DEVELOPING FOOD ANALYSIS METHODS USING BENCHTOP NMR SPECTROMETER

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DEVELOPING FOOD ANALYSIS METHODS USING BENCHTOP NMR SPECTROMETER

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

DEVELOPING FOOD ANALYSIS METHODS USING BENCHTOP NMR SPECTROMETER

Söyler, Alper
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The online monitoring of chemical reactions by using benchtop Nuclear Magnetic Resonance (NMR) spectroscopy has become increasingly attractive for the past few years. The use of quantitative online NMR spectroscopy is a promising alternative to traditional analytical methods with its rapid, quantitative and non-invasive nature that makes it applicable to complex and diverse biochemical mixtures like food systems.

In this dissertation, sucrose hydrolysis by invertase and milk lactose hydrolysis were chosen as model reactions for online monitoring. Reactions were performed in a continuous flow mode. Moreover, rather than conventional NMR spectroscopy experiments that mostly rely on the use of deuterated water, the benchtop setting allows working in protonated solvents, and tailored water suppression techniques were used to make quantification more accurate.

For the hydrolysis reactions, 10% sucrose solution, 5% lactose solution and a milk sample were hydrolyzed. The kinetic constant was determined by the fractional conversion model. Average rate constants for the hydrolysis reactions of sucrose, lactose solution and milk sample were found as 12.88×10^{-3} min^{-1}, 1.66×10^{-2} min^{-1}, 1.52×10^{-2} min^{-1}, respectively. All the results were comparable to the results obtained

in other studies found in the literature. Quantitative online NMR spectroscopy was seen as a promising tool for monitoring food processes in a continuous mode.

In addition to online monitoring of enzymatic hydrolysis reactions, authentication of different milk types was achieved using NMR spectroscopy and statistical methods (ANOVA and Discriminant Analysis). Lactose, fat and glycerol contents were obtained from the NMR spectra. Milks from Turkey and France were able to be differentiated with their glycerol content.

Lastly, honey adulteration by high fructose corn syrup has been studied by NMR spectroscopy combined with relaxometry techniques. Adulterated samples were discriminated by the relaxation times. Relaxation times of alpha anomer, sugar region, water and bulk sample were calculated using a monoexponential approach. A linear relationship was found between high fructose corn syrup concentration and T_2 relaxation times (R^2>0.92).

Benchtop NMR spectroscopy was seen as a promising, easy to use and affordable tool to analyze food samples either for academic research and for the industry. It has significant potential on developing new analysis methods.

Keywords: Food Analysis, Benchtop NMR Spectroscopy, Sucrose, Invertase, Lactose, Milk, Lactase, Honey, Adulteration, Authentication, Flow NMR, Continuous Mode Reaction Monitoring

ÖZ

MASATİPİ NMR SPEKTROMETRE KULLANARAK GIDA ANALİZ YÖNTEMLERİNİN GELİŞTİRİLMESİ

Söyler, Alper
Doktora, Gıda Mühendisliği
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Kimyasal reaksiyonların masa tipi Nükleer Manyetik Rezonans (NMR) spektroskopisi kullanılarak çevrimiçi izlenmesi, son birkaç yıldır giderek daha cazip hale gelmiştir. Kantitatif çevrimiçi NMR spektroskopisi gıdalar gibi karmaşık ve çeşitli biyokimyasal karışımlara uygulanabilir olması, bu n geleneksel analitik yöntemlere alternatif olarak kullanılabilmesine olanak sağlamıştır.

Bu tez çalışmasında, sükroz ve laktoz hidrolizi birer model reaksiyon olarak seçilerek, reaksiyonlar anlık izlenmiştir. Çoğunlukla dötoryumlanmış su kullanımına dayanan geleneksel NMR spektroskopi deneylerinden ziyade, masa tipi bir ekipman akış modunda kullanarak, su ile çalışmaya olanak sağlayan su sinyalini baskılama teknikleri ile kantitatif analizler yapılmıştır.

Hidroliz reaksiyonları için, %10 sükroz çözeltisi, %5 laktoz çözeltisi ve süt numunesi hidrolize edilmiş olup, kinetik modelleme amacı ile kesirli dönüşüm modeli kullanılmıştır. Sükroz hidrolizi, laktoz çözeltisi hidrolizi ve süt hidrolizi reaksiyonları için ortalama reaksiyon hız sabitleri sırasıyla 12.88×10^{-3} dk^{-1}, 1.66×10^{-2} dk^{-1}, 1.52×10^{-2} dk^{-1} bulunmuştur. Belirlenen bu kinetik sabitler diğer bilimsel çalışmalarda elde edilen sonuçlarla benzerlikler göstermiştir. Kantitatif çevrimiçi NMR spektroskopisi,

gıda proseslerini sürekli bir modda izlemek için umut verici bir araç olarak görülmüştür.

Enzimatik hidroliz reaksiyonlarının çevrimiçi olarak izlenmesine ek olarak, NMR spektroskopisi ve istatistiksel yöntemler kullanılarak farklı süt tiplerinin ayırt edilebilmesi NMR spektralarından laktoz, yağ ve gliserol içerikleri belirlenmiştir. Türkiye ve Fransa menşeli sütler, gliserol içeriği ile ayırt edilebilmiştir.

Son olarak, balın, yüksek fruktozlu mısır şurubu ile tağşişi, NMR spektroskopisi ve relaksometre teknikleri ile çalışılmıştır. Tağşiş edilen örnekler, relaksasyon süreleri analiz edilerek başarıyla tespit edilmiştir. Fruktoz şurubu konsantrasyonu ile relaksasyon süreleri arasın da lineer bir ilişki tespit edilmiştir (R^2>0.92).

Masaüstü NMR spektroskopisi, akademik araştırmalarda ve gıda endüstrinde, gıda örneklerini analiz etmek için gelecek vadeden, kullanımı kolay ve uygun fiyatlı bir metot olarak görülmektedir. Yeni analiz yöntemleri geliştirme konusunda önemli potansiyele sahiptir.

Anahtar Kelimeler: Gıda Analizi, Masa Tipi NMR Spektroskopi, Sükroz, Invertaz, Laktoz, Süt, Laktaz, Bal, Tağşiş, Orijin Belirleme, Sürekli Modda Reaksiyon Takibi

To my beloved family

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INTRODUCTION

1.1. History of Science of Food Analysis

History of food is as old as the history of humanity. Transition from raw to cooked food, from agricultural production through to industrial synthesis, from local to global and back took thousands of years. Today, impacts of food in economy, politics, culture and environment/ecology are much more than ever. 420,000 people die worldwide every year because of the food contaminated with bacteria, viruses, parasites, toxins or chemicals (World Health Organization, 2016). Nine million people die of hunger every year according to the World Health Organization (WHO) which is more than the people who have died during the wars in 21st century. These impede socioeconomic development by straining the healthcare systems, and harming the national economies, tourism and trade.

Importance of food analysis could be explored better through some historical examples.

In ancient times, it is known that Romans, especially elites, suffered chronic lead poisoning due to the excess amount of lead in their lined pots in which acidic foods were cooked and due to the channeled spring water into their houses through lead pipes. They have also used lead contaminated sugar to sweeten their wines. However, this was not being analyzed at those time. It is speculated that lead poisoning brought down the ancient Rome (Sumner, 2014).

The historians and scientists said that the ***witchcraft affair*** in Salem village (now Danvers), MA, USA was due to a disease known as *convulsive ergotism* (Matossian, Ergot and the Salem Witchcraft Affair: An outbreak of a type of food poisoning known as convulsive ergotism may have led to the 1692 accusations of witchcraft, 1982).

Ergot is the common name caused by the fungi of the genus *Claviceps*. When the victims eat fungi infected rye bread, they appeared dazed, were unable to speak, became manic and suffered from hallucinations and other distorted perceptions (Miedaner & Geiger, 2015). Totally 200 people were accused by practicing witchcraft and 20 were killed by hanging. Now, scientists understand from Salem court records that all these people showed the symptoms of Ergotism and The Salem Witch Trials stays as a dark time in American history (Matossian, Poisons of the Past: Molds, Epidemics, and History, 1991).

In the year of 1900, a mysterious outbreak of disease appeared in Manchester area in UK. The symptoms were paralysis of muscles and loss of function in sensory nerves which typically resulted from chronic alcohol poisoning. Among the victims, there was even a two-years-old girl whose father was working in the pub and she got little sups of beer from the kindly-disposed customers at the bar. She developed the symptoms of poisoning too. After the examinations and chemical tests done in the brewery, the glucose and invert sugar were found to be arsenical and consequently the beer was also contaminated with arsenic. The source of the contamination was the sugar refinery where sulphuric acid containing arsenic was used in the sugar processing. Over 6,000 people suffered from the poisoning and at least 70 people died (Kelynack & Kirkby, 1901). Neither sugar refinery nor beer factory were using food analysis techniques during their processes to detect arsenic in the sugar.

It can be thought that these examples are quite old enough and they occurred before the technology era. However, even with the current technological advancements, it is quite difficult to avoid possible contamination and/or adulteration in the foods. Hundreds of incidents happen each year.

In the early 1980s in Spain, 20,000 of people were reported ill from *Toxic Oil Syndrome*. This was the result of the adulterated industrial oil imported illegally from France and re-refined and sold as olive oil. This intoxication caused the death of over 1,800 people till the end of 1997 (Posada de la Paz, Philen, & Borda, 2001).

In 2012, over 250,000 eggs were recalled in Germany after laboratory testing revealed the high level of poisonous chemical, dioxin. The contamination of dioxin was found in the animal feed. This contamination killed of thousands of animals and the closure of hundreds of farms temporarily (Astley, 2012).

The above historical and current examples of the food related outbreaks show us clearly why we need food analysis methods to analyze its quality, composition, safety, authentication and adulteration during the production processes and post-production. Today, food is considered not only a source of energy but also an affordable way to prevent future diseases. Therefore, while the global food industry continues to expand, development of novel foods, functional foods and foods for special diet (e.g. gluten-free foods, lactose-free foods etc.) also created a pressure for the development of analytical methods.

Food is a complex mixture of diverse and numerous biochemical substances (McGorrin, 2006). Analysis of food requires continuous development of more sensitive, robust, rapid, and low-cost methods to maintain the quality, traceability and safety of foods in compliance with the legislations (Cifuentes, 2012). Although food is a vital part of human life, food analysis is benefited from the technical advancements which were initially developed for other industries such as chemical, pharmaceutical and petroleum industry.

Until the 19th century, food analyses were being conducted by the traders by their organoleptic skills. The first municipal laboratory for the food analysis was established in Brussels in 1856, followed by Paris in 1878 (Scholliers, 2007; Atkins & Stanziani, 2007) . These laboratories served to the traders who were suspicious of their suppliers. These laboratories were performing 'wet chemistry' methods for food analyses. These techniques rely on the filtration, evaporation, distillation and solvent extraction. As an example, moisture content was being measured by drying, fat content was being determined by diethyl ether extraction, and the protein content by Kjeldahl method. The methods like Kjeldahl became a gold standard for estimation of protein

content of foods for over 100 years because of its precision and reproducibility (Nollet, 2004).

Subsequent discoveries of vitamins, minerals, lipids, proteins and further understandings of their roles in human nutrition between 1900-1940, brought the need for new analytical techniques. Therefore, after 1950s, burdensome and extremely hard 'wet chemistry' methods began to abandon. Despite the fact that many classical techniques are still extensively used today, they were replaced with high-throughput instrumental methods which have high analyte specificity and lower detection limits.

Traditionally, analytical methods have been classified according to their working principle which are chromatographic, spectroscopic, electrophoretic, immunoassay, microbiological and genetics methods.

1.1.1. Chromatographic Methods

Chromatography is a method for separating mixtures into their components based on the differences in their equilibrium constants in a diphasic system; mobile phase and a stationary phase (Günzler & Williams, 2001).

After the invention of ***Liquid Chromatography (LC)*** by a Russian botanist Mikhail Tswett in 1901, the development of chromatography gave the deepest impact and numerous applications to the food analysis field (Ettre, 2003). In 1967, James Waters made an important breakthrough by developing ***High-Performance Liquid Chromatography (HPLC)*** (McDonald, 2008).

Shortly after being awarded with the Nobel Prize in Chemistry in 1952, A.J.P. Martin invented ***Gas Chromatography (GC)*** in partnership with A.T. James with an application for the separation and quantification of C_1-C_{12} volatile fatty acids (James & Martin, 1952).

Another chromatographic technique introduced in 1938 and widely used in food analysis is ***Thin-Layer Chromatography (TLC)***. Although TLC, GC and HPLC are

highly complementary, each has their own advantages regarding simplicity, sensitivity, cost effectivity depending on the intended application. Food additives, antioxidant and food dye analysis can be performed by TLC method (Sherma, 2000). Current analyses of polysaccharides, amino acids, vitamins, antibiotic residues would not be as rapid and productive as without HPLC. Similarly, fatty acids, flavors, pesticide residues are routinely analyzed by GC.

1.1.2. Spectroscopic Methods

Spectroscopic techniques rely on the fact that atoms and molecules interact with electromagnetic radiation. Chemical, physical, structural, qualitative and quantitative information of the compounds can be received with spectroscopic methods. The wavelength or frequency can be detected in the emitted or absorbed energy spectrum to obtain this information.

Spectroscopic techniques are highly used in food analysis because they provide fast analysis, require minimal sample preparation, give direct measurement of the food components, do not require of toxic solvents, allow to apply multiple tests on one sample. Also, they are non-destructive and noninvasive methods, therefore, can be used in process lines.

Mass Spectroscopy (MS) is an analytical method that determines the chemical composition of a sample based on their mass-to-charge ratio. J.J. Thomson was the premier scientist who recorded the first mass spectra of a simple low-molecular weight sample in 1912 (Thomson, 1912). Francis Aston manufactured the velocity focusing mass spectrometer in 1919 and was awarded for the Nobel Prize in 1922. Over the past decades, MS has primarily been used for direct determination and quantification food constituents and contaminants by the hyphenation of MS to capillary gas chromatography (GC-MS) and liquid chromatography (LC-MS) (Careri, Bianchi, & Corradini, 2002). Flavor mixtures have been characterized by GC-MS instruments since 1960s (Reineccius, 1991).

Ultraviolet Visible (UV-Vis) Spectroscopy is a sensitive method that uses ultraviolet and visible light between the wavelengths of 200 nm and 780 nm (Roberts, Power, Chapman , Chandra, & Cozzolino, 2018). UV-Vis spectroscopy relies on the interaction between light and matter which results in emission, absorption, and scattering. UV-Vis Spectrometer is also very commonly used method for food quality control method.

IR region of the electromagnetic spectrum was discovered by Sir William Herschel in 1800. After almost 150 years later Peter Fellgett obtained the first IR spectrum by using ***Fourier Transform Infrared (FTIR) Spectroscopy*** in 1949. This method uses the wavelengths between 2,500 nm and 25,000 nm and then, converts the measurement data into a useful output (Fourier Transform) (Nawrocka & Lamorska, 2013). It is particularly useful for testing liquid samples such as screening abnormalities in milk and measuring phenolic compounds that contribute to the flavour and colour of wine.

Raman Spectroscopy is a vibrational spectroscopy technique based on the Raman effect which was discovered in 1928 by the Indian scientist Sir C.V. Raman (Gardiner & Graves, 1989). Raman Spectroscopy can be used for quantification of the degree of unsaturation in lipids (Sadeghi-Jorabchi, Hendra, Wilson, & Belton, 1990); determination of cis and trans isomers content (Sadeghi-Jorabchi, Wilson, Belton, Edwards-Webb, & Coxon, 1991); identification of adulteration in oils (Baeten, Meurens, Morales, & Aparicio, 1996) and to monitor the rate of olive oil oxidation (Guzman, Baeten, Fernandez Pierna, & Garcia-Mesa, 2011).

Among the spectroscopic methods, ***Nuclear Magnetic Resonance (NMR) Spectroscopy*** is the most recently developed technique and discovered shortly after the Second World War. The first successful NMR experiments were done independently by Felix Bloch at Stanford and Edward Purcell at Harvard in 1946. Then, they were jointly awarded the Nobel Prize in 1952 (Thomas, 1991). NMR analyses offer a comprehensive characterization of foodstuff from the different points

of view including structural, compositional and functional aspects. In food analysis, NMR spectroscopy is used for quality control, to monitor shelf life, to detect adulteration, contamination and to analyze moisture content, fats and oils.

1.1.3. Electrophoretic Methods

The migration of electrically charged particles or ions in a solution as a result of an applied electric field is defined as electrophoresis. The ability to separate very similar substances like different proteins has increased especially since 1950 by introducing zone electrophoresis in paper and later gels of polyacrylamide and agarose (Vesterberg, 1989).

Electrophoretic techniques have been utilized in food analysis firstly using ***Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)*** method which allows protein separation by mass in electrical field. The current version of SDS-PAGE was described by Ulrich K. Laemmli in 1970 (Laemmli, 1970). SDS-PAGE has been commonly used in the separation of cereal and milk proteins (Strange, Malin, Van Hekken, & Basch, 1992).

After the commercial introduction in 1989, SDS-PAGE was replaced by ***Capillary Electrophoresis*** to develop new protein analysis methods in food samples (Lindeberg, 1996). This technique separates ions by their electrophoretic mobility in an applied voltage. Capillary electrophoresis has been used in important applications in food analysis such as the detection of food-borne pathogens (Shin, Hwang, Chung, & Jung, 2010), contamination (Vallejo-Cordoba & Gonzalez-Cordova, 2010), and genetically modified organisms (Guo, Qiu, Xiao, & Chen, 2009).

1.1.4. Immunoassay Methods

Immunoassay techniques have been developed by using highly specific and sensitive nature of immunological reactions. They have been used in food industry for detecting

naturally occurring constituents, pesticide residues, antibiotics and microorganisms. Immunoassays are based on the use of the biological mechanism to produce specific antibodies that interact with foreign substances (Samarajeewa, Wei, Huang, & Marshall, 1991).

The ***Enzyme-Linked Immunosorbent Assay (ELISA)*** was first developed in 1972 by Peter Perlman and Eva Engvall at Stockholm University in Sweden (Engvall, 1972). ELISA is an alternative technique to the use of costly, sophisticated equipments and is still maintaining the sensitivity and specificity with its short analysis time. It has been widely used for allergen analysis in foods (Poms, Klein, & Anklam, 2003); determination of aflatoxins (Leszczynska, Maslowska, Owczarek, & Kucharska, 2001) and detection of *Salmonella* in meat ((Farzan, Friendship, & Dewey, 2007)

1.1.5. Microbiological and Genetics Methods

Microorganisms are very important in food systems. In addition to their role in the production of fermented products like wine, beer, yoghurt and bread etc., microbes are the major focus in food safety. Pathogens are easily transmitted through food. Louis Pasteur is considered as the founder of food microbiology. In 1857, he showed that the souring of milk was caused by microbes (Shetty, Paliyath, Pometto, & Levin, 2005). By means of his works on microbiology, the food microbiology field began to develop more rapidly. Traditional microbiological methods rely on the growth of pathogens in culture media, then the isolation and the identification of the species by biochemical and serological methods. These methods are mostly time consuming and laborious (Fratamico, Bhunia, & Norfolk, 2005).

In the past 30 years, the advancements in technology let the researchers to develop rapid nucleic acid based genetic methods. The most important of these nucleic acid-based methods are Polymerase Chain Reaction (PCR) and Real-Time PCR. PCR based techniques rely on the amplification of the target DNA and the detection by gel electrophoresis, fluorescent probes (Nemati, Hamidi, Dizaj, Javaherzadeh, &

Lotfipour, 2016). The development of PCR technique has revolutionized molecular biology research in every field. And afterwards, with real-time PCR, the quantitative detection of microorganisms in a very short time became possible.

1.2. Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful and effective analytical technique which has been widely used to study the structure and the confirmation of molecules, interaction and dynamics between different molecules, the composition of chemical and biochemical solutions qualitatively and quantitatively.

NMR spectroscopy has several advantages over the commonly used analytical techniques like high pressure liquid chromatography (HPLC), Raman spectroscopy, mass spectroscopy (MS), gas chromatography (GC). NMR spectroscopy allows non-destructive, quantitative analysis of liquid and solid samples requiring very small sample volumes. It is also characterized by a short analysis time and a high reproducibility (Kumar, Singh, Bauddh, & Korstad, 2015).

The size of the molecules that can be analyzed by NMR spectroscopy may vary from a small organic molecule to proteins which are several kDA in molecular weight.

1.2.1. Basic Theory of NMR

The basic principle of nuclear magnetic resonance relies on the magnetic properties of the nucleus. If a sample is placed in a magnetic field and is exposed to radiofrequency (RF) pulse, the nuclei of the sample absorbs the energy. Absorption of this energy depends on the type of the nucleus (i.e. ^{1}H, ^{13}C etc.) and the types of the chemical environment of nucleus, because different protons of the nucleus (e.g. methyl and hydroxyl group of methanol) absorb the energy at different frequencies.

Positively charged nucleus spins and has a magnetic moment which creates a magnetic field (Figure 1.1). Nuclear spin is a form of angular momentum possessed by the nuclei. The nuclear spin value is characterized by a spin number. The nuclei of an even number of neutrons and protons have zero nuclear spin. However, the nuclei which have an odd number of protons and neutrons have non-zero spin numbers. For example, the nuclei which are used in the most practical applications of NMR have ½ spin numbers. If the magnetization is not parallel to the magnetic field, the magnetization precesses about the direction of the magnetic field with an angular frequency of ω ($\omega=2\pi\nu$, where ν is the frequency in Hz), which is called Larmor frequency (Mlynarik, 2017).

If the nucleus is put in a larger magnetic field, magnetic direction of the nucleus flip from one direction to the other. The absorbed energy by the nuclear spins generates a voltage and this voltage is amplified, detected and the signal is displayed as a Free Induction Decay (FID). Then, the frequency domain spectrum can be obtained by computing the Fourier transform of the signal-averaged FID (Figure 1.2).

