Chapter 7
Nuclear Magnetic Resonance Spectroscopy in Food Analysis

Ghulam Mustafa Kamal, Jalal Uddin, Muhammad Suleman Tahir, Muhammad Khalid, Sarfraz Ahmad, and Abdullah Ijaz Hussain

Abstract Nuclear magnetic resonance (NMR) spectroscopy is a powerful spectroscopic technique, conventionally used for structure verification, elucidation, and purity analysis in chemistry. NMR is a fast, reproducible, and nondestructive technique to provide detailed information of the compounds with little or no treatment required in the food samples. This makes NMR a suitable technique for the food analysis and is still a relatively underutilized methodology in the area of food science. It is due to its high cost, relatively low sensitivity, and required skills. The aim of this chapter is to explain NMR methodologies in the field of food analysis. This chapter covers the basic principles of NMR and its methodologies followed by their applications in food quality control and authentication (i.e., discrimination of foods based on different raw materials and its origin). In addition, a description of the chemometrics is provided considering combined NMR and multivariate statistical analysis. This combination is a powerful approach for addressing modern challenges in food quality control and authentication. In addition to standard NMR tech-

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niques, sophisticated NMR applications especially in hyphenation with other techniques are presented.

Keywords NMR spectroscopy · Hyphenated techniques · Metabolomics · Chemometrics · Data analysis · Statistical analysis · Food analysis · Food adulteration · Nutrition · Food fraud

1 Introduction

In 1946, two groups of scientists while working independently at Harvard University (Purcell, Pound, and Torrey) and Stanford University (Hansen, Bloch, and Packard) observed signals of proton resonance from paraffin water and wax, respectively [1, 2]. Bloch and Purcell were mutually awarded with the Noble Prize in Physics in 1952 for their invention. In 1950, when low-resolution NMR determined the moisture in food and milk analysis, it was the first time when NMR was used in the food science [3]. Due to inadequacy in instrumentation and convolution in food matrices, the application of NMR in food science began in the 1980s. Since then, it is being used effectively and systematically employed in food analysis and authentication. This usage has seen a dramatic increase in research and review articles involving NMR applications in food science. The popularity of NMR application in food science is evident from Fig. 7.1, where Pubmed results show data of last 5 years (2016 to 21 May 2020).

In domestic and international conferences, various oral and written communications have also been proposed. Every two years, in Europe, an International Conference on the applications of NMR in foodstuff is organized. The conference

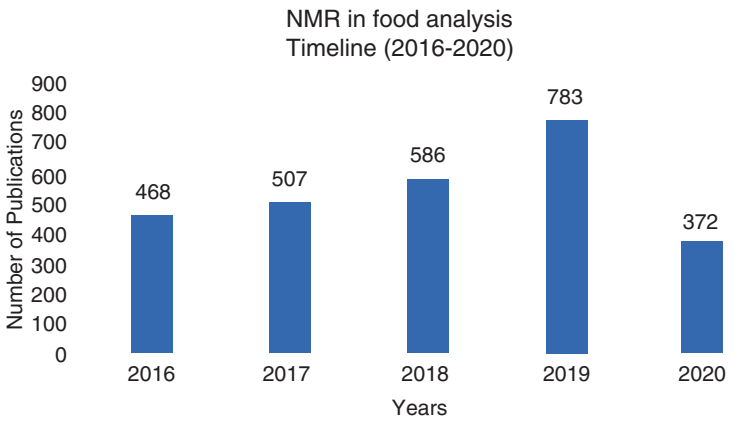

Fig. 7.1 PubMed search result of the number of publications with "NMR in food analysis" as a keyword

commenced in 1992 provided the opportunity for scientists from all over the world to propose new applications of NMR in food science and technology. European Union has approved NMR methods as official methods (e.g., determination of wine fraud) [4]. This development comprises of several reasons: (a) availability of the increased smoothness and user-friendly NMR instrumentation; (b) increased requirements of the industry to comprehend and formulate its products and processes; and (c) needs of efficient analytical techniques for the quality control and authentication of foods [5, 6] and thereby augmentation of the apposite constitution. Foods are very complicated and exceedingly heterogeneous matrix that contain a very great number of chemical compounds, whose constituents fluctuate significantly under specific conditions (e.g., slaughter or agronomical practices, industrial processes, storage, and maturation) [7]. Therefore, NMR could play a significant role in analyzing and characterizing foods.

2 Basic Principle and Theory of NMR

NMR spectroscopy is a technique based on the magnetic properties of certain nuclei. The ^{1}H-NMR and ^{13}C-NMR spectroscopy are important applications related to food analysis. The nuclei of certain atoms are considered to have a spin. These spinning nuclei possess a spin angular momentum (I) and a magnetic moment (μ). For each nucleus with spin, the number of allowed spins is quantized and can be determined by its I value by the formula "$2I + 1$" with integral differences ranges from $+I$ to $-I$. The nuclei possess an odd mass number, I becomes half-integer values (+1/2), whereas nuclei with an even mass number but odd atomic number I take integer values such as ^{13}C isotope. These spinning nuclei have $I \neq 0$, generate a magnetic moment called μ along the axis of spin, and act like a tiny magnetic bar. The nuclei have $I = 0$ are not NMR active nuclei, i.e., ^{12}C isotope. The magnitude of μ is directly proportional to (S) and gyromagnetic ratio (γ), which is a natural property of any nuclei and determines its magnetic strength ($\mu = \gamma S$). The nuclear magnetic moment, μ, interacts with the external magnetic field (B_0) generated by the magnets of NMR for understudied nuclei. For the nuclei like proton (^{1}H), magnetic moments show two alignments in the presence of applied B_0: either in the same direction (+1/2) or in opposite direction (−1/2) of the external magnetic field (Fig. 7.2). The aligned nuclei are more stable and of lower energy than nonaligned nuclei. In the presence of applied external magnetic field, there is a transfer of energy from original state to higher energy state. The wavelength at which it corresponds to the radio frequencies transfer of energy takes place and when the spin returns to its ground level, there is the emission of energy at the same frequency. This frequency is measured in many ways and processed to produce NMR spectra of corresponding nuclei [8].

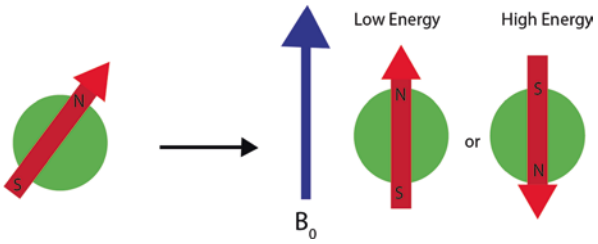

Fig. 7.2 Basic theory and principle of NMR [9]

2.1 Parameters of NMR

NMR gives valuable structural information of the compounds, which helps in quantitative and qualitative analysis. The valuable results are obtained from parameters like chemical shift, spin-spin coupling, and signal intensity.

2.1.1 Chemical Shift

Chemical shift (δ) is a plot of signal intensity (y-axis) versus frequency (x-axis) in one-dimensional (1D) NMR spectrum and expressed as ppm (part per millions). Universally, tetramethylsilane (TMS) or trimethylsilylpropanoic acid (TSP) is used as an internal standard because their protons are more shielded, and it is marked one end of the range to set the spectrum at 0 ppm. Chemical shift provides important information about the chemical environment of a nucleus in any molecule and assists in locating the signal in the NMR spectrum. Since in a compound not all nuclei may have the same chemical environment and therefore, they do not resonate at the same frequency. Moreover, chemical shift is proportional to the applied magnetic field, and nuclei absorb energy at various resonance frequencies due to the differences in the electron density among various nuclei. Nuclei with higher electron density around them have higher opposing magnetic field B_0 from electron and tend to resonate in lower chemical shifts (up field) and the nuclei are shielded when electron density around a nucleus decreases such as presence of electromagnetic element in the vicinity. The shielded proton experiences more B_0 and resonates at higher chemical shift (downfield). Chemical shift provides valuable information about the chemical environment and functional group of a molecule [9].

