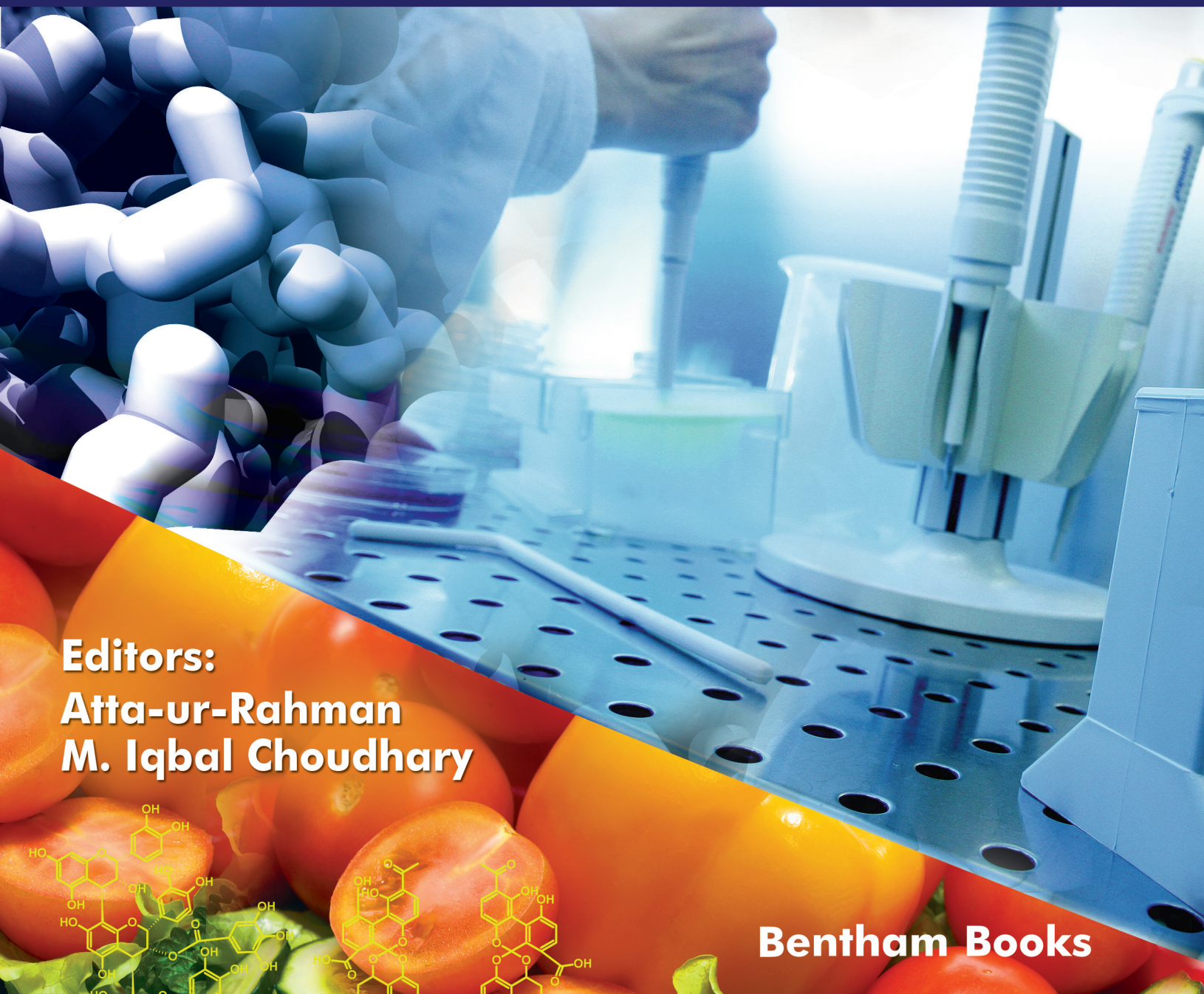
APPLICATIONS OF NMR SPECTROSCOPY



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Applications of NMR Spectroscopy

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PREFACE

Over the years NMR spectroscopy has emerged as a versatile and robust tool for a variety of research and industrial applications. NMR's capacity to analyse changes in microelectronic environment of molecules, and recent developments in magnet and probe technologies and resulting improvements in sensitivity and resolution made this a technique of choice for a variety of applications, including medical imaging to food quality monitoring, and from metabolomics to structural biology and chemistry. Literature on various applications of NMR spectroscopy is expanding with a dizzying pace, and it is often difficult to remain updated about the recent frontiers in this dynamic field.

The 9th volume of the book series, "*Applications of NMR Spectroscopy* is an excellent compilation of five comprehensive reviews, focussing on various applications of NMR spectroscopy. These reviews, written by leading practitioners of NMR, provide most updated and notable events in NMR applications.

