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# An introductory review of nuclear magnetic resonance spectroscopy: analysing meibomian secretions

**WDH Gillan***

*Department of Optometry, University of Johannesburg, PO Box 524, Auckland Park, 2006 South Africa*

< wgillan@uj.ac.za>

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In discussing new approaches to the limitations of characterizing meibomian lipids Butovich[1] states the following:

*"...the necessity to analyze fragments of complex molecules instead of whole intact lipids, no matter how large and complex they are - and start putting together the pieces of the great meibomian puzzle, both figuratively and literally. These are truly exciting times indeed".*

Tears are a complex layer of fluid that cover the cornea and conjunctiva and are "maintained, secreted, distributed and excreted" by various systems of the lacrimal apparatus[2,3]. The tears are separated into three distinct layers: the outer oily layer, the middle aqueous layer and the inner mucous layer[4, 5]. The outer oily layer is produced primarily by the meibomian glands (also known as the tarsal glands)[4]. The glands of Moll, considered to be modified sweat glands, also play a role in producing the outer oily layer[6]. The outer oily, meibomian, layer provides a hydrophobic barrier that prevents tear overflow onto the lids, provides a water-tight seal when the lids are closed during sleep and retards evaporation of tears[7]. Other functions of the meibomian layer include: protection, lubrication, nutrition, antimicrobial activity and results in a smooth optical surface (thus being linked to visual acuity)[1, 8-11].

Dry eye is defined as: "a multi-factorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface"[12]. The prevalence of dry eye in the general population varies between 5.5%[13] and 64%[14] depending on the subjects in the study and the criteria used to diagnose dry eye. Meibomian glands and their secretions (when abnormal) have been implicated in causing or exacerbating dry eye and blepharitis-like conditions[11, 15-18]. The evidence that meibomian secretions play a role in the development of dry eye has resulted in many investigations into the formation, secretion and composition of such secretions. Numerous methods of investigating tears have been used in the past in an attempt to elucidate the composition of the tears and meibomian secretions. Among methods used to analyze tears and meibomian secretions are the following (in a more or less time-related sequence): thin layer chromatography[7, 11] (TLC) and gas liquid chromatography[7] (GLC), high performance liquid chromatography [9, 20] (HPLC), evaporimetry[8], gas chromatography electron impact ionization mass spectrometry[21] (GC/EI-MS), electrospray tandem mass spectrometry[22] (ESI-MS/MS), atmospheric pressure ionization mass spectrometry[23], infra-red and fluorescence spectroscopy[24], nuclear magnetic resonance spectroscopy[25] and meibomian gland expressibility[16], among others. As the methods used to analyze meibomian secretions have developed and advanced so too have the accuracy and sensitivity of the methods improved. The aim of this

*DipOptom DPhil(RAU) CAS(NewEnCO) FAAO FIACLE

review is to introduce nuclear magnetic resonance spectroscopy (NMR), and its use in analyzing meibomian secretions, to the broad optometric community.

## Nuclear magnetic resonance spectroscopy

What follows is a superficial discussion relating to theory that underlies NMR. The interested reader who requires a more in-depth exposition of this technique is referred to specialized texts on the subject of NMR[26-30]. Electrons have intrinsic angular momentum which is known as spin (electrons do not physically spin but have an intrinsic angular momentum as if they were spinning, a quantum phenomenon)[31]. Some nuclei also have the quality of spin as a result of the nuclear components (neutrons and protons) having spin[31]. In NMR it is the nucleus that one is interested in. Whether a nucleus has an overall spin or not can be determined by the following rules[32]:

" * If the number of neutrons and the number of protons are both even, then the nucleus has no spin.

* If the number of neutrons plus the number of protons is odd, then the nucleus has a half-integer spin (eg: ½).

* If the number of neutrons and the number of protons are both odd, then the nucleus has an integer spin (eg: 1)".

The nucleus of ordinary hydrogen, for instance, has a spin quantum number (I) of ½ that can exist in two states, namely +½ and -½. Some nuclei have a quantum spin number of I = 0 meaning that these nuclei do not produce an NMR spectrum[33]. Protons are electrically charged and when spinning they create a magnetic moment that coincides with the axis of spin (essentially the magnetic moment gives the protons bar-magnet properties)[31, 33]. When no external magnetic field exists the magnetic moment of each proton is randomly oriented. However, when placed in an applied external magnetic field protons can assume one of two possible orientations, the proton may align with (low energy state) the magnetic field or it may align against (high energy state) the magnetic field. The spin states mentioned earlier determine how the protons orient in a magnetic field. Energy, in the form of electromagnetic radiation in the radio frequency range, is needed to flip a proton from a low energy state to a high energy state. When the protons absorb this energy the nuclei are said to be in resonance with the externally applied energy. The energy required to flip protons is proportional to the strength of the applied magnetic field[29-30, 33]. The stronger the magnet is in a NMR spectrometer, the more sensitive the instrument (a 300 MHz instrument is less sensitive than a 600 MHz instrument for instance).