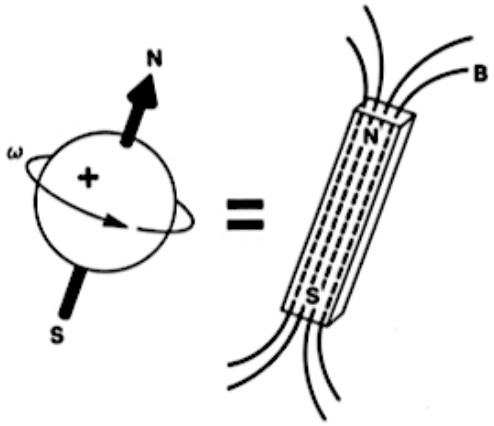


Figure 1.1. The charged nucleus rotating with angular frequency creates a magnetic field B and also spin at a frequency associated with the magnetic field strength

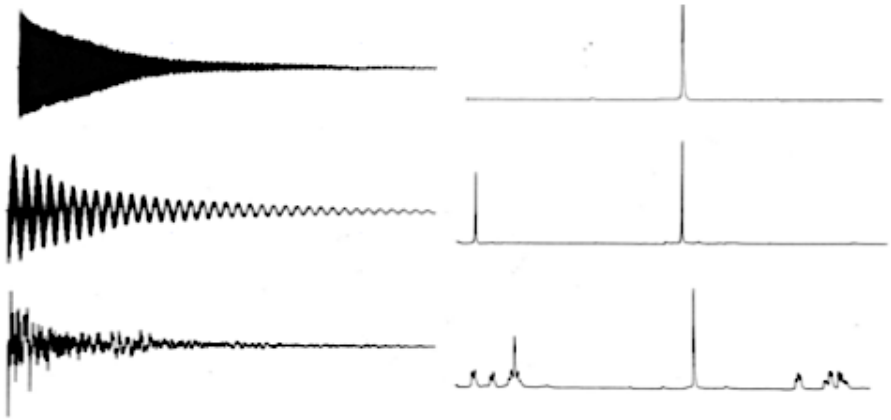


Figure 1.2. The sample FIDs are on the left side and their Fourier transforms are on the right side

1.2.2. NMR Instruments

NMR instruments can be categorized according to their resolution (or magnetic field strengths), high-field NMR and low-field NMR. Resolution is dependent on the homogeneity of the magnetic field in the region that the sample is placed and also on the magnetic field strength. Low cost, low field instruments which are based on the permanent magnets, are generally restricted to about 2 T. The need for the higher resolution and sensitivity has speeded up the development on the superconducting magnets since 1970s and approached the current 23.5 T (1 GHz) stable magnetic field.

1.2.2.1. High-Field NMR Instruments

High field NMR spectrometers have superconducting magnets which provide better field strength and better homogeneity. These properties make the high field NMR spectrometers very expensive instruments. The most important parts of the high field instruments are the magnet, the probe-head, the transmitter, the receiver and the computer as shown in Figure 1.3 (Friebolin, 2005).

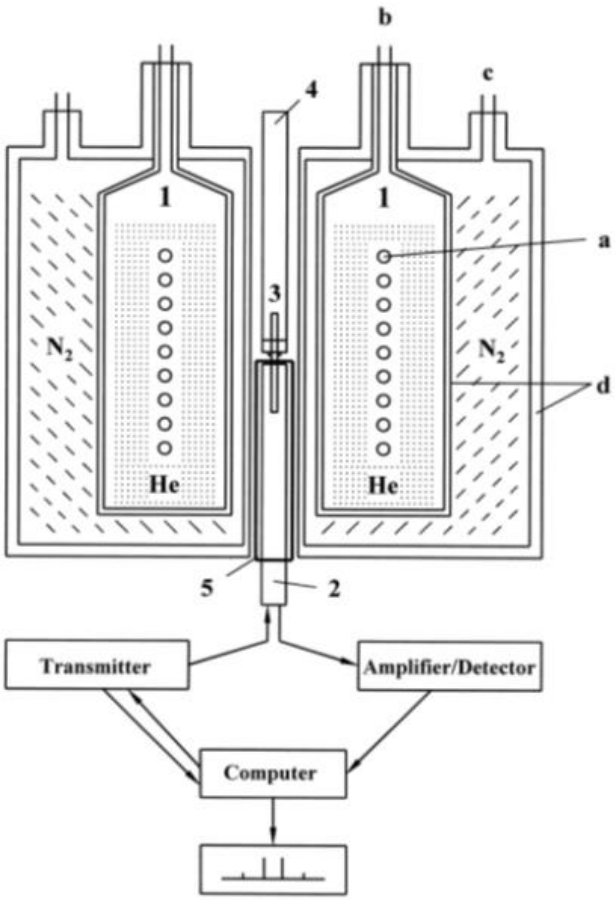


Figure 1.3. Components of an NMR high-field instruments with cryomagnet. 1, cryomagnet assembly; a, magnet coils; b and c, filling columns for liquid helium and liquid nitrogen respectively; d, vacuum jackets of the inner and outer Dewar vessels; 2, probe-head; 3, sample tube; 4, sample changer; 5, shim coils (Friebolin, 2005).

The transmitter generates the radio frequency which is used to excite the spins. Then the FID signal is amplified by the phase-sensitive detector. This FID signal is digitized and then Fourier transformed to obtain NMR spectrum.

High-field NMR spectrometers provide invaluable chemical information (i.e. composition and structure) in the analysis of foods. However, kilometers of

superconducting wires are used to manufacture high-field magnets which may cost up to $1 million. At the same time, these superconductors require very low temperature to operate and need cryogenic fluids like liquid nitrogen and liquid helium (Becker, 2000). Because below superconducting critical temperature, the winding material turns a superconductor from a normal resistive state. This causes high operating cost, makes need of bigger space and highly trained staff.

These drawbacks remarkably prevent the use of high-field NMR spectrometers in food related research and production factories.

1.2.2.2. Low-Field NMR Instruments

Instead of superconducting magnets, low-field benchtop spectrometers use permanent magnets which do not need any cryogens. Instead of the first two parallel plate design of the permanent magnets, the cylindrical Halbach design became more popular in the design of benchtop NMR spectrometers (Figure 1.4) (Mitchell, Gladden, Chandrasekera, & Fordham, 2014; Halbach, 1980; Gouilleux, Thesis: Gradient-based methods on a benchtop spectrometer. New perspectives for low-field NMR spectroscopy, 2017)

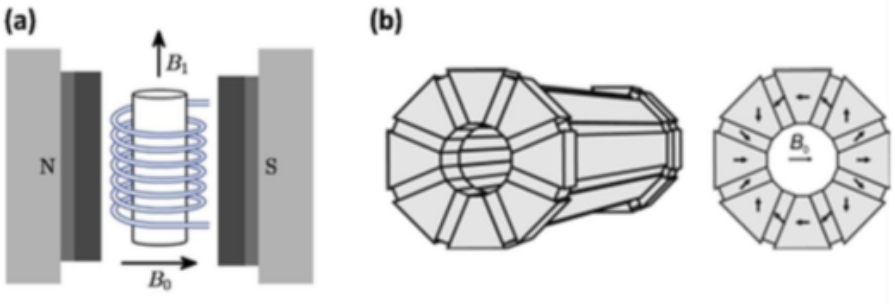


Figure 1.4. a) Parallel plate magnet design. The gap between the plates determines the magnetic field strength, B_0. The sample is placed in a solenoid radio frequency (RF)

coil. B_1 field is created to the vertical direction of B_0 field *(Mitchell, Gladden, Chandrasekera, & Fordham, 2014)*. b) Cylindrical Halbach design. Series of small polarized magnetic blocks are placed in a circular pattern *(Danieli, et al., 2014)*.

Benchtop NMR spectrometers are compact and cheaper than the high-field NMR instruments. Because they don't require cryogens, they have lower operating and maintenance costs. They are also very robust instruments. Because of the use of low static magnetic field, spectral resolution is lower and the peaks in the spectrum are shown broader and overlapped. However, being more reachable in the laboratories and production sites, benchtop NMR spectrometers are becoming more and more important for the process monitoring and food analysis such as composition, adulteration, and authentication analysis.

1.2.3. Use of NMR as an Analytical Technique

1.2.3.1. NMR Relaxometry

1.2.3.1.1. Time Domain NMR

Time Domain NMR (TD-NMR) is based on the same principle with pulsed NMR but it studies the relaxation behavior of materials directly from FID signal without Fourier transform (Mauri, et. al, 2013). Compositional changes during processing and storage occur in time in food samples. The study of these time-dependent changes requires TD-NMR and the most suitable parameters for monitoring these evolutions are the longitudinal (T_1) and transverse (T_2) relaxation times (Spyros & Dais, 2013). Because the TD-NMR are non-invasive, non-destructive experiments, and the devices are easy-to-use, low cost and portable, it is widely used in food analysis in recent years (Kirtil, Cikrikci, McCarthy, & Oztop, 2017).

TD-NMR devices were used to detect adulteration in vegetable oils by used frying oil (Zhang, Saleh, & Shen, 2013), adulteration of honey by high fructose corn syrup (Ribeiro, et al., 2014) and urea and hydrogen peroxide adulteration of milk samples

(Santos, Pereira-filho, & Colnago, 2016) by examining the changes in T_2 relaxation times. Also, solid fat content measurements of milk products (Alekseev & Khripov, 2015) and the determination of moisture content of meat products (Pereira & Colnago, 2012) by TD-NMR devices became very standardized methods in food industry.

1.2.3.1.2. Fast Field Cycling NMR

Fast field cycling (FFC) NMR has been introduced only couple of years after Felix Bloch and Edward Purcell performed first NMR experiments. However, it is being more popular in recent years as the new applications are being discovered. FFC NMR relaxometry measures the longitudinal spin relaxation rate, $1/T_1$, over a wide range of frequencies, from 0.01 MHz to 42 MHz. FFC NMR relaxometry provides important information on the molecular motion of water molecules in different environments (Anoardo, Galli, & Ferrante, 2001). It has very wide applications in food industry such as monitoring of spoilage of milk by measuring denatured proteins or monitoring the loss of water in meat during storage (Steele, Korb, Ferrante, & Bubici, 2016; Bajd, Gradisek, Apih, & Sersa, 2016).

1.2.3.2. NMR Spectroscopy

1.2.3.2.1. 1D and 2D NMR

1D and 2D experiments can be done using different nuclei such as ^{1}H, ^{2}H, ^{13}C, ^{19}F, ^{31}P. In 1D NMR, there is preparation period and the detection period. It is the fact that nuclei (^{1}H, ^{13}C, ^{31}P etc.) in the same molecule have slightly different resonance frequencies. Because the nuclei are surrounded by the electrons and in the static magnetic field, these electrons cause shielding effect which decreases the resonance frequencies of nuclei. This varies in every structural fragments of molecules (e.g. CH_2, CH_3 etc.) and their substituents (e.g. COOH, OH, NH etc.). The difference in the resonance frequencies is defined as chemical shift and expressed in ppm scale which

is independent of the spectrometer operating frequency (Mlynarik, 2017). A sample spectrum can be seen in Figure 1.5.

Spin-spin coupling or *J*-coupling is one of the phenomena that affects the appearance of NMR spectra. It determines the number of lines in multiplets. The number of spectroscopic lines in multiplets is defined as n+1, where n is the number of neighboring nuclei. As it can be seen in Figure 1.5, the number of neighboring protons is 3 for CH_2 group and 2 for CH_3 group. Therefore, there will be 4 (n+1) spectroscopic lines for CH_2 and 3 (n+1) spectroscopic lines for CH_3. *J* is the coupling constant.

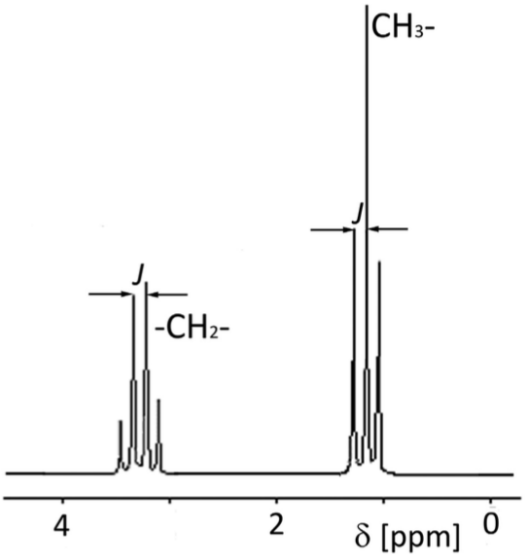


Figure 1.5. The NMR spectra of ethyl (CH_3CH_2) group *(Mlynarik, 2017)*

In situations where the samples are complex 2D NMR experiments could become more helpful. The introduction of an evolution and mixing time period between the preparation and the detection periods of 1D experiments is the basis of 2D NMR

experiments (Spyros & Dais, 2013). 2D NMR experiments consist of series of 1D experiments. For example, to get a 2D-COSY spectra, a series of 512 1D spectra is recorded. Afterwards, the data in each time period are subjected to two Fourier transformations and stacked to obtain 2D NMR spectrum (Macomber, 1998) (Figure 1.6). The most important advantage of 2D NMR spectroscopy is to see the interactions between different nuclei. By this method, it can be found which nuclei couple with which in 1D NMR spectra.

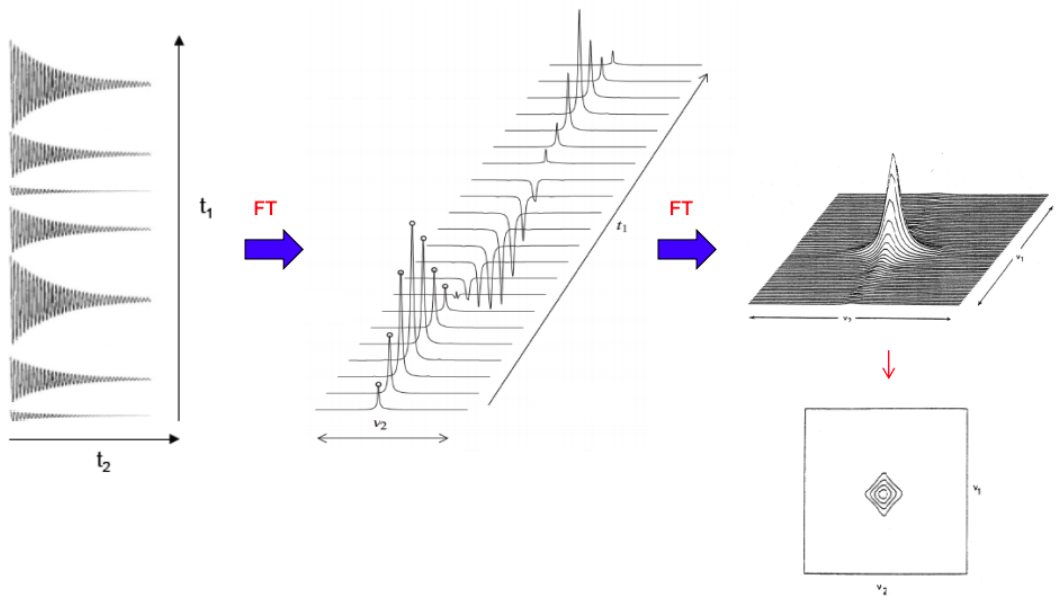


Figure 1.6. 2D NMR experiments procedure (Department of Chemistry, Syracuse University, 2018)

There are several types of 2D NMR spectroscopy such as Correlation Spectroscopy (COSY), Total Correlation Spectroscopy (TOCY) for 2D homonuclear correlations

and Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Quantum Coherence (HMQC) for 2D heteronuclear correlations.

2D-COSY NMR spectra of a sample molecule (1,3-dinitrobenzene) can be seen in Figure 1.7. The black line shows the diagonal peaks where 2D spectra match with 1D spectra. Blue and red squares indicate the cross peaks where coupled protons are found. H_1 proton is coupled to H_3 proton and H_3 proton is coupled to H_4 proton.

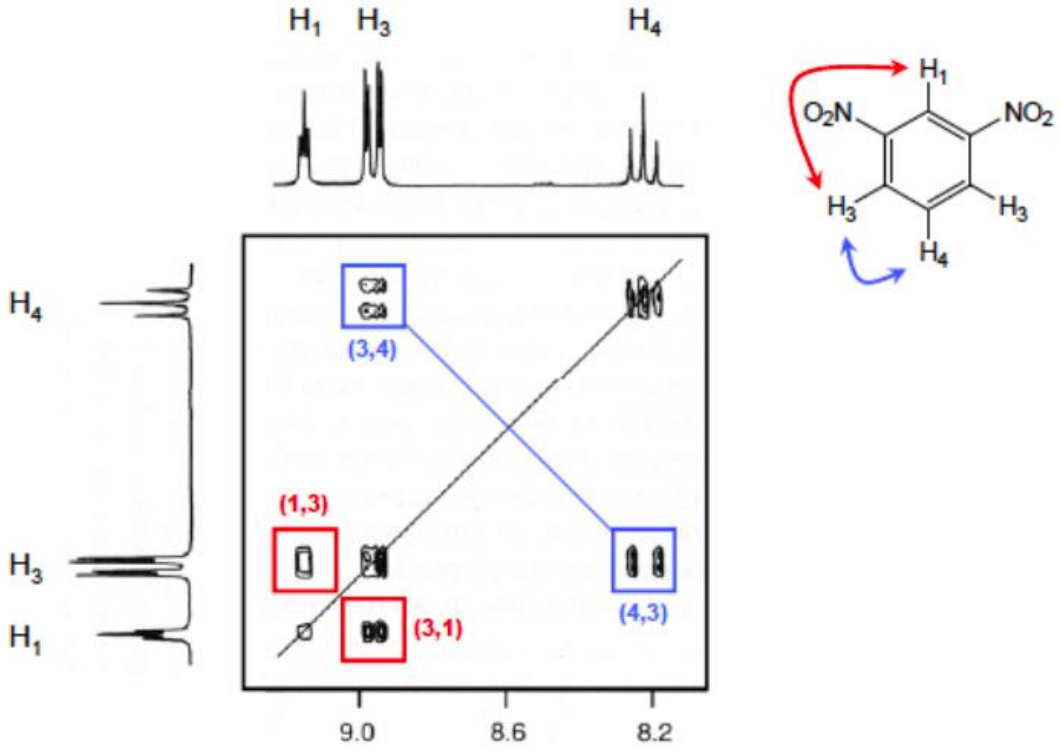


Figure 1.7. The sample spectra for 2D-COSY NMR experiment (Department of Chemistry, Syracuse University, 2018)

1.2.3.2.2. Gradient-Based Solvent Suppression Methods

Deuterium is the isotope of hydrogen (^2H) and resonates at very different frequency than the normal hydrogen and it is not seen in a ^1H spectrum. Since the precession frequency is different from the normal hydrogen precession frequency and rf coils are usually designed specific to a proton, in a regular ^1H experiments deuterium protons are not excited and thus do not produce a signal. Therefore, deuterated solvents such as D_2O are preferred to be used in NMR experiments to prevent overlapping between the solvent peak and the peak of interests. In that regard solvent suppression methods are crucial in NMR experiments of food samples because of the high water content of the majority of the food products. Solvents like water are generally small symmetrical molecules and have long T_1 relaxation times, therefore, have strong signals. Because of the limitations of the analog-to-digital converter, higher intensity of the solvent signal may cause problems in the detection of less concentrated molecules (Hatzakis, 2019).

Numerous solvent suppression methods have been developed in the literature. All these methods have three steps that contain preparation, excitation and readout blocks.

The most common suppression method is the saturation block (Figure 1.8.a) (Hoult, 1976). This block is efficient for solvent peaks which are located far from the peak of interest. The other popular preparation blocks are WET (water suppression enhanced through T_1 effects) block (Figure 1.8.b) and WET-180 block (Figure 1.8.c) (Ogg, Kingsley, & Taylor, 1994). They have series of selective pulses to suppress water signal.

90° hard pulse (Figure 1.8.d), composite 90° pulse (Figure 1.8.e) and Sat-180 block (Figure 1.8.f) and 1D version of NOESY (nuclear Overhauser effect spectroscopy) pulse sequence (Figure 1.8.g) are the most common types of excitation blocks. These excitation blocks are used to overcome the faraway solvent effect and to get narrower solvent peak (Gouilleux, Charrier, Akoka, & Giraudeau, 2017).

The FID (Figure 1.8.h) is the most common used readout block to detect signals except the suppressed solvent signal.

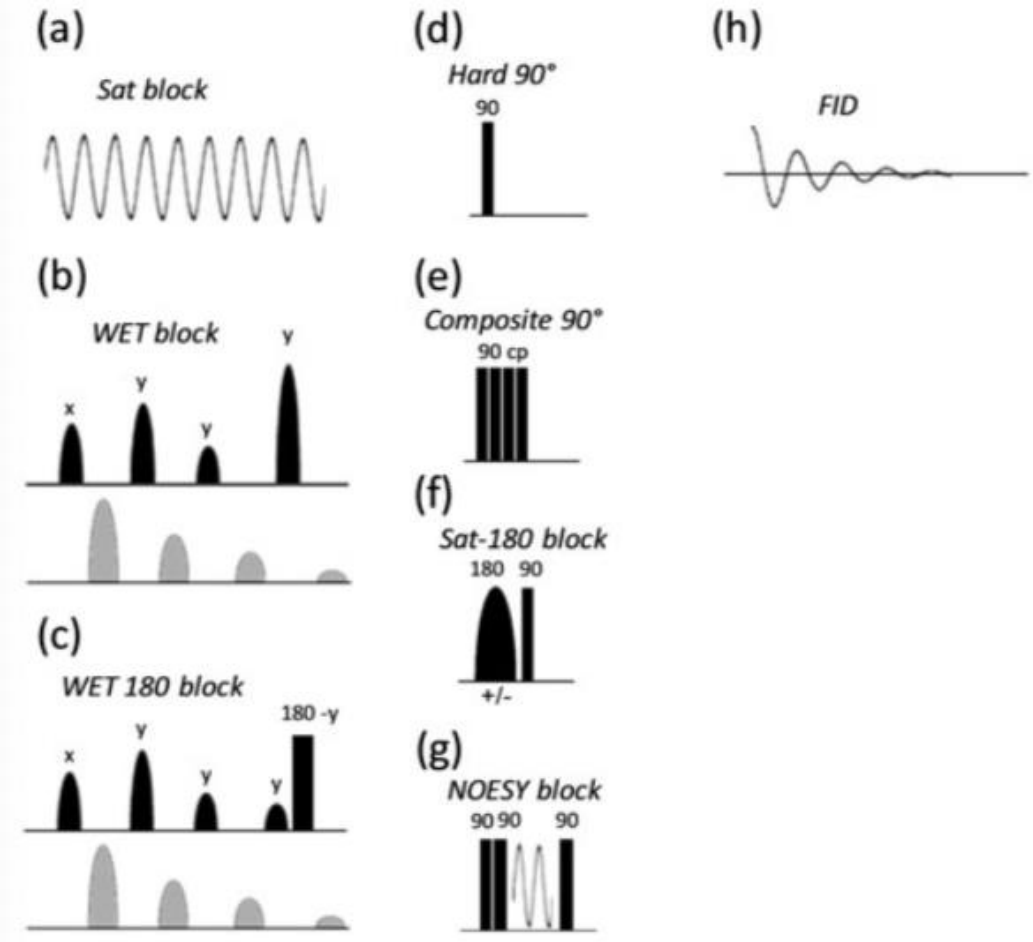


Figure 1.8. The most common preparation, excitation and readout blocks used in solvent suppression methods (Gouilleux, Charrier, Akoka, & Giraudeau, 2017).