Sinha *et al* have contributed a review on the applications of various newly developed NMR techniques in the identification of secondary metabolites from cyanobacteria with photoprotective properties. They have also discussed the general applications of NMR techniques in metabolomic profiling, and bioassay-guided isolation of secondary metabolites from natural sources. Coqueiro *et al* has reviewed the applications of NMR methods in metabolomic studies of coffee products, primarily with the objective of quality control, origin, authenticity, and sensory characteristics of this most consumed natural recreational substance. They also have discussed characteristic differences in the constituents of roasted and green coffee beans through NMR based methods and associated data processing techniques. Lignins are important natural products with various industrial and pharmacological uses. They are often present in biowaste of food processing. Avellino and Lomonaco contributed an excellent review on various NMR methods used for the structural identification and quality control of lignins, produced during the refining of coconut oil. NMR thus play an important role in the production of lignin-based value added products from a common industrial bio-waste. Pharmaceuticals are highly regulated for enantiomeric purity as the adverse effects of racemic mixtures have been well documented. Vashistha *et al* have exhaustively detailed the use of NMR as a key tool to determine the chirality and stereo-isomeric purity of drugs. They describe NMR's capacity to distinguish between two closely related compounds. Junior et al have written a detailed article about recent developments in NMR-based metabolomics and their applications in various fields. The delineated various stages of sample processing, choice of appropriate NMR techniques, and statistical tools. Their review provides convincing arguments on the advantages of NMR techniques for metabolomics and system biology studies over other hyphenated techniques.

We are pleased to extend our felicitation to all contributors, as well as production team of Bentham Publishers for the timely compilation of the 9th volume of this *e*book series. Contributions of Ms Asma Ahmed (Assistant Manager Publications), and leadership of Mr. Mahmood Alam (Director Publications) are duly acknowledged. We sincerely hope that like the previous volumes of this useful book series, the current compilation will contribute in better understanding of the applications of NMR spectroscopy.

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CHAPTER 1

NMR Spectroscopy for the Characterization of Photoprotective Compounds in Cyanobacteria

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Abstract: Cyanobacteria are ubiquitous in nature as they efficiently tolerate various extreme climatic conditions for survival, such as increasing effects of solar radiation, salinity, temperature, *etc*. Cyanobacteria are important sources of secondary metabolites, which enable them to withstand these harsh environmental conditions. Small-molecular-weight secondary compounds are primarily implied in the defense mechanisms in case of biotic and abiotic stresses. Various beneficiary secondary compounds are extracted from cyanobacteria, such as UV-screening pigments (mycosporine-like amino acids, scytonemin, carotenoids, *etc*.), phytohormones, cyanotoxins and antioxidants. Bioactivity-directed isolation techniques are used to identify these molecules from complicated matrices in pharmacognosy (discovery of biologically active compounds from natural sources). NMR spectroscopy has appeared as a specific major analytical technique applied in metabolomics. The easy sample preparation, the expertise to evaluate metabolite quantity, the notable investigational reliability, and the innately non-destructive quality of NMR spectroscopy have made it the first-line option for significant scientific metabolic analyses. Unlike some mass spectrometry methods, NMR is not discriminatory, depending on the metabolites' precursors or their ionization potential. Screening of metabolites needs maximum sensitivity, and it is a process with a broad scope. In this chapter, we have discussed the usage of NMR spectroscopy in the identification of photoprotective compounds and its advantages and disadvantages for metabolomic studies. We have also explored several new NMR techniques that have recently become available in order to fortify its advantages and overcome its inherent limitations in metabolomics applications.

Keywords: Cyanobacteria, Metabolomics, Nuclear Magnetic Resonance Spectroscopy, Scytonemin, Secondary Metabolites, Ultraviolet Radiation.

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INTRODUCTION

UV-radiation levels on Earth's surface are increasing due to CFCs and reduced cloud cover [1]. Cyanobacteria are the principal source of many metabolites, such as alkaloids, carbohydrates, flavonoids, pigments, phenols, saponins, steroids, tannins, terpenes and vitamins, which could be exploited in the biotechnology and industrial sectors [2]. Cyanobacteria are able to survive in high UV radiation due to several photoprotective mechanisms. Quenching strategies are being used by distinct species, which frequently imply the DNA repair system, antioxidative enzymatic activities, and UV-screening compounds in combination.

UV-induced damaged DNA can be repaired by photoreactivation, excision and mismatch repair. Photoprotective compounds (PPCs) having UV-absorbing properties such as mycosporine-like amino acids (MAAs) and scytonemin, are produced by cyanobacteria, which help in the protection against excessive UVR. Various environmental factors like the variations in the intensity and wavelength of UVR, nutrient deficiency curb, and a number of stresses affect the biosynthesis of these compounds [3].

