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APPLICATIONS OF NMR SPECTROSCOPY

Volume 5

Editors:
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Applications of NMR Spectroscopy

(Volume 5)

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PREFACE

Nuclear Magnetic Resonance (NMR) Spectroscopy is one of the most powerful analytical tools that probes the nature and properties of molecular structures. The NMR experiment provides tremendous information about the types and amount of atoms present in a sample, specific environments of atoms within a molecule, the purity and composition of a sample, and structural information about a molecule, including constitutional and conformational isomerisation. Certain characteristics of NMR spectroscopy make this the *technique of choice* for a wide range of research, analytical and medical applications. These characteristics include its capacity to analyse atoms, such as ^{1}H, ^{2}H, ^{13}C, ^{11}B, ^{15}N, ^{19}F, ^{31}P and ^{195}Pt, thus covering all major classes of chemical compounds, its non-destructive nature, the requirement of only a small quantity of material, and the ease of sample preparation in a whole range of solvents, as well as in the solid state and in living systems. Applications of NMR spectroscopy are spread over a wide range of disciplines, and the development of new NMR methods for various applications has had a profound impact on molecular research in general. New applications of NMR spectroscopy are being vigorously pursued in tandem for the development of newer hardware and software. This has created a need of a book series which reviews the most recent literature in this exciting field in terms of applications and developments. The 5th volume of the book series "*Applications of NMR Spectroscopy*", comprises six reviews focussing on three broad fields of NMR applications, *i.e.* molecular identification of natural and synthetic compounds, medical diagnosis and food sciences. These reviews have been contributed by the leading practitioners in this field.

A number of bioactive peptides have been isolated from fermented and processed food. These peptides have immense physiological significance. The relationships between structural features and functional activities have been extensively studied. Many bioactive peptides of dietary origin possess relatively short peptide residue lengths and hydrophobic amino acid residues. The review contributed by Reddy *et al.,* is an excellent overview of the applications of various NMR techniques in structural characterization of food derived bioactive peptides in their native state. The authors have discussed key structural differences between the food derived peptides and other classes of naturally occurring peptides, followed by a description of various NMR techniques, which are especially suited for their structural studies. In general, this review focuses on the application of NMR in food chemistry, taking examples of various classes of food derived peptides.

Tinnitus is the ringing or buzzing in the ears, caused by short-term exposure of high level sound. This common health issue is only poorly understood at the molecular level, and as a result, no successful treatment has been developed yet. Animal models of tinnitus have been

developed to study the complex changes which may occur in the central nervous system in tinnitus conditions. This may involve changes in the regulation of neurotransmitters, such as gamma-aminobutyric acid (GABA), glutamic acid (Glu), acetylcholine (Cho), *etc*. Brozoski *et al.,* have reviewed the application of a variant of NMR, high resolution proton magnetic resonance spectroscopy (^{1}H-MRS) to record the brain spectra of tinnitus model animals in order to determine the GABA, Glu and Cho concentrations. This review therefore demonstrates the power of ^{1}H-MRS in direct determination of biomarkers in animal models for a better understanding of the chemical changes in this complex disorder. ^{1}H-MRS has thus been demonstrated to be an excellent technique for the study of the biochemical composition in living systems, including living cells.

Dibyendu S. Bag focuses on the applications of NMR spectroscopy in the characterization of polymers, their monomers and copolymers. The NMR spectra of polymers are generally broad and featureless due to large molecular sizes and serious overlapping of signals. The solubility of large polymers in deuterated solvents is also an issue. Solid state NMR spectroscopy provides a viable alternative, but it has its own limitations. This review highlights recent applications of various NMR techniques in the identification of monomers, their corresponding polymers and co-polymers. The author has also discussed the NMR based characterization of polymers containing nanomaterials, such as fullerene and carbon nanotubes.

The review by Voronov and Ushakov focuses on the application of NMR spectroscopy in the study of structures and intermolecular dynamics of paramagnetic molecules, particularly the large proteins. Paramagnetism arises due to the presence of unpaired electrons in a molecular system. These unpaired electrons influence nuclear spins in biological molecules, and the resultant forces affect the NMR spectra in the solid state. Paramagnetism can thus provide long-range information about the structural and dynamic characterization of biomolecules and their intermolecular interactions. The review explains in great detail the NMR phenomena in paramagnetic systems, followed by a discussion of an NMR method especially suited for the study of structure and dynamics of complex molecular systems. This is very useful for researchers who wish to apply NMR in the structural biology of multi-electron protein and other such biomolecules.

