750, and 1000 μM for each component. Proton NMR spectra were acquired and processed using the same procedure as outlined above for the test samples. Spectra from the calibration mixture are shown in Figure 4. For each component or class of components, as in the case of cholesterol esters and wax esters, least squares regression was used to fit a linear model to the peak area versus the concentration data, each converted to logarithmic scale.

Results showed strong correlations between bilateral measurements completed within a subject and region and good reproducibility of parameters at 3-hour timed measurements. The distribution of the squalene, cholesterol esters, triglycerides, and wax esters in sebum from the scalp region of male subjects at a 3-hour time measurement is shown in Figure 5. The distribution is shown in mole fraction units calculated by dividing the concentration of a single component by the total concentration of squalene, cholesterol esters, triglycerides, and wax esters.

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Results demonstrated that the method of the present invention is suitable for quantifying the distribution of selected lipids in human sebum and determining the effect of treatment on lipid composition.

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EXAMPLE 3

Determination of meibum components in human tear films collected on
Sebutape® by proton NMR spectroscopy

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The proton NMR spectroscopy method of the current invention was used to determine the concentration profile of meibum lipids in human subjects.

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Meibum samples were collected from the eye of a female volunteer and analyzed for squalene, triglycerides, cholesterol esters, and wax esters content. Samples were collected using Sebutape® from lower eyelids (ciliary line) by touching the Sebutape® to the inner side of the eyelids for 5-10 seconds. The Sebutape® was removed from the cardboard backing using tweezers and placed into a 1 dram glass vial. The samples were stored at – 70 °C until sample extraction and analysis.

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The samples were removed from the freezer and allowed to come to room temperature in capped vials without assistance. Vials were uncapped and 600 uL of deuterated cyclohexane was added to each vial. Samples were capped and agitated for 15 minutes. The entire solution was transferred to a 5 mm NMR tube while the Sebutape® remained in the vial.

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Within 24 hours of sample preparation, the NMR tube was inserted into a spinner, and placed in an autosampler. The autosampler was interfaced with a Bruker Avance NMR spectrometer operating at 599.98 MHz for proton. The spectrometer was outfitted with a triple resonance cryogenically-cooled probe equipped with a z-axis gradient. The acquisition parameters included a pulse width of 85 degrees, acquisition time of 1.8 seconds, a relaxation delay of 1 second, a spectral width of 15 ppm, 32K data points, and scanned 512 times. All data were acquired at temperature of 25 °C. Spectra were processed using Metabonomi, a proprietary software package (See International Patent Application WO2004038602) in which NMR data are Fourier transformed. Within the software, the resulting spectra were phased, baseline corrected, referenced to the low levels of protonated cyclohexane present in the solvent at 1.38 ppm, and standardized to 32K data points in the region from 10 to –0.5 ppm. Using

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Metabonomi, the area of spectral regions were measured including regions assigned to cholesterol esters (CE) (4.52 to 4.60 ppm), wax esters (WE) (3.96 to 3.99 ppm), triglycerides (TG) (4.21 to 4.30 ppm), and squalene (5.06 to 5.13 ppm). A representative NMR spectrum of human meibum is shown in Figure 6.

5 Peak areas measured in the NMR spectra were converted to concentration values using a calibration function relating NMR peak areas to concentration. The calibration function was constructed from NMR data on six concentrations of a mixture of calibration standards. The calibration mixture was prepared from an equimolar, four-component stock solution prepared from
10 commercially available reference compounds dissolved in deuterated cyclohexane. The reference compounds were chosen to include at least one component from each class of compounds being measured with the NMR method. The reference compounds included squalene, triolein (a triglyceride), cholesteryl palmitate (a cholesterol ester), and palmitic acid stearyl ester (a wax
15 ester). The concentrations included 5, 50, 100, 500, 750, and 1000 μM for each component. Proton NMR spectra were acquired and processed using the same procedure as outlined above for the test samples. For each component or class of components, as in the case of cholesterol esters and wax esters, least squares regression was used to fit a linear model to the peak area versus the
20 concentration data, each converted to logarithmic scale.

The lipid concentrations detected in a meibum sample from a female volunteer are summarized in Table 3.

Table 3

Selected Lipid	μM Concentration in Sebutape® Extract (600 μL in volume)
Squalene	20.0
Cholesterol Esters	174.5
Triglycerides	23.5
Wax Esters	321.1

25 Results demonstrated that the method of the present invention is suitable for quantifying the selected lipids in human meibum.