In the past twenty years, quick and effective characterization of secondary metabolites has become a great challenge for the investigation of novel bioactive compounds and drug research. Many secondary metabolites have been identified by conventional techniques in cyanobacteria. Compounds were generally isolated from cyanobacterial extracts by semi-preparative liquid chromatography and identified using classical spectrometric techniques, namely ultraviolet-visible (UV-Vis) spectrophotometry, infrared (IR) spectroscopy, mass spectrometry (MS) or nuclear magnetic resonance (NMR). However, these processes are time-consuming. In order to expedite the isolation and purification of compounds from cyanobacterial extracts, various techniques are being used in metabolomics such as gas chromatography-mass spectroscopy (GC-MS), liquid chromatography mass spectroscopy (LC-MS), and high performance liquid chromatography (HPLC) often in combination with mass spectroscopy (HPLC-MS), or nuclear magnetic resonance (HPLC-NMR) [4 - 10]. Each technology has its own advantages and limitations. The selection of technology is subject to the focus of the inquiry and the nature of the samples and is also determined by the evaluation and its expertise availability. The metabolites are usually not recognized and evaluated by a single technology and mostly, various technologies are used for a complete study.

NMR is an extensively used technique for studying secondary metabolites from natural cyanobacterial extracts. It is the most effective technique for the structural identification of unknown compounds in a mixture. However, the isolation and at

least partial purification of compounds have to be carried out prior to NMR spectroscopy. To reduce these time-consuming steps, physical coupling of liquid chromatography and NMR has been used in the last two decades. In practice, the routine applications of HPLC-NMR have been successfully applied only in the last ten years. NMR spectra of HPLC purified fractions from biological samples are now possible due to the introduction of flow-through probe heads.

Compared to other technologies, NMR has several ascendancies in being non-destructive, non-adherent, easily quantifiable; it does not require chromatographic separation, sample treatment, chemical derivatization and identification of novel compounds. NMR is fully automated and surprisingly reproducible, allowing high throughput [11], large-scale metabolic studies that can be done easily by NMR spectroscopy in comparison to LC-MS or GC-MS. NMR is also used for the characterization of sugars, organic acids, alcohols and highly polar compounds. NMR analysis is not only used for biofluids or tissue extracts but is also applicable for whole tissues, organs, and other samples by using solid-state NMR (ssNMR) and magic-angle sample spinning (MAS) NMR [12 - 15]. Furthermore, NMR is exploited in the metabolite imaging of live samples *via* magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI) [16 - 20]. Real-time metabolite characterization of living samples can be done through NMR spectroscopy [21, 22]. Molecules can be studied at atomic level, not only based on ^{1}H atoms, but several other biologically reactive groups, including ^{13}C, ^{15}N and ^{31}P can be studied [23 - 28]. Lack of sensitivity is a limitation of NMR as it only provides information on 50 to 200 recognized metabolites with concentrations greater than 1 μM. NMR spectroscopy requires large amounts of samples, as compared to HPLC, GC, MS, GC-MS and LC-MS. Nevertheless, this is typically not a major problem in microbiology. This chapter provides an overview of the utilization of NMR spectroscopy in the profiling and structural elucidation of photoprotective compounds in cyanobacteria.

PRINCIPAL PHOTOPROTECTIVE COMPOUNDS IN CYANOBACTERIA

Scytonemin and MAAs are the chief UV-screening pigments biosynthesized by cyanobacteria mainly in response to harsh UV-irradiation since they are considered powerful UV-absorbing biomolecules.

Mycosporine-Like Amino Acids (MAAs)

MAAs are UV-absorbing uncoloured and water-soluble small molecules with molecular weights between 188 amd 1050 Dalton. They are characterized by a cyclic-hexenone or cyclic-hexenimine chromophore combined with nitrogen or alternatively an amino acid or its imino alcohol, with absorption maxima between

CHAPTER 2

Coffee Assessment Using ^{1}H NMR Spectroscopy and Multivariate Data Analysis: A Review

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Abstract: The importance of nuclear magnetic resonance (NMR) spectroscopy for the food industry, including the coffee industry, has led to several studies using this analytical tool to assess coffee in different scenarios. In this context, this chapter presents an overview of ^{1}H NMR-based metabolomics analysis used in coffee quality control. Several articles using ^{1}H NMR associated with multivariate data analysis have been published to investigate aspects related to quality, authenticity, sensory quality, production processes, and origin of coffee. In addition, the present review reports the main chemical shifts of the constituents present in the extracts of green and roasted coffee beans. The results presented confirm the potential and versatility of ^{1}H NMR combined with chemometric tools for coffee analysis, playing an important role in distinguishing and identifying the origins, quality, and purity of coffee. Exploratory data analysis, classification, and calibration are the most used chemometric methods in metabolomics applied in the coffee field. The results show the potential of NMR and multivariate data analysis for coffee authentication.