The application of Magnetic Resonance Spectroscopy (MRS) as a powerful disease diagnostic and grading tool for non-invasive measurement of metabolites is further presented in the review by Stecco *et al*. The authors have focussed on two types of NMR based techniques *i.e.* multivoxel and single voxel acquisitions, used for the diagnosis and grading of brain gliomas based on the analysis of biomarkers and metabolites. They have explained the strengths of the two brain NMR spectroscopy techniques as non-invasive tools, and related the levels of various metabolite ratios with the stages/grades of glioblastomas. The brain NMR

spectroscopy can be used for the determination of the effect of chemotherapy, as well as to identify the sub-types of the cancer.

Tischer and Mali have contributed an article focussing on the development of cross polarization-magic angle (^{13}C CP-MAS) solid state NMR spectroscopic technique as a robust tool for the characterization of proteins, lipids and starch in complex food matrices, and other samples of biopolymers. Solid state NMR spectroscopy has many advantages, including being operationally less expensive, versatile, reproducible, quick, and capable of analysing samples in the native state, without multi-step sample preparation needed for liquid state NMR. The authors have exhaustively reviewed the most recent developments in the field of CP-MAS NMR spectroscopy, including hardware and software developments. They discuss various NMR parameters which influence the outcome of CP-MAS NMR based analysis of a variety of food constituents. Such analysis of food for their key ingredients can help in improving the organoleptic and taste properties of food preparations and raw material.

We wish to express our gratitude to the authors of the above cited articles for their excellent scholarly contributions for the 5th volume of this well-known series. We also greatly appreciate the efforts of the entire team of Bentham Science Publishers for efficient processing and timely management of the publication. The efforts of Ms. Fariya Zulfiqar (Assistant Manager Publications), Mr. Shehzad Naqvi (Senior Manager Publications) and leadership of Mr. Mahmood Alam (Director Publications) are especially praiseworthy. We also hope that like the previous volumes of this internationally recognized book series, the current compilation will also receive wide readership and appreciation.

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Section I: Molecular Identification of Natural & Synthetic Compounds

CHAPTER 1

Application of NMR Spectroscopy for Structural Characterization of Bioactive Peptides Derived from Food Protein

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Abstract: Traditionally, food has not only been the source of human nutrition but also an excellent factor for deriving nutraceuticals and pharmaceuticals. The biopolymers such as protein and polysaccharides derived from food sources have special significance as they are important part of human diet. Several important bioactive peptides have been discovered from food protein hydrolysates and fermented products that have shown to possess a spectrum of beneficial pharmacological activities. This chapter deals with food protein derived bioactive peptides and their structural characterization employing NMR spectroscopy. It is well known from the vast literature on bioactive peptides that NMR spectroscopy is an indispensable tool to fully characterize the structures of bioactive peptides in their inherent physiological state. However, limited literature exists on detailed structural characterization of food derived bioactive peptides. It is therefore very important to review the literature on structural aspects of food peptides and provide an appraisal of NMR techniques suitable for comprehensive structural characterization of this class of bioactive peptides.

This chapter is aimed to introduce NMR spectroscopy and its applications to a wide range of readers in the field of food chemistry in general and to the food peptide researchers in particular. The main objective is to review the literature on food derived bioactive peptides and highlight the potential of NMR spectroscopy to understand structure-activity relationship of bioactive peptides in order to boost further developments in this important field. With a view to make this chapter readable to

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beginners in the field, basic principles of NMR spectroscopy will also be included.

Application of solution NMR spectroscopy to bioactive peptides will then be described. Salient features including the size and structural differences of food bioactive peptides when compared to the other classes of bioactive peptides will then be discussed in order to make an informed assessment of choice of NMR techniques suitable for their structural characterization.

Keywords: ACE inhibitory peptides, Antimicrobial peptides, Antioxidative peptides, Bioactive peptides, Chemical shift index, Food proteins, Immuno-modulatory peptides, Multidimensional NMR Spectroscopy, Nutraceutical value, Secondary structure, Structure activity relationship.

1. INTRODUCTION

1.1. Bioactive Peptides

Food proteins and peptides acquired functionality by their amino acid constituents and their sequences. The sequences of peptides and proteins in turn dictate the secondary, tertiary and quaternary structures [1, 2]. These protein and peptide sequences can be cleaved through enzymatic hydrolysis and produce smaller peptides that may physiologically be active (bioactive peptides).