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CLAIMS

What is claimed is:

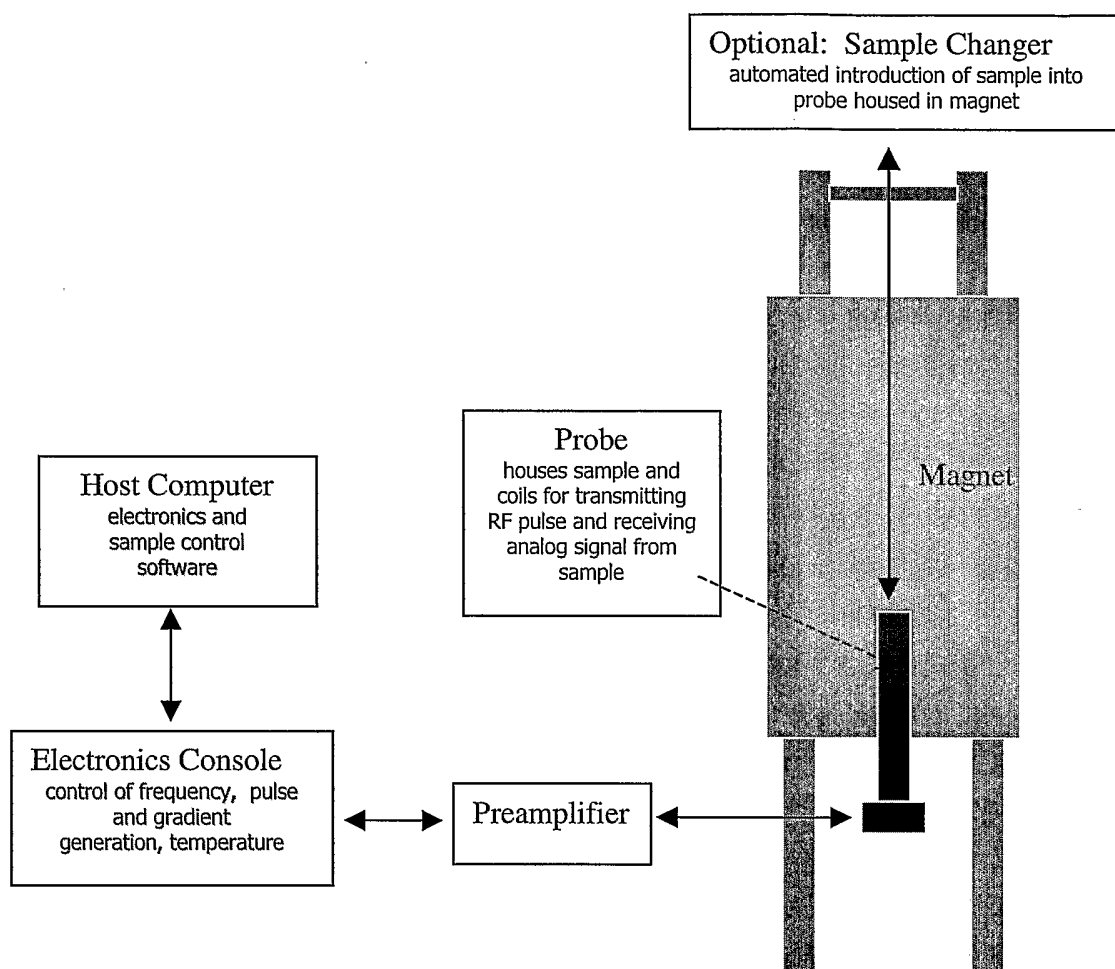
- 5 1. A method for identifying a compound that modulates sebum production, the method comprising:
 - a. contacting a sample from a treated subject with a nuclear magnetic resonance spectrometer;
 - b. generating a NMR spectrum for at least one lipid component of said sample; and
 - 10 c. comparing the amount of said one or more lipid components generated in step b with the amount of said lipid in a control sample.
- 15 2. The method of claim 1 or claim 15 further comprising integrating the area of the spectral regions for said lipid component.
- 20 3. The method of claim 1 or claim 15 wherein said amount of said lipid is determined by comparing the peak area of the spectral region for said one or more lipid components generated in step b with the peak area of a control sample.
- 25 4. The method of claim 1 or claim 15 wherein said amount of said lipid is determined by comparing the intensity of an NMR resonance assigned to one or more lipids to the intensity of an NMR resonance of said lipids in sample from a control subject.
- 30 5. The method of claim 1 wherein said NMR spectrometer has a host computer, an electronics console, a preamplifier, and a magnet, a probe and optionally an automated sample changer.
- 35 6. The method according to claim 5 wherein said magnet is selected from the group consisting of an electromagnet and a superconducting magnet.
7. The method according to claim 6 wherein said superconducting magnet has a strength above 11.7 Tesla.