Figure 7.3 shows a schematic diagram identifying major components of an NMR system. Under the influence of external magnetic field, electrons around a proton produce their own magnetic field. This provides the shield to the proton. Thus, some protons are more shielded and some are less due to the different number of electrons around them. Due to this magnetic field of electrons, the influence on external magnetic field may increase or decrease on the nucleus. Thus, nuclei resonate at different frequencies due to the difference in their chemical environment. Shielding and de-shielding cause a shift to the frequency of the absorption of proton either left or

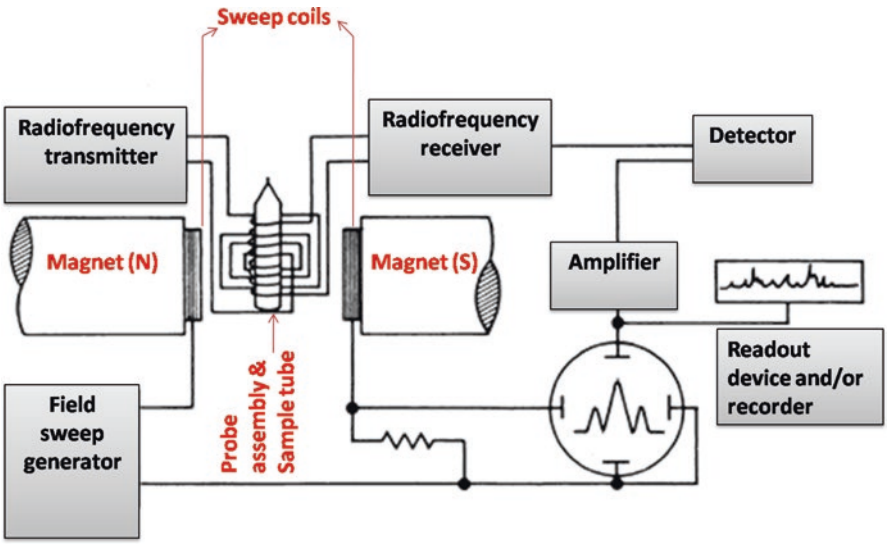

Fig. 7.3 Block diagram of NMR spectrometer [9]

right when compared to the position of the bare proton. This shift is called chemical shift. The resonance signals could overlap if two electrons present are in the same chemical environment and hence experience different chemical shift [10].

2.1.2 Spin–Spin Coupling

Spin–spin coupling is also called *J* coupling and responsible for the signal splitting in the NMR spectra. It is primarily arising from the interaction between neighboring spins mediated through the bonding electrons. It is very important for structural characterization, as its magnitude depends on the distance and relative orientation between the nuclei; however, *J* coupling further increases the complexity of the NMR spectra. The *J* coupling is measured in Hertz (Hz), and it can be either positive or negative and does not depend on the strength of the external magnetic field. The spin–spin splitting pattern can be explained empirically with $n + 1$ for spin ½ nuclei, whereas $2nI + 1$ rule is generally used for nuclei with integer values, where n represents the number of protons in neighbors and I is the nuclear spin quantum number [10, 13].

This splitting gives the information about the number of protons coupled, and its *J* value gives information about the relative orientation of adjacent C–H bonds. For example, ethyl iodide (CH_3–CH_2–I) is considered as a two spin system, where methylene proton after splitting appears as a quartet signal and methyl proton appears as a triplet in NMR spectrum. If a proton has no adjacent proton, then singlet appears in spectra that show no coupling [11].

2.1.3 Signal Intensity

The signal intensity is generally the area taken by peak in the NMR spectrum, and it is measured by digital integration. The intensity of an NMR signal is directly proportional to the number of equivalent nuclei in the sample, and it is used for spin multiplicity and quantitative analysis [12].

2.2 NMR Spectrometer

There are two basic types of NMR spectrometer currently in practice: the continuous waves (CW) and Fourier Transform (FT). In CW instruments, the magnetic field continuously varied from downfield (left) to the up field (right) end of the NMR spectrum for scanning. In this type of NMR spectrometer, one type of isotope nuclei under observation can be excited at a time. Another approach commonly used in modern sophisticated instruments is to use a short powerful burst of energy called a pulse that excites all the magnetic nuclei in the molecule at the same time. Since the molecule contains many different nuclei, many different frequencies of electromagnetic radiation are emitted simultaneously. This emission is called free-induction decay (FID)**.** FID is a time-domain signal of individual frequencies of different nuclei, which later converts into frequency domain signal using a computer and a mathematical method called as Fourier Transform (FT). For example, in an organic molecule, all the ^{1}H nuclei are induced to undergo resonance simultaneously, and at the same time, the pulsed FT instrument has several advantages compared to the CW instrument. For instance, it is more sensitive and can measure weaker signals. FT-NMR instrument are more sophisticated than CW instrument and require more complex program for signal detection. Before 1970, all experiments of NMR were taken by CW-NMR technique [13].

2.2.1 Magnet

The magnet of NMR is the most expensive part of the instrument, and it is considered as the main component. It is also called the heart of NMR instrument. In early spectrometers, permanent as well as electromagnets were used. They can produce a magnetic field of up to 2.3 T. Today, superconducting magnets are used which give high resolution in NMR experiments, and their field strength ranges from 6 to 23.5 T. In NMR spectrometer, active magnet shielding is present to avoid the effect of stray magnetic field surrounding the magnet [13, 14].

2.2.2 Shim Coils

In the high-resolution NMR spectroscopy, the homogeneity of magnetic field should be better than 1 ppb over the sample. Shims coils are used to narrow the spectral width and to remove the inhomogeneity, which is necessary for good quality spectral images. In most of the spectrometers, shim coil can be managed by computer. The computer finds out the best shim value using an appropriate algorithm.

2.2.3 Field Lock

After obtaining the required homogeneity using shim coils, stability of the homogeneous field is achieved by a field lock. The field strength varies over time due to temperature fluctuations and aging of the magnet. In a field lock deuterium resonance, the position is measured by spectrometer. Lock transmitter frequency is swept about 1 MHz as it searches the resonance frequency. When it detects resonance response, it stores these values and searches for another resonance response in other direction. When it detects response, it stores this frequency and jumps to midpoint of the stored values. It locks this frequency of midpoint as the resonance frequency of deuterium.

2.2.4 Probe

The probe is a core element in the NMR instrument, and it provides the interface for Radio frequency (RF), magnetic field, and samples. The sample is inserted into the probe and placed inside the magnet field, where it excites the nuclei and detects the NMR signal. RF coils are divided into three categories: (a) transmit and receive coils, (b) receive only coils, and (c) transmit only coils. In modern spectrometers, both transmit and receive coils are present. They act as transmitter of the magnetic field and receiver of RF energy from relaxed nuclei. Different types of probes like dual probe, broadband probe, triple resonance probe, inverse probe, cryogenic probe, micro coil probe, flow probe, solid probes, and MRI probes are used according to the type to experiment to be conducted [14].