Keywords: Coffee Compounds, Coffee Review, NMR-Based Metabolomics, ^{1}H NMR, Chemometric Tools.

INTRODUCTION

Coffee is one of the most consumed beverages in the world, enjoyed by millions of people. As a result, coffee achieved high economic values, being the most prominent commodity traded after oil [1]. In the past decades, extensive research on coffee has been conducted, showing significant progress. However, everyday coffee professionals face new challenges in implementing new methods of coffee quality control. Several studies on the metabolic composition of coffee have been

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published, investigating aspects such as quality, authenticity, sensory profile, geographical origin, among others.

In this sense, Nuclear Magnetic Resonance (NMR) spectroscopy has emerged as an essential tool, showing itself as an efficient analytical technique and a valuable alternative to other methods used for quality control, such as gas and liquid chromatography coupled to different detectors. Among the instrumental techniques used in food quality control, NMR is unique in providing a considerable amount of information about the molecular structure. NMR along with multivariate data analysis has become popular in the field of food science and has been applied in the study of different food products, such as beverages, for example in orange juice to detect adulteration [2], mango juice for discrimination of cultivars [3], and pomegranate juice for differentiating cultivars and detecting adulteration [4]; in black tea for altitude classification [5], green tea for geographical and climatic classification [6], and chamomile tea for geographical classification [7]; in milk for validation of nutrition labeling [8], for classification of cow and sheep milk [9], for differentiation of organic and conventional milk [10], for geographical classification [11], and for detecting adulteration of powdered milk [12]; in beer for brand classification [13], for discrimination of origin [14], for discrimination of lager Brazilian beers [15], and for discrimination of craft and industrial beers [16]; in wine, to obtain the metabolic profile of rice wines [17], for classification of the geographical origin and year of vintage [18], also for classification of the type and variety of Czech wine [19]; it has also been applied to meat, for the geographical classification of beef [20] and for the classification of wild fish or farmed gilthead sea bream [21]; in olive oils for classification based on cultivation areas [22], for discrimination of cultivars [23], and for detection of adulteration [24, 25]; and in fruits and vegetables such as apples for geographical classification [26], lettuce for discrimination of transgenic *versus* conventional types [27], potatoes for transgenic *versus* wild type [28], tomato for classification of varieties [29], and bananas for changes in metabolites during postharvest senescence [30]; among many other applications.

The increasing use of NMR for quality control is due to its robustness, reproducibility, speed of execution, low amount of organic solvent required, and quantitative analysis. Moreover, it is an elective technique to analyze complex mixtures without the need for sample separation and/or pre-purification steps. The low resolution of NMR, at first, limited its use for the analysis of mixtures. However, with the new high-resolution NMR spectrometers with high field magnets, multinuclear/multidimensional and solvent suppression techniques, improvements in NMR hardware, including cryoprobes, and more user-friendly software, the NMR application in food science has become more popular in recent

years. Although the use of NMR has increased, it is not yet fully explored in food science; mainly due to the high cost of the equipment, low sensitivity when compared to mass spectrometry, and sometimes the lack of NMR databases and insufficient NMR expertise among food scientists [31, 32].

The importance of NMR spectroscopy for food science, including the coffee sector, has led to the design of several studies that describe the potential of this powerful technique for the analysis of coffee. In this context, this chapter reviews the state-of-the-art ^{1}H NMR spectroscopy application combined with multivariate data analysis to evaluate coffee in terms of quality, authenticity, origin, sensory quality, and process monitoring, among other applications. Herein, we review the literature on modeling of ^{1}H NMR data sets with chemometric tools for coffee assessment.

Metabolomics aims to provide a global snapshot of all small molecules in a given organism or biological sample, which allows the determination and simultaneous comparison of thousand metabolites. Therefore, it requires the use of multivariate data analysis to extract helpful information from comparing a large number of spectra. NMR metabolomics and different chemometric tools have been combined to better analyze food for different purposes.

Several instrumental techniques and multivariate data analysis have been used for several purposes, such as to detect adulteration in high-quality coffee, authenticate coffee beans, discriminate between different species, classify coffee according to its geographic and genotypic origin, or even determine its sensory quality. Here, however, we focus on detailing the approaches using ^{1}H NMR-based metabolomics.

Of the two most important coffee species, Arabica (*Coffea arabica*) is the most valuable and expensive due to its superior organoleptic properties. Arabica is considered milder, acidulous, and fruitier, while Robusta (*Coffea canephora* – Conilon and Robusta botanical varieties) is considered neutral, with a weak flavor and occasionally has pronounced bitterness [33, 34].