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8. The method according to claim 5 wherein said NMR spectrometer operates at proton frequencies above 400 MHz.
- 5 9. The method according to claim 5 wherein said probe is selected from the group consisting of probes having electronics that operate at ambient temperatures and probes having electronics that operate at cryogenic temperatures.
- 10 10. The method according to claim 5 wherein said probe is selected from a conventional probe and a flow probe.
11. The method of claim 1 wherein said sample is selected from the group consisting of a sebocyte cell sample, a sebum tissue sample, a skin surface sebum sample and a tear fluid sample.
- 15 12. The method of claim 11 wherein said tissue sample is selected from the group consisting of a human tissue sample and a hamster sebaceous gland sample.
13. The method of claim 1 wherein said lipid component is selected from the group consisting of triglycerides, cholesterol, cholesterol esters, wax esters, and hydrocarbons.
- 20 14. The method of claim 13 wherein said hydrocarbon is squalene.
15. A method for identifying a compound that modulates the amount of at least one individual lipid component of a sebum sample, the method comprising:
- 25 a. contacting a sample from a treated subject with a nuclear magnetic resonance spectrometer;
- b. generating a NMR spectrum for at least one lipid component of said sample; and
- c. comparing the amount of said one or more individual lipid components generated in step b with the amount in a control sample.
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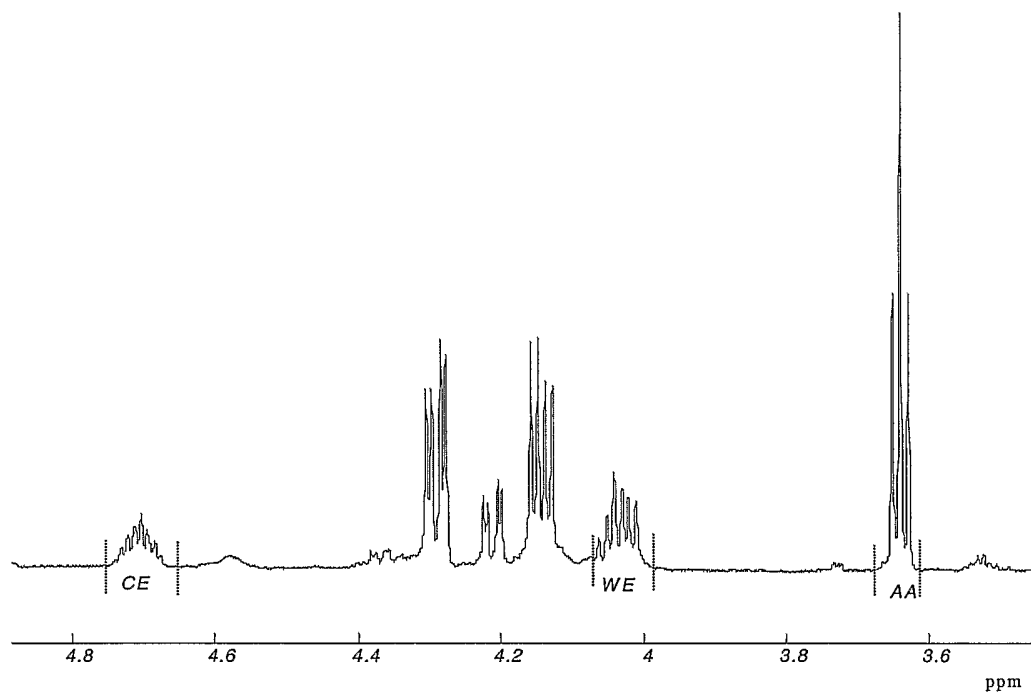
1/6