Bioactive peptides are food components that have beneficial effects on human health. They are derived from animal and plant sources; such as milk, eggs, cheese, meat, fish, soy, rice, wheat and legumes. Food protein consumed by humans exhibits physiological actions in the body either directly or after hydrolysis by gastrointestinal enzymes. Enzymatic hydrolysis of food protein results in the release of bioactive peptides [3 - 13] that consist of two or more amino acid residues joined together by peptide linkages. The process of protein hydrolysis to produce bioactive peptides is of several types, namely, (i) gastrointestinal digestion by digestive enzymes [14], (ii) fermentation by microbial enzymes [15 - 18], and (iii) *in vitro* hydrolysis by commercial enzymes [19 - 22]. Many proteinases such as alcalase, trypsin, chymotrypsin, pancreatin, pepsin and thermolysin as well as several other enzymes from bacterial, fungal

and plant sources have been employed in several *in vitro* studies to produce important bioactive peptides from various food proteins [6, 7, 20, 23]. Recently, an *in silico* approach (a chemoinformatics approach) has been developed to identify potential bioactive peptides that can be derived from food proteins of known sequences [24 - 27]. Sometimes, this approach is used in combination with quantitative structure-activity relationship (QSAR) study to predict the activity of possible bioactive peptides that can be produced by this method [25]. This approach also provides an insight into suitable enzymes needed to cleave the proteins to produce potential bioactive peptides in optimal yield. The procedure is then followed by experimental enzymatic production employing the identified enzymes, then by purification and characterization [24]. The peptides so produced have been proven to exhibit extremely important health beneficial properties [5, 13, 28].

Most of the bioactive peptides derived from food proteins contain two to twenty five amino acid residues that exert various therapeutic activities. It has been demonstrated that peptides derived from food proteins display antihypertensive, osteoprotective, antioxidant, antimicrobial, anti-inflammatory, anticancer, growth promoting and many other therapeutic effects on human body when they are absorbed from intestinal tract or *via* receptors [8, 18].

A few examples are highlighted below to exemplify the importance of the process of enzymatic hydrolysis or fermentation to derive health beneficial peptides from food protein. Milk and its products (cheese and yoghurt) are considered as the most beneficial for human health. Literature demonstrates that the bioactive peptides obtained from fermentation and enzymatic hydrolysis of these food products display superior antihypertensive (angiotensin-I converting enzyme (ACE)-inhibitory), antioxidant, antimicrobial, calcium-binding and immuno-modulatory activities [7, 17, 20, 29 - 35]. The spectrum of activities displayed by these peptides make them suitable for nutraceutical/pharmaceutical applications. For instance, a strong antimicrobial peptide, lactoferricin, has been obtained by gastrointestinal digestion of lactoferrin, an iron-binding protein from milk that protects the host against microbial infections. The potency of lactoferricin is 100 to 1000 times higher than its parent protein (lactoferrin) and is active against Gram positive, Gram negative and certain yeast and fungi [17, 36].

CHAPTER 2

Determination of GABA, Glutamate and Choline in the Auditory Pathway of Animals with Tinnitus, Using High Resolution Proton Magnetic Resonance Spectroscopy (^{1}H-MRS)

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Abstract: Following high-level sound exposure, central auditory changes are evident long afterward. Homeostatic central mechanisms appear to compensate, and often overcompensate, for loss of peripheral sensitivity resulting from insults. Over-compensation may produce the sensation of sound without a physical correlate, *i.e.*, tinnitus. However not everyone exposed to auditory trauma develops tinnitus. Similarly, in a controlled laboratory environment, not every animal exposed to high-level sound develops tinnitus. Despite more than two decades of effort, tinnitus pathophysiology is incompletely understood. Contributing is the unexpected complexity of tinnitus' central nervous system profile. Compensatory neural changes, such as increased spontaneous activity, have been identified, but they occur in the context of many other changes. Underlying mechanisms are also poorly understood. They may involve down-regulation of inhibitory neurotransmission mediated by γ-amino butyric acid (GABA), and/or up-regulation of excitatory neurotransmission, mediated by glutamic acid (Glu), or modulation by other systems, such as acetylcholine, involved in functions such as attention. Neural systems are integrated and well-regulated.

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Therefore compensatory changes in one system can produce reactive changes in others. Some or all may be relevant to tinnitus, and they may contribute to the failure to develop generally effective tinnitus therapeutics. In this context the potential roles of GABA, Glu and acetylcholine (indirectly indicated *via* choline, Cho) were quantified in the auditory pathway of rats with and without tinnitus, using high-resolution proton magnetic resonance spectroscopy (^{1}H-MRS). Brain volumes of interest (VOI) were the dorsal cochlear nucleus (DCN), inferior colliculus (IC), medial geniculate body (MGB), and primary auditory cortex (A1). VOI spectra were obtained using a vertical bore Varian Unity/Inova 600 mHz NMR spectrometer with a 14.1 T magnet. A hybrid short-pulse and short echo time sequence was used for microvolume localized ^{1}H-MRS. The pulse sequence was optimized for signal acquisition in the spectral band containing the neurochemicals of interest. Signals were further optimized using a tunable pickup coil. Brain spectra were compared to external calibration spectra for determination of GABA, Glu, and Cho concentrations (mM) in each VOI. Chronic tinnitus was produced by a single high-level unilateral sound exposure, and was quantified using a psychophysical procedure sensitive to tinnitus. Contrary to expectation, significant decreases in GABA (*i.e.*, loss of inhibition) were not found in tinnitus animals. Glu levels were found to be significantly elevated in the contralateral A1, as were GABA levels. In exposed animals without tinnitus, GABA levels were uniquely elevated in the ventral MGB, suggesting that in those animals inhibitory compensation in the MGB might counter overcompensation. Cho levels were also found to be elevated in the contralateral A1 of tinnitus animals. The observed local concentrations of GABA and Glu may reflect a distributed alteration of inhibitory-excitatory equilibrium. These results suggest that targeting multiple neurotransmitter systems when developing therapeutics could improve outcomes.