2.2.5 Console

In spectrometer, a console is present next to a magnet, which supports the recording of the NMR spectrum. It provides three channels for radiofrequency that include observe, lock, and decoupling channels. These frequencies are controlled, amplified, pulsed, and transmitted to the probe head. In spectrometer, signals are amplified and then mixed, and NMR signal is attained using quadrupole phase detection. The two signal components are digitized in analog to digital converter (ADC) and fed into the computer memory [15].

3 1D NMR

1D NMR spectroscopy involves ordinary (^{1}H) Proton, ^{13}Carbon, and spectra of other cores. 1D NMR spectrum consists of two dimensions: The frequency axis corresponds to the x-axis (the chemical shifts) and the intensity corresponds to the y-axis (Fig. 7.4). Each one-dimensional NMR experiment consists of two portions: preparation and detection. While preparing, the spin system is fixed to a specific condition. The subsequent signal is recorded during detection. In the least complex case, the preparation is a 90° pulse (in our model connected along the x pivot) that turns the equilibrium charge Mz onto the y pivot (My). Each spin continues with its own Larmor frequency around the z-axis and generates a signal in the collector coil after this pulse. The signal decays because of T_2 relaxation and is named as free induction decay (FID).

As a rule, the analysis is directed ordinarily and the information is recorded to amplify the signal to noise ratio. After summation, the information is Fourier Transformed to give the last 1D spectrum. To terminate the signals of large molecules, one-dimensional methods including simple pulse-acquired 1-dimensional 1-Proton experiment and Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence are employed. This method is also utilized to identify hetero-nuclei, like ^{13}C and ^{31}P [16].

A lot of structural and quantitative information in the form of parameters like signal intensities, coupling constants, and chemical shifts can be achieved by utilizing one-dimensional solid or liquid-state NMR spectroscopy of high resolution. Researchers can choose different nuclei, such as H^{1}, C^{13}, P^{31}, and F^{19},for the same sample to reveal the most important nuclei that can allow to study food samples under different prospects and gather the maximum information. Sample pretreatment and separation of many food components are not required [17].

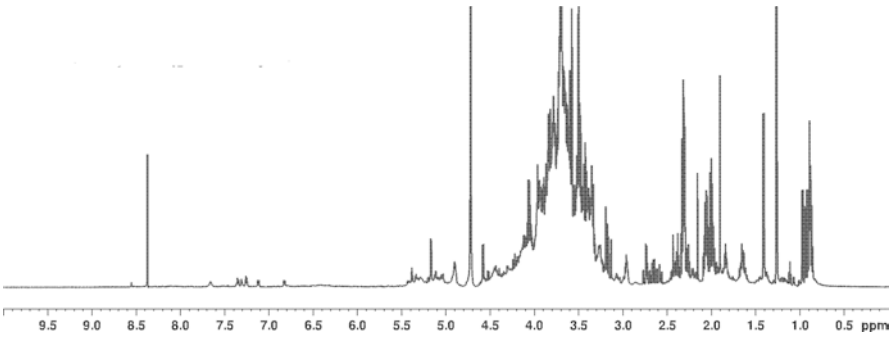

Fig. 7.4 A representative 1D NMR spectrum [9]

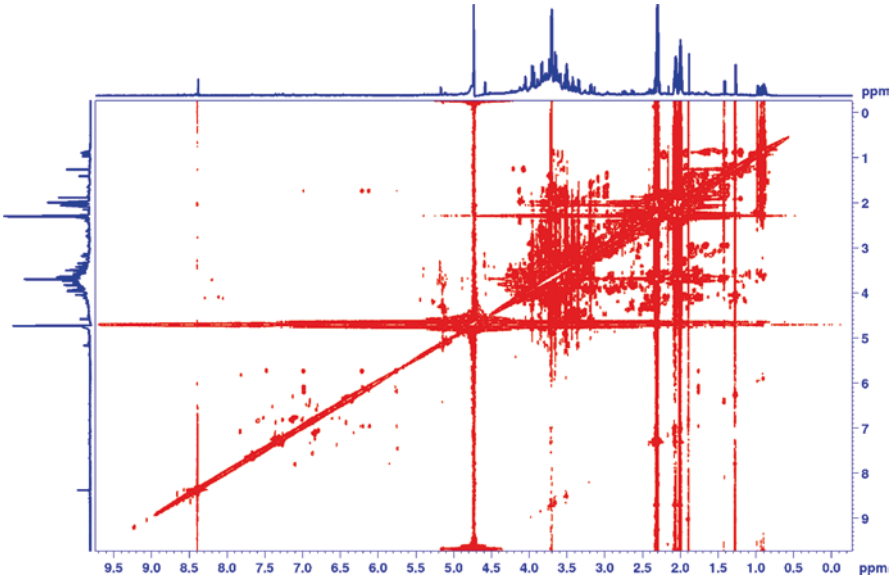

Fig. 7.5 A representative 2D ^{1}H–^{1}H TOCSY NMR spectrum [9]

4 2D NMR

A two-dimensional nuclear resonance spectrum consists of two frequency axes. Intensities represent the third axis and are commonly shown as contour plots (i.e., similar to the demonstration as utilized in geographical maps). The horizontal axis is termed as F2 (direct dimension) and the vertical axis as F1 (indirect dimension) as shown in the typical 2D NMR spectrum in Fig. 7.5. If both dimensions consist of chemical shifts, the experiment is termed as shift-correlated 2D-NMR, if one dimension indicates scalar couplings, the spectra are termed as *J* resolved.

With the assistance of 2D NMR spectroscopy, multipulse groupings are used to give extra data which are otherwise not achievable from 1D spectrum. The extensively employed types of 2D NMR spectroscopy give the relation between proton signals based on them, extensively indirect dipole-dipole coupling (C–C, H–H, or H–C), dipolar coupling like Nuclear Overhauser Effect (NOE interaction), and relative diffusion rates like diffusion ordered spectroscopy (DOSY).

4.1 COSY

COSY (correlation spectroscopy) is the first method of 2D NMR described by Jeener in 1971. It is two-pulse sequence that consists of two 90° pulses. Correlation spectroscopy enables association of all coupled protons (H^1–H^1 correlation) and

three bond H–H coupling that provides undeniable proof of proton assignments. COSY is helpful when peaks overlap in H^1 NMR and when many similar coupling constants are present. COSY also has a number of limitations. First due to polarization transfer between two spins, it cannot be used for all metabolites simultaneously. Second, it cannot provide 3D spatial localization. Third, it is not capable of identifying coupling constants [18].

4.2 HMBC

Heteronuclear multiple bond coherence (HMB) allows the correlation of protons and attached carbons over a long range. The spectra of HMBC can be used to determine the long-range H-C coupling constant. It determines association over 2 and 3 bonds. It is more sensitive than direct detection methods and provides information about the position of quaternary carbon. It gives useful information about the structure of molecule, but it is very complex [19].

4.3 NOESY

Nuclear Overhauser Effect Correlation Spectroscopy (NOESY) spectra provide us information about the protons that are close in space but not connected by chemical bonds. It uses the trough space interaction. It tells about the configuration of molecules. By NOESY, it is possible to find out the space relationship between all protons of a molecule. The disadvantage of this method is that it is time-consuming because all protons have to be radiated one by one. It also creates problems for protons which have very close chemical shifts [19].

4.4 TOCSY

Total correlation spectroscopy (TOCSY) yields homonuclear proton correlation and is based on scalar coupling between protons. It transfers magnetization of spin A on to spins B, C, etc. in a chain by relaying coherence from one proton to another. Principally, it can correlate all the protons present in a spin system. TOCSY spectrum shows all possible correlations even between spins that are not directly coupled. Another important experiment homonuclear Hartmann-Hahn (HOHAHA) spectroscopy (used in solid-state NMR spectroscopy) is identical to TOCSY because both have similarity in cross-polarization (transfer of polarization from proton to carbon nuclei). Therefore, TOCSY is also referred as HOHAHA [20].