The nuclei (for example a hydrogen nucleus) within a sample spin about the magnetic field axis of the applied magnetic field in a similar fashion to a top gyrating around its axis. This gyration is known as precession. Figure 1 shows a vector diagram indicating the precession of a nucleus around its axis (Z).

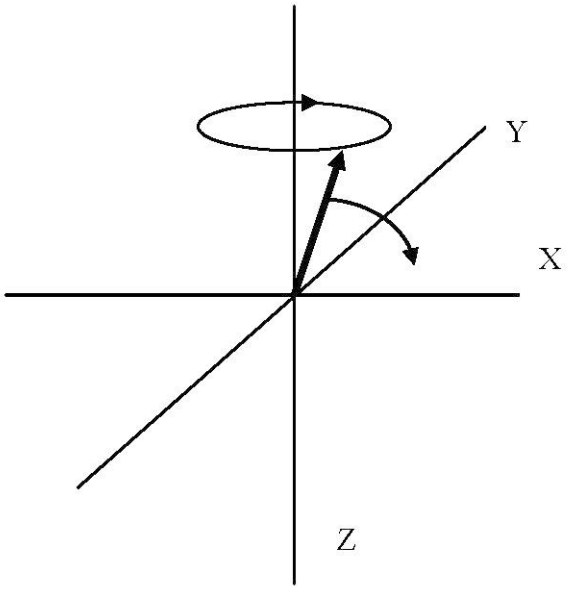


**Figure 1.** A schematic showing the precession of the vector (bold, thick arrow) around the z-axis. The vector tilts towards the X-Y plane when a radio frequency pulse is applied to the sample (modified from Solomons *et al*[33]).

When an external source of energy, in the form of a pulsed radio frequency, is applied and which matches the precessional frequency of the nuclear magnetic moment, then the magnetic vector of the nucleus tips away from the axis of the applied magnetic field (Z in Figure 1) and lies in the X-Y plane. A receiver coil situated around the sample will detect this rotation of the precessional axis as an oscillating magnetic field (in the form of an electric current). Within seconds or less, the nucleus releases the absorbed energy allowing the nucleus to return to its ground energy state. As it does this the oscillating electric current that was detected by the receiver coil deteriorates exponentially. Fourier transforms are then used to convert the

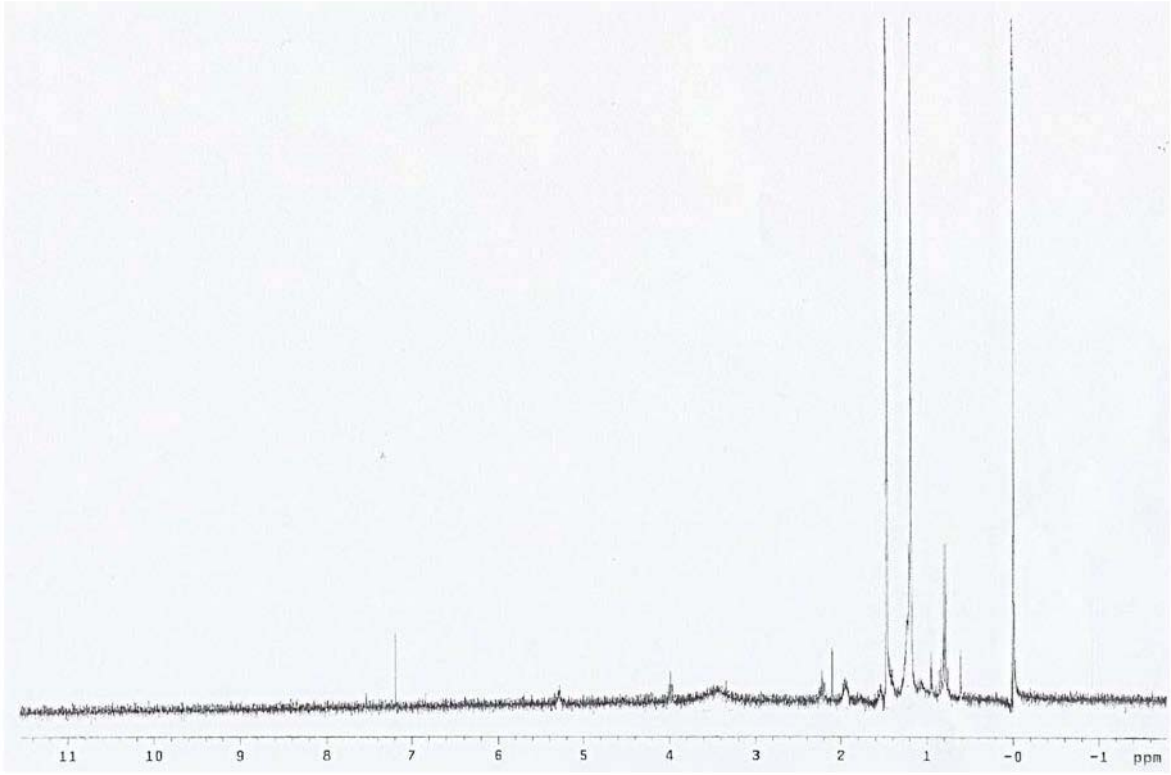


**Figure 2.** A 300 MHz NMR spectrum of meibomian expression. The signal above 0 ppm indicates the TMS signal.

current into a signal. It is this signal that is used to produce the NMR spectra[28-30, 33].