Due to its high added value, coffee is susceptible to adulteration. Roasted coffee can suffer several types of adulteration as a cost reduction strategy. Often, adulteration is practiced by diluting it with less expensive and lower quality coffee or less valuable materials. The practice of a fraudulent economic gain when replacing high-quality coffee with lower quality coffee is evident, as it is challenging to distinguish them after the roasting and grinding processes. The strategy of mixing coffee with less valuable materials is also used to reduce the price, impacting its sensory quality and, for the consumer, whether economic or health-related [35]. A schematization of the ^{1}H NMR-based metabolomics

CHAPTER 3

Evaluation of Structure-property Relationship of Coconut Shell Lignins by NMR Spectroscopy: From Biorefinery to High-added Value Products

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Abstract: Nuclear magnetic resonance (NMR) spectroscopy is one of the most important spectroscopic methods for lignin characterization, playing a key role in its structural elucidation and the choice of its suitable application. This chapter intends to cover a wide range of matters regarding the NMR utilization in the lignin field from the biorefinery up to lignin valorization through different chemical modifications in order to produce high-added-value products with potential technological applications. The chapter is divided into four main sections, in which the first and second ones will provide a review of the main structural properties, NMR experiments used for lignin characterization, and the recent advances of this technique. The third section is composed of topics about biorefinery with a focus on microwave-assisted organosolv delignification (MWAOD) and how the main pulping parameters affect the lignin structure, besides the use of NMR as a quality control tool for MWAOD up-scaling. Finally, the fourth section discusses NMR as a tool for determining structure-property relationship by performing some chemical modifications in lignin structure aiming its valorization, such as glycidylation, microwave-assisted phosphorylation, microwave-assisted selective acetylation, and the performance of lignins from different pulping processes as antioxidant compounds and the influence of their structure on it. Therefore, this chapter contributes to the development of the lignin field by reviewing the recent advances provided by NMR spectroscopy.

Keywords: Lignin Valorization, Lignochemical Platform, Structural Elucidation.

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INTRODUCTION TO THE LIGNIN CHEMISTRY

Lignin is the second most abundant natural polymer, after cellulose, and is also the major source of aromatic compounds on Earth. It has a structural function in the plants, conferring rigidity to their wall cells and protecting cellulose from the hydrolytic attack of pathogenic microorganisms [1].

In terms of chemical structure, lignin is a tridimensional amorphous polymer with a hydrophobic character formed by the random polymerization of phenylpropanoid units derived from three monolignols, such as *p*-coumaryl, coniferyl, and sinapyl alcohols. These alcohols originate from the monomeric and phenolic substructures known as *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), and the difference between them is the degree of methoxylation in their structures, as shown in Fig. (**1**).

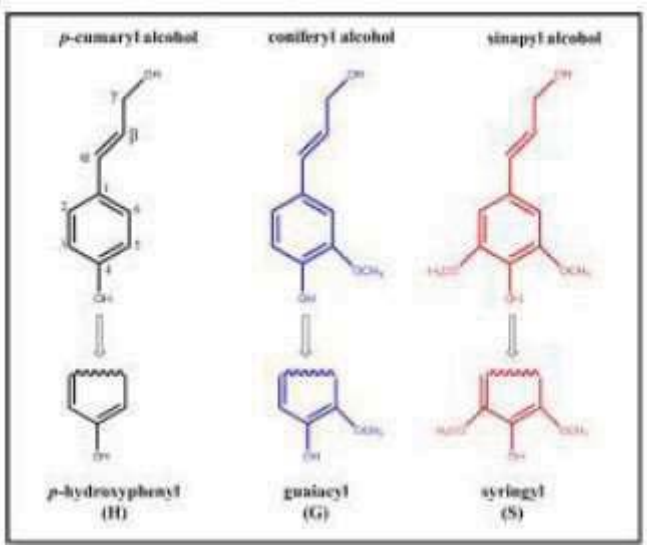

Fig. (1). The monomeric substructures present in lignin molecule.

The monomeric components of lignin molecule are linked to each other in a random way through different types of bonds, such as C-C and C-O-C, being β-1, β-β, 5-5' and α-O-4, β-O-4, and 4-O-5, the main types of linkages, Fig. (**2**). These substructures can be linked to each other as a result of the polymerization between the monolignols, forming a complex, branched, and tridimensional molecule Fig. (**3**) in which the main linkages are highlighted. The proportion of monolignols is strongly dependent on the plant species. Generally, softwood lignin (pine, spruce) is formed exclusively by G units, whereas hardwood lignin (birch, poplar, eucalyptus) contains both G and S units. On the other hand, lignin from

herbaceous biomass is composed of all three monolignols, although the H-content is generally low (< 5%) [2].