1.2.3.2.3. Quantitative NMR

Quantitative NMR (qNMR) was first introduced as an analytical tool for quantitative analysis in 1963 (Jungnickel & Forbes, 1963). ^{1}H qNMR is the most widely used

technique for the quantitative measurement of multi-components in complex mixtures without separation of the individual components (Bharti & Roy, 2012). The measurement uncertainty for qNMR has been reported as 1.5% for 95% confidence interval (Malz & Jancke, 2005). This is quite acceptable for precise and accurate quantification. For accurate quantification, pulse sequence, acquisition parameters (repetition time, acquisition time, signal-to-noise ratio, shimming, temperature, solvent suppression techniques) and post-processing parameters (phase correction, baseline correction, integration, deconvolution) should be chosen very carefully.

1.2.3.2.3.1. Referencing Techniques in qNMR

In qNMR analysis, a reference material is required to measure the concentration of the analyte. This analyte should be in very pure form, inert, stable, non-volatile and not expensive. There are 3 different referencing techniques which are internal standard, external standard and calibration curve method.

1.2.3.2.3.1.1. Internal Standard

One of the most used referencing techniques in qNMR is to use an internal standard (Giraudeau, Tea, Remaud, & Akoka, 2014). That also removes the need to construct a calibration curve for the experiment. In this method, a known concentration of a reference material is dissolved in a known volume of analyte solution for quantitative analysis (Bharti & Roy, 2012). The most important parameters for choosing the internal reference are its solubility, the absence of chemical interactions and the similar relaxation times with the analytes and a distinct chemical shift.

The most widely used internal standards are 3-(trimethyl-silyl)-1-propane sulfonic acid sodium salt (DSS), Tetra methyl silane (TMS), 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP), maleic acid, sodium acetate and formic acid.

1.2.3.2.3.1.2. External Standard

External standard is used when the addition of the reference material is avoided into the analyte solution. For this purpose, special NMR tube with an extra capillary insert is used. This extra capillary is filled with the standard material (Figure 1.9) (Bharti & Roy, 2012).

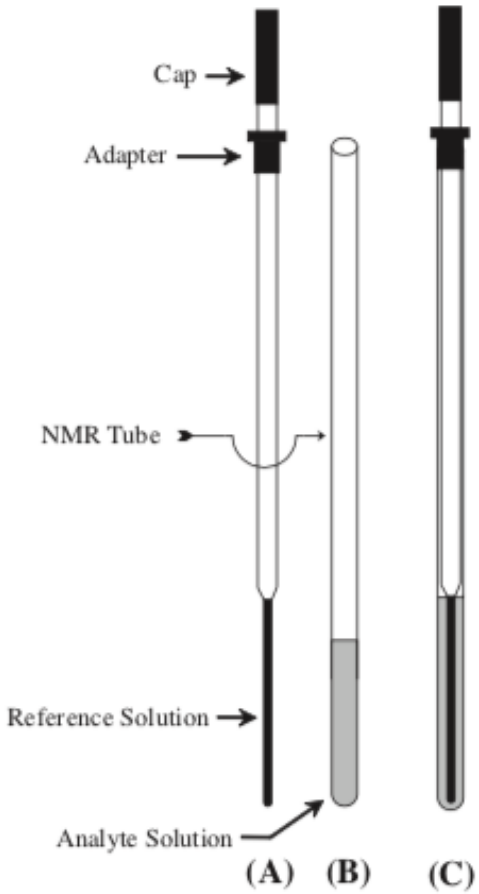


Figure 1.9. An extra capillary insert and NMR tube for external standard method, (A) capillary insert, (B) NMR tube, (C) assembled for the experiment.

1.2.3.2.3.1.3. Calibration Curve

In calibration curve method, analyte solution itself is used instead of internal and external reference material. A calibration curve is plotted and the NMR experiments is performed with different dilutions of the analyte solution. The calculated integral areas from these experiments are compared with the calibration curve to find the concentration of the analyte.

1.2.3.2.3.2. Quantification Methods

1.2.3.2.3.2.1. Relative Quantification Method

For the quantification, integral areas of the peaks (area under the peak) are used. The molar ratio M_X/M_Y of the compounds is calculated by using the integral areas *A* and the number of contributing protons *N* (Eq. 1) (Figure 1.10).

$$\frac{M_X}{M_Y} = \frac{A_X}{A_Y} * \frac{N_Y}{N_X} \qquad \text{(Eq. 1)}$$

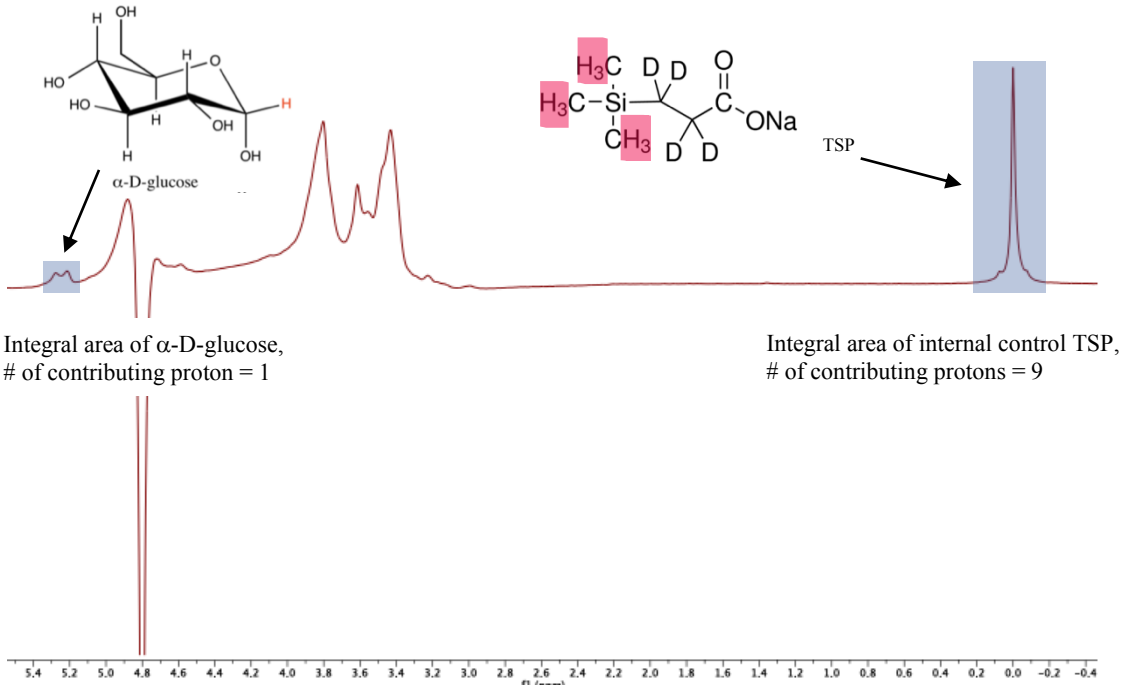


Figure 1.10. NMR spectra of glucose with 43 MHz (^{1}H) benchtop NMR

The relative quantitation method is mostly used in the quantitation of the ratios of isomers. The molecular weights of the compounds are not needed to be known (Holzgrabe, 2010).

1.2.3.2.3.2.2. Absolute Quantification Method

In the absolute quantitation method, internal standard or external standard (Figure 1.10) are added gravimetrically to calculate;

$$C_x = \frac{A_x/N_x}{A_{Std}/N_{Std}} * \frac{MW_x}{MW_{Std}} * C_{Std} \qquad \text{(Eq. 2)}$$

C, *A*, *N*, and *MW* are the concentration in mg/g, the integral area in a fully relaxed ^{1}H NMR spectrum, the number of hydrogens contributing to the signals, and the molecular weight. The *x* index refers to the analyte and the *Std* index refers to the internal or external standard.

1.2.4. Applications of NMR Spectroscopy in Food Systems

Despite the fact that NMR instruments date back to 1940s, because of the complexity of food matrices, lack of the expertise of food scientists, high equipment cost, operating expenditures, size and infrastructure needs, NMR found its place in food research and industry after the first benchtop NMR relaxometry instrument was introduced by Bruker in 1973. Although benchtop relaxometry instruments have been in use in food research over 40 years, benchtop NMR spectrometers are very recent and their potential in food industry is largely unexplored (Blümich & Singh, 2018). Benchtop NMR spectrometers can be used for process control purposes either by online-monitoring in a flow cell or in an NMR tube (Dalitz, Cudaj, Maiwald, & Guthausen, 2012; Meyer, Kern, Zientek, Guthausen, & Mainwald, 2016).

Due to the fact that foods contain organic compounds, with protons originating, e.g., from water, carbohydrates, fat and proteins; proton NMR has become the most common type of NMR to determine these abundant food components. Its non-destructive and non-invasive nature has made NMR spectroscopy very popular in the fields of food chemistry, food microbiology and food packaging (Pentimalli, et al., 2000; Nestor, et al., 2010; Picone, et al., 2011). The most studied areas are water/moisture contents, lipid content, adulteration and authentication of foods (Keeton, et al., 2003; Chen, Wei, & Zhang, 2010; Hu, Wang, Wang, & Lu, 2017; Gouilleux, Marchand, Charrier, Remaud, & Giraudeau, 2017).

Keeton et al. (2003) determined the moisture and fat content of meat products by NMR spectroscopy. Chen et al. (2010) compared differential scanning calorimetry and NMR

to explore the role of water during extrusion cooking of soybeans, and they found the distribution of water and the water content results were consistent between two methods. Hu et al. (2017) investigated the adulteration of paprika powders by Sudan dye I by NMR spectroscopy. Sudan dye I is carcinogenic and added to spices illegally to give fresh appearance. Gouilleux et al. (2017) authenticated 6 different edible oils by benchtop ultrafast 2D NMR spectroscopy.

1.2.5. NMR Spectroscopy in Continuous Mode

1.2.5.1. Flow NMR

It is crucial to understand the reaction kinetics, its products and by-products, completion time, efficiency and the yield of the reactions (Elipe & Milburn, 2016). NMR is becoming very important tool to monitor chemical and enzymatic reactions. By its quantitative and non-destructive nature, NMR spectroscopy can provide this detailed information during the processes. Sampling can be done during the reaction and measurements can be made in an NMR tube. However, it is labor intensive and also it neglects many effects like mixing and stirring rate. Also, reaction continues during sampling. Thus, the best option is to use NMR spectroscopy in continuous mode by using a flow cell. By this method, NMR spectroscopy can be directly connected to reactors.

Flow NMR spectroscopy was mostly used in reaction monitoring in flow chemistry for organic synthesis (Foley, et al., 2014). It has very limited applications in food analysis. Rezzi et al. classified olive oils according to their geographical origin and processing by high throughput flow ^{1}H NMR (Rezzi, et al., 2005).

There are three factors that affect to set up an experiment in flow NMR. These are in-flow effect, out-flow effect and polarization build-up.

1.2.5.2. Factors Affecting Flow NMR

1.2.5.2.1. In-Flow Effect

During flow NMR, sensitive volume in measured area always changes while excited nuclei are flowing out and unexcited ones are flowing into. This causes high signal reduction and a wrong quantification by integral area measurements. This is called in-flow effect (Hall, et al., 2016). Therefore, at the beginning of the flow NMR spectroscopy experiments, it is very important to determine the experiment parameters like repetition time and the flow rate.

1.2.5.2.2. Out-Flow Effect

Out-flow effect impacts the resolution of NMR spectra. The excited nuclei show the transverse magnetization and pass the sensitive volume before the completion of the acquisition. This causes higher transverse relaxation rate and line-broadening in spectra (Gouilleux, Thesis: Gradient-based methods on a benchtop spectrometer. New perspectives for low-field NMR spectroscopy, 2017).

1.2.5.2.3. Polarization Build-up

Increasing the flow rate decreases the time spend in the sensitive volume of the flow cell. This causes shorter T_1 in flow conditions than static conditions and creates sensitivity enhancements. However, this is true as long as the excited spins are refreshed by the fully polarized ones. Polarization build-up is seen when the sample flows through the pre-polarization volume. The polarization volume needs to be considered carefully in the NMR instrument (Gouilleux, Thesis: Gradient-based methods on a benchtop spectrometer. New perspectives for low-field NMR spectroscopy, 2017).

1.3. Objective of The Study

Benchtop NMR spectrometers have been in the market for some time. Magritek, Oxford Instruments, Thermo Fisher Scientific, Nanalysis are some of the leading manufacturers of these systems. However, utilization these benchtop NMR spectrometers for food analysis is still not that common. Water signal being a challenge in NMR spectroscopy is an important reason for that and most of the food-omics studies that deals with structural characterization of foods involves High Resolution NMR instruments with deuterated solvent. In this dissertation, the goal is to use benchtop NMR spectroscopy in different systems both in continuous and static modes and show the potential of these systems in further applications. Four different systems were evaluated for this purpose. The specific objectives of the study could be listed as follows:

1. To monitor an enzymatic reaction (invertase catalyzed sucrose hydrolysis) by NMR spectroscopy in continuous flow mode
2. To monitor lactose hydrolysis in a model system and regular milk using NMR spectroscopy in continuous flow mode
3. Use the information obtained in objective 2 to find out the differences between 36 milk samples obtained from Turkey and France through multivariate analysis methods that were applied on NMR spectroscopy data

To use a combinatorial approach of spectroscopy and relaxometry for evaluating honey samples adulterated with fructose syrup

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

D-(+)-Glucose, D-(−)-Fructose, Sucrose sugar and 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid (TSP) were provided from Sigma-Aldrich Co. (St. Louis, MO, USA). HPLC grade water from a water purification system (Nanopure Infinity, Barnstead International, IA) was used for the preparation of the solutions. Acetic acid glacial from VWR, Radnor, PA, USA was used for the pH adjustment. Invertase (≥300 units/mg solid) for the sucrose hydrolysis was supplied from Sigma-Aldrich Co. (St. Louis, MO, USA).

D-(+)-Lactose monohydrate were purchased from Fluka BioChemika, Switzerland. Lactase enzyme (Maxilact® LGi 5000) for lactose hydrolysis was supplied from DSM, (Heerlen, The Netherlands). Imidazole and D-(+)-Galactose were provided from Sigma-Aldrich Co. (St. Louis, MO, USA). Organic whole milk was supplied from the local French supermarket for the lactose hydrolysis experiments. All the materials were weighed by Ohaus® Discovery DV215CD Analytical Balance (Ohaus® Europe GMBH, Greifensee, Switzerland).

For the authentication of milks, 36 different samples of milk (whole milk, organic whole milk, semi-fat milk, lactose-free milk, goat milk) were purchased from local Turkish and French supermarkets.

For honey adulteration experiments, organic honey was purchased from local French supermarket. High fructose corn syrup (F42) was supplied from Mondelez International, Turkey.

2.2. Methods

2.1.1. Real-time Benchtop NMR Spectroscopy for the Online Monitoring of Sucrose Hydrolysis

2.2.1.1. Sample Preparations

10% (w/w) glucose, fructose and sucrose solutions and their mixtures (Glucose + Fructose+ Sucrose with a concentration of 10% (w/w)) were prepared separately by dissolving the sugars in non-deuterated HPLC grade water in a 50 mL tube. During solution preparation, 1% (w/w) TSP was added to all sugar solutions as the internal standard. All solutions were stirred for 2 minutes for complete dissolution of sugars and TSP. pH of the sugar solutions was adjusted to optimum working pH of the invertase enzyme (pH=4.5) with glacial acetic acid. TSP with ^{1}H chemical shift of 0.0 ppm was chosen as the chemical shift and reference concentration.

All NMR spectroscopy experiments for this dissertation were completed in the Laboratory CEISAM (Interdisciplinary Chemistry: Synthesis, Analysis, Modelling) at the University of Nantes, France. A part of the funding of the research came from EURELAX COST Action through Short Term Scientific Mission program.

2.2.1.2. NMR Experiments

NMR experiments were performed on a low-field spectrometer operating at a 43 MHz frequency with a compact permanent magnet based on the Halbach design (Magritek Spinsolve, Wellington , NZ) This spectrometer is equipped with a gradient coil through the transversal plane of the NMR tube that can produce a maximum field gradient of 0.16 $T.m^{-1}$ and also has an external lock system which allows the use of non-deuterated solvents. For on-line monitoring, the system includes a glass flow cell with 5 mm outer diameter, a peristaltic pump (Reglo Digital, Ismatec, Wertheim, Germany) and PEEK tubing (Figure 2.1). The inner diameter of the flow cell is 4 mm in the measurement region (10 mm in length) and decreases to 1 mm in upper and

lower regions of the measurement region to shorten the flow time during the experiment (Figure 2.2). A heating plate (RCT Basic, IKA-Werke GmbH & Co. KG, Staufen, Germany) was added to the system to control the temperature of the hydrolysis reaction. The flow rate was also optimized as will be explained afterwards.

Figure 2.1. System setup for online reaction monitoring

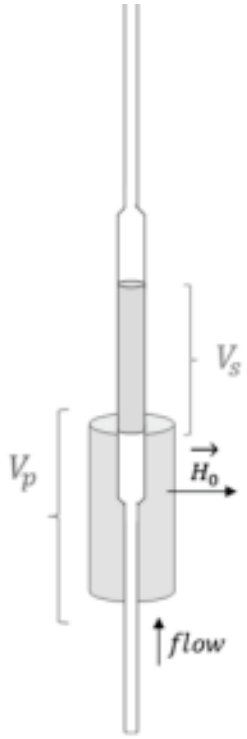


Figure 2.2. Scheme of the flow cell used in the NMR experiments. H_0 represents the static magnetic field vector. V_s and V_p are the sensitive volume and pre-polarization volume, respectively.

2.2.1.2.1. Spin-Lattice Relaxation Time (T_1) Measurements

An inversion recovery sequence was used for measuring longitudinal relaxation times, T_1 using an inversion time range, *t* of 0.1-10,000 ms with 15 points. The recovery curve was fitted using Equation 3.

$$I = I_O(1 - 2 \cdot exp\left(-\frac{t}{T1}\right)) \quad \text{(Eq. 3)}$$

2.2.1.2.2. NMR Spectroscopy Experiments

NMR experiments were carried out at 0.5 ml/min flow rate. That rate was found after preliminary experiments as will be discussed later. The experiments were performed at 29 °C since this corresponded to the temperature at which the magnet stability was optimal. The determination of appropriate solvent suppression pulse sequence was crucial while working with non-deuterated solvents especially water. The gradient coil in the spectrometer enabled us to use the recently developed solvent suppression methods like the WET-180-NOESY pulse sequence (Figure 2.3) (Gouilleux, Charrier, Akoka, & Giraudeau, 2017). This sequence gives an optimal solvent suppression for small molecules on a benchtop spectrometer, leading to a lower and narrower water signal with a clean phase with a minimal impact on nearby peaks.

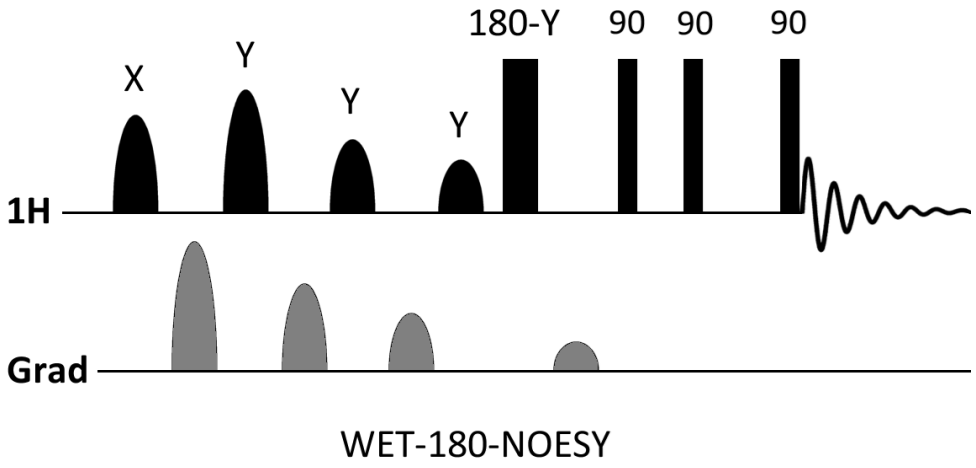


Figure 2.3. WET-180-NOESY pulse sequence used for solvent suppression

The Signal-to-Noise Ratios (SNR) were calculated using the SNR calculation script of MestReNova software (Mestrelab Research Version 12.0.3, Spain) by measuring

the height of the peaks of interest and dividing these values by the noise level. The SNR has been calculated and measured as 260 at the beginning of the experiments. During the reaction, the concentration of sucrose decreased to 50 mg/g water starting from 300 mg/g. SNR at this concentration was calculated as 125. The limit of detection was found as 20 mg/g –corresponding to an SNR of 50– water by extrapolation. Here, 128 scans were found sufficient to yield an acceptable SNR for quantification. The 1D ^{1}H spectra were obtained with 128 scans for a total experiment time of 12 min. The 90^{o} pulse angle was accomplished by a pulse length of 6.7 µs at 0 dB. The FIDs were obtained with 16 K points, a dwell time of 200 µs, and a repetition time of 6 s, corresponding to 5 times the longest T_1 (see below). The 1D data were processed with MestReNova software. All spectra were processed with a 0.2 Hz exponential apodization, an automatic phase correction and an automatic baseline correction via a Whittaker smoother algorithm. To align all the spectra correctly, TSP was used for ppm-scaling reference and set at 0 ppm. The resulting ^{1}H spectra from the different time points of hydrolysis experiment were stacked too see the progress of hydrolysis.

2.2.1.2.3. Quantitative Analysis

For the quantitative NMR measurements, the glycosidic proton peak of sucrose and the anomeric proton peak of glucose were chosen for hydrolysis follow up and for the quantification. Before peak integration, manual phase correction and manual baseline correction were performed in addition to the automatic corrections. This procedure was found necessary to obtain a similar baseline for all the peaks that were used for integration. The resulting spectra from hydrolysis experiments were rearranged as a superimposed plot. The integral areas of the internal reference (TSP), sucrose and glucose were calculated by integration with the MestReNova software. Deconvolution tools were also evaluated but yielded a slightly lower performance than integration, probably due to the non-ideal line shapes. The integral areas of sucrose and glucose were normalized according to the integral area of TSP. Concentrations of sucrose and

glucose for each time point were calculated using equation 4. The number of protons contributing to the signal for TSP was taken as 9, for glucose and sucrose, it was taken as 1.

$$C_x = \frac{A_x/N_x}{A_{TSP}/N_{TSP}} * \frac{MW_x}{MW_{TSP}} * C_{TSP} \qquad \text{(Eq.4)}$$

C, *A*, *N*, and *MW* are the concentration in mg/g, the integral area in a fully relaxed ^{1}H NMR spectrum, the number of hydrogens contributing to the signals, and the molecular weight. The *x* index refers to the analyte (sucrose or glucose) while the *TSP* index refers to the internal reference.

