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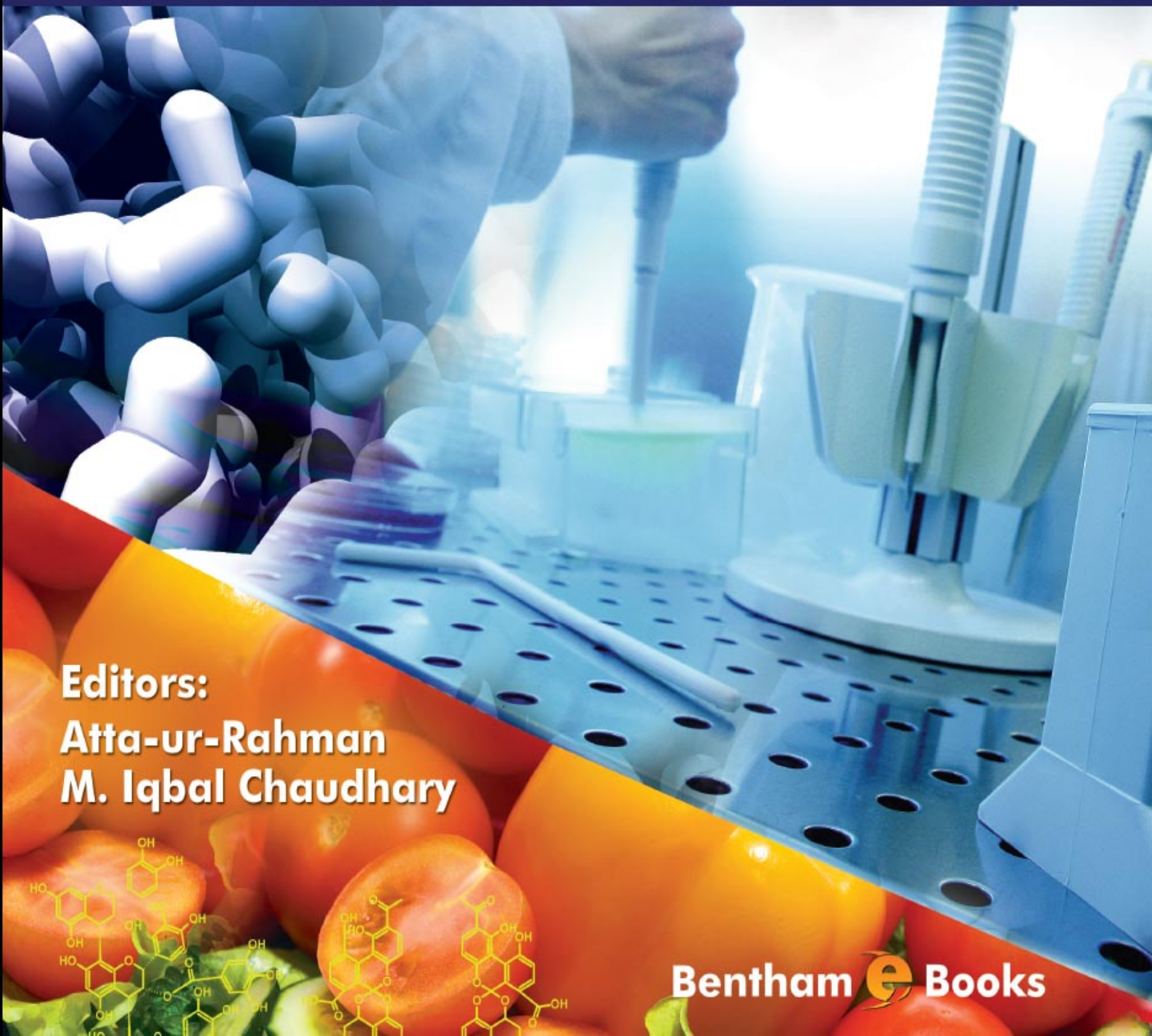
Volume 4

APPLICATIONS IN FOOD SCIENCES



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APPLICATIONS OF NMR SPECTROSCOPY

Applications in Food Sciences

(Volume 4)

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Applications of NMR Spectroscopy

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PREFACE

Volume 4 of the ebook series *Applications of NMR Spectroscopy* is mainly focussed on the use of NMR spectroscopy as a key method for food and beverage analysis and characterization. Food presents a complex mixture of many different compounds with different chemical structures, concentrations, solubilities, properties, and nutritional values. Any change in food composition can lead to a gross change in its quality, taste, and calorific value. The present book is based on six well-written reviews, each focussing on a unique set of applications of NMR spectroscopy in food analysis. In each of these articles, the optimum use of this powerful technique with reference to the field of food science is introduced in an easy to understand manner. The real strength of the book is its highly practical approach in describing both the concepts and applications of NMR spectroscopy for various purposes.

Review contributed by Melado-Herreros *et al* provides practical applications of several NMR techniques used in the multi-component analysis of food samples. Apart from introducing the concept of multi-component analysis with reference to food items, the authors have also explained the use of NMR techniques such as ^{1}H HR-MAS (High Resolution Magic Angle Spinning) for solid state analysis, MRI (Magnetic Resonance Imaging) and CSI (Chemical Shift Imaging) for physiological analysis of fruits and vegetables. The NMR technique called "relaxometry mapping" (relaxation time measurement) gives important information about water compartmentalization, structure and integrity.