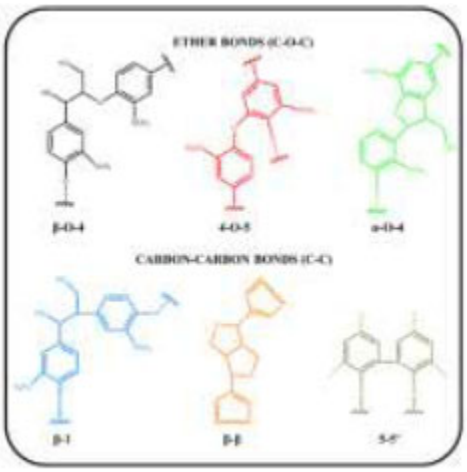

Fig. (2). The main types of carbon-carbon and ether linkages present in lignin structure.

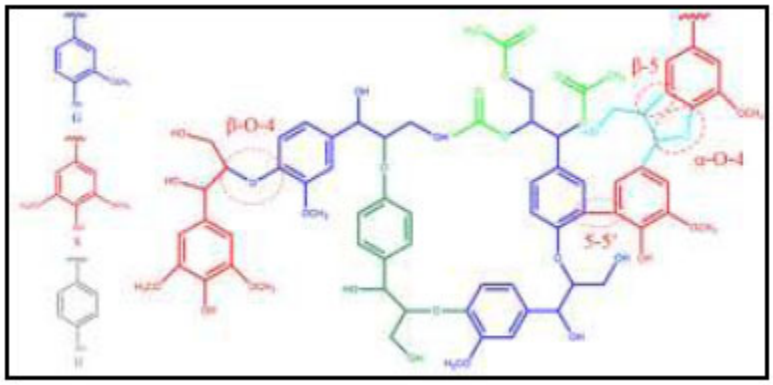

Fig. (3). Representation of the lignin structure.

For understanding the complex chemical structure of lignin, a detailed and careful characterization must be performed using different analytical techniques, such as wet, chromatographic, and spectroscopic methods. However, among them, the nuclear magnetic resonance (NMR) is the one that provides the information about the lignin skeleton, such as the interunit linkages (the type of bonds existent between the monomers), the monolignol composition, and the presence of other types of functional groups (acetyl, formyl, *etc*.), which will be discussed in the next topic.

CHAPTER 4

Application of NMR Spectroscopy in Chiral Recognition of Drugs

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Objective:

The objective of this chapter is to highlight the current developments in NMR spectroscopy for chiral recognition of pharmaceuticals reported during the past five years.

Introduction

NMR spectroscopy is an effective technique to contextualize structural characteristics and chirality discrimination of various compounds. During the past few decades, extensive studies have been carried out on chirality recognition (CR) of organic compounds, including pharmaceuticals. The assessment of enantiomeric drugs and related methods have become a customary assignment in most NMR labs, enabling the identification of molecular connectivity as well as the development of conceptual frameworks.

Background

Various NMR active nuclei (^{1}H, ^{13}C, ^{19}F, & ^{31}P, *etc*.) can be used to strengthen the CR abilities of NMR spectroscopy. NMR active nuclei are isochronic in the optically inactive environment and thus unable to exhibit CR. However, certain nuclei are anisochronic in a chiral atmosphere making CR feasible. CR capabilities of NMR are dependent on the application of chiral discriminating agents such as chiral

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derivatizing agents (CDAs), chiral lanthanide shift reagents (CLSRs), and chiral solvating agents (CSAs) that essentially contain a chiral auxiliary. CDAs tend to bind covalently with a functional group of the analytes, whereas CSAs bind *vis* non-covalent associations, like dipole-dipole and ion-pair, *etc*. However, CLSRs, like CSAs, do not bind covalently with the enantiomers of analytes. However, CLSRs, like CSAs, do not bind covalently with the enantiomers of analytes rather they comprise a paramagnetic center that can bring chemical shift difference of diastereotopic nuclei.

Method

Herein, the recent developments in methods based on NMR spectroscopy for CR of certain chiral drugs using various chiral discriminating agents are discussed.

Application

The methods described herein can be an assessment tool to provide details of molecular geometry and the construction of spatial relationships of chiral molecules.

Keywords: Baclofen, Betaxolol, Chiral Recognition, Chiral Derivatizing Agent, Chiral Solvating Agent, Chiral Drugs, Ibuprofen, Ketamine, NSAIDs, Rasagiline.

INTRODUCTION

Stereochemistry plays a vital part in biological functions and the development of new drug substances [1], asymmetric synthesis [2], stereoselective catalysis [3, 4], and supramolecular chemistry [5 - 10]. The enantiomeric purity of chiral drugs is an important consideration that establishes their therapeutic activities. Thus, chiral recognition (CR) involving assessment of chiral purity (in terms of enantiomeric excess, *i.e.*, *ee*) and absolute configuration (AC) of drug entities is a major area of research in drug development. Over the past few decades, different techniques were proposed for the evaluation of CR for a wide range of organic molecules which includes colorimetric [11 - 13] circular dichroism [14], high-performance liquid chromatography (HPLC) [15 - 18], thin layer chromatography (TLC) [19, 20], and nuclear magnetic resonance (NMR) spectroscopy, *etc*.