Keywords: Animal model, Choline, GABA, Glutamic acid, ^{1}H-MRS, PRESS, STEAM, Tinnitus.

TINNITUS

Chronic subjective tinnitus is the persistent perception of sound ("ringing in the ears") in the absence of objective sound. Across a range of definitions, survey methods, and societies, 10 to 15 percent of the adult population report having chronic subjective tinnitus [1]. Of those, 10 to 15 percent seek medical advice or treatment for the condition [2]. Unfortunately there are no generally effective therapeutics for tinnitus, and while a standard of care has been established, actionable recommendations focus on counseling and acceptance [2]. Lack of generally effective treatments may in part be attributed to lack of knowledge and

an unexpectedly complex pathophysiology [3]. Following high-level sound exposure, central auditory changes are evident long afterward [4]. Homeostatic mechanisms appear to compensate, and in some instances to overcompensate for peripheral insults [5]. Overcompensation may produce the sensation of sound without an objective physical correlate, *i.e.*, tinnitus. Underlying mechanisms are not well understood. They may involve down-regulation of inhibitory neurotransmission mediated by γ-amino butyric acid (GABA) [6], and/or up-regulation of excitatory neurotransmission mediated by glutamic acid (Glu) [7 - 9], or modulation by other systems, such as acetylcholine [10 - 12]. It also seems likely that tinnitus dysfunction is distributed across the central auditory system [3, 13, 14] and involves areas well outside of the auditory pathway [15, 16].

Volume localized proton magnetic resonance spectroscopy (^{1}H-MRS) is well suited to quantifying regional brain levels of compounds participating in neurotransmission. Tinnitus therapeutic development could benefit from understanding patterns of regional neurotransmitter alteration associated with tinnitus. Since tinnitus-related pathophysiology is distributed, heterogeneous region-specific transmitter alterations are likely [17]. ^{1}H-MRS was used in the present experiments to quantify GABA, Glu, and choline (Cho) in rats with psychophysically determined evidence of chronic noise-induced tinnitus. GABA and Glu were examined because they serve, respectively, as major inhibitory and excitatory neurotransmitters in the auditory pathway [18, 19] as well as elsewhere in the brain. Cho was examined because cholinergic systems appear to play a major role in attentional mechanisms [20 - 24]. Although Cho is not a neurotransmitter, it has been shown to be a serviceable surrogate for acetylcholine [25]. The process of attention has been of interest in tinnitus research because a meaningless background sound such as tinnitus should habituate and not be attended to over time [26 - 30]. Despite this theoretical prediction, individuals who are bothered by their tinnitus have their attention uncontrollably drawn to it.

MAGNETIC RESONANCE SPECTROSCOPY (MRS)

MRS and MRI are based on a nuclear magnetic resonance (NMR) phenomenon. NMR was discovered in 1946 in the US by Edward Purcell and Felix Bloch. Initially, NMR was used only in physics to measure nuclear magnetic moments.

CHAPTER 3

NMR Spectroscopy for the Characterization of Polymers

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Abstract: Nuclear Magnetic Resonance (NMR) spectroscopy is one of the most simple and reliable techniques for structural elucidation of polymers. This chapter summarizes the application of NMR spectroscopy for identifying monomers and polymers, and also elucidation of constitutional, configuration and conformational structures of polymers and copolymers. The determination of various polymer structures includes polymer tacticity, polymer molecular weights and molecular weight distributions, copolymer composition, monomer reactivity ratios, monomer sequence distribution in the copolymer chains. Examples are cited in most of the cases to explain the polymer characterization from the NMR spectra. It also illustrates the characterization of polymers containing nanomaterials like fullerene (C_{60}) and carbon nanotubes (CNTs).

Keywords: Copolymer composition, Fullerene (C_{60}), Molecular weight distribution, Monomer reactivity ratios, Monomer sequence distribution, Monomers, Nanomaterials containing polymers, NMR spectroscopy, Polymers, Polymer characterization, Polymer molecular weight, Polymer tacticity.