Lipid oxidation/peroxidation is a key issue in the storage, and processing of edible oils and oil containing and oil-based food. This undesirable series of complex reaction leads to the development of off-flavour, odour, and degradation of the overall quality. It is therefore important to accurately and correctly measure the quantity and types of oxidized products. Hwang and Bakota have contributed an excellent review on the applications of various NMR techniques (^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR) for the analysis of the types, and extent of oxidative changes during the processing and manufacturing of oil-based food as well their storage. They demonstrate that NMR can be effectively used for determining the oxidative stability of lipids and oils, and their products.

Hermosin-Gutierrez *et al* review the various NMR techniques used for the study of the structures and dynamic properties of various classes of pyranoanthocyanins. Anthocyanins are plant-based pigments, which have the ability to protect against a myriad of human diseases. They frequently interact with other phytochemicals and give rise to new classes of compounds which are often difficult to decipher. Pyranooanthocyanins are however fairly stable compounds of complex structures. They occur as glycosides and exhibit complex structural variations in the flavone skeleton which often makes their structure determination

quite challenging. NMR spectroscopy is especially suited for elucidating such structures. Pyranoanthocyanins also have a special significance in the color of foods and beverages, and their dynamic properties are important to be studied.

The article by Géan *et al* describes various key developments in NMR spectroscopy as a powerful tool for the study of the structures of tannins, their relationship with the taste of wine, as well as their health protective effects. Their anti-oxidant properties and their protective effect on membranes against lipid oxidation have been discussed.

Quantitative ^{1}H-NMR (qNMR) is an application of NMR spectroscopy for the determination of the concentrations of one or more chemical species in a solution with a very high level of precision. It is simple and rapid, yet an elegant technique in which the area of an NMR signal is directly proportional to its concentration and this "response" is the same for all molecules. Sugimoto *et al* have comprehensively reviewed the concept of qNMR methods, and their applications in complex food analysis, such as purity assessment, and quantification of mycotoxins, pesticides, preservatives, phytosterols, etc.

Kralicek and Ozawa have critically reviewed various NMR spectroscopic methods used in structure determination of large cell free proteins, and their complexes. The authors describe the various cell-free expression systems used for the rapid and cost effective production of target proteins with required isotope labeling.

At the end we would like to express our gratitude to all the contributors for their excellent contributions. The entire editorial team of Bentham Science Publishers, particularly Ms. Fariya Zulfiqar (Assistant Manager Publications), Mr. Shehzad Naqvi (Senior Manager Publications) and team leader Mr. Mahmood Alam (Director Publications) deserve our deep appreciation for compiling such an excellent volume which should prove to be of wide interest to the readers.

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CHAPTER 1

Application of NMR to Resolve Food Structure, Composition and Quality

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Abstract: Food is a complex system formed by several chemical compounds and physical structures at different organization levels. For food analysis and characterization, it is not only important the study of the chemical composition, which will define the nutrient content, but also the physical distribution of the different compartments and structures that will define the physical properties of food products. Physical properties of food will define the palatability and texture of the food product and thus, the acceptance by the consumers. When talking about Nuclear Magnetic Resonance (NMR) spectroscopy we refer to several techniques that study the interaction of electromagnetic radiation with matter. Nuclear magnetic spectroscopy is the use of the NMR phenomenon to study physical, chemical and biological properties of matter, from the microscopic to the macroscopic. NMR spectroscopy is a very successful and multipurpose technique which is very suitable combined with chemometrics, for the analysis of food products [1]. In this chapter, we will review several NMR techniques that are related to both chemical and physical characterization. Such techniques are 1H High-Resolution Magic Angle Spin (1H HR-MAS), which provides a high resolution chemical spectrum without component extraction [2], relaxometry, which gives information about the water compartmentation, structure and integrity [3], magnetic resonance imaging (MRI) and chemical shift imaging (CSI), which is an efficient tool for the physiological analysis of fruit and vegetables [4]. The following chapter will address, first of all, what needs to be measured on food, as well as several NMR techniques that have been used for the analysis of food products.

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These techniques are 1H High Resolution Magic Angle Spin (1H HR-MAS), MRI, 1D and 2D relaxometry, relaxometry mapping and chemical shift imaging. We further focus on the explanation of multicomponent analysis and finally offer some remarks about prospects in the field.

Keywords: Chemical properties, Chemometrics, Food structure, Macrostructure, Microstructure, Physical properties, Structure, Texture.

FOOD DIMENSIONS

To start with this chapter, we would like to offer an introduction of different food dimensions in order to understand the different parameters that affect the food. There are both chemical and physical characteristics that influence the nutrient composition, shelf life, texture, structure... and that are important to take into account when studying and/or designing a food product. These characteristics may be measured and controlled by means of nuclear magnetic resonance (NMR).

Composition

According to Skov *et al.* [5], from a physical, chemical and biological perspective, food matrices are complex multifactorial systems containing mixtures of heterogeneous classes of molecules (nutrients), and complex physical structures.

Nutrients

Nutrients are basically classified into macronutrients (fats, proteins and carbohydrates) and micronutrients (vitamins, minerals, phytochemical, zoochemicals, fungochemicals and bacteriochemical) [6]. Macronutrients provide energy to the body and are required for growth, metabolism and other functions. Macronutrients differ in the energy density, being highest for fat (above 12 kcal g^{-1}) than for protein (9 kcal g^{-1}), and last for carbohydrates (4 kcal g^{-1}). Carbohydrates provide the glucose used by all cells as fuel, as well as deliver the fiber intake. Proteins are the unique source of nitrogen, basic for amino acids and tissue repair, and fat provides a mean for the absorption of fat soluble micronutrients [7].