NMR spectroscopy is a potential technique used not only for the determination of chemical structure but also for CR. Among the active NMR nuclei, ^{1}H is commonly utilized for structural elucidation, differentiation between two closely related compounds, and other associated applications. In addition, it has a natural

abundance of 99.98%, and its better sensitivity to atmospheric changes make it enormously handy in CR studies [21, 22].

The assessment of CR has become a routine work in most synthetic laboratories to provide details of molecular geometry and the construction of spatial relationships. Three main approaches are developed for CR through NMR spectroscopy depending on the application of chiral discriminating agents such as (i) chiral solvating agents (CSAs) [23 - 27], (ii) chiral lanthanide shift reagents (CLSRs) [25, 28], and (iii) chiral derivatizing agents (CDAs) [23, 24, 28]. All three NMR approaches essentially involve the development of diastereomeric pair of the chiral substances with the chiral discriminating reagent to create two asymmetric entities without any symmetric relationship. As soon as the enantiomeric species are converted into diastereomeric pairs either by covalent bonding (as in the case of CDAs) or by non-covalent associations (in case of CSAs or CLSRs), the difference in chemical shift ($\Delta\delta$) for the two enantiomers would start appearing. The degree of difference of chemical shift is a measure of chiral differentiation; the greater the $\Delta\delta$ value, the better would be the chiral differentiation. The presence of a suitable chiral auxiliary is required for the formation of the diastereomeric complexes for NMR discrimination [27]. Three different approaches are described below.

Chiral Solvating Agents (CSAs)

CSAs are the chiral substances to be mixed with two isomers of the analyte to form in-situ diastereomeric complexes *vis* the formation of H-bonds, dipole-dipole, or π–π interactions, *etc*. The application of the NMR technique, in particular, provides a broad range of CSAs that can be employed to assess CR [29, 30]. During the past few years, the design and synthesis of novel CSAs for the discrimination of enantiomers are considered very crucial and imperative for prochiral analytes and have gained significant attraction. Nevertheless, the use of many of the commercial CSAs is not practically feasible because of the poor shifting of $\Delta\delta$ values that result in hard to recognize baseline resolution, thus hinder the enantiomeric analysis.

One significant technique that demonstrates dissimilar reactive orientations uses non-covalent interaction between an achiral host and two chiral guests to form a "host-guest complex" [31, 32]. The two diastereomers are formed when the guests are capable of π-interaction with the host. The corresponding NMR spectra of diastereomers show two different resonance frequencies for protons on the achiral host ring (that are equivalent in the absence of chiral guests). The magnitude of the difference in the two frequencies can be used to determine the enantiomeric excess (ee) of chiral analytes.

CHAPTER 5

NMR-Based Metabolomics: General Aspects and Applications in Cancer Diagnosis

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Abstract: Metabolomics is defined as a matrix of studies on the methodologies and analytical technologies related to data analysis in microorganisms, plants, and animals. In humans, about 50–200 metabolites have been identified, improving our understanding of the metabolome and genomics. A broad range of fields commonly apply metabolomics studies, including those related to the effects of drugs, cellular function, and drug discovery, as well as to the search for biomarkers of diseases. It is possible to divide a well-designed metabolomics study into phases, including ethical approval, sample collection and preparation, sample analysis (Liquid or Gas Chromatography Coupled to Mass Spectrometry - LC-MS/GC-MS, High or Ultrahigh Performance Liquide Chromatography - HPLC/UPLC, or Nuclear Magnetic Resonance - NMR), identification/quantification of metabolites, statistical analysis, and biochemical interpretation. In studies involving biological samples, the use of NMR has increased, in comparison with GC-MS and LC-MS, because NMR presents advantages, including the fact that it is nondestructive to samples, no chromatographic separation is needed, and there is a possibility of determining unknown metabolites. In addition to discussing NMR-based metabolomics studies involving biological samples from humans, in this chapter, we discuss essential aspects in early stages, including sample preparation in phosphate buffer, utilization of internal standards, the importance of water suppression, and the use of 1D and 2D NMR spectroscopies for identification of metabolites.

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Thereafter, the most often-used multivariate analysis in chemometrics will also be presented, demonstrating which OPLS-DA method is the best option for group discrimination.Some metabolites identified in human biofluids will be described, especially those associated with pathological conditions or dysregulation in metabolic pathways for different types of cancer. Finally, we present the results of a preliminary NMR-based metabolomics study involving patients with colorectal cancer and healthy volunteers.

Keywords: Breast cancer, Colorectal cancer, Lung cancer, Metabolomics, Nuclear Magnetic Resonance, OPLS-DA, Pancreatic cancer.