4.5 HSQC

Heteronuclear single quantum coherence or heteronuclear single quantum correlation (HSQC) is the NMR experiment that generates a signal of NMR active nuclei that are bonded together [21]. It is employed for tallying chemical shift from proton (showed in F-2 pivot) to ^{13}C (in "roundabout," F-1 pivot) and about their straightforward connected carbons through $^{1}J_{CH}$ coupling. Multiplicity-edited HSQC usually used for the discrimination between carbons comprising of an odd (CH or CH_3) or even (CH_2) hydrogen's number [22].

5 NMR Hyphenated Techniques

The coupling of separation methods and NMR spectroscopy was proposed in the late 1970s when the implication of NMR in organic chemistry had already become extensive [23]. In the hyphenated technique, two different analytical techniques are combined by a proper interface. Mainly spectroscopic techniques are combined with chromatographic techniques. Hirsch Feld in 1980 precisely proposed the coupling of at least two instrumental analytical methods in a single output. For the coupling, the main reason is to attain data-rich and powerful information using a single analytical strategy [24]. The advantages of this system are: (a) it is rapid, (b) more prominent level of robotization, (c) larger sample throughput, (d) high reproducibility, and (e) it decreases contamination because of closed system [25].

5.1 LC-NMR

For coupling of NMR with liquid chromatography (LC) methods, optimization of chromatographic steps is needed to increase analyte concentration in the detection cell. High column loading and narrow elution are needed in order to maximize analyte concentration and tally the NMR detection [26]. One can perform liquid chromatography–nuclear magnetic resonance (LC-NMR) experiments in continuous-flow as well as in stop-flow manner. Other than NMR and HPLC instrumentation, the real need for online LC-NMR is constant-flow probe. To achieve NMR spectra of consistent or ceased-flow, a valve before the probe is inaugurated [27]. For liquid chromatography operation, UV-vis is also employed as a primary detector. In LC-NMR coupling, magnetic field strength of more than 9.4 T is suggested [28].

5.2 LC-NMR-MS

Liquid chromatography–nuclear magnetic resonance–mass spectroscopy (LC-NMR-MS) has been applied to detect the organic compounds in various fields, including natural products, drug metabolism, drug discovery and drug development, combinatorial chemistry, impurity profiling, metabolomics, and metabonomics [29]. The wide range of applications of LC-NMR-MS has been assigned as another optional technique used in academic and industrial laboratories, especially in the food analysis fields [30].

On-flow and stop-flow are the commonly available interfaces for LC-MS-NMR. With stop-flow, terminating the flow on the desired chromatographic peak being detected by NMR can be influenced by the MS instrument. In the loop collection mode, for conditions when the UV detector is not the most suitable. This is because of a deficiency of chromophores in the sample, and MS of the LC-MS-NMR system may also use to record the catching of the chromatographic peak inside the loop [31].

6 NMR Data Analyses of Foods

Foodstuff is considered as a complex mixture, comprising a wide range of metabolites produced by the metabolism of plants, bacteria, and animals. These metabolites have different chemical properties and concentrations. This complexity and the variability of food's metabolism, structure, and composition are evident in their NMR spectra. These NMR spectra comprise a vast amount of information that is not easy to extract and interpret by traditional univariate statistical methods, where the focus is on one variable at a time. Moreover, signal overlap often occurs in this type of NMR spectra, and it hindered the identification and/or quantification of the compounds of interest. It increases the uncertainty in the selection of molecules that are linked to certain food properties [13]. The combination of NMR spectroscopy with multivariate statistical methods is the most efficient way of analyzing the complex NMR spectral data of food samples. NMR spectra of a large number of samples could be used for statistical pattern recognition techniques (supervised or unsupervised) for determining spectral fingerprints. This method usually in combination with multivariate statistics and chemometrics approach to identify and quantify the metabolites in any system is termed as metabolomics [32]. Alternatively, this approach is also known as NMR-based food metabolomics (foodomics). Metabolomics does not require the determination of individual signals in the spectrum but follows to explore the precise spectral features that can distinguish and clearly indicate the existence of useful biomarkers or metabolites in the foodstuff [33].

6.1 NMR-Based Metabolomics

Every technique has its own advantage and disadvantage which results in different levels of authentication of metabolomics data. Therefore, the choice of well-suited instrumentation is very important to address a specific question in metabolomic analysis. Many factors are involved in the proper selection of instrumentation, including the availability of these analytical platforms and the possibility to combine different techniques in order to obtain a comprehensive metabolic profile. NMR is a powerful spectroscopic methodology, traditionally used for structure verification, elucidation, and purity analysis in chemistry [34]. NMR is a robust and reliable technique for metabolomic applications in which high reproducibility is paramount [35]. It offers rapid detection of structurally diverse metabolites at the same time and provides a metabolic "snapshot" of the sample [36]. There were some issues in the sensitivity of NMR which now have been efficiently overcome by advancements in NMR spectrometer hardware including the use of cryoprobes, cold-probes, or by using high field instruments, i.e., 800 MHz. Use of high-field instruments improves both the sensitivity and the resolution, but cost of the instrumentation is still an issue [37].

Depending on the aim and the information required to be generated from the experiment, we can use NMR-based metabolomics in two different ways. If the analyst is interested in a limited set of certain metabolites, the approach of choice is usually known as "targeted metabolomics," whereas in cases where the interest of analyst is to examine as many metabolites as possible, then it is known as "untargeted" approach [32]. Figure 7.6 shows a typical NMR-based metabolomics workflow in food analysis.

6.1.1 NMR-Based Targeted Metabolomics

NMR-based targeted metabolomics is used for the quantitative analysis of a few to several selected known metabolites. This involves the ability to differentiate the targeted metabolites from other intrusive metabolites, which may be achieved based on the chemical shift in an NMR spectrum [38]. Conventionally, quantitative analysis by NMR has been restricted to relatively simple mixtures with minimal peak overlap. This can be achieved by 1D ^{1}H-NMR because its peaks scale is linear with the concentration of the analytes in NMR spectrum. This makes NMR a natural choice for targeted metabolomics due to its analytical precision which usually does not depend on the chemical properties of target molecules [39, 40].