Despite the generally accepted consideration of the role of macro- and micro-

nutrients, the analysis of a limited number of compounds narrows the whole view of food. Bordoni *et al.* [8] pointed out that some foods contain more than 25,000 compounds with concentrations varying according to variety, breeding, season, and geographic origin, among the major factors. Human metabolome contains about 50,000 different detectable compounds with 20% circadian variations.

Throughout World War I and II, the main concerns in nutrition were the vitamin and mineral deficiencies, while in 1960's the concern focused to the excess of nutrients (fat, cholesterol or sodium) or the imbalance in the intakes of fat and carbohydrates. In the 1980's interest turned to fiber, vitamin A, C and E and selenium, and it was only in 1998 that the first recognition of functional food was set as those with proactive health path beyond the basic (adequate) nutritional functions. Also, functional foods are not considered as pills or capsules [9].

Functional foods can be classified into conventional (*e.g.,* fruits and vegetables), modified (fortified, enriched or enhanced), medical (formulated by and to be used only under medical supervision, such as oral supplements in the form of phenyl ketonuria formulas free of phenylalanine, and diabetic, renal, and liver formulations), and special dietary (gluten-free, lactose-free…). It is expected that modifying foods through biotechnology for improving their nutritional value or health attributes will increase the number of new functional foods into the markets [9]. The above highlights the need for analytical methods that would allow a global analysis of food composition, as well as a specific assessment of selected nutrients.

Moreover, a recent discovery shows that individuals with different genotypes in a population may not benefit (or may even suffer) from increased level of nutrients in functional food [6]. It is estimated that human genome contains approximately 10 million of single nucleotide polymorphisms (SNP) which would lead to substantial differences in nutritional response among individuals. That is why there are large efforts involved toward linking nutrition science and genomics into a discipline called nutrigenomics.

Alternatively, food digestion may be manipulated to enhance nutrient absorption by changing digestion rate, and the site of absorption. The main methodologies for

CHAPTER 2

NMR Spectroscopy for Evaluation of Lipid Oxidation

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Abstract: During storage and use of edible oils and other lipid-containing foods, reactions between lipids and oxygen occur, resulting in lipid oxidation and the subsequent development of off-flavors and odors. Accurate and timely assessment of lipid oxidation is critical for effective quality control of food products. NMR spectroscopy techniques including ^{1}H, ^{13}C, and ^{31}P NMR have been used to determine the oxidation stage and quality of lipids, to elucidate chemical structures of oxidation products, and to verify oxidation mechanisms. NMR spectroscopy methods have been successfully employed to identify oxidation products, including primary oxidation products such as hydroperoxides and conjugated dienes and secondary products such as aldehydes, alcohols, epoxides and their derivatives. ^{1}H NMR can also be used to determine the extent of lipid oxidation during frying and storage by monitoring the decrease in peak area of protons located in reactive sites of oil molecules including olefinic, bisallylic and allylic protons. ^{13}C NMR has been used to identify oxidation products along with ^{1}H NMR, gas chromatography-mass spectroscopy (GC-MS) and other methods. ^{31}P NMR also has been utilized to assess the oxidation of edible oils along with ^{1}H and ^{13}C NMR spectroscopies. These methods correlate well with traditional methods and offer highly reliable, non-destructive, fast analysis of lipid oxidation. These analytical methods will be summarized in this chapter.

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Keywords: Conjugated diene, ^{13}C NMR, Diels-Alder reaction, DEPT, Frying, Hydroperoxide, ^{1}H NMR, Lipid oxidation, NMR spectroscopy, Oxidation products, ^{31}P NMR.

INTRODUCTION

NMR spectroscopy is a very reliable, convenient, fast, non-destructive method to identify and elucidate chemical structures of organic compounds. Therefore, NMR has been used for analyses of a variety of beverages, fruits, vegetables, and other food products, including beer, wine, soy sauce, vinegar, coffee, tea, fruit juice, mandarin orange, kiwifruit, mango, black raspberry, melon, watermelon, tomato, lettuce, Brassica rapa, potato, carrot, maize, wheat, milk, cheese, butter, margarine, honey, fish, and meat [1 - 3]. Although liquid NMR spectroscopy, in which the sample is dissolved in a deuterated solvent, is the most widely used method, solid-state NMR techniques, such as the HRMAS-NMR (high resolution magic angle spinning-nuclear magnetic resonance) is also utilized to determine ingredients in food products [4].

NMR spectroscopy has also been found to be a very reliable tool to analyze edible oil. For example, the fatty acid profile [5 - 8] and the unsaturation level of edible oil [9] can be determined by ^{1}H NMR. The amount of free fatty acids in a lipid ingredient can be determined by measuring the intensity of the carboxylic group proton (COOH) peak in the ^{1}H NMR spectrum, which was found to be very well correlated to a conventional acid value test method [10]. ^{1}H NMR spectroscopy provides information on unsaponifiable materials such as alcohol, sterol, hydrocarbon, and tocopherols, and thus the information could be used to reveal the geographical origin of olive oils [11]. Furthermore, it has also been extensively utilized for the analysis of olive oil, which is a frequent target for adulteration due to its high market value relative to commodity oils. Olive oil is often diluted with less expensive oils and labeled as pure olive oil. Therefore, tools that can elucidate the chemical composition of the constituent oils are in high demand. NMR has been a valuable tool for the quality assessment and authenticity of these oils [12].