SYSTEMS BIOLOGY, METABOLOME AND METABOLOMICS

Systems Biology

Humans start life from a single cell, as do all mammals. In adulthood, there are trillions of cells and thousands of cell types. The entire process of cell replication occurs based on two kinds of biological information: (*i*) digital data from the genome; and (*ii*) environmental data, including metabolites secreted on the cell surface or chemical gradients. Environmental data can be classified into two distinct types, (*i*) deterministic data, where the consequences of the signals are predetermined, and (*ii*) stochastic data, where chance dictates the result. Both types of signals generate significant noise in biological system; but however, they occur only in particular cases, in which, the noise converts into signals [1].

In this context, we can define Systemic Biology as the computational integration of data generated by sets of genetic, transcriptomic, proteomic, and metabolic platforms. Systemic biology supports the understanding of function at various biomolecular organization levels. In particular, it is a quantitative analysis of how the components of a biological system interact in an organism. This analysis is performed using multidisciplinary teams, using robust data generation tools, as well as analysis and interpretation of results, typically involving Data Science teams. This interdisciplinary approach, using molecular, computational, mathematical, and statistical tools, led to the creation of quantitative models that integrate the various levels of information to describe and predict the behavior of living organisms in response to environmental disturbances [2].

Systemic Biology seeks to understand biological organisms at all levels, from the characterization of their constituent parts (genes, RNAs, proteins, metabolites, *etc*.), to the elucidation of the interconnections among the various members of these networks of interactions, to the understanding of organism as a whole [3]. In recent years, this branch of biology has become part of a more extensive process

(the system) that enables the construction of models that predict the behavior of systems and that respond to external stimuli, disturbances, or changes in composition. For example, all reactions that occur inside a human cell (or in any cellular microorganism) are part of a more extensive system, the functioning of the cell, as are the several external and internal environmental factors that influence these reactions [4].

Biological systems are dynamic, using complex cellular circuits to perform functions such as cell growth, differentiation, and reproduction. Genomic-scale technologies that explore transcriptome, proteome, protein-protein, protein-DNA, and protein-RNA interactions data, among others, are powerful tools for systemic analysis. Nevertheless, none of these individual data sets presents a global view of cellular behavior, because the complexity of living organisms is an emerging property inherent not only in genes, RNAs, proteins, or metabolites but also as a consequence of their actions and interactions [3].

Metabolome

In the mid-1920s, Fritz Kahan published a series of books involving the term Man-machine. As an enthusiast of technology and physiology, Kahan created fascinating illustrations through analogies between the human body and industrial machines developed in the early 20th century. Seventeen years later, humanity entered the "*omics era*", which was stimulated by massive advances in technology, permitting the systematic and quantitative characterization of the molecular machinery of the cell (Genome, Transcriptome, Proteome, and Metabolome). Consequently, what was previously not possible to characterize in a human cell, suddenly emerged in all directions in the forms of cellular and molecular quantification (Fig. **1**) [5].

Among the types of characterization performed by systemic biology, one merits particular attention: the Metabolome, which we define as the composition of all small molecules present in an organism [6]. By contrast, Rinschen and collaborators (2019) [5] stated that the metabolome represents small chemical entities (metabolites) that connect the genome and the proteome, representing the final stage in a dynamic system, the metabolism. In other words, metabolites consist of the end products of cellular metabolism, which are considered the final phase of all cellular processes [7]. Abdelnur and collaborators (2014) [6] stated that metabolites constitute a diverse set of atomic arrangements when compared to proteomes and transcriptomes, providing a wide range of cellular physical and chemical properties.

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Prof. Atta-ur-Rahman, Ph.D. in Organic Chemistry from Cambridge University (1968) has 1,232 international publications (45 international patents and 341 books). He received the following awards: Fellow Royal Society (FRS) London (2006), UNESCO Science Prize (1999), Honorary Life Fellow Kings College, Cambridge University (2007), Academician (Foreign Member) Chinese Academy of Sciences (2015), Highest Civil Award for Foreigners of China (Friendship Award, 2014), High Civil Award Austria ("Grosse Goldene Ehrenzeischen am Bande") (2007), Foreign Fellow Chinese Chemical Society (2013), Sc.D. Cambridge University (UK) (1987), TWAS (Italy) Prize (2009). He was the President of Network of Academies of Sciences of Islamic Countries (NASIC), Vice President TWAS (Italy), Foreign Fellow Korean Academy of Science & Technology, President Pakistan Academy of Sciences (2003-2006) and (2011 – 2014). He was the Federal Minister for Science and Technology of Pakistan (2000 – 2002), Federal Minister of Education (2002) and Chairman Higher Education Commission/Federal Minister (2002-2008), Coordinator General of COMSTECH (OIC Ministerial Committee) (1996-2012), and the Editor-in-Chief of Current Medicinal Chemistry.

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