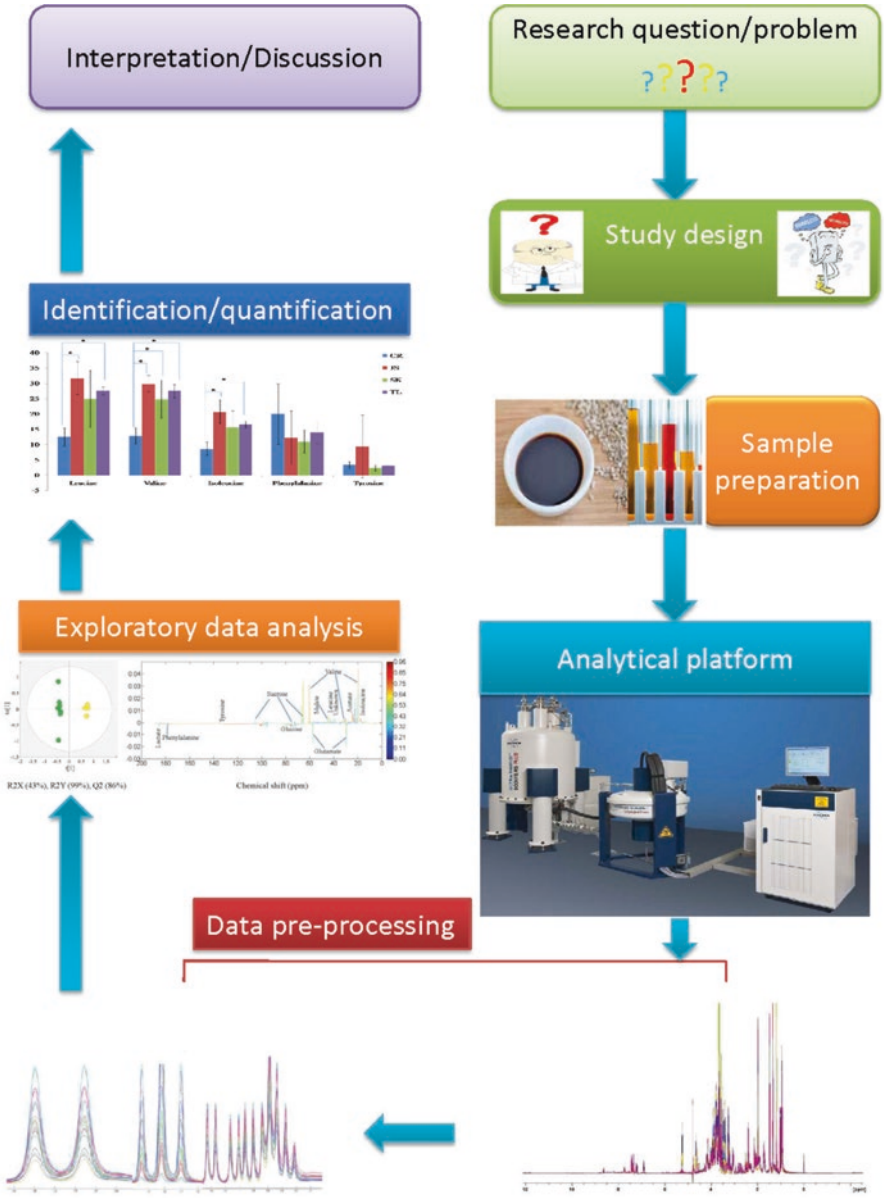

Fig. 7.6 NMR-based metabolomics workflow for application in food analysis [9]

6.1.2 NMR-Based Untargeted Metabolomics

The comprehensive analysis of all the quantifiable metabolites in a biological or food sample is known as the untargeted metabolomics. This approach is unbiased and thus makes it global in scope and sometimes is also termed as global metabolite profiling. Due to the comprehensive nature of untargeted metabolomics approach, it must be coupled to advanced chemometric techniques, such as multivariate statistical analysis which is useful in reducing the large datasets into a smaller set of manageable variables [40]. Several solvents and extraction methods should be applied and compared between the groups of samples because the nature of many compounds of interest in untargeted metabolomics is unknown [41].

6.2 Chemometrics in NMR

Svante Wold was the man who first introduced chemometrics in 1972. Isenhour and Jurs further contributed to its progress when they published the first pattern recognition article in 1972. However, the first real article on chemometrics was published by Kowalski, Massart, and Wold [42]. Chemometrics is a rather new technique, but it has a huge impact on the processing of spectroscopic data. An extended chemometric software is nowadays an essential part of a laboratory and process instrumentation. Chemometrics methods are still under development, and the definition may need to be modified from time to time in order to include all future developments [43]. In the 1980s, John explained the use of chemometrics (multivariate data analysis) coupled with NMR spectroscopy. Later on, further advancement was made by Nicholson and his group in the field of NMR-based metabolomics [44]. Chemometrics technique is commonly a complement to the analytical methods [45]. The simplest definition of chemometrics employs the use of mathematical and statistical methods to select optimal methodology and experiments to determine chemical information by scrutinizing the data [46]. The objectives of chemometrics at scrutinizing the NMR spectra in food matrix is threefold: (a) to classify and differentiate among groups of food samples, for example as a function of geographical origin or botanical origin (e.g., olive oil, honey, and wine); (b) to examine the relation between physiochemical properties and composition; and (c) to establish calibration prediction models to determine unfamiliar samples and additionally control the nourishment processes [47].

Chemometrics works with NMR data in two ways: (a) targeted or metabolic profiling and (b) chemometrics approach or metabolic fingerprinting [48]. Targeted analysis needs very little data for pretreatment, while metabolic fingerprinting needs a large amount of data, and chemometrics needs to be employed to draw useful information [49]. Figure 7.7 shows a workflow of chemometrics for NMR spectral data. One of the important approaches in chemometric-based NMR data analysis is targeted metabolic profiling, where all metabolites of interest in the NMR spectra are targeted and defined, followed by integrating the corresponding signals [40].

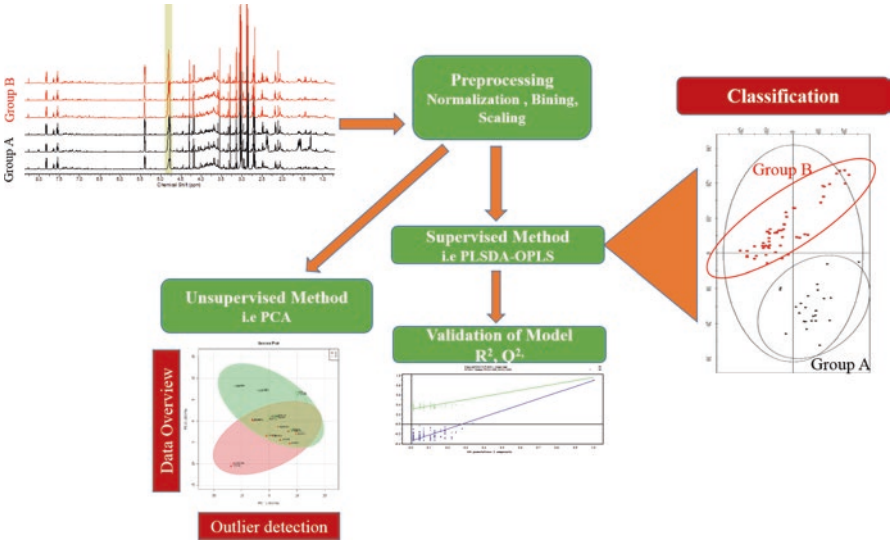

Fig. 7.7 A typical chemometric workflow in an NMR-based metabolomic approach [51]

The signal intensities or the calculated concentrations of a food's metabolites can then be employed as input variables into multivariate statistical methods. Not all the variables extracted from the spectra are used for statistical analysis. Variables with poor predictive performance and/or poor discriminatory power should be abolished. Data exploration is the first stage for data reduction, which provides information about the quality and suitability of each variable for succeeding statistical analysis [50].

6.2.1 NMR Data Processing in Chemometric

The chemometric approach does not require any chemical shift assignment. Upon applying the technique of buckets or bins, the spectra are prepared for chemometric analysis. Each spectrum can be pretreated and split into *(N)* number of buckets of 0.01 to 0.05 ppm width. Each bucket is integrated separately, and the number of buckets *N* and *S* values of integrals forms an $N \times S$ matrix. The total data matrix of (*n*) number of spectra, $n \times N \times S$, can go directly to chemometric analysis using a number of multivariate statistical techniques, and this could correlate the spectral data with constituents (variables) of a food system. The final step is the exploration of the ability of the statistical techniques to locate spectral regions (spectral fingerprints) that could be able to differentiate food samples with different physicochemical properties, for example, originated from different geographical areas or varieties. These spectral patterns are used subsequently to construct classification and prediction models for unknown samples. The main benefit of the chemometric approach

is its susceptibility to automation. However, it is unable to identify and/or quantify useful metabolites and biomarkers [48].