2.2.1.3. Kinetic Modelling

The change of concentration of sucrose by time during hydrolysis was modelled using a fractional conversion model kinetics approach (Equation 5) to find the rate constant.

$$\frac{C-C_\infty}{C_o-C_\infty} = e^{-kt} \qquad \text{(Eq. 5)}$$

C represents the concentration at time *t*, C_0 and C_∞ represents the initial and equilibrium concentration respectively, and k represents the rate constant.

2.2.2. Monitoring Production of Lactose-Free Milk in a Continuous Flow System by Quantitative Benchtop NMR

2.2.2.1. Sample Preparations

Hydrolysis reactions were performed both for a model system and a real milk sample. As the model solution, a solution of glucose, galactose and lactose was used. 5% (w/w) glucose, galactose and lactose solutions were prepared separately by dissolving in non-deuterated HPLC grade water in a 50 mL tube. As the milk sample, an organic, UHT whole milk (Lactel, France) bought from a grocery store in France was used.

During the model solution preparation, 0.2% (w/w) TSP was added to all sugar solutions as the internal standard. All solutions were stirred for 5 minutes for complete dissolution of sugars and TSP. TSP with ^{1}H chemical shift of 4.81 ppm was chosen as the chemical shift and concentration reference for model lactose hydrolysis experiments.

Because of the complex nature of milk, TSP was masked with the peaks of the fats present in the milk. Therefore, instead of TSP, imidazole with ^{1}H chemical shifts of 7.256 ppm and 8.11 ppm was chosen as the concentration reference for milk lactose hydrolysis experiments. 0.5% (w/w) imidazole was added to milk as the internal standard.

2.2.2.2. 1D NMR Spectroscopy Experiments

Continuous flow system on benchtop NMR instrument was installed as explained before in section 2.2.1.2.

NMR experiments were carried out at 0.5 ml/min flow rate at 29 °C and WET-180-NOESY pulse sequence was used as explained before in section 2.2.1.2.2.

The Signal-to-Noise Ratios (SNR) were calculated as explained before in section 2.2.1.2.2. Here, for model lactose solution hydrolysis and milk hydrolysis, 64 scans

were found sufficient to yield an acceptable SNR for quantification. The 1D ^{1}H spectra were obtained with 64 scans for a total experiment time of 6 min for model lactose solution hydrolysis and 7 min for milk hydrolysis. The 90^{o} pulse angle was accomplished by a pulse length of 6.7 µs at 0 dB. The FIDs were obtained with 16 K points, a dwell time of 200 µs, and a repetition time of 6 s for model lactose solution hydrolysis and 7 s for milk hydrolysis, corresponding to 5 times the longest T_1. The 1D data were processed with MestReNova software. All spectra were processed with a 0.2 Hz exponential apodization, an automatic phase and baseline corrections by a Whittaker smoother algorithm. The signal of the TSP was used for ppm-scaling reference in model lactose solution hydrolysis and the signal of the imidazole was used for ppm-scaling reference in milk hydrolysis and set at 0 ppm. The resulting ^{1}H spectra from the different time points of hydrolysis experiment were stacked too see the progress of hydrolysis.

2.2.2.3. 2D TOCSY NMR Experiments

Since milk is a complex system consisting of many nutrients, to understand the chemical structures better, 2D spectroscopy experiments were also conducted.

2D TOCSY experiments of milk sample was performed with WET-180-NOESY pulse sequence and with ZQ filtering. 4096 x 200 points with 16 scans per experiment were used with a dwell time of 200 µs, with a mixing time of 80ms for the TOCSY spinlock. The 90^{o} hard pulse angle was accomplished by a pulse length of 10.5 µs. The WET 180 NOESY block composed of a Gaussian selective pulse of 90 ms centered on the water signal in combination with 2.5ms trapezoid shaped gradient (Gouilleux, Charrier, Akoka, & Giraudeau, 2017).

ZQ filtering uses 180° CHIRP pulses of 5ms and 2.5kHz of sweeping frequency. A cosine function was applied in the 2 dimensions of the 2D map prior to a double Fourier transform.

2.2.2.4. Quantitative Analysis

The quantitative NMR measurements were performed for hydrolysis follow up by choosing beta anomeric proton peak of lactose, the combined peak of formed glucose and galactose. Before peak integration, manual phase correction and manual baseline correction were performed in addition to the automatic corrections. The resulting spectra from hydrolysis experiments were rearranged as stacked spectra and superimposed plots.

The integral areas of beta anomeric proton peak of lactose, the combined peak of produced glucose and galactose and the internal references, TSP and imidazole for model lactose solution hydrolysis and milk hydrolysis, respectively, were calculated by integration with the MestReNova software. The integral areas were normalized according to the integral area of internal references for each type of experiments.

Concentrations of glucose, galactose and lactose for each time point were calculated using equation 6.

$$C_x = \frac{A_x/N_x}{A_{TSP}/N_{TSP}} * C_{TSP} \qquad \text{(Eq. 6)}$$

C, *A*, and *N* are the concentration in mM, the integral area in a fully relaxed ^{1}H NMR spectrum, and the number of hydrogens contributing to the signals. The *x* index refers to the analyte (glucose, galactose or lactose) while the *TSP* index refers to the internal reference.

The number of protons contributing to the signal for TSP was taken as 9, for imidazole for two peaks in total, it was taken as 3. Because the peaks of glucose and galactose were overlapped, these peaks were surgically phased and separated as explained in results and discussions section to calculate number of protons contributing to the signal and the concentration of glucose and galactose separately.

2.2.3. Authentication of Milks by Benchtop ^{1}H-NMR Profiling and Multivariate Analysis

2.2.3.1. Sample Preparations

0.5% (w/w) imidazole was added to the 36 milk samples (Table 2.1) in 2 mL microcentrifuge tubes as the internal reference. All the samples were stirred for 5 minutes for complete dissolution of imidazole in milk. Then, the samples were transferred into 5 mm NMR tubes and stored in dark and refrigerated before analysis to prevent any microbial growth and milk degradation.

Table 2.1. Types of milks used in the authentication experiments

Sample Name	Country	Fat Status	Sugar Status	Milk Type	Marketing	Processing
WMF-1	France	Whole	Regular	Cow	Conventional	UHT
WMF-2	France	Whole	Regular	Cow	Conventional	UHT
WMF-3	France	Whole	Regular	Cow	Conventional	Pasteurized
WMF-4	France	Whole	Regular	Cow	Conventional	Pasteurized
OMF-5	France	Whole	Regular	Cow	Organic	UHT
OMF-6	France	Whole	Regular	Cow	Organic	Pasteurized
OMF-7	France	Whole	Regular	Cow	Organic	Pasteurized
HFMF-8	France	Half	Regular	Cow	Conventional	UHT
OHFMF-9	France	Half	Regular	Cow	Organic	Pasteurized
HFMF-10	France	Half	Regular	Cow	Conventional	Pasteurized
HFMF-11	France	Half	Regular	Cow	Conventional	Pasteurized
HFMF-12	France	Half	Regular	Cow	Conventional	UHT
OHFMF-13	France	Half	Regular	Cow	Organic	UHT
WMF-14	France	Whole	Regular	Cow	Conventional	UHT
OHFMF-15	France	Half	Regular	Cow	Organic	UHT
HFMF-16	France	Half	Regular	Cow	Conventional	UHT
OMF-17	France	Whole	Regular	Cow	Organic	Pasteurized
OHFMF-18	France	Half	Regular	Cow	Organic	UHT
GMF-19	France	Half	Regular	Goat	Conventional	UHT
GMF-20	France	Whole	Regular	Goat	Organic	UHT
GMF-21	France	Whole	Regular	Goat	Organic	UHT
GMF-22	France	Whole	Regular	Goat	Organic	UHT
WMT-23	Turkey	Whole	Regular	Cow	Conventional	UHT
WMT-24	Turkey	Whole	Regular	Cow	Conventional	UHT
WMT-25	Turkey	Whole	Regular	Cow	Conventional	UHT
WMT-26	Turkey	Whole	Regular	Cow	Conventional	UHT
WMT-27	Turkey	Whole	Regular	Cow	Conventional	UHT
WMT-28	Turkey	Whole	Regular	Cow	Conventional	UHT
OMT-29	Turkey	Whole	Regular	Cow	Organic	UHT
GMT-30	Turkey	Whole	Regular	Goat	Conventional	UHT
LFMF-31	France	Half	Lactose-Free	Cow	Conventional	UHT
LFMF-32	France	Half	Lactose-Free	Cow	Conventional	UHT
LFMT-33	Turkey	Half	Lactose-Free	Cow	Conventional	UHT
LFMT-34	Turkey	Half	Lactose-Free	Cow	Conventional	UHT
LFMT-35	Turkey	Half	Lactose-Free	Cow	Conventional	UHT
LFMT-36	Turkey	Half	Lactose-Free	Cow	Conventional	UHT

2.2.3.2. NMR Spectroscopy Experiments

NMR experiments were carried out in 5 mm NMR tubes and WET-180-NOESY pulse sequence were used as explained before in section 2.2.1.2.2. NMR spectroscopy experiments were repeated 3 times for each milk sample.

NMR spectroscopy experiments were conducted as explained in section 2.2.1.2.2. Repetition time was set to 7 s, corresponding to 5 times the longest T_1. The 1D ^1H spectra were obtained with 128 scans for a total experiment time of 12 min. The signal of imidazole at 7.256 ppm was used for ppm-scaling reference.

2.2.3.3. Quantitative Analysis

For the quantitative analysis, glycerol peak, lactose peak and fat peak were chosen. Manual phase correction and manual baseline correction were performed in addition to the automatic corrections

The integral areas of selected peaks were calculated by integration with MestReNova software and normalized according to the integral area of internal reference, imidazole.

2.2.3.4. Multivariate Analysis

Considering the factors in Table 2.1 (country (TR/FR), milk type (Goat/Cow), marketing (Conventional/Organic), processing (UHT/Pasteurized), fat and sugar status) Analysis of Variance (ANOVA) was applied on the responses of glycerol; lactose and fat contents to see if any of the responses are showing differences with respect to the factors. In addition, to obtain some grouping info on the data set a multivariate analysis technique, *Discrimination Analysis* was conducted. General Linear model and Quadratic model tools in MINITAB (Version 16, 2017) were used for ANOVA and discrimination analysis respectively. To confirm the applicability of

ANOVA, normality and equality of variance assumptions were checked at 95% significance level by Anderson Darling and Bartlett's tests respectively. Results of ANOVA were given in Appendix A.1. Tukey comparison at 95% significance level was used to compare the different levels of each factor. Results of discriminant analysis were given in Appendix A.2.

2.2.4. Determination of Honey Adulteration by NMR Relaxometry

2.2.4.1. Sample Preparation

Samples were prepared by dissolving 100 mg of organic blossom honey in 500 μL water containing 1% TSP. To test adulteration, mixtures were prepared using organic blossom honey and high fructose corn syrup (F42). Adulteration model mixtures were prepared by adding high fructose corn syrup to pure honey at a ratio of 0%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% by weight. The samples were stirred for 3 mins for mixing honey and high fructose corn syrup properly.

2.2.4.2. NMR Spectroscopy Experiments

^{1}H NMR spectrum was recorded in a 5 mm NMR tube for each pure honey and adulterated sample on a 43 MHz (^{1}H) Magritek Spinsolve NMR spectrometer.

NMR spectroscopy experiments were conducted as explained in section 2.2.1.2.2. Repetition time was set to 6 s, corresponding to 5 times the longest T_1. The 1D ^{1}H spectra were obtained with 32 scans for a total experiment time of 12 min. The signal of TSP at 4.81 ppm was used for ppm-scaling reference. Pure honey spectrum is seen in Figure 2.4.

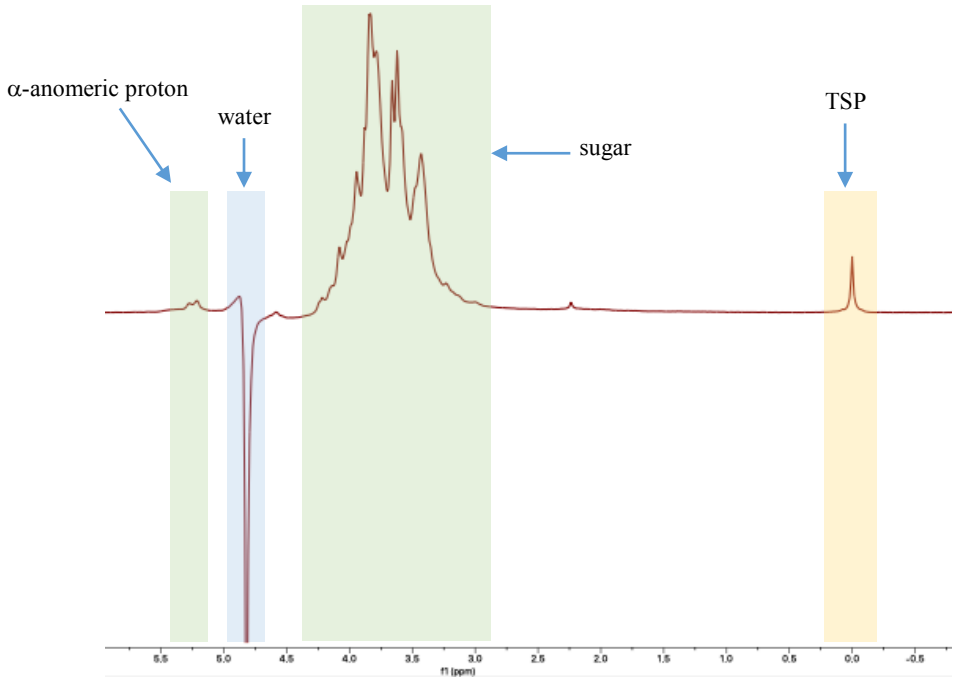


Figure 2.4. Pure honey spectrum obtained at 43 MHz (^{1}H) benchtop NMR spectrometer

2.2.4.3. NMR Relaxometry Experiments

The transverse relaxation time (T_2) was measured with a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (Carr & Purcell, 1954; Meiboom & Gill, 1958) All samples were analyzed triplicates. T_2 data were acquired with 4 scans, 4096 points, 6.7 µs 90^o pulse length, 0.5 s echo time, 16 echoes and 3 s repetition time.

In conventional benchtop NMR relaxometry CPMG signal is obtained from the whole spectrum since spectral resolution is not possible due to inhomogeneity. However, using a spectroscopy system like the one used in this study, T_2 of specific sections in the spectrum could be calculated. That is why the approach followed here could be considered as combinatorial approach of spectroscopy with relaxometry. Considering the honey spectrum shown in Fig. 2.4 T_2 of bulk, sugar, alpha anomeric proton and

water peaks of honey at different adulteration rates were analyzed by using MATLAB. Sample T_2 graph for different regions of the spectrum can be seen in Figure 2.5.

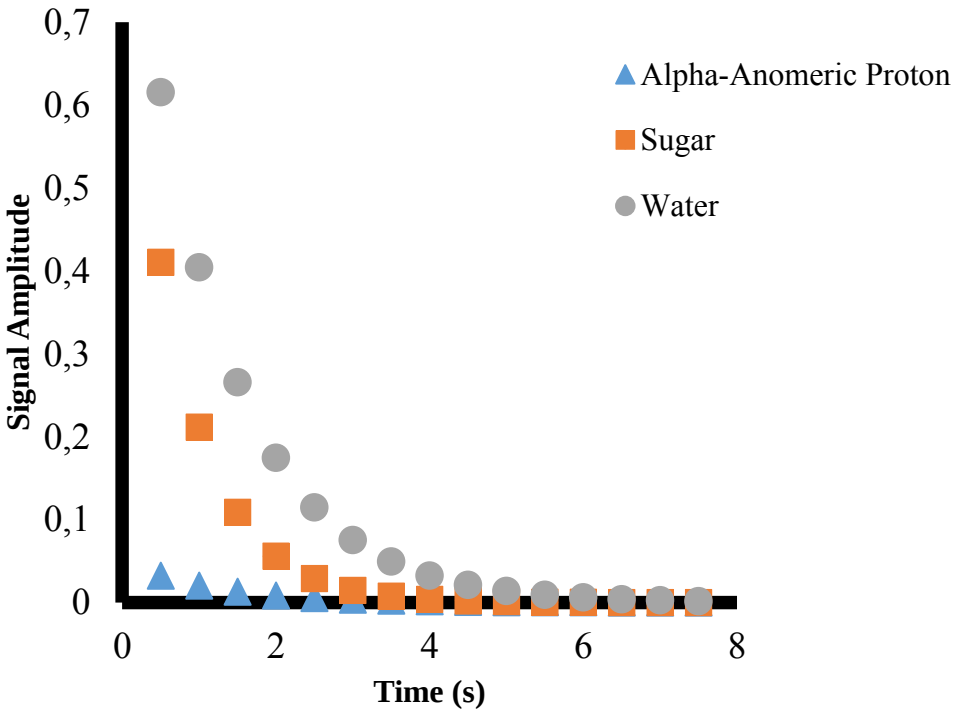


Figure 2.5. Sample T_2 graph for different regions of the spectrum for honey

2.2.4.4. Statistical Analysis

Regression analysis was conducted to predict the adulteration rate using the relaxation times. Assumptions of the model and lack of fit was checked at 5% significance level.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Real-time Benchtop NMR Spectroscopy for the Online Monitoring of Sucrose Hydrolysis

3.1.1. Exploring the 1D NMR Spectra

1D NMR experiments were first conducted to identify the peaks which could be used to monitor the sucrose hydrolysis reactions. As a result of the sucrose hydrolysis, glucose and fructose are produced (Figure 3.1).

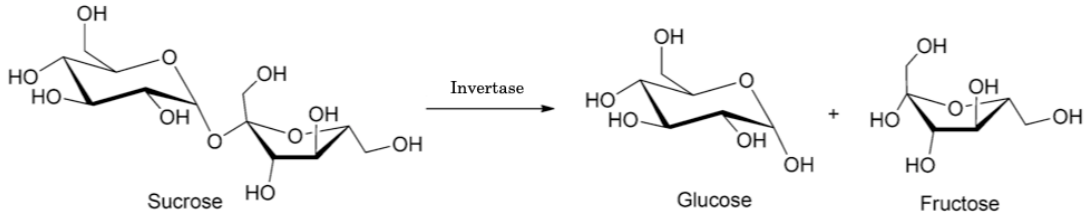


Figure 3.1. Hydrolysis of Sucrose into Glucose and Fructose

Figure 3.2 shows the spectra of sucrose, glucose and fructose solutions and the mixture of all sugars. Because of the relatively low magnetic field, the resulting spectrum suffered from numerous overlaps between 3-4.5 ppm. The suppressed water peak can be seen at 4.9 ppm. Suppressing the water signal is critical especially at low-field because overlapping issues become more stringent due to the reduced spectral width

at low frequency. Although the beta anomeric proton of glucose is overlapped with the water peak, the alpha anomeric proton of glucose is clearly seen at 5.3 ppm. In addition, the glycosidic proton of sucrose at 5.48 ppm is very distinct. These two peaks can also be seen clearly in the NMR spectrum of the mixture solution. Therefore, they were chosen to monitor the hydrolysis of sucrose by the invertase enzyme.

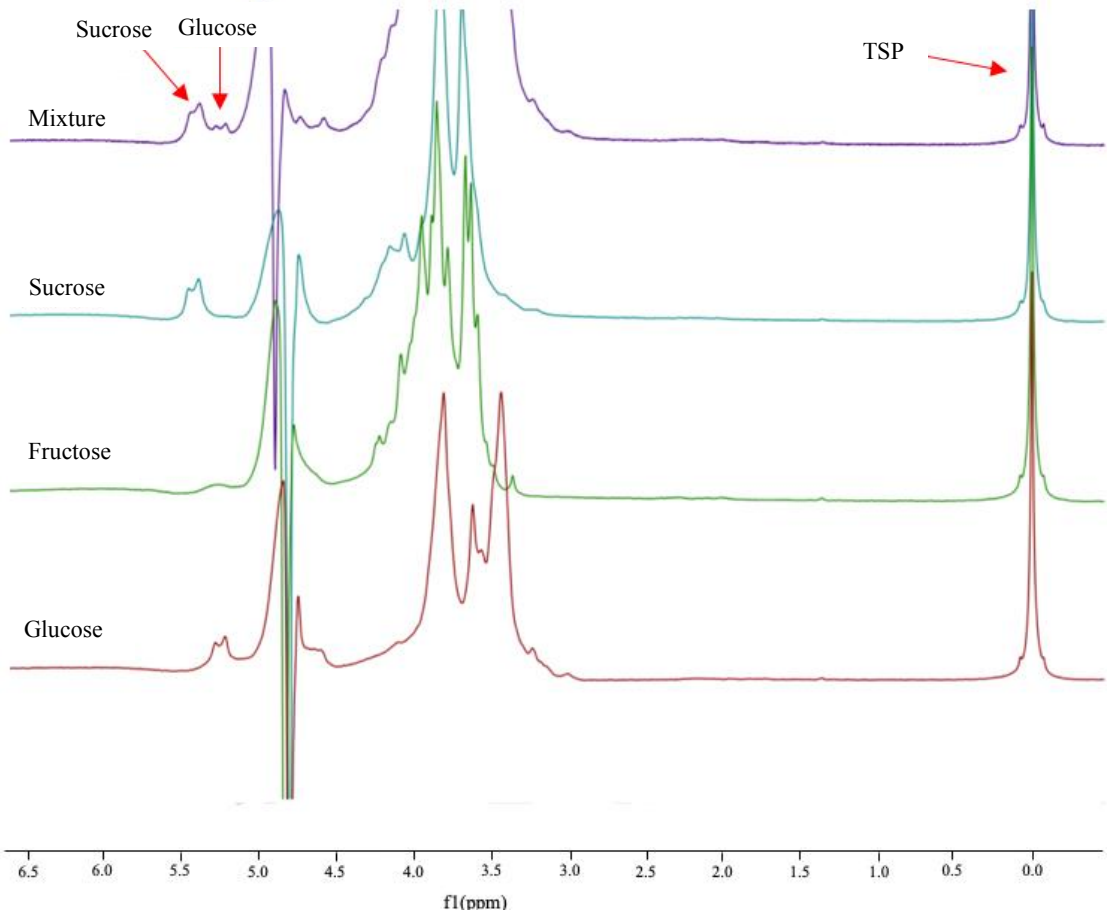


Figure 3.2. 1D ^{1}H NMR spectra of sucrose solution, glucose solution, fructose solution and mixture of all sugars recorded at 43 MHz with water signal suppression

3.1.2. Determination of Optimal Flow Rate

The choice of the optimal flow rate is one of the critical parameters in flow NMR experiments as the signal becomes broader and the resolution decreases with increasing flow rates. In flow NMR, 'inflow' and 'outflow' effects need to be considered carefully. During the experiment, the excited spins in the sensitive volume are refreshed by unexcited ones, leading to shorter apparent T_1. This 'inflow' effect allows shorter repetition times (T_R) to reach quantitative conditions (Nordon, McGill, & Littlejohn, 2001). But when the flow rate is significantly high, excited spins leave the sensitive volume before the acquisition of the FID is finished. This is called 'outflow' effect. This causes severe line broadening and affects the resolution of the NMR spectra (Gouilleux, Charrier, Akoka, & Giraudeau, 2017). To determine the optimum flow rate to be used in hydrolysis experiments, NMR spectra of the model mixture were recorded in non-flow conditions and in flow with 0.5 ml/min, 1 ml/min and 2 ml/min flow rates. As observed in Figure 3.3, when the flow rate increases from 0 ml/min to 2 ml/min, the peaks became broader and the glucose peak overlapped with the water peak, as a consequence of the outflow effect. Therefore, it was decided to use 0.5 ml/min flow rate in the online monitoring of hydrolysis reactions, which resulted in a modest reduction of longitudinal relaxation times resulting from the inflow effect.