The spectra produced by NMR provide information regarding molecular structure that enables one to identify a compound or elucidate the structure of an unknown compound. Three important aspects of the NMR spectra are useful in providing the types of information mentioned above. They are: chemical shift (the position of the signal peak on the X-axis), the area under the peak (the integral giving an indication of concentration) and signal splitting (coupling).

Figure 2 is an NMR spectrum obtained by conducting a 300 MHz investigation of meibomian secretions obtained from one of my colleagues in the Department of Optometry. It was not a successful attempt at elucidating the sample as the sensitivity of the instrument was inadequate. The spectrum shown in Figure 2 cannot be used to evaluate individual lipid structures and can only be used to determine a "fingerprint" of classes of molecules that might be present in meibomian secretions. The spectrum obtained, however, is useful for indicating some of the aspects of importance mentioned above. The X-axis is labelled ppm (parts per million). Chemical shift (expressed in Hertz) is proportional to the strength of the applied external magnetic field. To maintain uniformity (for example when a sample is evaluated in instruments of different sensitivity) it is necessary to represent the chemical shift in a manner that is not dependent on the frequency of the instrument[33]. The label "ppm" is a ratio determined by dividing the chemical shift by the frequency of the spectrometer[33]. The chemical shift (ppm value) that one notes on an NMR spectrum is dependent on the environment surrounding each individual nucleus (in other words the shift reflects protons, in this instance, that are in different chemical environments). The signal above 0 ppm is produced by a compound known as tetramethylsilane (TMS) which acts as a reference of proton absorption against which other signals are compared. TMS is commonly used for $^{1}H$ and $^{13}C$ spectroscopy. The positions of other signals are all relative to the TMS signal. A signal that occurs further to the left of another is "downfield" while a signal further to the right is "upfield". Upfield versus downfield relates to the strength of the magnetic field (higher versus lower respectively) needed to bring the nuclei into resonance. The position of a signal relative to the TMS signal is known as its chemical shift. The practical significance of the chemical shift is that it gives an important indication of

the structure of the molecule in question[33]. In Figure 2 a number of signal peaks exist between 0 and 2 ppm. These peaks are due to molecular components that are present in the solvent used to liquefy the meibomian expressions, namely deuterated chloroform and are not helpful in attempting to analyse samples. The area under a specific signal (the integral) is proportional to the number of hydrogen atoms producing that specific signal. Comparing the area under one signal with that found under another signal (in the form of a ratio) gives the ratio of the areas for the specific signals and represents the number of hydrogen atoms producing one signal as compared to the other[33]. The presence of clustered, multiple peaks in one signal is known as signal splitting (or coupling). Coupling is a result of the magnetic effect that non-equivalent hydrogen atoms (that are within two or three bonds of the hydrogen producing the signal) have on the hydrogen atom producing the signal[33]. In other words coupling results from the effect that neighbouring protons have on the proton producing the signal. Making use of the chemical shift, area and coupling of signals one is able to start elucidating the structure of compounds[33]. Tables and charts are available that allow one to begin associating the chemical shift of a sample with possible structural environments for the nuclei that produced the signal[27, 33]. The interested reader is referred to specialized texts for a more in-depth explanation of how unknown compounds are analyzed[26-30].

## NMR analysis of meibum

Butovich[1] is of the opinion that NMR is one of the more informative spectroscopic techniques that are available today and that NMR has become an indispensable tool in the structural and conformational analysis of organic molecules. Butovich[1] also notes that there is a paucity of literature relating to the use of NMR in the analysis of human meibum. A literature search of several prominent databases (Science Direct, Pubmed, Investigative Ophthalmology and Vision Science, Archives of Ophthalmology) produces little literature relating to human meibum and NMR. Robosky *et al*[25] have recently shown that NMR used in conjunction with cryogenically cooled probes is adequately sensitive to detect lipids in meibomian secretions of humans. Robosky *et al*[25] made use of a 600 MHz instrument and were able to detect, and quantify, squalene, cholesterol esters, triglycerides and wax esters in human meibomian secretions. NMR use is in its infancy regarding the analysis of human meibum. However, statements like: "…NMR will become a routine procedure in lipodomic studies of complex mixtures"[1] and "NMR spectroscopy is arguably the most important analytical method available today"[27] suggest that NMR will become an important tool in the quest to understand at least one aspect of the tear layer, namely the oily outer layer. As Butovich[1] stated: "These are truly exciting times indeed.".

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