6.2.2 Univariate Statistical Analysis

Univariate statistical (UVS) analyses are used to test a hypothesis for individual variables to assess, whether a significant difference between groups exists. The most commonly used UVS parameter in chemometrics is analysis of variance (ANOVA). It is a statistical method in which the total variability present in a data set is divided into its individual components in order to highlight the contribution of specific variables [52]. ANOVA comprises a group of suitable statistical methods, such as one- and two-way ANOVA. One-way ANOVA checks the statistical importance of the mean parameter values of two or more groups of samples that are affected by a single independent variable. The statistical importance is assessed using the Fisher *F*-test, which investigates the null hypothesis of two or more specimens belonging to similar group. High value of *F*-test for each variable proposes the null hypothesis to be wrong, the samples belonging to different groups, and that specific variable may be exploited for classification. Two-way ANOVA ameliorates the statistical analytical ability of one-way ANOVA by considering the effect of two independent variables on the response, in addition to the possible interactions between these two variables [53].

It has been suggested that ANOVA could be replaced with multivariate analysis of variance (MANOVA) for large spectroscopic data. The ANOVA is used for the dataset where only one dependent variable is considered, while the MANOVA method includes multiple, dependent variables. For instance, ANOVA tests for the difference in means between two or more groups, while MANOVA tests for the difference in two or more vectors of the means [54]. MANOVA is superior to ANOVA because it discloses correlations between variables and reveals differences not shown by ANOVA.

6.2.3 Multivariate Statistical Analysis

Multivariate statistical analysis (MVA) is used for data reduction and visualization or for discrimination and classification of variables to be considered. In the first step of MVA, an unsupervised analysis is to be performed without prior information of neither the nature nor the group membership of the samples. This is often performed using principal component analysis (PCA) [55]. Exploratory treatment performed after data is cleaned or pre-processed. On the contrary, unsupervised methods such as partial least square (PLS) and orthogonal partial least square discriminant analysis (OPLSDA) are examples of exploratory analyses. It is used to identify the trends or clustering in data set, but does not attempt to relate observations to a class label or response. It is also of vital importance that models obtained from supervised

analysis must be validated [56]. Some of the multivariate statistical methods are described in the following sections.

Principle Component Analysis (PCA)

Principal component analysis (PCA) is the extensively employed multivariate analysis method for metabolomic and, in fact, chemometrics in general [57]. It was invented by Karl Pearson in 1901 [58], and later it was further developed and named by Harold Hoteling in the 1930s [59]. In the analysis of NMR data, PCA is the most common unsupervised method employed, particularly by the chemometric approach. It remains the principal statistical tool for inceptive analysis of big data sets to survey trends (similarities), classifications, and identification of outliers [48]. PCA is an analytical technique that employs an orthogonal transformation to change a set of observations of feasibly correlated variables into a set of linearly uncorrelated variables called principal components. If there are ṅ observations with variables, then the quantity of specific principle components can be determined. The modification is conveyed in such a manner that the primary principal component depicts the largest possible variance, and each following component has the exorbitant variance under the restrictions that it is orthogonal to anteceding components. The resulting vectors (each being a linear combination of the variables and containing *n* observations) are an uncorrelated orthogonal basis set. The relative scaling of the original variables makes PCA sensitive [60].

Partial Least Squares Discriminant Analysis (PLS-DA)

Unlike PCA, PLS-DA is a supervised method employed for optimizing segregation among different groups of samples that are achieved after joining two data matrices Y (i.e., groups, class membership, etc.) and X (i.e., raw data) [61, 62]. In these circumstances, a more focused evaluation and analysis of the data are possible. The basic principle is similar to PCA, but in PLS-DA, a second piece of information is used, namely, the labeled set of class identities. The major benefit of this technique is accessibility and managing extremely noisy and collinear information, which are extremely ordinary results from metabolomics experimental methods [63]. This technique gives an optical elucidation of complicated data sets via low-dimensional, feasibly explainable outcomes plot that demonstrates differentiation among various groups [64].

Orthogonal Partial Least Square Discriminant Analysis (OPLSDA)

OPLSDA was proposed as a refinement of the PLSDA method to differentiate two or more groups (classes) utilizing multivariate data [65, 66]**.** A regression model between the multivariate data and a response variable that only comprises class

information is calculated in OPLSDA. The benefit of OPLSDA compared to PLSDA is that a sole component is employed as a predictor for the class, while the other components explain the variation orthogonal to the first anticipating component [67].

7 NMR Applications in Food Analysis

Food adulteration and food fraud are common in foods. Due to the recent advancement of food fraud-related methods, it is gaining awareness in public, and problems are highlighted in public. It can involve public health risks due to the increasing ratio of economically motivated adulteration [68]. Adulterated food is an official term, which means that a food product fails to comply with the stated standards. Adulteration usually refers to fractious with health or safety standards. With the increasing number of food products, adulteration incidents are also increasing and result in serious economic and health issues. The food fraud issue has become a global threat for the food chain supply with its lethal destructive potential, particularly when many product ingredients are derived from different countries. Therefore, sometimes, it is difficult to detect and trace source of contaminants and related food safety concerns and even more difficult to detect and trace back cases of deliberate product fraud, especially in highly processed foods that are imported from multiple suppliers [69]. Food ingredients commonly associated with food frauds include oil, fish, honey, milk and dairy products, meat products, grain-based food products, wine and alcoholic beverages, and organic food. Many analytical techniques have been used to detect adulteration and fraud in food [68].

Present-day NMR instrumentation does not hold the disadvantages of the early days of NMR spectroscopy that include low sensitivity and high cost of analysis. Modern hardware consisting of a strong magnetic field of up to 23.5 T and cryogenic probes, which aid in the easy identification of components of food at microgram and nanogram levels. The screening of a large number of samples (overnight run) and decrease in experimental time to few minutes even for low sensitive nuclei (e.g. ^{13}C, ^{15}N). It is possible due to the development of sophisticated software and innovation in automation [70]. NMR has found applications in many fields of food science. Some of the important applications of NMR spectroscopy in the analysis of different foods are given in the following sections.

7.1 Fruits and Vegetables

Fruits and vegetables are complex, and their consumption is encouraged because they possess health benefits. They consist of a diverse range of compounds, which are responsible for several biological activities. NMR, being a nondestructive and powerful technique, can analyze this diversity in fruits and vegetables [17]. Among fruits, strawberry is an important fruit due to its lovely flavor and substance

properties including sugar, organic acids, amino acids, and essential metabolites. These metabolites are significant in strawberry during maturation [71], and these provide the essential support for its organoleptic properties, with sugar influencing the sweetness while amino acids and organic acids affecting the taste or aridity. A research has concentrated on disposing the impacts of PGRs (Plant Growth Regulator) on the yield of strawberry plant. A nuclear magnetic resonance-based methodology combined with multivariate analysis and pathway investigation has been utilized to assess the impact of Gibberellins, forchlorfenuron, and brassinolide associated with two diverse development stages [71]. A similar study demonstrated the application of NMR-based metabolomics with matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and transcriptomics assistant [72].