Challenges in Assessing Lipid Oxidation

There are many methods used to assess oxidation of lipids. However, a lack of consistency in results from different analytical methods is still common. The major reason for this inconsistency is that there are numerous oxidation products, and any single analytical method measuring only one kind of oxidation product does not, by definition, measure other oxidation products and therefore cannot give a satisfactory description of lipid oxidation throughout the entire course of oxidation [13]. For this reason, in general, several different indications of oxidation are measured at the same time to assess the level of lipid oxidation. Pignitter and Somoza [14] have evaluated current lipid oxidation analysis methods and stressed that a method that combines the concomitant detection of primary and secondary oxidation products was necessary for a more consistent assessment of lipid oxidation.

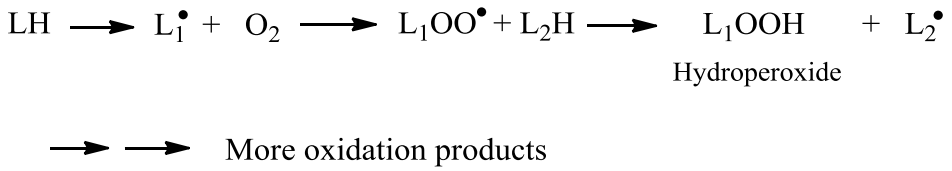

Fig. (1). Formation of hydroperoxide.

Peroxide value is one of the most widely used methods to measure lipid oxidation [15]. Lipid hydroperoxides are primary oxidation products that are formed almost immediately after lipid oxidation begins by the reaction of the lipid radicals and oxygen (Fig. **1**). The concentration of hydroperoxides serves as an important marker for the initial oxidation of lipids. Fig. (**2**) shows one of the most widely used methods to determine the concentration of hydroperoxides (peroxide value).

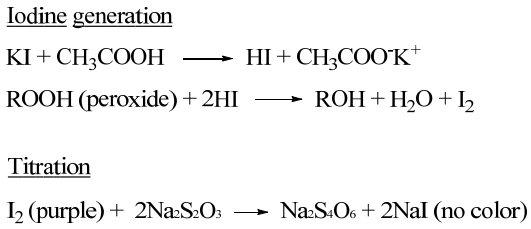

Fig. (2). Determination of peroxide value.

CHAPTER 3

The Application of NMR Spectroscopy to the Study of Pyranoanthocyanins: Structural Elucidation, Solution Equilibria and Exhibited Color in Foods and Beverages

M. Victoria Gomez[1], Sergio Gómez-Alonso[1,2], Isidro Hermosín-Gutiérrez[1,*]

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Abstract: Anthocyanins are an important class of natural pigments widely distributed throughout the Plant Kingdom and foodstuffs. They are rather reactive compounds that are involved in several equilibria in aqueous solution and give rise to new classes of anthocyanin-derived pigments. This reactivity creates changes in the exhibited color that is especially interesting with regards to foods and beverages. Among the anthocyanin-derived pigments, pyranoanthocyanins have gained attention in the last two decades because of their higher stability, mainly the resistance to discoloration by changing pH or after the addition of bisulfite, in comparison to their precursor anthocyanins. One of the first challenges regarding pyranoanthocyanins was their structural elucidation, leading to an increasing difficulty due to the finding of several new subclasses of chemical structures, beginning with one the first discovered, relatively simple, vitisin-type pyrano-anthocyanins and continuing with other more complex structures like those of flavanyl-vinyl-pyranoanthocyanins or pyranoanthocyanin dimers. Multidimensional Mass Spectrometry (MS^n) and Nuclear Magnetic Resonance (NMR) spectroscopy have been crucial tools for the structure elucidation of pyranoanthocyanins. In addition, NMR spectroscopy has contributed to the gaining of

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knowledge about the aqueous solution equilibria in which pyranoanthocyanins are involved, thus helping the interpretation of the molecular basis behind the higher stability of such anthocyanin-derived pigments. This review introduces the pyranoanthocyanins by means of a description of the anthocyanin reactivity and the formation of anthocyanin-derived pigments in foods and beverages. The review continues with an overview of the current knowledge about different structures of pyranoanthocyanins, their main properties related to exhibited color, especially their behavior against changing pH and bisulfite bleaching, their reactivity, and the occurrence of pyranoanthocyanins in foods and beverages. Finally, this review deals with the application of NMR spectroscopy to several interesting issues related to pyrano-anthocyanin chemistry, namely the structure elucidation of the different classes of pyrano-anthocyanins, the behavior of these compounds in aqueous equilibria of hydration and proton transfer, and the formation of new pyranoanthocyanin-related compounds.

Keywords: Anthocyanin, Bleaching resistance, Color, Food, Fruit, Hydration, Juice, NMR, Pigment, Pinotin, Portisin, Pyranoanthocyanin, Vitisin, Wine.

PYRANOANTHOCYANINS: AN INTERESTING CLASS OF PIGMENTS DERIVED FROM ANTHOCYANINS

Introduction

Anthocyanins constitute the largest and probably most important group of water soluble natural pigments [1]. To date, there have been more than 635 anthocyanins identified in nature, and such a versatile group is responsible for the vivid blue, purple, and red color of many fruits, vegetables, and flowers [2]. In fact, the word anthocyanin is derived from the two Greek words *anthos* and *kyanos*, meaning flower and dark blue respectively [3]. Very likely, the physiological role played by anthocyanins in plants is to attract animals by their interesting color, leading to seed dispersal and pollination. Owing to strong absorption of light, not only in the visible but also the UV wavelengths ranges, they may also be important in protecting plants from UV-induced damage [4].