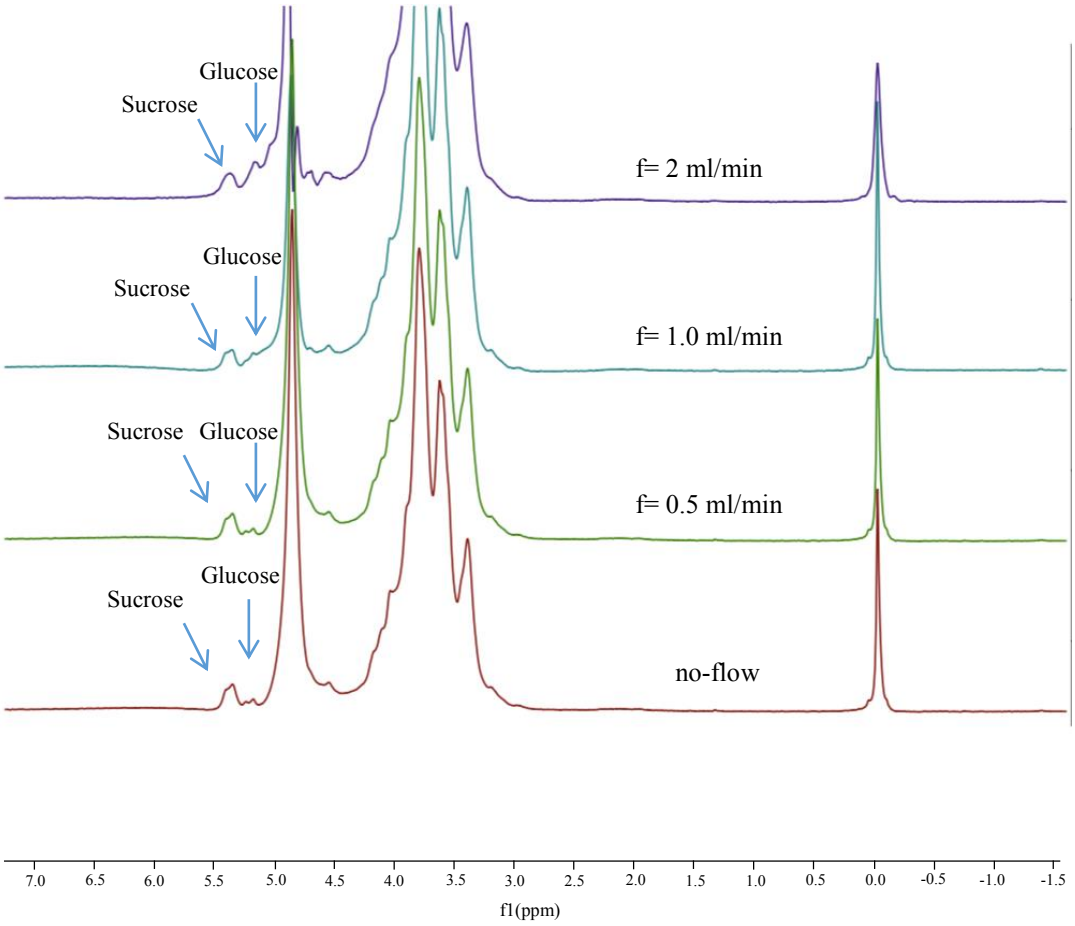


Figure 3.3. 1D ^{1}H NMR spectra of model mixture solution recorded at 43 MHz in different flow rates of 0 ml/min, 0.5 ml/min, 1.0 ml/min, 2 ml/min, with water signal suppression

3.1.3. Spin-Lattice Relaxation Time (T_1) and Repetition Time (T_R) Measurements

As stated before, spin-lattice relaxation times (T_1) were measured by the Inversion Recovery pulse sequence. For quantitative measurements, the peak of interest with the longest T_1 is the limiting factor, in our case it was the TSP. For the corresponding

peak, T_1 values were measured as 1.2, 1.1, 0.97 and 0.93 s in non-flow and in flow conditions with the flow rates of 0.5 ml/min, 1 ml/min and 2 ml/min, respectively. Here, the choice of a 0.5 ml/min flow rate resulted in a negligible reduction of the relaxation time ($p<0.05$), but was necessary to avoid significant broadening. In order to ensure a 1% accuracy on the quantitative measurements, the repetition time T_R was set to about 5 times the longest T_1, leading to $T_R = 6$ s.

3.1.4. Online Monitoring and Kinetic Modelling of Sucrose Hydrolysis with qNMR Measurements

In Figure 3.4, the consumption of sucrose and the production of glucose in the course of the hydrolysis are clearly observable. At time around 240 min, the integral area of sucrose reaches to a constant value and does not change after this time.

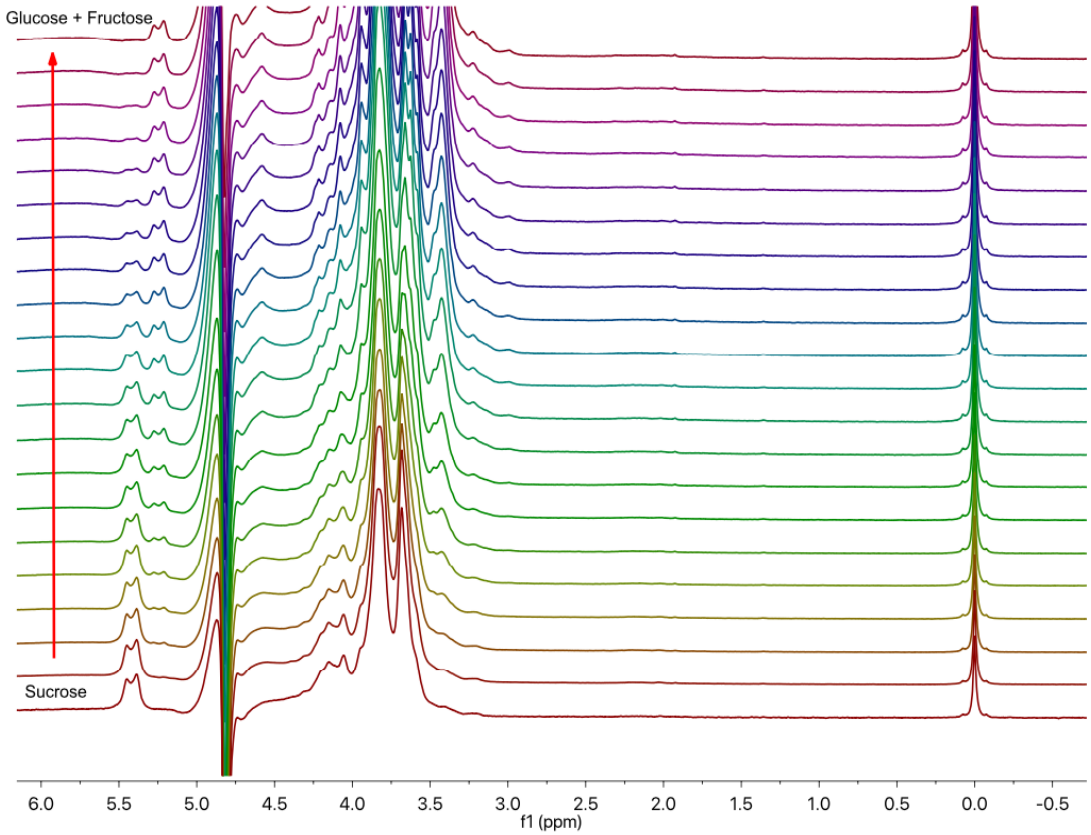


Figure 3.4. 1D ^{1}H NMR stacked spectra of sucrose hydrolysis recorded at 43 MHz

Hydrolysis experiments were repeated 3 times and the change of the mean concentrations of sucrose and glucose as a function of time can be seen in Figure 3.5, showing the good repeatability of the experiments. In the figure, it is seen that 1/6 of the sucrose was not converted. This might have been caused by invertase optimum temperature and substrate inhibition. The optimum temperature for the activity of invertase is between 50 °C -60 °C. However, the experiments were performed at 29 °C because this corresponds to the temperature at which the magnet stability is optimal. This may affect the reaction rate and the amount sucrose converted. Also, the activity of invertase is affected by the substrate concentration. The maximum rate of hydrolysis is obtained with a sucrose concentration of 5 to 10%, and then with higher concentrations, the rate was shown to decrease (Bergmeyer, 1965). In the experiments, 10% sucrose concentration has been used for quantification purposes and this might have also affected the amount of sucrose being converted.

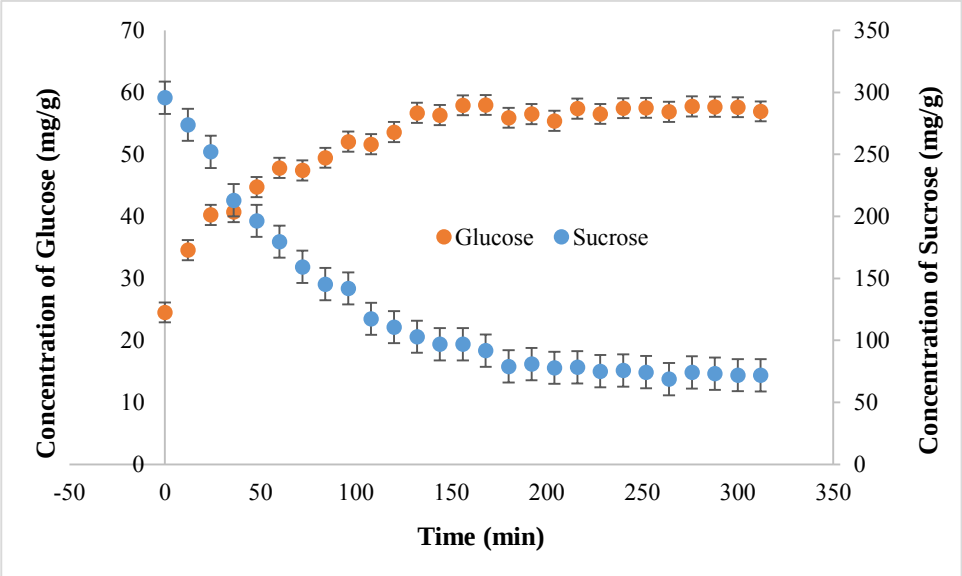


Figure 3.5. Sucrose and glucose concentration changes in the course of sucrose hydrolysis by invertase, monitored by quantitative ^{1}H NMR benchtop spectroscopy.

The mean concentrations of TSP were also plotted as a function of time to demonstrate baseline variation of the experiments (Figure 3.6). The mean curve was plotted for sucrose concentration and the data were fitted by equation 5. The average rate constant was found as k=[12.88±1.96]x10^{-3} min^{-1} (Figure 3.7) at 29°C.

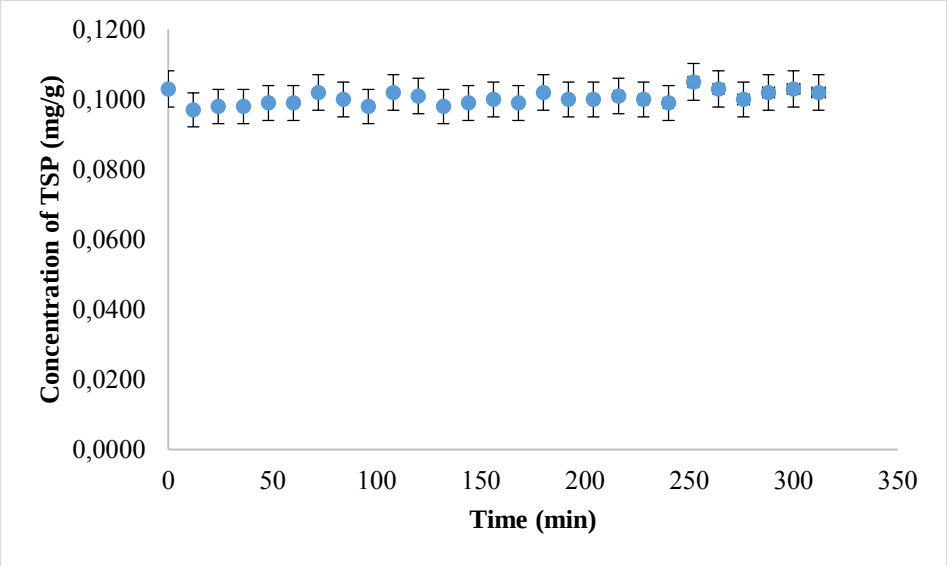


Figure 3.6. The mean concentration changes of TSP as a function of time.

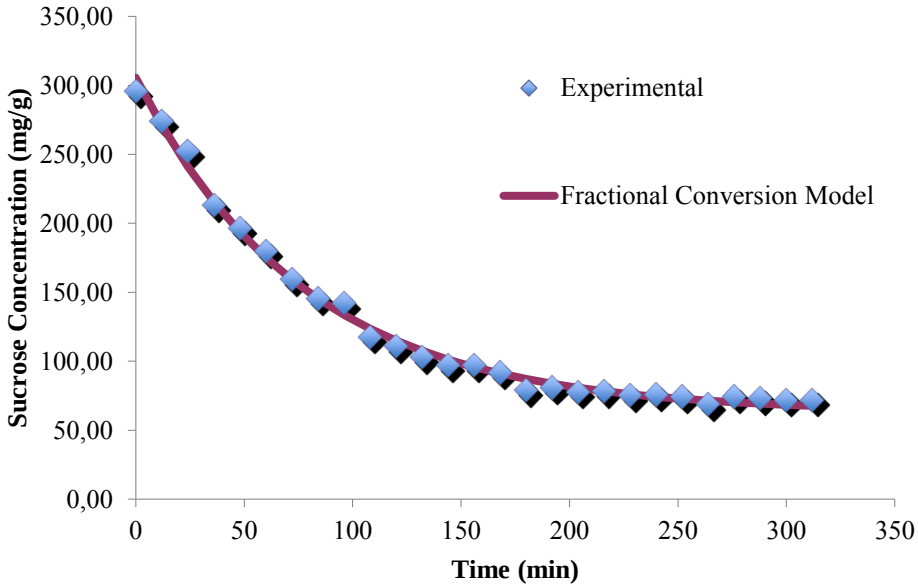


Figure 3.7. First order reaction kinetics obtained by adjusting the time evolution of sucrose concentration by a fractional conversion model

Vu and Le (2008) studied the kinetics of the immobilized and free invertase and found the kinetic constant for free invertase as $1.8x10^{-3}$ min^{-1}, $4.1x10^{-3}$ min^{-1}, $3.2x10^{-2}$ min^{-1} at 50°C, 55°C, 60°C respectively (Vu & Le, 2008). In the aforementioned study, although a different approach (a new enzyme-catalyzed reaction was performed every time to see how the activity changed with respect to the initial enzyme-catalyzed reaction) was followed to calculate the activity of the enzyme, in terms of order of magnitude, values were found to be reasonably close to the ones obtained in this study ($\sim10^{-3}$). The differences might have been caused by the temperature, the initial concentration or the activity of the enzyme used in this study.

3.2. Monitoring Production of Lactose-Free Milk in a Continuous Flow System by Quantitative Benchtop NMR

3.2.1. 1D NMR Spectra

Hydrolysis of lactose by lactase enzyme gives two products, glucose and galactose (Figure 3.8).

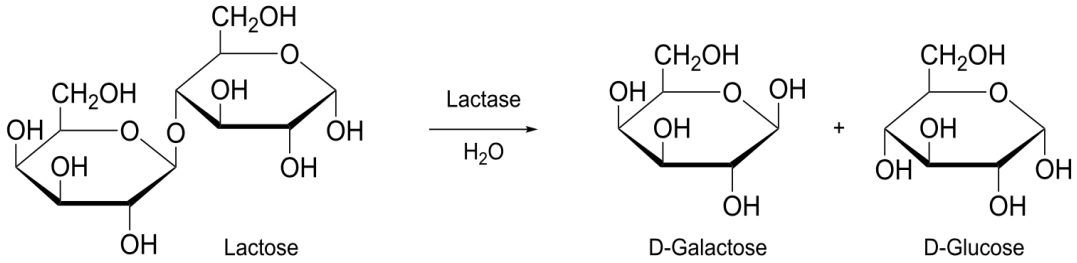


Figure 3.8. Hydrolysis of Lactose into Glucose and Galactose

1D ^{1}H NMR spectroscopy experiments were carried out to see whether there were possible distinctive peaks of glucose, galactose and lactose to follow up the hydrolysis reaction.

Figures 3.9 shows the stacked spectra of glucose, galactose and lactose. Because the experiments were performed in low field benchtop spectrometer, the spectra of the sugars were highly overlapped.

As can be seen from Figure 3.9, alpha anomeric proton peaks of glucose, galactose and lactose are overlapped at 5.3 ppm. Although beta anomeric proton peaks of glucose and galactose are masked by water peak, beta anomeric proton peak of lactose

can be seen as a distinctive peak at 4.4 ppm. This peak was chosen to monitor the lactose hydrolysis by the enzyme.

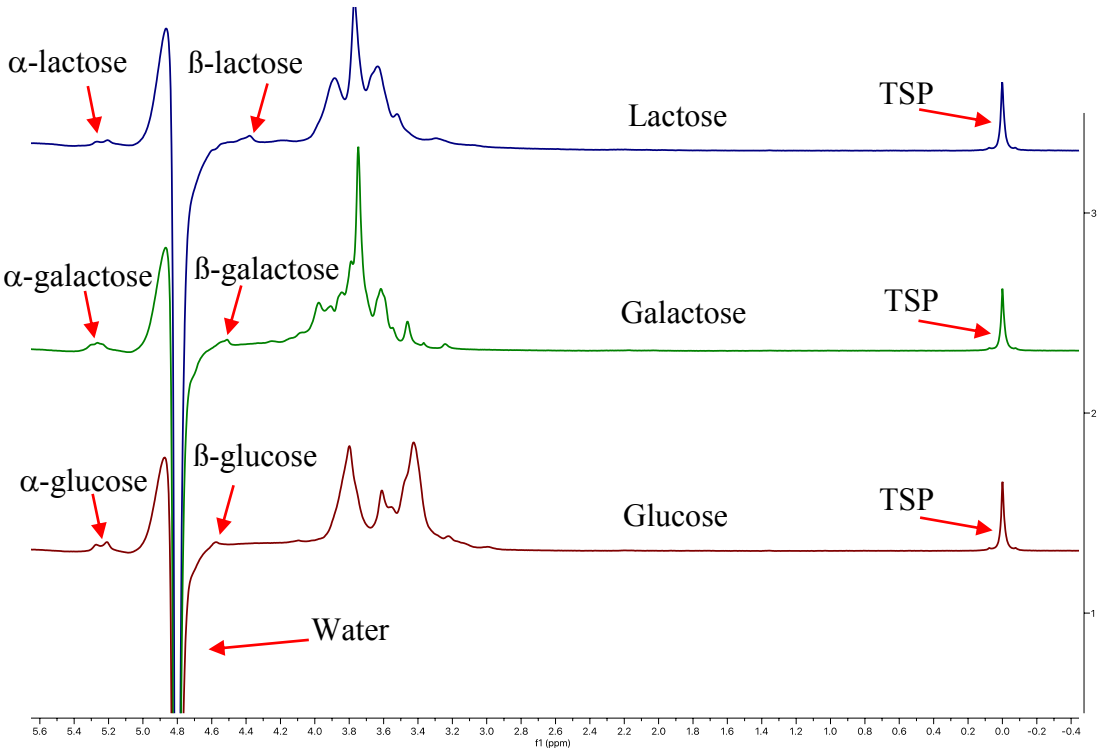


Figure 3.9. Stacked spectra of glucose, galactose and lactose at 43 MHz (^{1}H) benchtop NMR spectrometer

Milk is a much more complex fluid than the model lactose solution. The major constituents of milk are water, fat, proteins and lactose. Milk also contains trace amounts of vitamins, minerals, organic acids, enzymes. Therefore, 1D ^{1}H NMR spectrum of milk will be different than model lactose solution.

As can be seen from Figure 3.10, milk spectrum has very broad fat peaks between 0.5 – 2.5 ppm. Because the peak of TSP overlaps with fat peaks in milk, imidazole at 7.256 ppm and 8.11 ppm was used as the internal reference material for quantification. Glycerol peak has been observed next to ß-lactose peak between 4.0 – 4.25 ppm. Also, α-lactose peak overlapped with unsaturated fat content of milk.

There are two reasons for the presence of glycerol in the milk. Firstly, glycerol is used for the treatment of ketosis which is a metabolic disease caused by the negative energy balance during lactation (Johnson, 1954). Other one is glycerol is used in the feed as an energy source. Expansion of the biodiesel industry decreased the availability of corn for animal feed and increased its prices. Therefore, glycerol which is also a by-product of the biodiesel industry has started to be used as an energy source for cows (Carvalho, Schmelz-Roberts, White, Doane, & Donkin, 2011). The energy concentration of glycerol (1.98-2.29 Mcal/kg) is almost equal to corn starch (Schroder & Sudekum, 1999).