Figure 1 – Schematic of NMR spectrometer



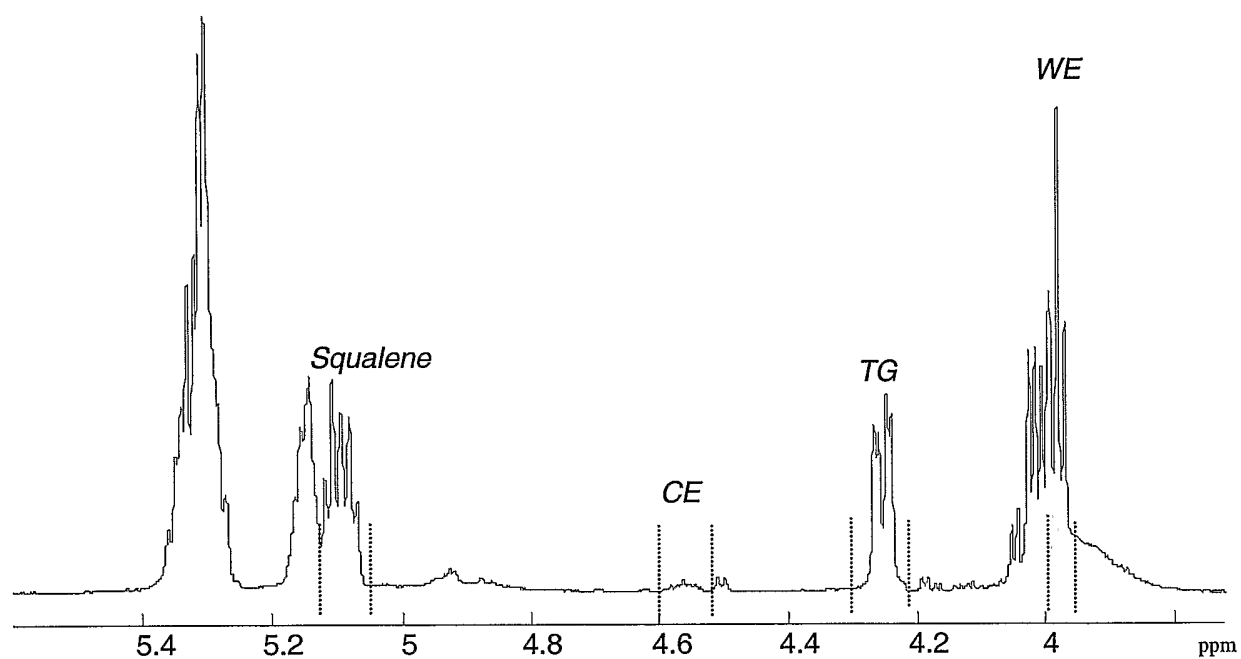
2/6

Figure 2 – Proton NMR Spectrum of Hamster Ear Extract



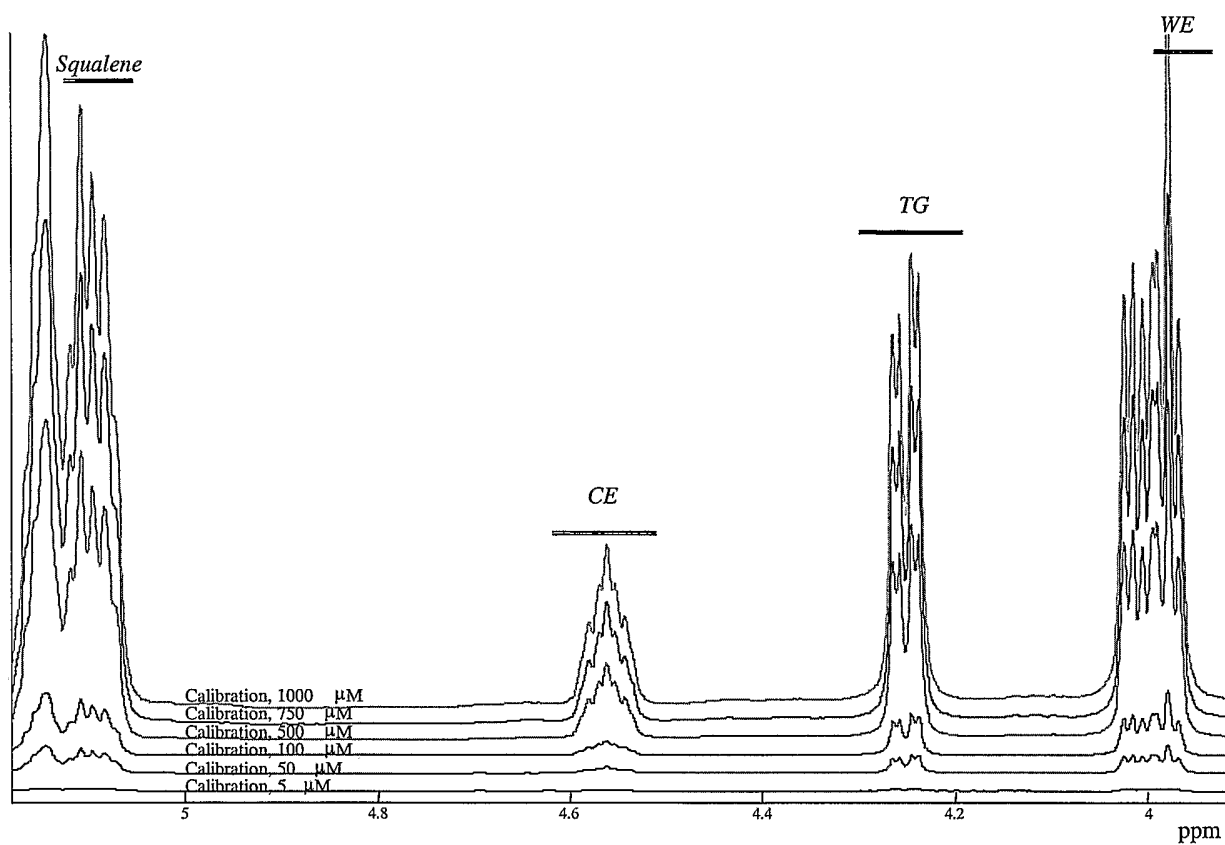
3/6