Dietary consumption of anthocyanins is high compared to other flavonoids, owing to their wide distribution in plant materials. Many studies have reported evidences of the bioactivities associated with anthocyanins that, together with their

bioavailability, should be a focus of future research regarding their putative health-promoting effects [5]. In addition, anthocyanins are used as food colorants primarily in the beverage industry. As public concern about synthetic food dyes has increased recently, consumers and food manufacturers desire colorants from natural sources. Synthetic dyes commonly used in the food industry have been suspected to cause adverse behavioral and neurological effects [6]. However, anthocyanins are rather reactive compounds and their use as food colorants is limited by several factors like the acidity of the foodstuff or the development of various reactions involving anthocyanins leading to discoloration.

The color of anthocyanin-rich foodstuffs involves different kinds of mechanisms that stabilize the original anthocyanins or transform them into other pigments thus changing the food color [7]. Not all anthocyanin-derived pigments are more stable than their precursors and many of them tend to form polymeric pigments that finally become insoluble and precipitate. Among the most recently discovered anthocyanin-derived pigments, those resulting from the formation of a new pyrano ring over the anthocyanin flavonoid core, the so-called pyrano-anthocyanins, have received increasing interest for a number of reasons: they are deeper colored pigments than their precursors at the medium acidic to neutral pH ranges usually found in foodstuffs, they are more stable towards the reactions leading to anthocyanin discoloration, and also because polymeric pigments do not remain in solution during storage [8].

The properties and structures of pyranoanthocyanins are closely related to those of their precursors, the anthocyanins. In addition, the statement that pyrano-anthocyanins are more stable pigments is made by comparing them to the relatively high reactivity shown by anthocyanins. Therefore, an introductory overview of the chemical structures and properties of anthocyanins is necessary for a better understanding of the behavior of pyranoanthocyanins.

Chemical Structure of Anthocyanins

Anthocyanins belong to a large group of polyphenolics named flavonoids which are secondary metabolites synthesized by higher plants. Their aglycones share a C-6 (A-ring)–C-3 (C-ring)–C-6 (B-ring) carbon skeleton [9]. The aglycones are

CHAPTER 4

NMR Spectroscopy: A Powerful Tool to Investigate the Role of Tannins in the Taste of Wine and their Health Protective Effect

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Abstract: Tannins in the skin and seeds of grapes used to make red wine are responsible for the two dominant sensory perceptions of astringency and bitterness. Astringency is a tactile sensation causing a dry, rough and puckering mouth-feel, while bitterness triggers an unpalatable harsh taste. Although these flavors are both associated with tannins, their mechanisms of action differ greatly. Astringency results from an interaction between the tannins and the saliva proteins, whereas bitterness is the result of an interaction between the tannins and the taste receptors located on the tongue. In the last decade, various studies using NMR spectroscopy have revealed new clues to the understanding of astringency perception at the molecular level. We now know the three-dimensional structure and the colloidal state of tannins are key factors in the

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mechanism of tannin-saliva protein interactions. Although the latter are undeniably related to astringency, it is only very recently we have learned that the lipids of oral cavity membranes and a fortiori provided by fat foods could also play a role in this complex sensory phenomenon. Indeed, strong interactions between tannins and membrane lipids have been highlighted in recent research supported by a fluidizing effect on membranes depending on the tannin structure. These findings show lipids interfere with tannin-saliva protein and tannin-taste receptor interactions involved in astringency and bitterness respectively. In addition to their role in taste, tannins as antioxidant molecules and in a larger extent polyphenolic compounds provided by foods are strongly suspected to have a positive role in many pathologies. Whereas their antioxidant properties have been widely demonstrated, their protective effect on membrane against lipid oxidation has been shown for the first time by NMR investigations. New insights into the location of tannins within the membrane have been proposed to explain their inhibitory effects on free radicals. Moreover, a synergistic effect has been evidenced proving the beneficial effect of food polyphenols as shown by epidemiological studies.

Keywords: Antioxidants, Astringency, Bitterness, Lipid oxidation, Membrane location, Molecular dynamics, NMR, Polyphenols, Synergistic effect, Tannin-lipid interactions, Tannin-saliva protein interactions, Taste, Wine tannins.

INTRODUCTION

Tannins are polyphenolic compounds largely distributed in the vegetal kingdom. They are found in large quantities in wood and plants. Tannins are also widespread in foods (*e.g.* fruits, vegetables, legumes, cereals) and beverages like wine and tea [1]. The present chapter concentrates on wine tannins and their polyphenolic subunits coming from grape berries. Grape tannins, also called condensed tannins, are polymers of flavan-3-ols or catechins (Fig. **1**). The basic structural units are two stereoisomers: (+)-catechin and (-) -epicatechin. The molecular diversity of tannins is due to i) the number and position of hydroxyl groups on aromatic rings, ii) the stereochemistry of C2 and C3 chiral carbons on the pyran ring, iii) the presence of a galloyl moiety on C3 position, and iv) the number and the nature of the interflavane bonds (C4-C8 and C4-C6) in oligomeric forms. In strong acid and aerobic medium, wine condensed tannins decompose into cyanidin, a red pigment, that is to why they are commonly known as procyanidins [2]. Tannins and their polyphenolic subunits present in grape skin