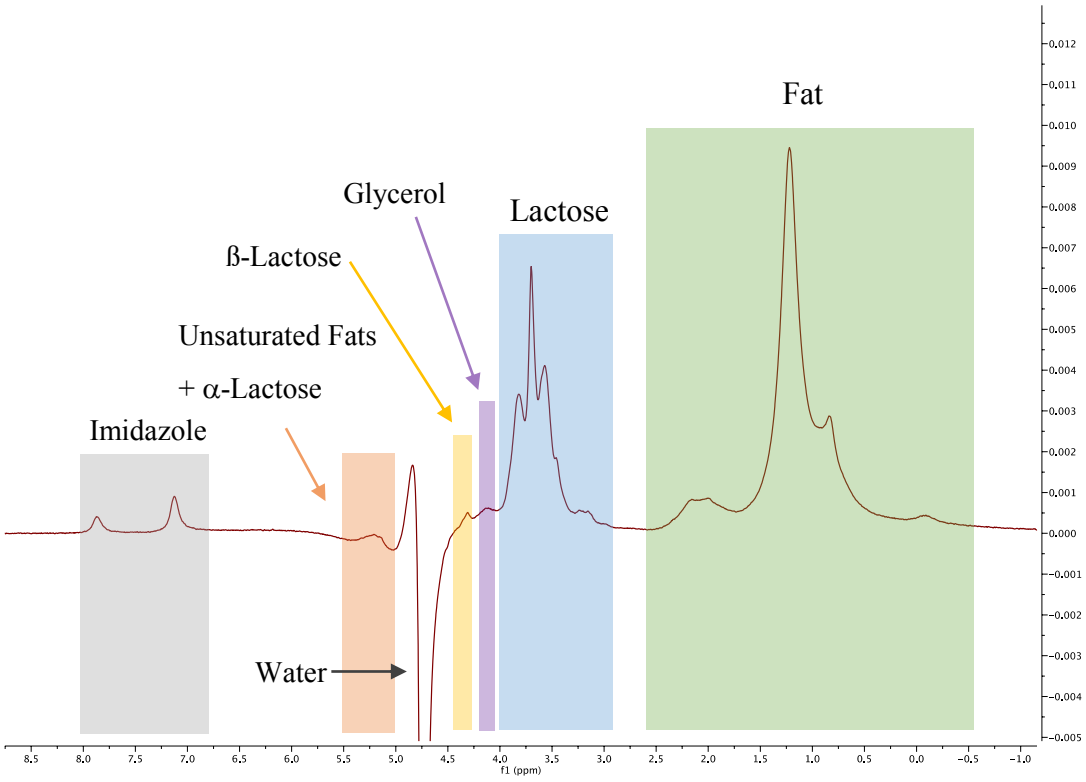


Figure 3.10. 1D ^{1}H NMR spectrum of milk at 43 MHz (^{1}H) benchtop NMR spectrometer

3.2.2. 2D TOCSY NMR Spectra

2D TOCSY NMR spectroscopy experiment was performed on a milk sample to enhance the low resolution of 1D NMR experiments and to correlate ^{1}H of molecules in order to characterize milk constituents. 2D TOCSY experiment has been conducted also at the 43 MHz (^{1}H) benchtop NMR spectrometer. As can be seen from the Figure 3.11, 2D TOCSY experiment results were correlated with 1D NMR experiments. Signals with red color indicate high positive intensities, orange signals indicate average positive intensities and blue signals indicate negative intensities.

Because of the low resolution, big molecules like proteins cannot be detected with benchtop NMR spectroscopy. Therefore, casein cannot be seen in the spectra. In 2D

TOCSY spectrum, fat region, lactose, α-lactose, β-lactose, water and imidazole and their contributing protons can be seen clearly. So, 1D spectra and the peak assignment has been confirmed by the 2D experiment.

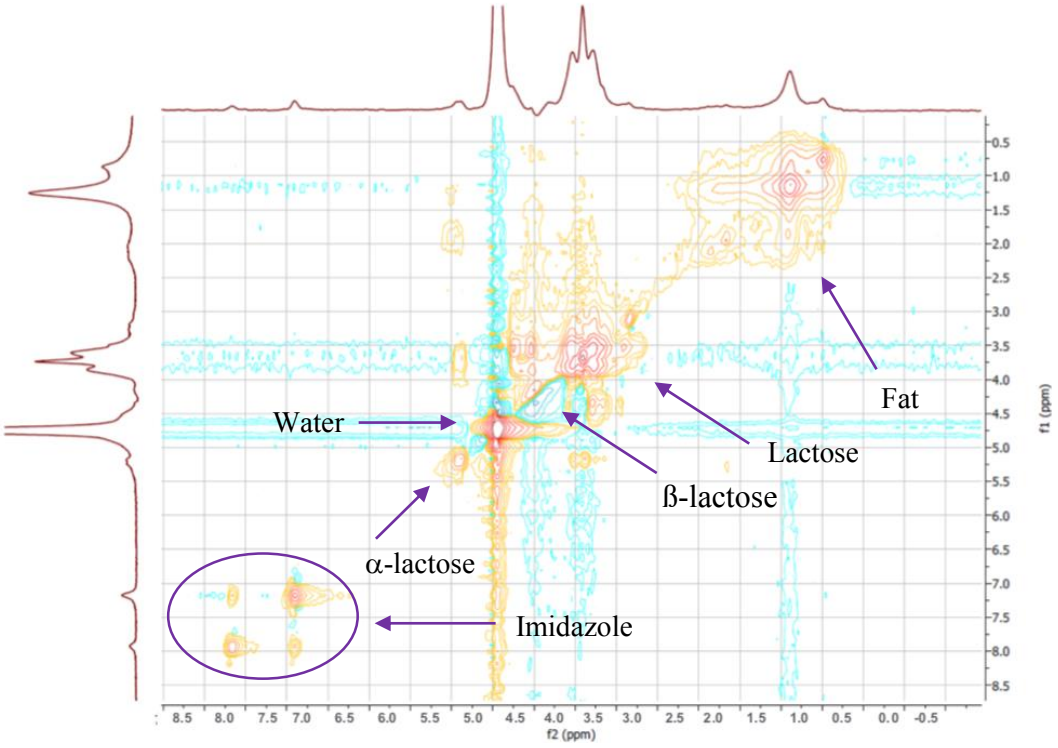


Figure 3.11. 2D TOCSY NMR spectrum of milk at 43 MHz (^{1}H) benchtop NMR spectrometer

3.2.3. Spin-Lattice Relaxation Time (T_1) and Repetition Time (T_R) Measurements

Lactose hydrolysis experiments were also conducted in flow conditions for model solution and milk. Determination of T_R is quite important for the flow measurement and basically it depends on T_1.

In flow conditions, water peak overlaps with other peaks and makes it difficult to use inversion recovery pulse sequence and to measure Spin-Lattice relaxation times (T_1). Therefore, an estimation of T_1 values was done by recording the experiments at different Repetition Times (T_R). Since longitudinal magnetization is affected from short T_R times, signal becomes lower if T_R is not set to 5 T1. Thus, recording the signal at different T_R's and finding the T_R that will make the signal constant was an approach to obtain the maximum signal.

The 1D ^1H spectra were obtained for model lactose solution and milk sample with different T_R values between 1,000-40,000 ms with 16 point at a flow rate of 0.5 ml/min. Obtained spectra were processed by manual phase correction and manual baseline correction in addition to the automatic corrections. 16 spectra were superimposed for integration. Because the longest T_1 should belong to the TSP and imidazole, integral areas of these peaks have been calculated by MestReNova software. The integral areas were plotted as a function of repetition times for model lactose solution (Figure 3.12). The same approach was followed for imidazole.

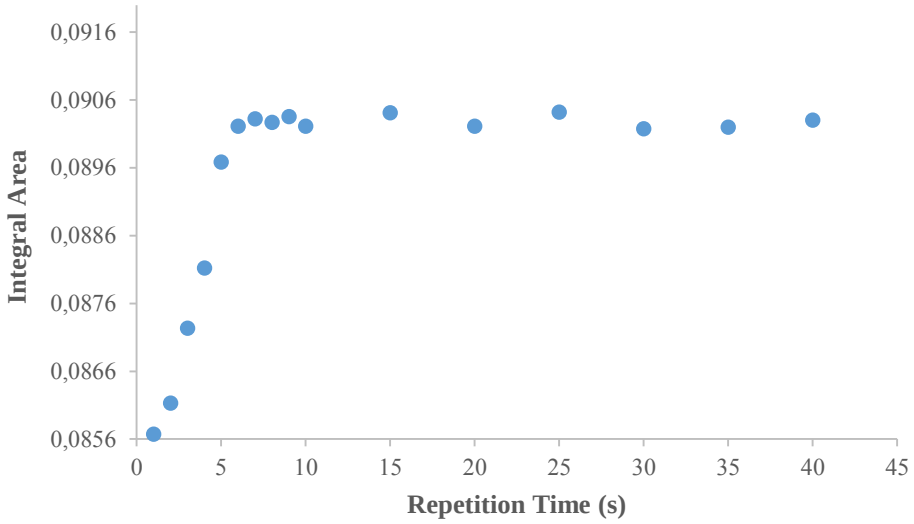


Figure 3.12. The integral area of TSP for model lactose solution as a function of T_R

T_R values for the qNMR measurements have been chosen as 6 s and 7 s for model lactose solution and milk sample, respectively. To reach the measurement of 99% equilibrium magnetization, T_R value should be at least 5 times higher than the T_1. Therefore, T_1 values can be estimated from T_R values. T_1 value is calculated as 1.2 s for model lactose solution and 1.4 s for milk sample for 0.5 ml/min flow rate.

3.2.4. Online Monitoring of Lactose Hydrolysis with qNMR Measurements

For model lactose solution hydrolysis, 5% lactose solution was hydrolyzed with 20 µl lactase enzyme. The NMR spectra were obtained at 6 min intervals for 240 minutes. In Figure 3.13, the consumption of lactose and the production of glucose and galactose can be seen from ß-lactose and glucose + galactose peaks.

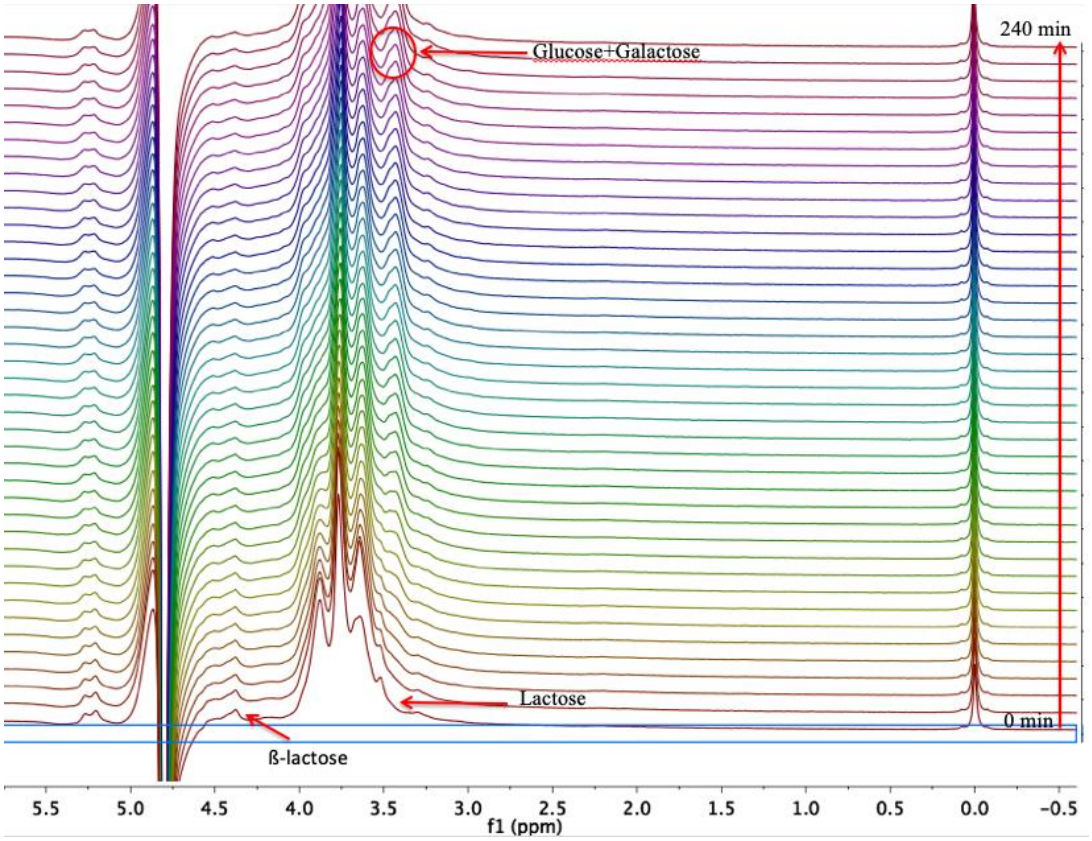


Figure 3.13. 1D ^{1}H NMR stacked spectra of lactose hydrolysis of model lactose solution recorded at 43 MHz (^{1}H) benchtop spectrometer

Superimposed spectra were plotted for all 41 experiments (Figure 3.14). The glucose + galactose peak detected in Figure 3.14 was seen as a very distinctive characteristic peak when the spectra were superimposed. From the separate 1D ^{1}H experiments of glucose, galactose, lactose (Figure 3.9), the only peak that was eligible to monitor hydrolysis was seen as the ß-lactose peak. However, from the superimposed spectra, it can be seen that glucose + galactose peak could also be used to monitor lactose hydrolysis in addition to ß-lactose peak.

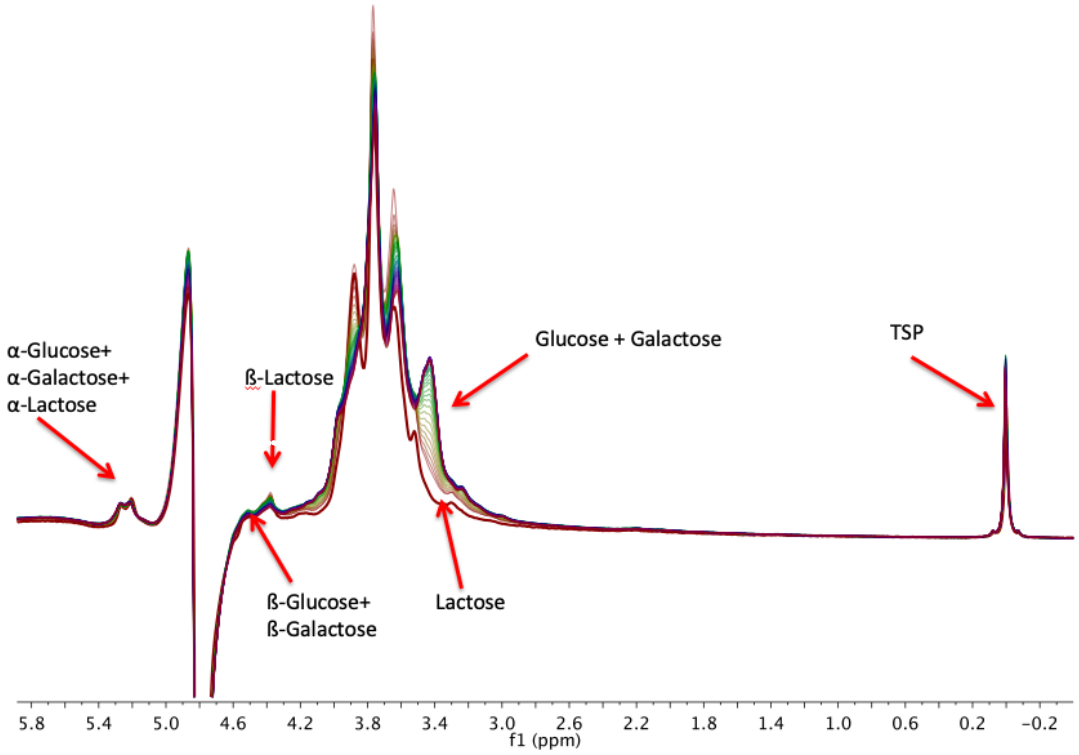


Figure 3.14. 1D ^{1}H NMR superimposed spectra of lactose hydrolysis of model lactose solution recorded at 43 MHz (^{1}H) benchtop spectrometer

For the quantitative NMR measurements, the glucose + galactose peak needs to be surgically separated because the percentage of the glucose and galactose in the peak and the number of protons contributing to the peak by glucose and galactose separately were not known. For the online monitoring of lactose hydrolysis, integral area of the glucose + galactose peak was followed up between the region 3.29 ppm to 3.52 ppm (Figure 3.15.a) and the ß-lactose peak (Figure 3.15.b).

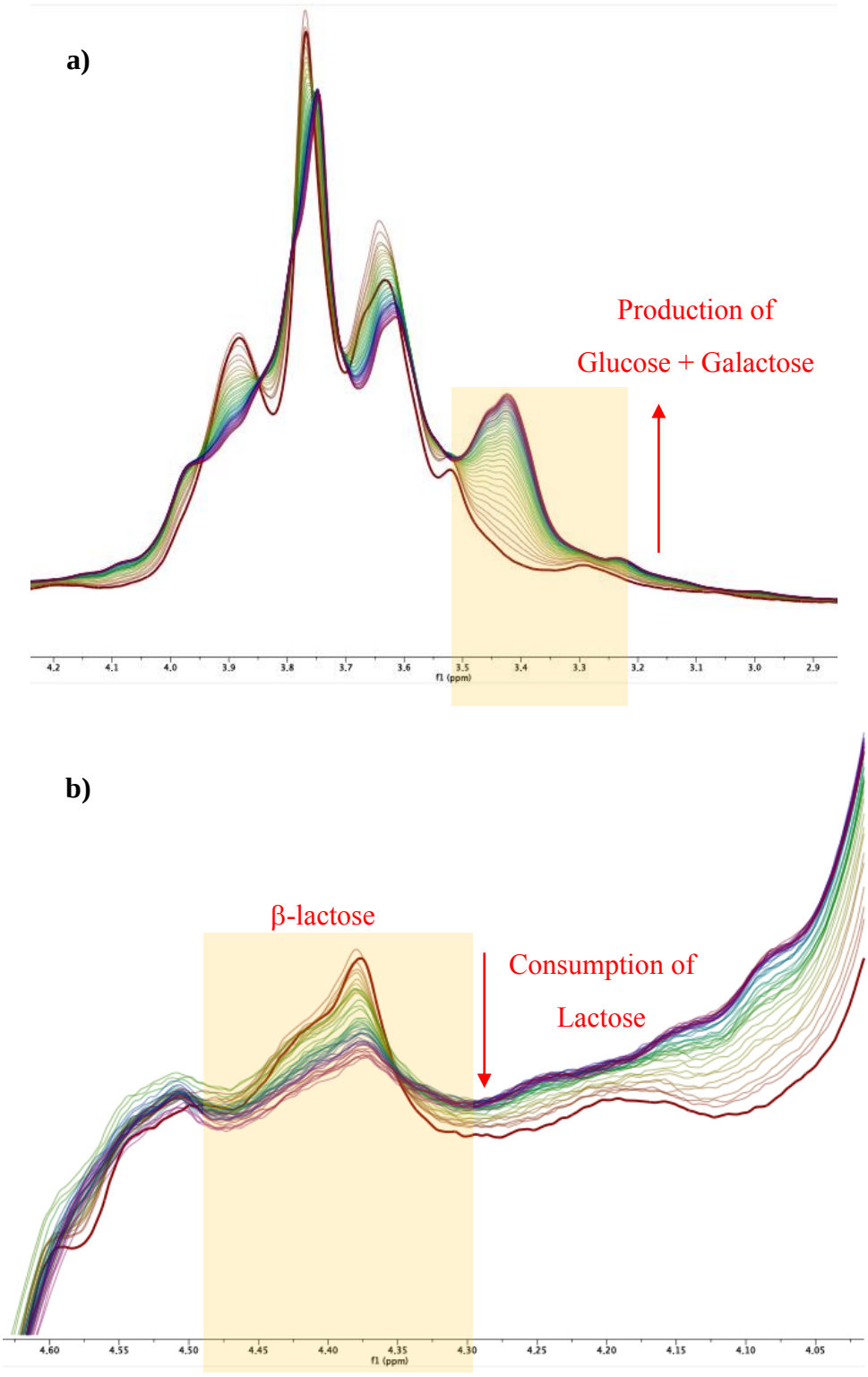


Figure 3.15. a) 1D ^{1}H superimposed spectra of lactose hydrolysis of model lactose solution focused on glucose + galactose region, b) ß-lactose region

Equations 7 and equation 8 were used to find the number of protons contributing to the peak by glucose and galactose and the percentage of glucose and galactose contributing to the integral area under the peak between 3.29-3.52 ppm, respectively.

$$N_x = \frac{A_x * N_{x\alpha}}{A_{x\alpha}} \qquad \text{(Eq. 7)}$$

$$\%A_x = \frac{1}{N_{Glucose} + N_{Galactose}} * N_x * 100 \qquad \text{(Eq. 8)}$$

A and N, denoted the integral area in a fully relaxed ^{1}H NMR spectrum and the number of hydrogens contributing to the signals respectively. The *x* index refers to the analyte (glucose or galactose) and the α index refers to the alpha anomeric proton of glucose or galactose.

The number of hydrogens contributing to the signal (3.29-3.52 ppm) were found as 5.0 for glucose and 0.78 for galactose. The glucose contributes to the integral area under the peak between 3.29-3.52 ppm by 86.5% and the galactose contributed by 13.5%.

Hydrolysis experiments were repeated three times and the change of the average concentrations of glucose, galactose and lactose from the experiments has been plotted as a function of time in Figure 3.16 which shows the good repeatability of the experiments.

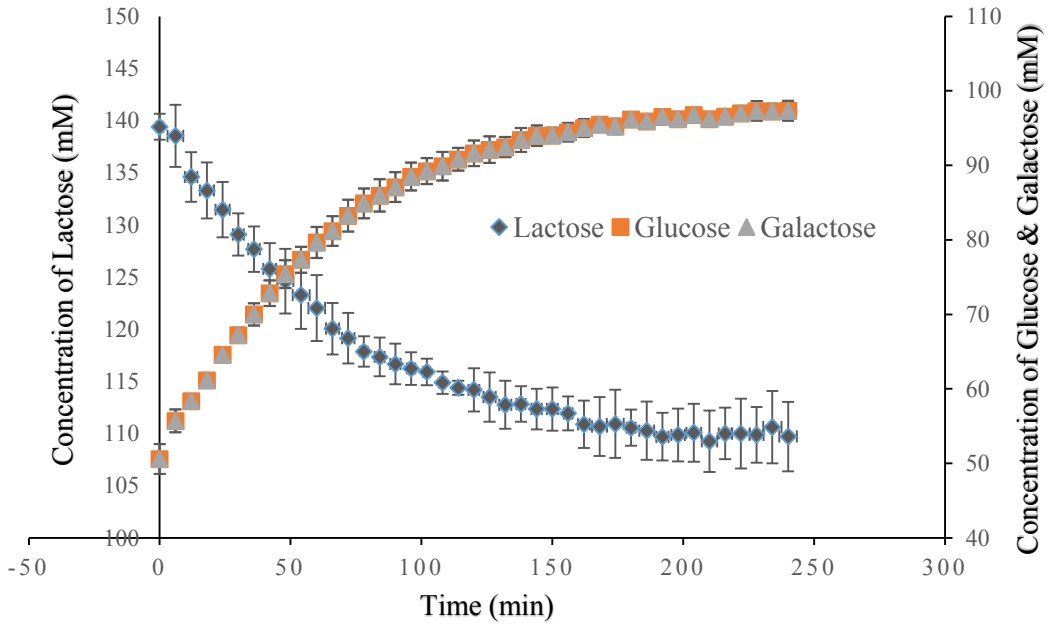


Figure 3.16. Glucose, galactose and lactose concentration changes in the course of model lactose solution hydrolysis by lactase, monitored by quantitative ^{1}H NMR benchtop spectroscopy.