INTRODUCTION

Nuclear Magnetic Resonance (NMR) spectroscopy is one of the most powerful and finest techniques in elucidating the functional groups and the structures of organic molecules. Its application in polymer chemistry has also been developed

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rapidly. It is now well-established as an important technique for identifying monomers and polymers, and also elucidation of constitutional, configuration and conformational structures of polymers [1 - 3]. Some of these structural analyses may be carried out by other techniques too. However, the method of NMR spectroscopy is a simple, convenient, rapid and reliable technique for analysis of organic molecules, monomers and polymers. This article describes the application of NMR technique for polymer analysis including identification of monomers and polymers and determination of various structures of polymer. The polymer structures include various stereochemical features of polymeric chains like tacticity, chain branching and crosslinking, copolymer composition, monomer reactivity ratios and monomer sequence distribution in copolymers, polymer molecular weight and molecular weight distribution, crystallinity *etc.* The characterization of nanomaterials especially fullerene and carbon nanotube containing polymers has also been discussed briefly. This chapter also provides a brief sketch of such illustrations with examples for better understanding of characterization of polymers using NMR spectroscopy.

'NMR' SPECTROSCOPY FOR POLYMERS

The nuclear magnetic resonance (NMR) spectroscopy was discovered in 1945 by Felix Bloch and Edward Mills Purcell. They were awarded Noble prize in Physics in 1952 for their discovery and the development of method for nuclear magnetic precision measurements. Nuclear magnetic resonance arises because of the nuclear spin of some atoms and the interaction of spinning nucleus (generates magnetic dipoles) with magnetic field [4]. There are (2**I** + 1) (where, **I** = the *nuclear spin quantum number*) possible orientations or states of the nucleus. These states are normally degenerate. But in the presence of magnetic field, the degeneracy is lifted resulting (2**I** + 1) different states or energy levels. The origin of NMR spectroscopy is basically due to this splitting up of degeneracy of energy levels under magnetic field, in which absorption of electromagnetic radiation from radio frequency (rf) range (typically 1-900MHz) promotes nuclei from low energy level to high energy level. Both ^{1}H and ^{13}C nuclei have the simplest situation and are widely studied. Both of them have half integral spin (I= ½). They have two energy states because of the alignment of spin either parallel with (low energy state) or against (high energy state) the external magnetic field. Once two energy

levels have been established, it should be possible to introduce quanta of energy hν (h is Planck's constant and ν is the frequency of radiation) to effect a transition between these energy states. The difference of energy between these two states depends upon the strength of the *external magnetic field (B_0)* at the nucleus and *magnetogyric ratio (γ)* of the nucleus. Thus the fundamental NMR equation is given by,

$$\nu = \frac{\gamma B_0}{2\pi} \qquad \textbf{(1)}$$

Where, ν is the resonance frequency proportional to both γ and B_0.

The magnetogyric ratio (γ) is a fundamental nuclear constant and is proportionality constant between the magnetic moment (μ) and the spin number (**I**): $\gamma = (2\pi\mu / \mathbf{I}h)$.

NMR spectra are obtained either by the variation of magnetic field or frequency, which are called field sweep or frequency sweep respectively. The absorptions of the sample are recorded as chemical shift values w.r.t the absorption of a reference compound. *Chemical shift* is universally expressed as the δ-scale which is defined by,

$$\delta = \frac{\nu_S - \nu_R}{\nu_R} \times 10^6 \qquad \textbf{(2)}$$

$$\delta = \frac{B_R - B_S}{B_R} \times 10^6 \qquad \textbf{(3)}$$

Where, $(\nu_S - \nu_R)$ is the difference in absorption frequencies of the sample and reference. The Bs and B_R are the field strengths corresponding to resonance for a particular nucleus in sample and reference respectively. The δ-scale is dimensionless and is independent of B and ν. Hence, a NMR spectrum with greatly improved resolution of absorption using very strong magnetic field can be

CHAPTER 4

Structure and Intramolecular Dynamics of Biologically Active Compounds: Analysis of NMR Spectra Transformed by Spin Labels

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Abstract: The present review summarizes current applications of the NMR phenomenon in paramagnetic systems for studying structure and intramolecular dynamics of multielectron molecular systems. The potential of spin labels for molecular identification was demonstrated in the example of structural biology issues. Discussion of molecular structure of specific organic heteroatomic compounds and their complexes is preceded by the section, where peculiarities of the NMR phenomenon in paramagnetic systems are considered. In opinion of the authors of the review, such consideration is needed for researchers who are not experts in the NMR theory. The separate section of the review is devoted to a method for investigation of structure and dynamics of complex molecular systems. This method is based on application of the NMR spectra transformed by complexes, first of all, paramagnetic ones (spin labels).