Figure 3 – Proton NMR Spectrum of Sebum Extracted into Deuterated Cyclohexane



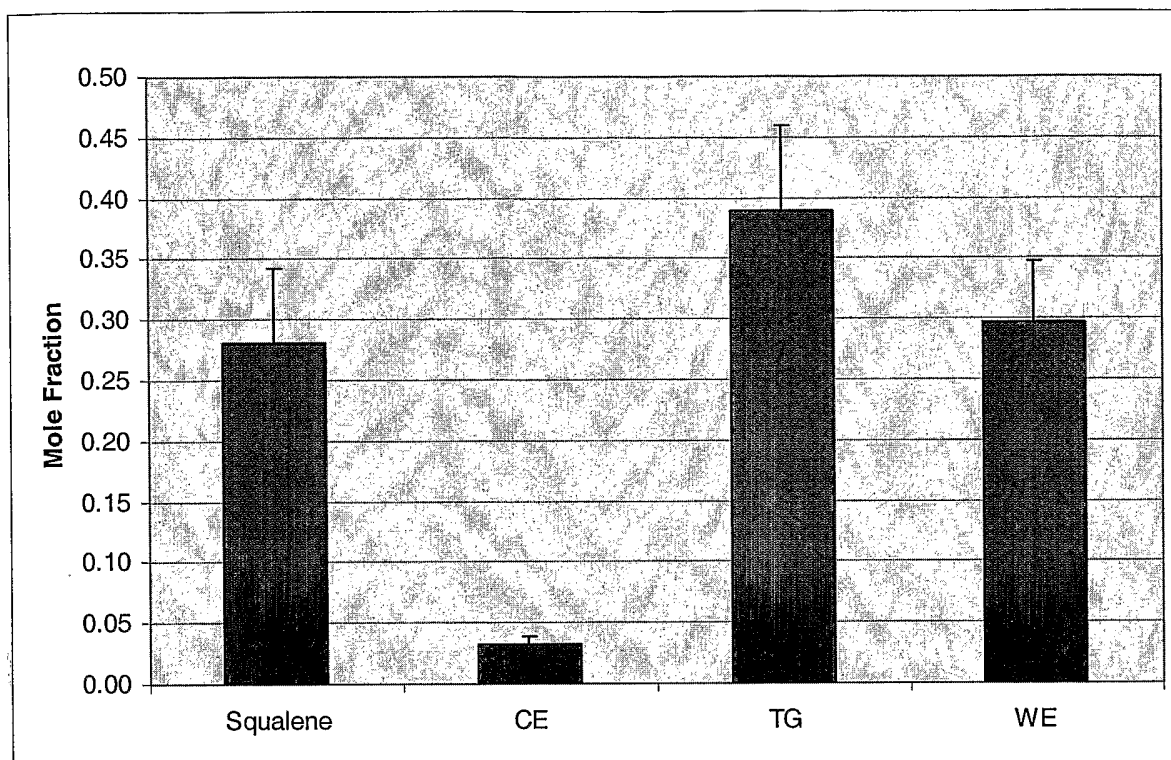
4/6

Figure 4 – Proton NMR Spectrum of Calibration Mixtures at 6 concentrations ranging from 5 μ M to 1000 μ M.