and seed are extracted and solubilized in wine during the maceration process. A large variety of species have been isolated and identified in red wine; four monomers: (+)-catechin, (+)-gallocatechin, (-)-epicatechin, (()-epigallocatechin, and (-)-epicatechin-3-O-gallate; 8 dimers (B_1, B_2, B_3, B_4, B_5, B_6, B_7, and B_8); some trimers (C_1, C_2, T_2) [2b, 3]. The presence of oligomers and polymers is highlighted by analytical methods such as liquid chromatography, nuclear magnetic resonance spectroscopy and mass spectroscopy [4]. However, their three-dimensional structure is up to now limited to trimers of catechin. Higher order polymers have not been resolved at atomic resolution [5]. The mean degree of polymerization (mDP) and the proportion of galloylated forms depend on the origin of tannins. Tannins from seeds have lower mDP than those found in grape skins (*ca.* 10 *vs.ca.* 30) but a higher proportion of galloylated oligomers (epicatechin gallate mostly) (*ca.* 30% compared to *ca.* 5% respectively) [6].

The total amount of condensed tannins depends primarily on the type of wine (red or white) but also on the variety, the terroir, and the wine-making process. In red wine, it typically ranges from 1-4 $g.L^{-1}$ with highest concentrations for monomeric catechins (+)-catechin and (-)-epicatechin, ca. 0.1-0.2 $g.L^{-1}$ and lower amounts for dimeric and trimeric procyanidins [7]. Unlike red wine, in white wine, quantities are much smaller (<0.5 $g.L^{-1}$) as it is prepared from the juice cleared of skins, seeds and stems.

A distinctive feature of condensed tannins and their polyphenolic subunits is their colloidal properties in aqueous solution [8]. Indeed, above a threshold concentration (critical micellar concentration, CMC), the molecules associate themselves to form nanometer-sized colloids, due to their self-assembly in aqueous medium. Below the CMC, molecules are in the free state, while above the CMC, they exist under the micellar form due to aggregation processes (Fig. **2**). The CMC values vary with their degree of polymerization (oligomers > monomers) and galloylation (dimers > galloylated dimers), as stated in (Table **1**). Considering reported CMC values, in most cases polyphenols are in the free state for concentrations found in wines (*ca.* 1.5-3 mM) [9], except for some monomers such as epicatechin.

CHAPTER 5

Applications of Quantitative ^{1}H NMR in Food-Related Analysis

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Abstract: Quantitative NMR (qNMR) is a powerful tool to quantify an analyte without the need for an identical standard, which is considered to be a primary ratio method. In particular, the use of ^{1}H NMR has been widely applied in the quantification of medicines, beverage components, and natural products in medicinal plant extracts because of its high sensitivity and the widespread presence of ^{1}H nuclei in organic molecules. Our group has previously reported a novel ^{1}H qNMR technique that is able to determine quantitative values of the analyte with metrological traceability to the International System of Units with a certified reference material as the internal standard (IS); this is called AQARI (accurate quantitative NMR with internal reference substance).

There are other advantages to qNMR using ^{1}H such as simple sample preparation, low sample consumption, and rapid, non-destructive measurement. Furthermore, it is widely recognized as a reliable technique due to the development of high-field magnets, and improvements in probe technology and gradient shimming techniques contributed to the enhancement of sensitivity, resolution, and precision. In Japan, qNMR using ^{1}H NMR has been set as an official method for assessing the purities of reference substances for pharmaceuticals and food additives.

In this review, we describe our recent studies regarding the applicability of qNMR using ^{1}H NMR for the purity assessment of organic compounds and the analysis of

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complex mixtures. The first half of the review introduces qNMR using ^{1}H NMR with IS, while the second half describes our recent purity assessments of commercial reagents for food analysis, and the quantification of several preservatives in processed foods by qNMR.

Keywords: AQARI, Acesulfame potassium, Acetamiprid, Carminic acid, Carthamin, Dichlorvos, Food additives, Flavonoid, Isoxathion oxon, Metrological traceability, Pesticides, Preservatives, Quantitative NMR, Steviol glycosides, Trichothecene mycotoxin.

INTRODUCTION

Nuclear magnetic resonance (NMR) can provide structural information about organic compounds from the chemical shift of the resonance signal on the spectrum as well as the signal multiplicity, spin-spin coupling patterns, and signal intensity (area). NMR is a non-destructive technique so is widely used to elucidate the structure of various organic compounds including natural compounds and chemical synthetics. NMR as a quantitative tool increased significantly after Jungnickel *et al.* [1] and Forbes *et al.* [2] reported the quantification of intra-molecular proton ratios in 26 pure organic compounds and commercial analgesic preparations. Quantitative NMR (qNMR) is regarded as a practical and effective tool for the quantification of organic components because of the improvements in the probes and the use of NMR spectrometer with high-field magnet, gradient shimming technique, and the establishment of accurate and precise data-processing and data-method [3]. In particular, qNMR using ^{1}H NMR has been used to study crude samples such as metabolites in urine or serum [4, 5], naturally occurring compounds in medicinal plants [6, 7], and organic compounds in pharmaceutical samples [8] and beverages [9 - 15]. The ^{1}H nucleus is superior to other NMR-active nuclei such as ^{13}C, ^{15}N, and ^{31}P in terms of sensitivity and general applicability.