Metabolomics is a powerful tool in the determination of changes in small molecules that emerge from the outer and internal environmental stimuli. A recent study shows the application of ^{1}H-NMR spectroscopy to investigate the metabolite profiling of Italian cherry tomatoes using multivariate analysis. In this study, cherry tomatoes were collected from different geographical origins and were analyzed by chemometrics [73]. NMR-based chemometrics approach was used successfully to characterize the cranberry supplements from whole cranberry powder as a reference standard. Compounds responsible for variation in metabolic profiling were ascertained using a chemometric approach. This includes citric acid and cranberry peel constituents ursolic acid, oleanolic acid, and hyperoside [74]. The classification of the Korean and Chines garlic and Chines and Korean onion was performed using ^{1}H-NMR spectroscopy approach with the conjunction of MVA. The results show that NMR can be used as a technique in tracking food origin and creates a possibility to tackle mislabeling of origin and thus providing a reliable technique in quality evaluation and fraud controls [75]. Allium genus (garlic and onion) is a profitable bioactive class of food crops with conceivably significant properties. Broad contrasts were seen between the sugar concentrations in onion species. Red onion contained a minimum measure of amino acids, and yellow onion contained the most elevated measure of amino acid. The level of flavanol was higher in yellow onions than in red onions, and garlic and leek contained a less measure of flavanols than the other allium species. ^{1}H-NMR together with HPLC-MS can be helpful in the identification and evaluation of the most metabolites, speaking to an effective way to pinpoint useful functional food ingredients from allium species [76]. NMR strategy is a quick and economical procedure and better than customary strategies, which are identified with variables, for example, taste, well-being, and security. ^{1}H-NMR together with HPLC-MS empowers the recognizable proof and measurement of an enormous number of metabolites from allium species [76].

7.2 Coffee and Tea

Coffee is the most consumed beverage in the world. The chemical composition of the coffee beans affects its flavor and quality. A high-resolution NMR is used to investigate espresso in aqueous solution and organic solvent. A detailed NMR investigation of fluid concentrates of green espresso bean was performed by Wei et al. [77]. Sixteen compounds were detected in the ^{1}H NMR spectrum [78]. Green coffee bean components were quantified using ^{13}C NMR. Coffee comprises of a complex blend of many different organic compounds ranging from traces up to 10% by weight [79]. A recent study of Brazil showed six different adulterants, namely, barley, corn, coffee husks, soybean, rice, and wheat in roasted coffee. They used ^{1}H-NMR with chemometrics as an assistant tool [80]. Okaru et al. [79] show the application of NMR spectroscopy for the routine screening of coffee for quality and authenticity. They investigated the influence of extraction time NMR device and nature of coffee. These parameters alter the level of caffeine, 16-O-methylcafestol (OMC), kahweol, furfuryl alcohol, and 5-hydroxymethylfurfural (HMF) [81]. Effect of roasting time on the structure of coffee melanoidins by NMR spectroscopy has been reported recently. This study shows that roasting affected the low molecular weight fractions of coffee melanoidins by incorporating with chlorogenic acid using 1D and 2D NMR [82].

Tea is also a well-known beverage consumed in the world. The quality of tea is evaluated through sensorial attributes, such as flavor and appearance. The tea is traditionally established using expert tea tasters. Numerous factors, for example, climatic conditions, soil, growth altitude and horticultural practices, plucking season, sorting of leaves, pressing, and storage, influence the flavor and chemical composition of the tea [83]. Le Gall et al. [86] have recognized 31 compounds in the ^{1}H-NMR spectrum of green tea extract. Metabolic profiles have been utilized to compare the high-quality Longjing teas and other Chinese teas. Longjing tea indicates a more elevated amount of theanine, gallic acid, caffeine, and other minor sugar compounds and lower levels of unsaturated fats and sucrose as compared to other teas. High-quality teas differ from low-quality ones considering the amount of caffeine, theanine, and catechins [83, 84]. NMR-based metabolomics approach showed the protective effect of tea polyphenols in sulfur mustard-induced injury in rats. In total, 13 biomarkers were identified related to sulfur mustard injury in rats [85].

7.3 Vinegar

Vinegar is a product utilized as a condiment for salad. It is produced by a variety of raw materials (e.g., grapes, apple, honey, orange, pineapple, and rice) by an alcoholic product pursued by acetic fermentation. The chemical composition of vinegar relies upon different factors, for example, manufacturing strategies and the

topographical environment of its production. Balsamic and traditional balsamic vinegar from Modena and Reggio Emilia are the most famous vinegar at the international level. ^{1}H-NMR spectrum of vinegar has been accounted for both using a D_2O buffered solution by Caligiani et al. [86, 87]. Wine vinegar is described by a high concentration of ethyl acetate, glycerol, methanol, and tartaric acid while ethyl acetate and glycerol signals are not perceptible in alcohol/agrin vinegars. Apple vinegar is rich in alanine although nectar vinegars and pineapple vinegar have highest amount of tartaric acid. Rice and orange vinegars are richer in lactic acid and contain less amount of methanol. Alanine signals are not noticeable in orange vinegar [88]. Recently, a comprehensive and nondestructive method based on ^{13}C isotopic evaluation was developed by Wang et al. [89] based on ^{1}H-NMR spectroscopy. The applied approach was successful to authenticate the quality of vinegar [89].

7.4 Oils

Olive oil is an integral part of the Mediterranean diet, and its fruit is used to extract natural juice. NMR spectroscopy has the potentials for controlling olive oil quality, authenticity, and geographical variations [90]. NMR spectroscopy coupled with multivariate analysis can differentiate between olive oils of different olive varieties as well as from different origins. ^{1}H-NMR has also been applied to the study of other vegetable oils (peanut, soybean, maize, and sunflower) and their mixture with olive oils [91]. Two main points are considered: olive oils are always classified by a high quantity of terpenes and a low quantity of saturated fatty acids. NMR metabolic profiling has been used to investigate the effects of different factors, such as altitude and irrigation [92]. A comparative study of oxidative products of commercially available refined and cold-pressed camellia oil, stored at room temperature for 1 year, was studied by ^{1}H and ^{31}P NMR spectroscopy [93]. Recently, solvent-based strategy was applied to improve the key parameter in edible fats and oils by ^{1}H-NMR spectroscopy. This methodology was successfully applied to the analysis of corn, sunflower, sesame, olive, and peanut oils. Moreover, the evolution of thermal oxidation and lipolysis of virgin olive oil and sunflower has been analyzed.

7.5 Fish

There is an increasing need for appropriate analytical techniques suitable for a complete snapshot of fish metabolome to assess the fish dietary quality. Fish is the main source of polyunsaturated fatty acids. A major application of NMR-based metabolic investigation of fish has been focused on the investigation of fish fatty acid composition mainly by using ^{13}C NMR spectroscopy. Various methods using ^{1}H NMR metabolomics are available to study fish-based foodstuffs [92]. Another study was conducted to assess the biomarker in biological fluid after fish consumption and to

examine the relationships with health parameters in a free-living population. The urine of the fish consumed population was compared to control and analyzed by NMR spectroscopy. This study determined trimethylamine-*N*-oxide (TMAO), dimethylamine, and dimethyl sulfone displayed a significant level in urine after fish consumption. Fish consumption yields a greater increase in urinary TMAO compared to red meat [94].