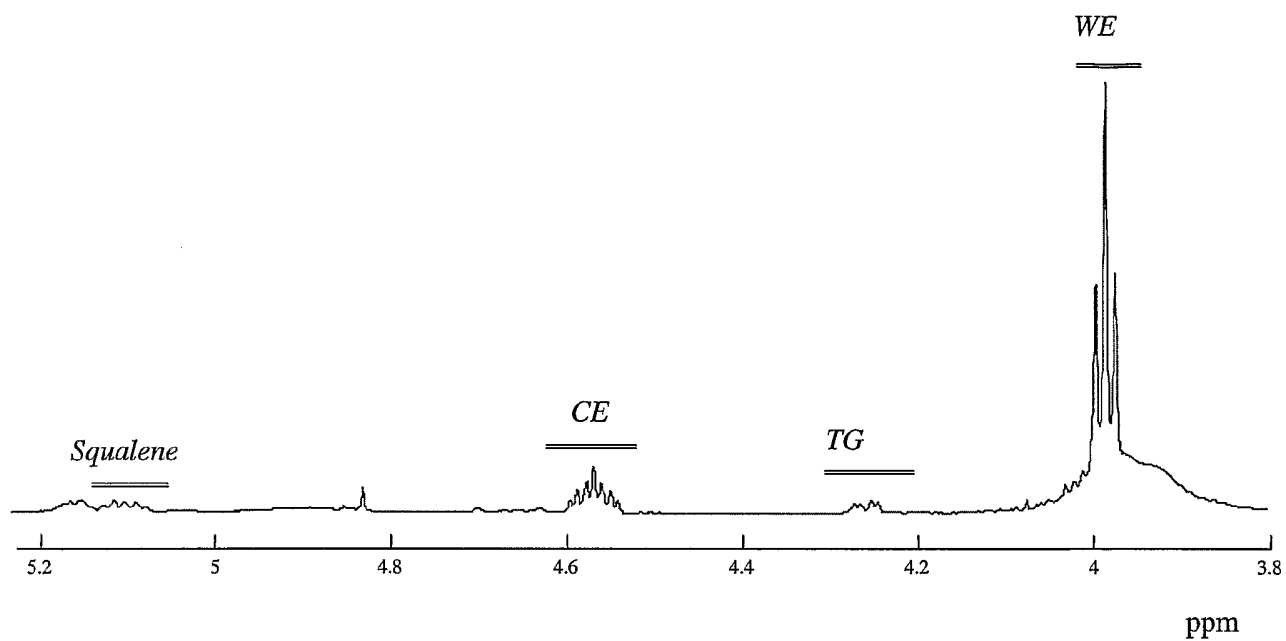
5/6

Figure 5. Distribution of Components in Sebum From Scalp of Male Subjects



One standard deviation unit is shown on the graph.

Figure 6 – Proton NMR Spectrum of Meibum Extracted into Deuterated Cyclohexane



INTERNATIONAL SEARCH REPORT

national application No
PCT/IB2006/000485

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01R33/46

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)
G01R

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

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

EPO-Internal, PAJ, WPI Data, INSPEC, BIOSIS, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6 180 669 B1 (TAMARKIN DOV) 30 January 2001 (2001-01-30) abstract column 13, paragraph 4 -----	1-15
A	US 5 207 715 A (FOSSEL ET AL) 4 May 1993 (1993-05-04) abstract claim 1 -----	1-15
A	SAINT-LÉGER D ET AL: "Stratum corneum lipids in skin xerosis." DERMATOLOGICA. 1989, vol. 178, no. 3, 1989, pages 151-155, XP002381852 ISSN: 0011-9075 abstract ----- -/--	1-15

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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"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

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Date of the actual completion of the international search

19 May 2006

Date of mailing of the international search report

03/08/2006

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INTERNATIONAL SEARCH REPORT

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PATENT ABSTRACTS OF JAPAN vol. 017, no. 369 (P-1572), 12 July 1993 (1993-07-12) & JP 05 060686 A (KAO CORP), 12 March 1993 (1993-03-12) abstract -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

ational application No

PCT/IB2006/000485

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 6180669	B1	30-01-2001	NONE	
US 5207715	A	04-05-1993	NONE	
JP 05060686	A	12-03-1993	NONE	