Keywords: Hyperfine coupling, Method of paramagnetic additives, Nuclear magnetic resonance, Paramagnetic systems, Structure and dynamics of molecules.

INTRODUCTION

Soon after its discovery, the phenomenon of nuclear magnetic resonance (NMR) has constituted a basis for a powerful method allowing structural studying of compounds and their properties, at least, in liquid phase. For a long time, diamagnetic compounds remained the main objects of the structural investigations

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using NMR technique (see, for example, [1 - 3] and the references cited therein). Despite the fact that this situation is still retained, there are strong grounds to believe that study of the NMR phenomenon in paramagnetic systems will also allow obtaining valuable information on molecular structure. This relates, as a rule, to paramagnetic complexes, the specifics of which are defined by unpaired electrons on *d*- and *f*- orbitals.

Electron-nuclear or hyperfine coupling (HFC) between unpaired electrons and nuclei of the paramagnetic molecule leads to characteristic shifts and broadenings, connected with spatial and electronic structure of the molecule, NMR spectrum of which is detected, by explicit functional dependence. In paramagnetic compounds, diverse relaxation effects can be observed that essentially expands research possibilities of the NMR method. The investigations of temperature dependence of paramagnetic shifts and signal broadenings enable to establish thermodynamic parameters of complex formation in solution and to elucidate stoichiometry of complexes and peculiarities intra- and intermolecular exchange processes. Hence it follows that the NMR spectra transformed by complex compounds (spin labels), the specific of which is defined by HFC, permit a considerable body of unique information on molecular structure to be obtained [4 - 16].

The present review deals with some ideas, the understanding of which would expand a circle of tasks related to problems of structural biology. In this line, general information on the NMR of paramagnetic molecules is briefly covered. Special emphasis is paid to the review [5] and the monograph [6] which contains references both to reviews and original publications of other authors studying the abovementioned problem. Also, the prospects of application of the NMR phenomenon in paramagnetic systems for studying molecular structure in a liquid phase, which very often become defining for existence of biological objects including humans, are considered. Examples of the specific spectra discussed in this review illustrate those methods, which need to be used for the purpose of obtaining information on molecular structure of both ligands and complexes from their NMR spectra transformed by hyperfine coupling.

NMR IN PARAMAGNETC SYSTEMS

A reason of specificity of the NMR phenomenon in paramagnetic systems can be rationalized as follows (see [5, 6] and the references cited therein). If resonant nucleus (for example, proton) is present in a paramagnetic molecule, *i.e.* molecule with uncompensated electron spin moment, it undergoes additional magnetic effect from the electron spin. This leads to broadening and paramagnetic shifts (contact and pseudo-contact ones) of resonance lines in comparison with width and position of lines in the NMR spectra of non-coordinated molecules. Contact shift is observed when the probability of unpaired electron location in the place of resonant nucleus differs from zero. This shift is proportional to a constant of proton-electron coupling and (in the case of insignificant spin-orbital interaction) is defined by the expression:

$$\omega_k = -A\frac{\gamma_e}{\gamma_n}\frac{g\beta S(S+1)}{3kT}, \qquad \textbf{(1)}$$

where γ_e and γ_n are gyromagnetic coefficients for proton and resonant nucleus; g is g-factor of paramagnetic compound; S is spin of complex; β- is Bohr magneton; T- is absolute temperature; k- is Boltzmann's constant; A- is constant of contact hyperfine coupling.

The A value, which can be both positive and negative, is defined by the magnetic moment of unpaired electrons of a paramagnetic particle and by orientation of the unpaired electron on this nucleus. According to Fermi's formula:

$$A = \frac{4}{3}\pi g\beta g_n\beta_n\rho(N), \qquad \textbf{(2)}$$

where g_n - is nuclear factor; β_n- is nuclear magneton; $\rho(N)$ - is density of unpaired electron spin on the resonant nucleus. Transfer of the unpaired electron from the coordinating ion to ligand and its distribution over the ligand molecule (*i.e.* the mechanism of its delocalization) is defined by specifics of electronic structure of the paramagnetic complex.

Pseudo-contact shift is caused by dipole-dipole hyperfine coupling between the

Section II: Medical Diagnosis

CHAPTER 5

NMR Spectroscopy in Brain Gliomas: Technique, Diagnosis, Grading and Follow-up after Therapy

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Abstract: Magnetic resonance spectroscopy (MRS) is a diagnostic technique that permits non-invasive measurement of metabolites in tissues.