7.6 Juices and Beverages

NMR technique has been applied for quality control of different fruit juices, such as apple, black currant, grapefruit, lemon, orange, peach, and pineapple. NMR-targeted and non-targeted metabolomics approaches have been applied in food analysis [95]. The application of NMR metabolomics to examine orange juice adulteration. Microbial growth in fruits and their products is of great attention as it causes the decay process, loss of organoleptic properties, and generation of toxic substances. Due to the low pH of many fruit products, fungi are often predominant microorganisms while some bacteria are also responsible for food decay [96]. An example of such a study conducted recently by Cusano et al. [95] is based on NMR metabolomics of fermented juices. This study deals with metabolic changes in ciders fermented by six yeast species, and it was a valuable tool for the identification of metabolites from yeast. PCA was performed on NMR data from all spectral regions, and only the aromatic region revealed the potential to discriminate the yeast action [97]. Alcoholic and nonalcoholic beverages are a complicated mixture of different classes of compounds with varied concentrations. Alcoholic beverages consist of a varying quantity of ethanol in comparison to nonalcoholic beverages [98].

Fermented beverage, such as beer, is prepared from malted grains, hops, yeast, and water. Other specific fruits and herbs can also be added to give distinct characteristics to the product. NMR coupled with multivariate statistics has allowed beers to characterize considering the type, brewing site, production date, and malt type. Hyphenated NMR, namely, LC-NMR/MS, has allowed the description of some aromatic compounds, 2-phenylethanol, tyrosol, present in extremely low concentration [99].

Mixture of many compounds containing water, ethanol, glycerol, organic acid, and inorganic ions are utilized to manufacture wine. The chemical conformation of grapes and their wine is affected by the environmental situations of the vineyard. The ^{1}H-NMR metabolic approach has provided interesting information on the grape variety, geographical origin, and fermentation of wine acquired at each fermentation stage and aging period [100].

7.7 Soy Sauce

The ^{1}H-NMR has been applied on Zivania, the standard Cypriot alcoholic beverage from different countries. The evolution in metabolites in Korean standard soy sauces during 12-year maturing period has been examined by NMR combined with multivariate statistical analysis. The global metabolite profile of the soy sauce allowed a superior assessment of soy sauce quality [100, 101]. An improved strategy has also been developed and successfully applied for the differentiation of Chinese and Asian soy types by using ^{13}C NMR spectroscopy coupled with multivariate statistics [9, 102].

7.8 Milk and Dairy Products

Dairy milk is an important constituent of human food used in fresh as well as processed forms [103]. Two significant supplements, like lipids and lactose, are present in milk. Being an organic liquid, the composition of milk is influenced by different factors, such as breed, season, individual metabolism of animals, health state, diet, and milky protocols [104]. Milk is a critical dietary constituent being an extraordinarily complex and nutritionally complete biological fluid. Currently, it has been observed that the milk composition can be modified to upgrade its profitable attributes as well as to search for parameters that could identify the animal species from which the milk comes. Milk is an intricate emulsion and good ^{1}H-NMR spectra and ^{13}C provided a route of separation between the milk of various animal species according to fatty acid composition [105]. Coimbra et al. reported the detection of formaldehyde in raw milk by time domain-NMR and chemometrics approach. Different refrigeration storage time 0 and 48 h was assessed to check the growth of formaldehyde. The whiteness index of the milk is associated with the increased level of formaldehyde, while the lightness values indicate an increase in the yellowing index when compared to the control samples. This study shows a successful application of time domain-NMR in dairy products such as raw milk [106].

7.9 Butter and Margarine

NMR-based metabolic profiling has also been applied to analyze polar and apolar extracts from the spread and margarine [107]. The polar portion includes numerous acids including benzoic, sorbic, citrus, lactic, butyric, and formic acid, while a polar fraction contains rumenic and linoleic acid. The rumenic acid, trademark ruminant fat, has been found in all the margarine tests. The degree of natural acids, lactose, has been suggested as a significant marker of quality control and production process [107].

7.10 Cheese

Cheese is a fermented dairy product, harboring diverse microbial communities (microbiota) that change over time and vary depending on the type of cheese. The cheeses are varied as a function of starter and adjunct cultures. The final product of the cheese depends on the microorganisms, and they play an important role in their quality, flavor, and safety. Many studies have focused to explore the composition of cheese microbiota and the molecular mechanisms involved in cheese ripening [108]. The geographical origin of mozzarella buffalo cheese and graviera has been traced by using NMR [109, 110]. A good discrimination of mozzarella cheese samples from different geographical origins has been acquired utilizing a coupling of IRMs with NMR data [111].

7.11 Honey

Honey is considered a complex natural food product with various physiochemical properties. It is mainly composed of sugars and other constituents, such as amino acids, organic acids, carotenoids, vitamins, enzymes, minerals, and aromatic substances. Cheap sweeteners can be added to honey for adulteration or indirectly honeybees can be fed with sugar. Therefore, detecting and quantifying methods of adulteration are of prime importance. Due to its demand and interest of the consumer, honey authentication is increasing, and it is necessary to check the acceptability. Honey producers and quality control laboratories are now requiring increasingly sophisticated methods of analysis. One of the best techniques for honey characterization is NMR. NMR-based method for honey quality control and traceability are very well established [112]. NMR-based screening provides a cost-efficient, complete analysis that can be used reliably to ensure honey quality [113]. Song et al. [114] used 147 authentic monofloral honey from China to investigate NMR and chemometrics. NMR data from δ 0.00 to 6.00 ppm is the most suitable region to determine the adulteration of pure acacia honey [115]. A similar study was performed by Schievano et al. [116] on acacia honey from Italy, where carbohydrate profile can be traced to the authenticity of acacia honey when compared to commercially available acacia honey in the market. In this study, sugar profiles in honey were used as a fingerprint to confirm the authenticity or revealed the adulteration of the product by sweetener addition. NMR spectroscopy is used in the composition analysis and authentication of Chinese honey with a combination of chemometrics. A total of 65 major and minor components in honey were identified and quantified from their NMR spectra [117].

7.12 Rice

Rice is one of the most valuable cereal food crops in the world. H-NMR spectroscopy combined with multivariate analysis methods such as principal component analysis (PCA) and discriminate analysis (DA) has been applied to the profiling of metabolites in numerous rice. ^{1}H-NMR-based fingerprint combined with multivariate statistical analysis is the best tool for the classification of rice grains by geographical origin as well as for the discrimination among different pigmented rice grains [118].

7.13 Wheat

Proton NMR spectroscopy has been widely used to detect the metabolic profile of different samples of wheat [119]. A combined approach including HR-MAS to study the hydrated wheat flour and liquid state and ^{1}H-NMR to study the corresponding aqueous extracts have been employed for enzymatic degradation caused by endogenous hydrolases. The kinetic parameters resulting from the best fit have been extracted and subjected to multivariate statistical analysis resulting in good discrimination between the hard and the soft wheat. PCA showed that the environmental factors pose a great impact on metabolic profile than genotype.

8 Conclusion

Recently, demographic and commercial trends have shown an increase in the production of food of both plant and animal origin. Therefore, health risks are also intensifying in parallel. In recent years, food fraud has emerged as a potential threat to various countries. Various techniques are available for the quality control and authentication of foods. However, the majority of those techniques are targeted and provide information about a single aspect of foods. NMR spectroscopy combined with multivariate statistical methods is a powerful tool for the quality control and authentication of foods. This chapter also demonstrates that NMR is a valuable tool for the investigation and authentication of foods. NMR is a robust method to identify food adulteration and food fraud and is used to trace the geographical origin of food, evaluation of toxicants of food, and the raw material of different food. It has wide applications in food, including, fruits, coffee, tea, beverages, butter & margarine, cheese, vegetables, milk & dairy products, honey, rice, wheat, maize, juices, fish, meat, vinegar, etc. NMR spectroscopy could be preferred over most of the other techniques because of its nondestructive, robust, efficient, and nontargeted nature.

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