In this review, we describe the acquisition principles and characteristics of qNMR using ^{1}H NMR. In addition, we describe recent studies that have used qNMR to determine the purity or content of commercial reagents, natural organic compounds, and food additives.

QUANTITATIVE NMR (qNMR)

NMR is a widely known method for determining the structure of organic compounds: because the atoms (nuclear spins) that comprise the molecules are measured directly, NMR also provides quantitative information. The quantitative potential of NMR has been recognized for a long time, since a report in 1963 on the application of NMR to determine relative ratios of specific components in pharmaceutical products [2]. The authors demonstrated the utility of NMR in comparison with other analytic methods (infrared and extraction methods). There are two applications for qNMR: it can be used to determine either the proportion or the absolute concentration of a target analyte in a sample. In this review, we will mainly focus on the latter application, assessment of the concentration of a target analyte. In addition, although a variety of nuclei such as ^{1}H, ^{13}C, ^{19}F, and ^{31}P can be used in qNMR, we will only be addressing the widely utilized ^{1}H nucleus.

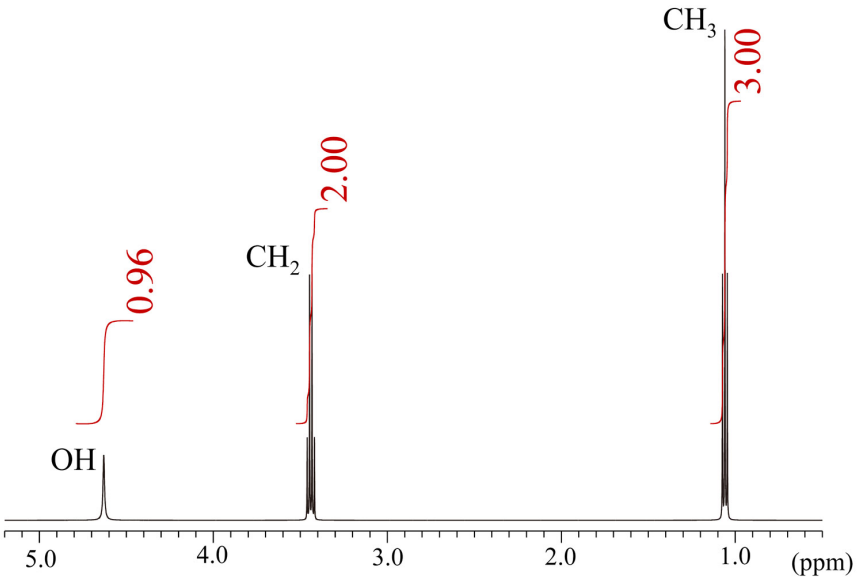

Fig. (1). NMR spectrum of ethanol.

Principles of qNMR

The basic principle of qNMR is described by formula (1). Generally, the integrated signal area (*I*) observed on the NMR spectrum is directly proportional

CHAPTER 6

Cell-Free Protein Synthesis for NMR Structural Analysis of Large Proteins and Complexes

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Abstract: Cell-free protein expression systems offer many advantages over cell-based approaches for the expression and isotope labeling of proteins for NMR analysis. Cell-free systems allow for the rapid single day production of target proteins at milligram production levels with inexpensive isotope enrichment. The open nature of these systems also allows the addition of molecules that can aid the folding and stabilization of target proteins. In this chapter we briefly discuss the available cell-free expression systems and whether they can be used for the isotope-labeling of a target protein, and how new PCR-directed cell-free expression approaches can aid the rapid identification of expression constructs with enhanced yield and solubility. We then focus on recent advances in the cell–free production of proteins for NMR structural analysis of large proteins and macromolecular complexes (including membrane proteins). The NMR spectra of such molecules are often problematic to assign because of their large number of cross peaks and line broadening resulting in loss in signal resolution and intensity. A range of selective, combinatorial and segmental isotope labeling strategies based on cell-free protein synthesis are now available to enable residue-specific and sequence-specific assignment of NMR spectra. Cell-free deuteration of target proteins can reduce line broadening issues, and stereo-array isotope labeled (SAIL) amino acids can be incorporated to provide NOE constraints for structure determination. Cell-free protein synthesis also allows the incorporation of unnatural amino acids which can act as NMR probes to provide long distance information.

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Keywords: Cell-free protein synthesis, Deuteration, Isotope labeling, Macromolecular complex, Membrane protein, NMR spectroscopy, SAIL, Unnatural amino acid.

INTRODUCTION

Despite the development of ^{13}C and ^{15}N stable-isotope labeling strategies and multidimen-sional (2D-4D) NMR approaches, large proteins and macromolecular complexes (> than 30 kDa) are still challenging prospects for NMR structural analysis. This is for two reasons, firstly the preparation of appropriately isotope-labeled proteins and complexes that are stable enough for NMR analysis can be a major bottleneck. Secondly, such molecules exhibit slower tumbling rates that enhance transverse relaxation causing increased line widths and reduced signal to noise [1]. This coupled with their associated large number of NMR signals results in spectral overlap and reduced signal intensity making it very difficult to assign their NMR spectra. However, advances in NMR techniques such as transverse relaxation-optimized spectroscopy (TROSY) triple resonance experiments, sparse labeling, long range measurements, and deuteration are making large proteins a more tractable proposition [2 - 4]. Also, cell-free protein expression approaches offer significant advantages over cell-based approaches for the preparation of large proteins for NMR analysis and can provide innovative solutions to help reduce spectral overlap and improve signal intensity.