Brain Spectroscopy for diagnostic use mainly relies on two techniques: multivoxel and single voxel acquisitions. The first allows a wider extension of the analysis of tumoral and peritumoral brain tissue, with a better mapping of metabolites distribution. This technique is time consuming and can be affected by several artifacts.

The single-voxel technique allows a more robust acquisition, more repeatable, and a better visualization of metabolites spectrum but is limited by the size and position of the voxel of acquisition, based upon operator choice.

Brain MR Spectroscopy is often an essential tool in differential diagnosis of undetermined brain mass. The cases in which spectroscopy can help in differential diagnosis are: gliomas versus metastasis, gliomas versus non-neoplastic lesions and glioma versus lymphoma. The neuronal metabolite pattern and the ratios between N-Acetyl-Aspartate, Creatine and Choline add diagnostic and functional information to the classic MR morphologic features of the brain lesion.

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A lymphoma and a Glioblastoma have very different natural history, prognosis and also therapeutic options. Grading assessment is fundamental in the subsequent decision and therapeutic menagement.

The WHO classifies brain tumors from the least aggressive (benign) to the most aggressive (malignant). Glioblastomas belong to astrocytic tumors, ranging from Grade I (least malignant, *e.g.*, I grade, pilocytic astrocytoma) to Grade IV (most malignant, *e.g.*, IV grade Glioblastoma)

Determining the classification and grade of a brain tumor can predict its likely behaviour.

The histopathological assessment after the biopsy or surgery is fundamental for characterizing the brain tumors. Not in every case biopsy is possible, depending on the localization of the tumor and conditions of the patient; in these cases, Brain spectroscopy can be as a non-invasive tool to hypothesize the tumor grading based on neuronal metabolites ratios. In the follow-up of low-grade gliomas, Spectroscopy allows, with diffusion and perfusion techniques, to find early signs of transformation into higher grades, with a consequent change in patient treatment and care. Moreover, after neurosurgery and chemo-radiotherapy, Spectroscopy has an important role in the differential diagnosis between radionecrosis, pseudoprogression and pseudoresponse.

In conclusion, Spectroscopy, although must be considered as a complementary MRI technique, gives an important contribution to the diagnosis, characterization, follow-up of brain gliomas.

Keywords: Brain, Choline, Gliomas, Lactate creatinine, MRI, NAA, NMR, Spectroscopy.

INTRODUCTION

Glioma represents about 70% of all primary brain tumors, characterized by different prognostic features and histological heterogeneity [1].

Diagnosis, relationship with surrounding anatomical structures and evaluation of tumor extent are crucial in evaluating prognosis and planning treatment. The gold standard for diagnosing the histological type and grade is stereotactic or open biopsy [2], but their diagnostic accuracy may be hampered by sampling errors or the presence of mixed malignant and benign histopathological features [2, 3].

TECHNIQUE

The fundamental principle of MR spectroscopy (MRS) is the electron cloud around an atom, shielding the nucleus from the magnetic field to different degrees, which determines slightly different resonant frequencies following the Larmor Equation and chemical shift effect and, therefore, a different signal.

Water suppression is an essential component of any MRS sequence, either through chemical shift selective (CHESS) or inversion recovery (IR). Otherwise, all spectra would be invisible because dominated by water. Sometimes water saturation may affect peaks close to water and provoke magnetization transfer effects due to saturation of bound protons.

Therefore, there have been attempts to acquire unsuppressed water signal as an internal reference to quantify the metabolite concentrations in each voxel [4, 5]. However, water suppression is the most common method used to solve this problem nowadays.

The primary sequence consists of a 90 degree RF pulse, with gradients, turned off and reception of the signal by the coil immediately after the single RF pulse.

The main difference toward a classical MR pulse sequence for imaging is that it is not used a “read-out” (frequency encoding) gradient during the receiving time of the signal from the tissue.

Identifying the different chemical compounds is possible by means of differences in various degrees in the electronic shielding of the atoms, allowing to identify different resonance frequency of the atoms.

MRS can be obtained from many nuclei, including, carbon (13C) and sodium (23Na), fluorine (19F), phosphorus (31P), although the mostly used for clinical MRS are protons (H-MRS), very abundant in human tissue. The organ more suitable to be studied is the brain, due to its lack of movement.