The mean curve was plotted for lactose concentration and the data were fitted by equation 5. The average rate constant was found as $k=1.66x10^{-2}$ min^{-1} at 29°C (Figure 3.17).

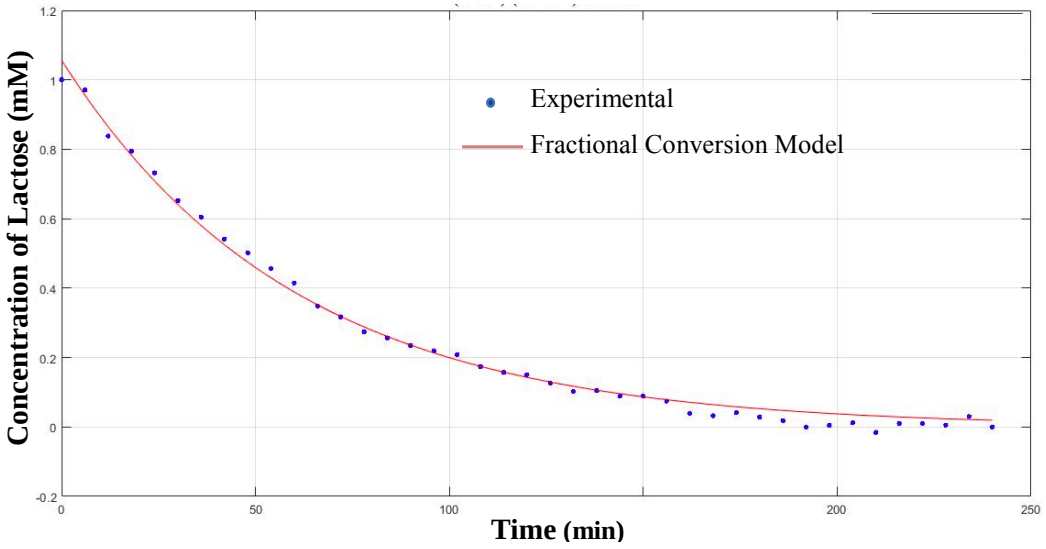
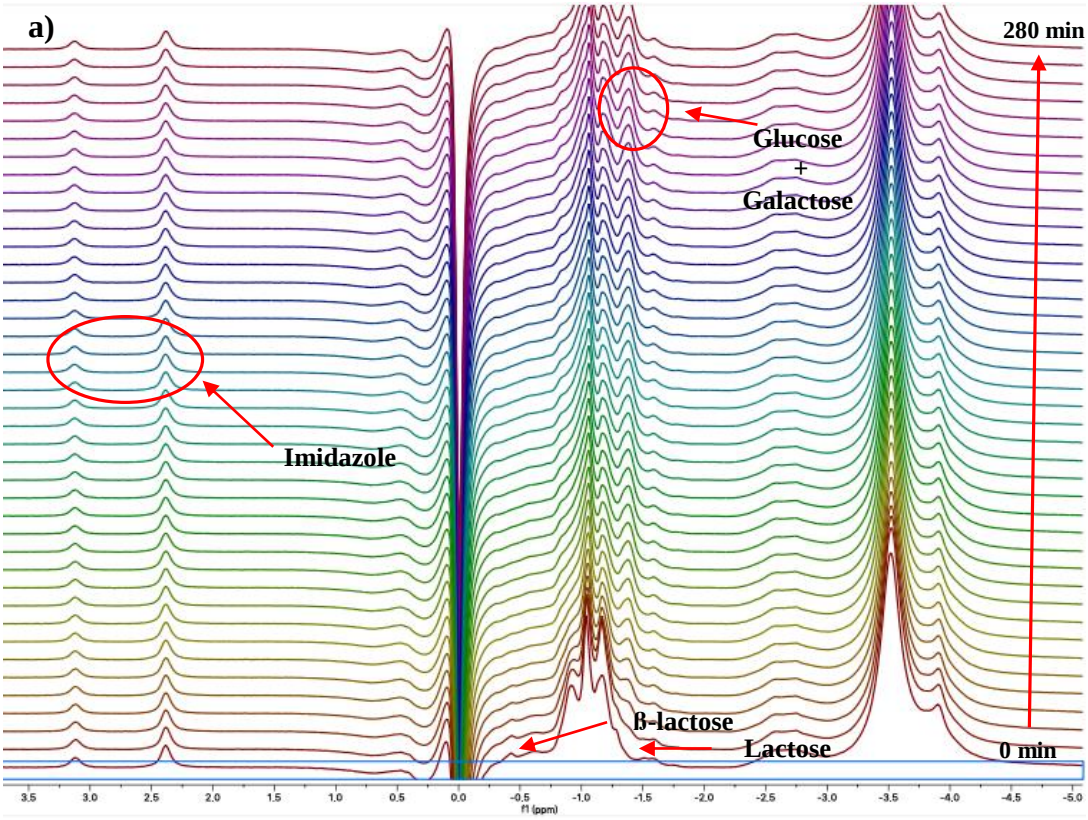


Figure 3.17. First order reaction kinetics obtained by adjusting the time evolution of lactose concentration in model lactose solution by a fractional conversion model

Panesar (2007) studied lactose hydrolysis and the kinetics of lactase enzyme from *Kluyveromyces maxrianus* (Panesar, 2007). He found the kinetic constant of lactase as 1.56×10^{-2} min^{-1}. It is consistent with the k value that was found in our experiments.

After obtaining good repeatability and consistent results with model lactose solution hydrolysis, milk was hydrolyzed with 20 µl lactase enzyme. The NMR spectra were obtained at each 7 mins for 280 mins. In Figure 3.18.a (stacked spectra) and Figure 3.18.b (superimposed spectra), the consumption of lactose and the production of glucose and galactose can be seen from ß-lactose and glucose + galactose peaks.


a)
280 min
Glucose
+
Galactose
Imidazole
ß-lactose
Lactose
0 min
f1 (ppm)

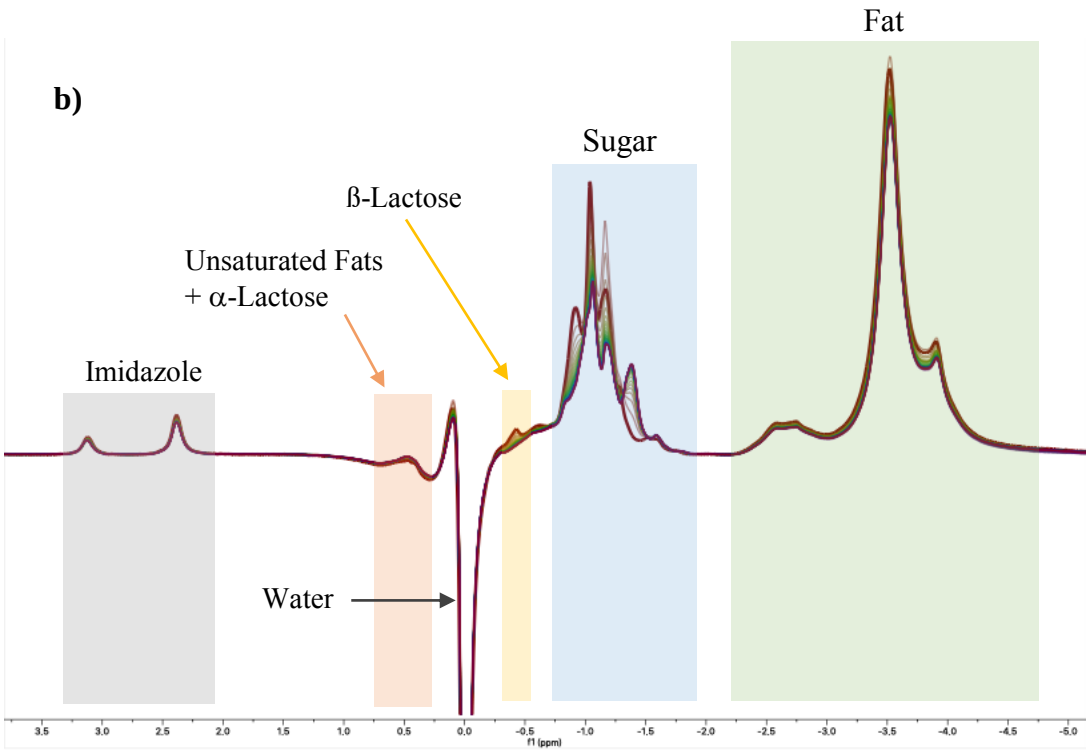


Figure 3.18. a) 1D ^{1}H NMR stacked spectra and b) superimposed spectra of milk lactose hydrolysis recorded at 43 MHz (^{1}H) benchtop spectrometer

For the quantitative NMR measurements, the glucose + galactose peak and ß-lactose peak were chosen and surgically separated as in model lactose hydrolysis. The experiments were repeated 3 times and the concentrations were calculated by Eq. 6. The change of the mean concentrations of lactose, glucose and galactose were plotted as a function of time (Figure 3.19). The experiments showed good repeatability.

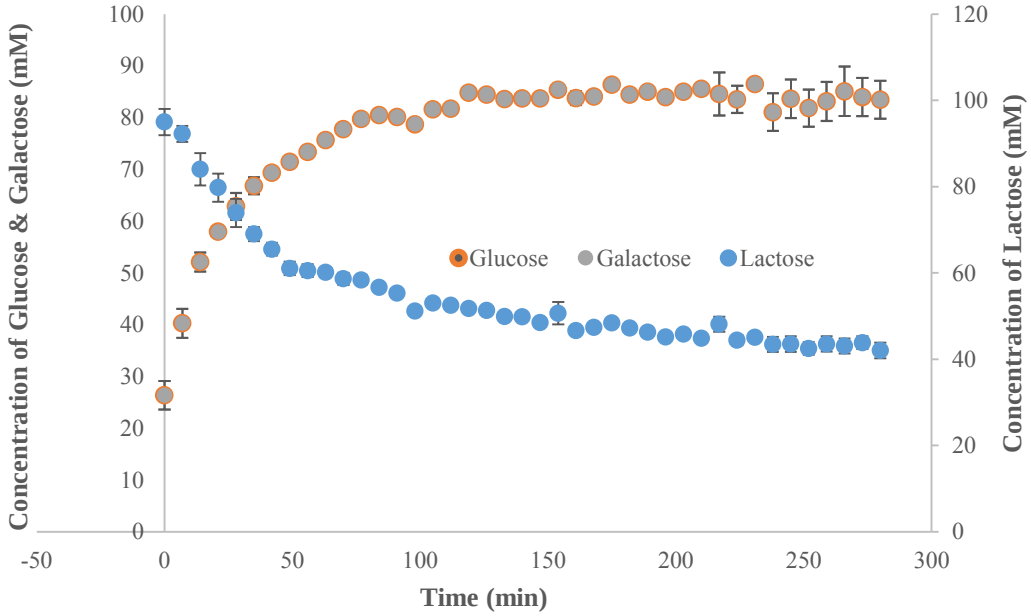


Figure 3.19. Glucose, galactose and lactose concentration changes in the course of milk lactose hydrolysis by lactase, monitored by quantitative ^{1}H NMR benchtop spectroscopy.

The mean curve was plotted for lactose concentration and the data were fitted by equation 5. The average rate constant was found as $k=1.52x10^{-2}$ min^{-1} at 29°C (Figure 3.20). The rate constant is very consistent with the rate constant that Panesar found (Panesar, 2007).

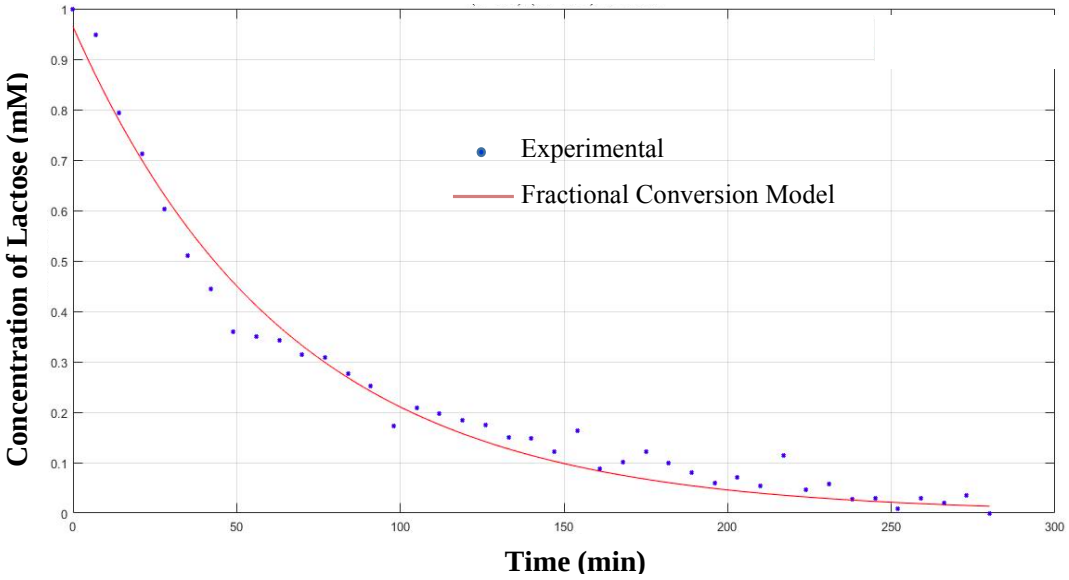


Figure 3.20. First order reaction kinetics obtained by adjusting the time evolution of lactose concentration in milk by a fractional conversion model

3.3. Authentication of Milks by Benchtop ^{1}H-NMR Profiling and Multivariate Analysis

3.3.1. 1D NMR Spectra

1D NMR experiments for 36 different types of milk samples were first conducted to identify the peaks which could be used for authentication studies. The sample spectrum of milk can be seen in Figure 3.21. After the phasing and baseline corrections of 36 spectra, alpha and beta lactose anomeric proton peaks of some of the milk samples were placed below the baseline. Hence, they gave the negative integration value. Therefore, for the authentication analysis, integral area of glycerol, lactose and fat peak were used. Imidazole peak's integral area was used for the normalization of other peaks. The normalized integral areas can be seen in Table 3.1.

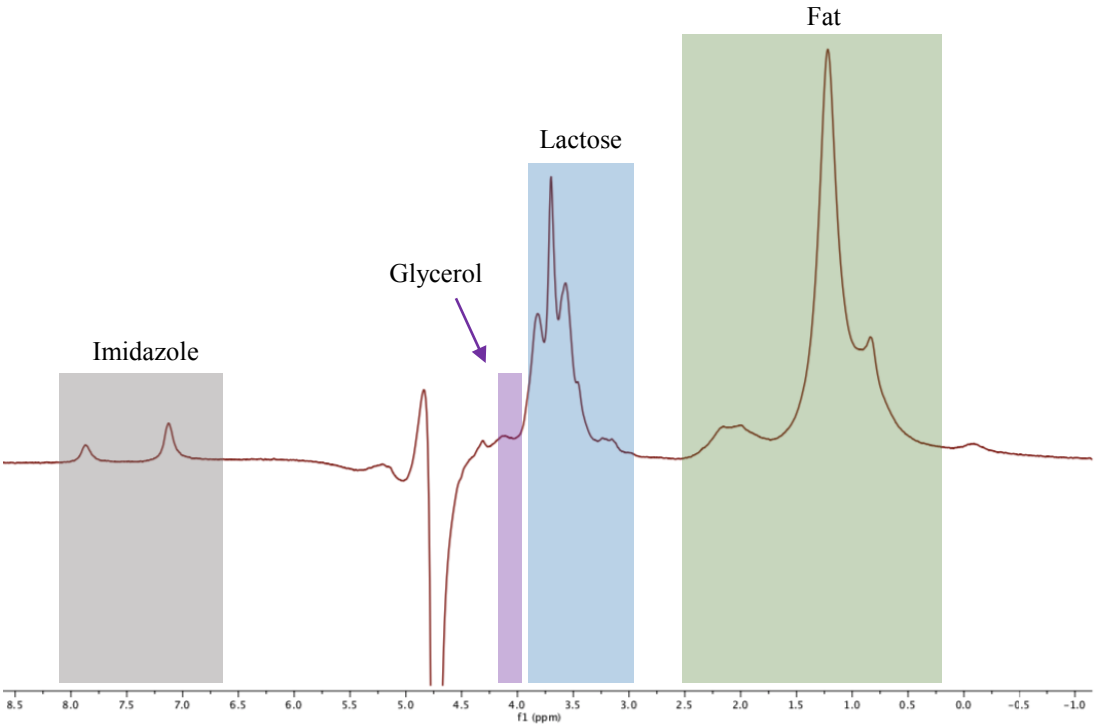


Figure 3.21. The sample spectrum for milk in 43 MHz (^{1}H) benchtop spectrometer

Table 3.1. Integral areas of glycerol, lactose and fat content of analyzed milks

Sample Name	**Glycerol**	**Lactose**	**Fat**
WMF-1	1.126	18.023	40.585
WMF-2	1.204	21.068	49.632
WMF-3	0.845	18.416	44.177
WMF-4	1.022	21.370	49.785
OMF-5	1.006	18.631	47.615
OMF-6	1.043	20.927	55.941
OMF-7	0.576	19.352	48.897
HFMF-8	0.687	20.311	25.845
OHFMF-9	0.275	19.461	20.380
HFMF-10	0.472	20.384	25.257
HFMF-11	0.195	22.737	24.772
HFMF-12	0.550	22.638	25.964
OHFMF-13	0.700	23.062	28.130
WMF-14	0.956	21.808	57.543
OHFMF-15	0.522	18.763	22.294
HFMF-16	0.502	18.866	22.594
OMF-17	0.817	22.858	57.398
OHFMF-18	0.482	20.006	26.536
GMF-19	0.723	20.100	27.899
GMF-20	0.749	17.719	47.585
GMF-21	0.841	20.372	46.935
GMF-22	1.006	18.727	52.168
WMT-23	0.863	19.080	44.018
WMT-24	1.007	19.483	48.226
WMT-25	0.279	22.412	45.022
WMT-26	0.428	18.615	41.632
WMT-27	0.772	18.911	44.755
WMT-28	0.230	19.651	44.747
OMT-29	0.175	22.332	46.650
GMT-30	0.163	19.955	44.893
LFMF-31	0.436	0.000	24.890
LFMF-32	0.123	0.000	24.288
LFMT-33	0.412	0.000	22.528
LFMT-34	0.297	0.000	23.290
LFMT-35	0.373	0.000	20.793
LFMT-36	0.059	0.000	27.991

Lactose-free milks (LFM(T/F)) were found to have no lactose and half-fat milks (HFM(T/F)) had nearly half fat content compared to whole milk samples.

3.3.2. Multivariate Analysis

3.3.2.1. Analysis of Variance (ANOVA)

To see whether the examined factors are significant on the determined response, ANOVA was conducted on the data set. Glycerol, fat content and lactose content values calculated from NMR spectra were the evaluated responses. For fat and lactose contents, only the fat and sugar status of the milk were found to be significant ($p<0.05$). Results of ANOVA are given in the Appendix A.1. Marketing type, country and processing were found to be insignificant on lactose and fat content ($p>0.05$). However, for glycerol, results were interesting. It was found that when the glycerol contents are compared, Turkish and French milks were found to be significantly different from each other; glycerol contents of French milks being higher ($p<0.05$). This was also observed in the multivariate analysis as will be explained in the next section. In addition to the countries of the milks, fat status of the milks was also found to be significantly different with respect to glycerol content. In other words, glycerol content was able to distinguish the fat status of the milk: whole or low fat ($p>0.05$)

3.3.2.2. Discriminant Analysis

Discriminant analysis is a multivariate separation method and is used to predict the membership of the samples to specific groups. The method uses the discriminant scores which are also called centroids. The discriminant scores are obtained from group means where the groups differ significantly from each other (Tillmanns & Krafft, 2017).

In this study, for the 36 milk sample data set, linear and quadratic discriminant analysis were applied separately to the factors in Table 2.1 (country, fat status, sugar status,

milk type, marketing, processing) with the three responses (glycerol, lactose and fat) obtained from the 1D NMR spectra.

Linear method was sufficient to discriminate whole milk and half fat milk samples (Appendix A.2, Table A.2.1) and also regular and lactose-free samples (Appendix A.2 Table A.2.2). As can be seen in Table 3.2, half fat milks (16 samples) and whole fat milks (20 samples) have been classified 100% correctly without any wrong classification.

Table 3.2. The summary of the classification of milk samples according to their fat content

	True Group	
Put into Group	**Half**	**Whole**
Half	16	0
Whole	0	20
Total N	16	20
N correct	16	20
Proportion	1.000	1.000

The linear discrimination function for half-fat group and whole fat group was able to be described by equation 9 and 10, respectively. So, the new set of milk samples will be classified according to their fat content with the following equations.

$$Half\ fat\ milk = -20.140 - 3.272 \times [Glycerol] + 0.121 \times [Lactose] + 1.631 \times [Fat]$$ (Eq. 9)

Whole $milk = -75.998 - 6.933 \times [Glycerol] + 0.131 \times [Lactose] + 3.227 \times [Fat]$
(Eq. 10)

Similarly, 6 lactose-free milk samples and 30 regular milk samples have been classified correctly with linear discriminant analysis (Table 3.3). The functions for the lactose-free group and regular milk group can be seen in equation 11 and 12, respectively.

$Lactose - free\ milk = -2.42 - 0.18 \times [Glycerol] - 0.01 \times [Lactose] + 0.20 \times [Fat]$
(Eq. 11)

$Regular\ milk = -109.96 + 15.42 \times [Glycerol] + 10.07 \times [Lactose] + 0.15 \times [Fat]$
(Eq. 12)

Table 3.3. The summary of the classification of milk samples according to their lactose content

	True Group	
Put into Group	**Lactose-Free**	**Regular**
Lactose-Free	6	0
Regular	0	30
Total N	6	30
N correct	6	30
Proportion	1.000	1.000

The linear discriminant analysis on the country factor was able to classify the milks from France and Turkey by 77.8% (28 correct results out of 36) (Appendix A.2, Table A.2.3). As it can be seen from the Table 3.4, 3 milks from France have been misclassified as the milks from Turkey and also 5 milks from Turkey have been misclassified as French milks.