Cell-free expression systems rely on cell extracts to provide the necessary components for translation (ribosomes, amino acetyl-tRNA synthetases, translation factors), protein folding (chaperones, disulphide isomerases), and energy metabolism. These extracts are supplemented with molecular building blocks (NTPs for mRNA synthesis, amino acids for protein production), salts, energy substrates (cAMP, ATP, GTP) and energy regen-eration systems (high energy phosphate donors with appropriate kinases) to drive protein synthesis from a DNA or RNA expression template [5]. The continuous supply of substrates and removal of inhibitory by-products using continuous exchange cell-free (CECF) approaches have enabled cell-free reactions to be prolonged for many hours resulting in the production of target proteins at the milligram per millilitre of reaction scale [6].

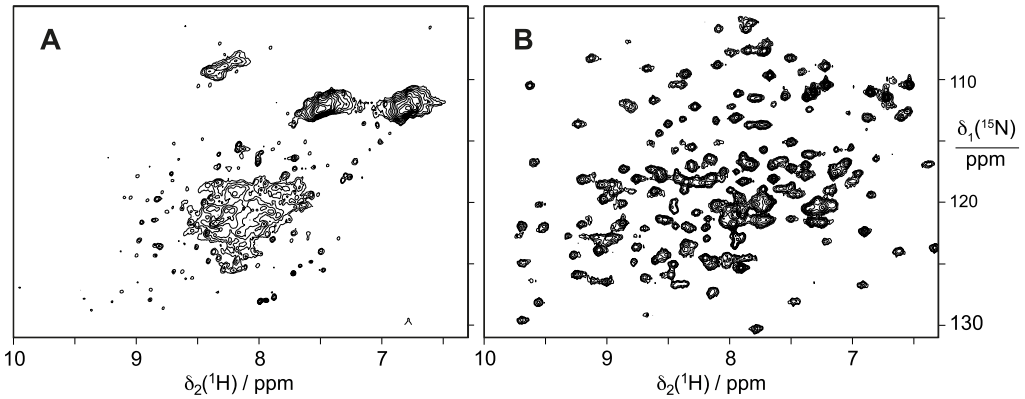

Fig. (1). Correctly folded ^{15}N- labeled α270 fragment of *E. coli* DNA polymerase III is readily obtained by cell-free protein synthesis but not by *in vivo* expression. ^{1}H-^{15}N HSQC spectra of (**A**) ^{15}N- α270 fragment synthesised *in vivo* in *E. coli*. (**B**) ^{15}N- α270 fragment synthesized in an *E. coli* cell-free protein expression system. Figure taken from Ozawa *et al.* (2013) (9), reproduced by permission of Oxford Journals.

In comparison to cell-based and chemical approaches, target proteins are more likely to fold correctly as cell-free expression has slower rates of synthesis due to the translational machinery being more dilute than in the cell [7], as a result any nascent polypeptide being synthesized has a greater probability of folding correctly as opposed to aggregating [8]. On the other hand, chemical synthesis is limited to peptides <40 amino acids in length on a scale suitable for NMR analysis [7], and cell-based approaches often suffer from protein degradation, aggregation and loss of template DNA. An example of the advantage of cell-free expression is shown in Fig. (**1**), which shows a ^{1}H-^{15}N HSQC spectra of the ^{15}N-labeled the N-terminal 270 residue fragment (α270) from the α subunit of *E. coli* DNA polymerase III (Pol III) [9]. These ^{1}H-^{15}N HSQC spectra of (A) ^{15}N-labeled α270 made *in vivo* and (B) ^{15}N-labeled α270 made by cell-free expression clearly indicate that properly folded α270 is readily obtained by cell-free protein synthesis but not by *in vivo* expr-ession. Cell-free systems are non-living which means they can express cytotoxic proteins such as antimicrobial and membrane proteins [10 - 12]. Cell-free systems also provide greater flexibility for facile selective and uniform ^{13}C/^{15}N isotope labeling, this can be achieved easily by replacing the amino acids in the reaction mix with their isotope-labeled equivalents [13]. These amino acids can be purchased individually or as labeled

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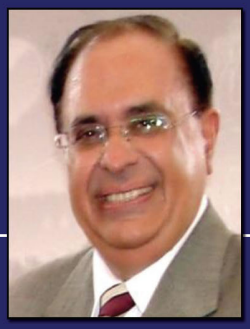

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Atta-ur-Rahman, Ph.D. in organic chemistry from Cambridge University (1968), has 1020 international publications in several fields of organic chemistry including 727 research publications, 37 international patents, 68 chapters in books and 188 books published largely by major U.S. and European presses. He is the Editor-in-Chief of eight European Chemistry journals. He is Editor of the world's leading encyclopedic series of volumes on natural products "Studies in Natural Product Chemistry" 48 volumes of which have been published under his Editorship by Elsevier during the last two decades.

Prof. Rahman won the UNESCO Science Prize (1999) and was elected as Fellow of the prestigious Royal Society (London) in July 2006. He has been conferred honorary doctorate degrees by many universities including (Sc.D.) by the Cambridge University (UK) (1987). He was elected Honorary Life Fellow of Kings College, Cambridge University, UK, conferred the TWAS (Italy) Prize and the Austrian government has honoured him with its high civil award ("Grosse Goldene Ehrenzeischen am Bande") (2007). He is Foreign Fellow of Chinese and Korean Academy of Sciences, Foreign Fellow of the Chinese Chemical Society and former President of Pakistan Academy of Sciences.

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