The aim of the technique is to find the subtle and weak signals from metabolites, so requiring at least a High Field MR scanner (1.5T and greater). The chemical shift is the change of the spin frequency typically for an atom depending on the

Section III: Food Sciences

CHAPTER 6

Solid State NMR of Food and Biopolymers

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Abstract: Nuclear magnetic resonance (NMR) is widely applied in chemistry and biochemistry, as well as in materials science. The capacity to elucidate new compounds or recognize structures by a fingerprint are common uses of liquid state NMR. Solid state NMR provides another added advantage to the analytical flux, as it can be performed without multistep sample preparation and in a non-destructive way; these aspects greatly speed up sample analysis. Research on mechanics and electronics in the recent years has led to the development of hardware for solid state NMR and a more versatile technique, cross polarization - magical angle spectroscopy (^{13}C CP MAS). These techniques are robust, operationally less expensive and reliable even when performed by non-highly trained experts. These developments have allowed this technique to be used for the analysis of food. Here, we present some technical considerations for the implementation of solid state NMR as a reproducible technique, considering typical NMR parameters such as the length of pulses and spectral calibrations. The potential target compounds in food analysis are discussed regarding the type of results that can be obtained; changes to the protein and lipid content and quality lead to different spectra as well as physical-chemical characteristics with an impact on the organoleptic and taste experience for consumers. The structure, crystal arrangement and uses of the starch flour were reviewed to explain the role of materials science tools and structural knowledge. Many techniques are used to perform state-o--the-art starch analysis and to assess its role in flour products; solid state NMR spectroscopy has considerable potential as a quick, reliable and cost-beneficial way to routinely validate processes and products. Finally, we suggest parameters for future standardized experiments and the possible comparison with other analytical methods.

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Keywords: Analytical methods, Biopolymers, Food chemistry, Hydrocolloids, Non-destructive method, Nuclear magnetic resonance parameters, Rapid method, Solid state NMR, Starch, Ultrastructural characterization.

1. BASIC CONCEPTS

1.1. Fundamentals

According to Cheng, Zhang, Zhao and Ouyang [1], "*food is any substance consumed to provide nutritional support for the human body. It is usually of plant or animal origin, and contains essential nutrients, such as carbohydrates, fats, proteins, vitamins and minerals*". Consequently, a food system is a complex mixture of different molecules that interact to result in the food structure.

Several aspects of food quality have to be observed during the development of a new product, or even during the production of a traditional one. Food quality involves external factors such as appearance (size, shape, color, gloss and consistency), texture, flavor, and other internal factors (chemical, physical and microbial), and several analytical techniques have been used to study the factors that affect the food quality [2, 3].

According to Bertocchi and Paci [4], the application of high-resolution NMR spectroscopy to the solid state was developed in the 1970s, but this technique progressively lost favor due to the difficulties arising from the complicated experimental technical aspects; the number of papers in the literature using magic angle spinning (MAS) and ^{13}C NMR with cross-polarization (CPMAS) increased. Reports after the 1980s increased quite slowly up to 1990 and increased rapidly from 1990 to 2000, particularly in food science. In recent years, *i.e.* 2000 to today, with the development of NMR instrumentation and improved programs to collect and analyze the data, the applicability of NMR has rapidly expanded in the field of food science and technology [5].

NMR can be applied to a wide range of matrices without altering the sample or producing hazardous wastes [5]. Additionally, an NMR spectrum contains a great amount of information that can be obtained in a short time period and employed as an alternative to many classic analytical procedures, such as such as high

pressure liquid chromatography (HPLC), gas chromatography (GC) and mass spectrometry (MS) [5, 6].

A wide range of NMR food-related research has covered various fields of food science, including food microbiology, food chemistry, food engineering and food packaging [5]. Some reviews have appeared in recent years showing how these tools for the study of solids by solid state NMR spectroscopy can be applied to solving specific chemical problems [4, 7 - 9].

Solid state NMR spectroscopy can provide chemical information about the material that will be analyzed, but also the chemical environment and ultrastructural details that are not easily accessible by other non-destructive high-resolution spectral techniques [10]. The information contained in an NMR spectrum can be obtained in a short time, and the different signals present in the NMR spectrum provide two kinds of information: the chemical shifts and the relative intensities. Additionally, NMR allows for the determination of a large number of components with very little or no manipulation of the samples, offering to both the industry and the consumers a very powerful analytical tool to control the composition, authenticity and quality of food products [6], as well as to study the interactions between food components.

Solid state NMR can be useful to both the food scientist and food processor, based on the requirements of the food industry to understand and innovate its products and processes, and on the pressure to develop new methods to enforce legislation and quality control [11]. Thus, several applications of solid state spectroscopy have provided valuable results to understand the chemical, physical, and biological phenomena that occur in foods. However, there are some points that must be mentioned: (i) the method is non-destructive and no derivatization is required to perform experiments; (ii) it has a very high potential to discriminate between crystallinity and polymorphic behavior in samples; (iii) starch and cellulose, food components that have crystalline and amorphous domains in different proportions, present more reliable results when studied by CPMAS NMR; (iv) foods containing proteins and fats are very difficult to crystallize, and in the solid state their spectra have been poorly resolved; (vi) the best results achievable are for saccharides and polysaccharides, both as the matrix and as

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