Table 3.4. The summary of the classification of milk samples according to their origin of country by linear method

	True Group	
Put into Group	**France**	**Turkey**
France	19	3
Turkey	5	9
Total N	24	12
N correct	19	9
Proportion	0.792	0.750

To improve the results of the linear method, quadratic discriminant analysis was applied and significant improvement was attained. Quadratic method predicted the origin of the country of the milks correctly with 86.1% (Appendix A.2, Table A.2.4). Only 2 milks out of 24 from France have been misclassified as Turkish milk and 3 milks out of 12 from Turkey have been misclassified as French milk (Table 3.5).

Table 3.5. The summary of the classification of milk samples according to their origin of country by quadratic method

	True Group	
Put into Group	**France**	**Turkey**
France	21	2
Turkey	3	10
Total N	24	12
N correct	21	10
Proportion	0.875	0.833

Discriminant analyses on the factors, milk type (cow, coat), marketing (conventional, organic), processing (UHT, pasteurized) did not show any significant classification rates and even did not improve by the quadratic method. The correct classification for these factors was around 50% (Appendix A.2, Table A.2.5-A.2.10).

3.4. Determination of Honey Adulteration by NMR Relaxometry

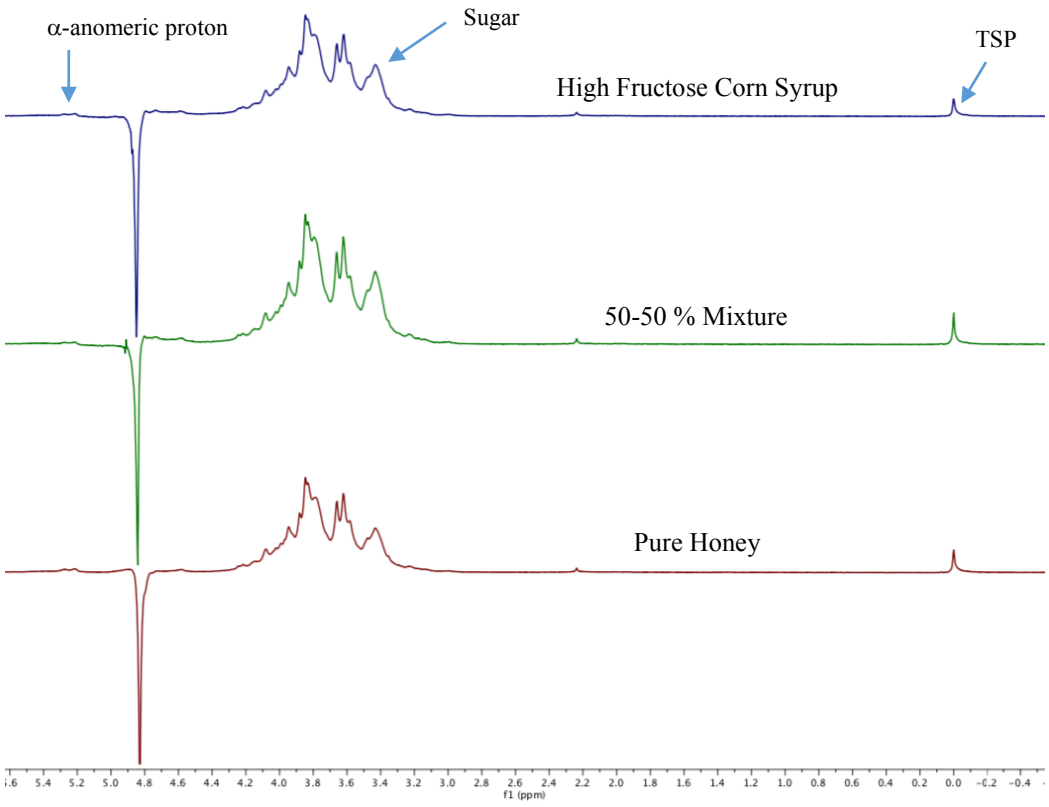
One of the most important methods of adulteration encountered in the honey industry is the addition of cheaper sweeteners such as refined sugar, high fructose corn syrup and maltose syrup (Ribeiro et al., 2014). Such adulteration may be detected by Stable Carbon Isotope Ratio Mass Spectrometry, NMR Spectroscopy, HPLC or Raman Spectroscopy or Near Infrared Spectroscopy (Arvanitoyannis, Chalhoub, Gotsiou, Lydakis-Simantiris, & Kefalas, 2005). In addition, a study examining the change on the T_2 relaxation times due to the contribution of high fructose syrup is available in the literature (Ribeiro et al., 2014). The basic logic is that by adding fructose syrup, the water binding behavior of the sugars in honey changes and accordingly the compositional changes shows the effect on the T_2 relaxation times. In this dissertation

NMR Spectroscopy was combined with relaxometry to explore adulteration on honey samples.

3.4.1. 1D NMR Spectra

1D NMR spectroscopy experiments were first conducted with pure honey, pure high fructose corn syrup and 50-50% mixture of pure honey and high fructose corn syrup to identify peaks that can be used for adulteration analysis. The stacked and superimposed spectra can be seen in Figure 3.22 (a) and (b) respectively.

a)



b)

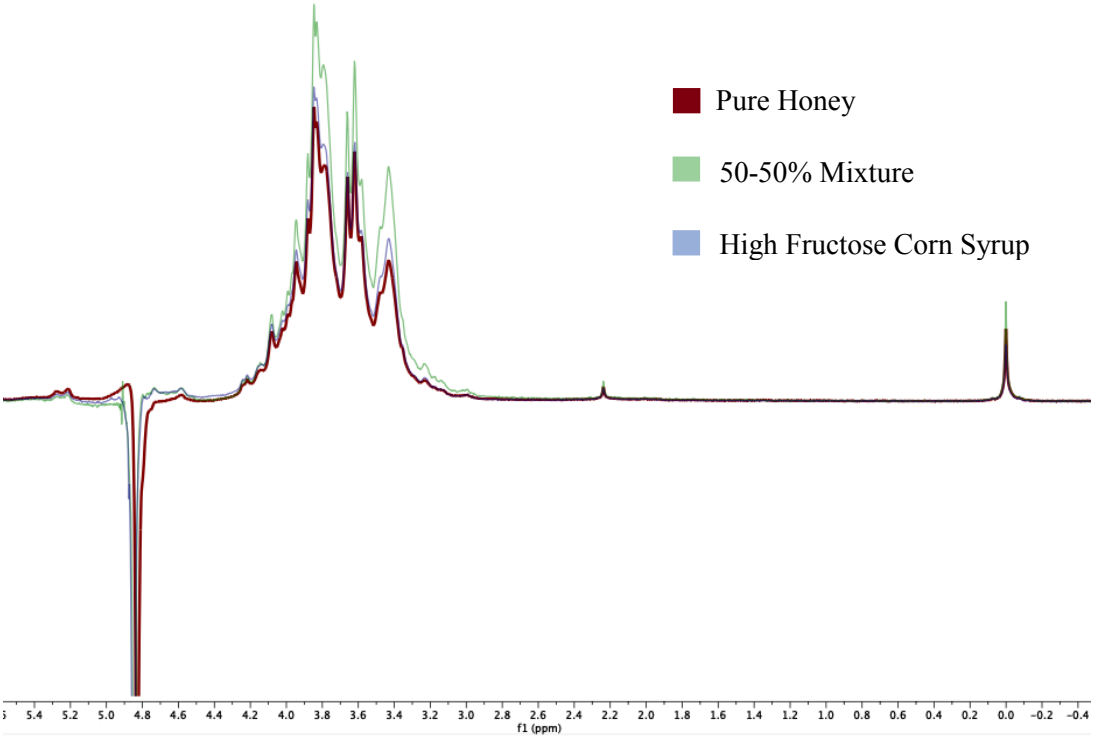


Figure 3.22. a) Stacked spectra of honey, 50-50 % mixtures, fructose syrup. b) Superimposed spectra of honey, 50-50 % mixtures, fructose syrup.

It can be seen from Figure 3.22 that there was no distinctive peak to differentiate pure and adulterated honey. Therefore, it was decided to make T_2 relaxometry measurements to differentiate adulterated samples instead of NMR spectroscopy. However, the spectra were used to obtain the T_2 values of different parts in the spectrum.

3.4.2. T_2 Relaxometry Measurements

T_2 relaxation times were calculated for water, sugar peak in total, alpha anomeric proton and for the bulk. Results are given in Table 7. Since the goal was to predict if

adulteration could be detected by using NMR relaxometry, regression analysis was conducted on the data. To explore the type of the regression, scatter plots were obtained for the data (Fig. 3.23). Except for the relaxation times of sugar regions, a linear pattern was observed and regression analysis was performed accordingly.

Table 3.6. T_2 relaxation times of water, sugar peak in total, alpha anomeric proton and for the bulk

% of Adulteration (Fructose)	T2 Bulk	Std Dev	T2 Alpha	Std Dev	T2 sugar	Std Dev	T2 water	Std Dev
100	1233.333	28.868	1379.333	21.385	903.500	0.424	1820.333	65.064
95	1230.000	26.458	1358.333	37.448	897.567	4.571	1833.333	75.831
90	1253.333	20.817	1412.000	30.406	896.367	3.523	1778.333	60.880
80	1220.000	17.321	1345.333	30.436	898.500	3.005	1771.333	63.058
70	1206.667	23.094	1348.667	33.710	893.667	4.216	1625.333	52.166
60	1176.667	23.094	1322.333	19.502	892.933	5.119	1583.667	59.811
50	1140.000	17.321	1300.333	17.954	887.600	4.071	1445.667	29.195
40	1113.333	20.817	1292.667	5.132	890.600	5.839	1469.000	47.655
30	1086.667	23.094	1271.000	35.595	890.067	4.716	1405.333	33.501
20	1070.000	17.321	1218.000	24.576	889.900	4.168	1359.667	27.025
10	1046.667	23.094	1220.000	5.568	888.700	5.333	1328.000	31.796
5	1046.667	23.094	1259.000	34.699	886.467	3.686	1319.667	26.083
0	1043.333	11.547	1174.333	40.154	881.933	5.160	1222.667	27.647

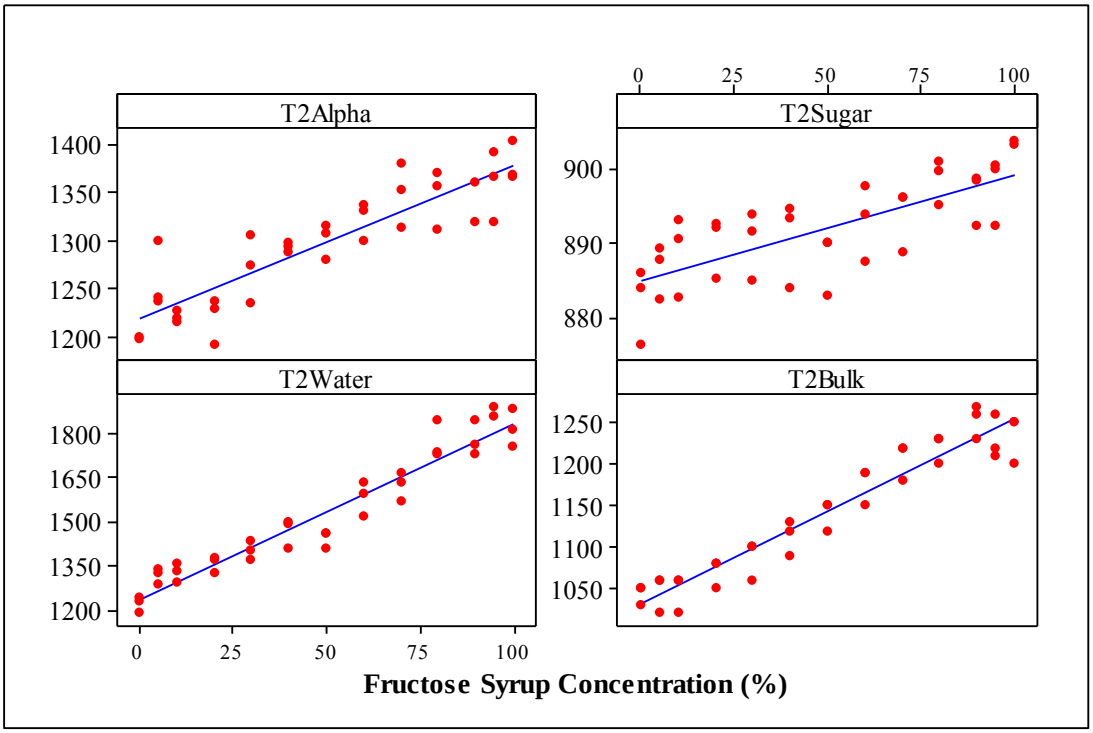


Figure 3.23. Scatter plots for T2 relaxation data of alpha anomeric proton, sugar, water and bulk regions

Regression analysis results are given in Appendix A.3. Equations and the corresponding R^2 values are also shown as follows.

T2Alpha = 1209.09 + 1.78587 x FS R^2=93.17% (Eq. 13)

T2Sugar = 885.871 + 0.133088 x FS R^2=57.34% (Eq. 14)

T2Water = 1243.1 + 5.87335 x FS R^2=94.64% (Eq. 15)

T2Bulk = 1030.35 + 2.29558 x FS R^2=93.47% (Eq. 16)

Lack of fit was also found insignificant for all models ($p<0.05$). Since the presence of fructose syrup was not identified from the spectrum and was overlapping with the sugars present in the honey as shown before, it was not possible to differentiate adulteration based on sugar content. However, for water, anomeric proton and accordingly for the bulk this was not the case.

Results showed that the combinatorial approach of spectroscopy and relaxometry could be used to understand the adulteration rate in honey. Further validation studies should be performed and different honey samples should be used to standardize the method.

CHAPTER 4

CONCLUSION

Developing food analysis methods are very challenging due to the complex nature of food systems. Cheap, affordable, fast and reliable methods have been the interest of food scientists and producers. Especially, nondestructive methods have gained attention since they do not require any pretreatment on the samples. In this dissertation, it was focused to examine benchtop NMR spectroscopy-based methods for different food science applications. Continuous reaction monitoring, authentication of products and adulteration were studied.

Two of the most widely used hydrolysis reactions in food industry are sucrose hydrolysis in sugar industry and lactose hydrolysis in dairy industry. For the quantitative NMR measurements, selecting the experiment parameters is very crucial. Therefore, T_1, T_R, flow rate, water suppression method and the NMR peaks that will be monitored during hydrolysis have been selected before the hydrolysis experiments. The hydrolysis reactions have been monitored successfully.

Food authentication has always been the interest of the consumers, food producers and regulatory authorities. It is getting more importance because of the food safety and food quality issues. In this dissertation, milk samples from the origins of France and Turkey have been analyzed for the authenticity analysis by benchtop NMR spectroscopy. The significant difference has been found their glycerol content. Milks from France origin contain much more glycerol than the milks from Turkey origin. Because the glycerol is the byproduct of the biodiesel industry and it is used as an energy source alternative to corn, it can be easily concluded that France is investing more on biodiesel industry than Turkey. Benchtop NMR spectroscopy can also be used to differentiate lactose-free and half-fat milks successfully.

Ambition to earn more money has directed people to food adulteration from the ancient times. They always searched the new methods, new substances to adulterate foods while the scientists search for new methods to analyze the adulteration. In this dissertation we have studied combinatorial approach of spectroscopy and relaxometry to find the rate of adulteration of honey by high fructose corn syrup. The results showed that T_2 relaxation times can be used to determine the adulteration rate of honey.

Benchtop NMR spectroscopy is seen as a promising, easy to use, non-invasive and affordable tool to analyze food samples either for academic research and for the industry. It has significant potential on developing new analysis methods.

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APPENDICES

A. STATISTICAL ANALYSES

A.1. Analysis of Variance for Milk

General Linear Model: Glycerol, Lactose, ... versus Country, Fat Status, ...

```
Factor       Type   Levels  Values
Country      fixed       2  FR, TR
Fat Status   fixed       2  Half, Whole
Sugar Status fixed       2  LF, Regular
Milk Type    fixed       2  Cow, Goat
Marketing    fixed       2  Conventional, Organic
Processing   fixed       2  Pasteurized, UHT


Analysis of Variance for Glycerol, using Adjusted SS for Tests

Source       DF   Seq SS   Adj SS   Adj MS      F      P
Country       1  0.63116  0.94559  0.94559  17.93  0.000
Fat Status    1  1.26309  0.96947  0.96947  18.38  0.000
Sugar Status  1  0.00003  0.00248  0.00248   0.05  0.830
Milk Type     1  0.01629  0.04408  0.04408   0.84  0.368
Marketing     1  0.05808  0.04587  0.04587   0.87  0.359
Processing    1  0.14374  0.14374  0.14374   2.73  0.110
Error        29  1.52925  1.52925  0.05273
Total        35  3.64163


S = 0.229636   R-Sq = 58.01%   R-Sq(adj) = 49.32%


Analysis of Variance for Lactose, using Adjusted SS for Tests

Source       DF   Seq SS   Adj SS   Adj MS       F      P
Country       1   216.06     0.24     0.24    0.11  0.747
Fat Status    1   564.60     2.18     2.18    0.97  0.332
Sugar Status  1  1262.73  1238.00  1238.00  552.16  0.000
Milk Type     1     3.29     1.82     1.82    0.81  0.375
Marketing     1     0.08     0.04     0.04    0.02  0.894
Processing    1     1.32     1.32     1.32    0.59  0.449
Error        29    65.02    65.02     2.24
Total        35  2113.10


S = 1.49737   R-Sq = 96.92%   R-Sq(adj) = 96.29%
```

Analysis of Variance for Fat, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Country	1	1.99	50.72	50.72	3.60	0.068
Fat Status	1	4925.93	3159.71	3159.71	224.26	0.000
Sugar Status	1	8.21	11.06	11.06	0.78	0.383
Milk Type	1	0.05	0.10	0.10	0.01	0.934
Marketing	1	11.46	10.59	10.59	0.75	0.393
Processing	1	3.13	3.13	3.13	0.22	0.641
Error	29	408.60	408.60	14.09		
Total	35	5359.38				

S = 3.75360 R-Sq = 92.38% R-Sq(adj) = 90.80%

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Grouping Information Using Tukey Method and 95.0% Confidence for Glycerol

Country	N	Mean	Grouping
FR	24	0.6	A
TR	12	0.2	B

Grouping Information Using Tukey Method and 95.0% Confidence for Glycerol

Fat Status	N	Mean	Grouping
Whole	20	0.6	A
Half	16	0.2	B

Grouping Information Using Tukey Method and 95.0% Confidence for Glycerol

Sugar Status	N	Mean	Grouping
Regular	30	0.4	A
LF	6	0.4	A

Grouping Information Using Tukey Method and 95.0% Confidence for Glycerol

Milk Type	N	Mean	Grouping
Cow	31	0.5	A
Goat	5	0.3	A

Grouping Information Using Tukey Method and 95.0% Confidence for Glycerol

Marketing	N	Mean	Grouping
Conventional	24	0.4	A
Organic	12	0.4	A

Grouping Information Using Tukey Method and 95.0% Confidence for Glycerol

Processing	N	Mean	Grouping
UHT	28	0.5	A
Pasteurized	8	0.3	A

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Grouping Information Using Tukey Method and 95.0% Confidence for Lactose

Country   N  Mean  Grouping
TR       12  10.0  A
FR       24   9.8  A



Grouping Information Using Tukey Method and 95.0% Confidence for Lactose

Fat
Status    N  Mean  Grouping
Half     16  10.2  A
Whole    20   9.6  A



Grouping Information Using Tukey Method and 95.0% Confidence for Lactose

Sugar Status N  Mean  Grouping
Regular     30  20.3  A
LF           6  -0.4    B



Grouping Information Using Tukey Method and 95.0% Confidence for Lactose

Milk
Type   N  Mean  Grouping
Cow   31  10.3  A
Goat   5   9.6  A



Grouping Information Using Tukey Method and 95.0% Confidence for Lactose

Marketing      N  Mean  Grouping
Organic       12  10.0  A
Conventional  24   9.9  A



Grouping Information Using Tukey Method and 95.0% Confidence for Lactose

Processing    N  Mean  Grouping
Pasteurized   8  10.2  A
UHT          28   9.7  A

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Grouping Information Using Tukey Method and 95.0% Confidence for Fat

Country   N  Mean  Grouping
FR       24  38.4  A
TR       12  35.2  A
```

```
Grouping Information Using Tukey Method and 95.0% Confidence for Fat

Fat
Status    N  Mean  Grouping
Whole    20  48.9  A
Half     16  24.7  B


Grouping Information Using Tukey Method and 95.0% Confidence for Fat

Sugar Status N  Mean  Grouping
LF           6  37.8  A
Regular     30  35.8  A
Grouping Information Using Tukey Method and 95.0% Confidence for Fat

Milk
Type   N  Mean  Grouping
Goat   5  36.9  A
Cow   31  36.7  A


Grouping Information Using Tukey Method and 95.0% Confidence for Fat

Marketing      N  Mean  Grouping
Organic       12  37.5  A
Conventional  24  36.2  A


Grouping Information Using Tukey Method and 95.0% Confidence for Fat

Processing    N  Mean  Grouping
Pasteurized   8  37.2  A
UHT          28  36.4  A
```

A.2. Discriminant Analysis for Milk

Table A.2.1. Linear Discriminant Analysis: Fat Status versus Glycerol, Lactose, Fat

Linear Method for Response: Fat Status

Predictors: Glycerol, Lactose, Fat

Groups

Group	Half	Whole
Count	16	20

Summary of Classification

	True Group	
Put into Group	Half	Whole
Half	16	0
Whole	0	20
Total N	16	20
N correct	16	20
Proportion	1.000	1.000

Correct Classifications

N	Correct	Proportion
36	36	1.000

Squared Distance Between Groups

	Half	Whole
Half	0.0000	36.0809
Whole	36.0809	0.0000

Linear Discriminant Function for Groups

	Half	Whole
Constant	-20.140	-75.998
Glycerol	-3.272	-6.933
Lactose	0.121	0.131
Fat	1.631	3.227

Table A.2.2. Linear Discriminant Analysis: Sugar Status versus Glycerol, Lactose, Fat

Linear Method for Response: Sugar Status
Predictors: Glycerol, Lactose, Fat

Groups

Group	Lactose-Free	Regular
Count	6	30

Summary of Classification

	True Group	
Put into Group	Lactose-Free	Regular
Lactose-Free	6	0
Regular	0	30
Total N	6	30
N correct	6	30
Proportion	1.000	1.000

Correct Classifications

N	Correct	Proportion
36	36	1.000

Squared Distance Between Groups

	Lactose-Free	Regular
Lactose-Free	0.000	208.885
Regular	208.885	0.000

Linear Discriminant Function for Groups

	Lactose-Free	Regular
Constant	-2.42	-109.96
Glycerol	-0.18	15.42
Lactose	-0.01	10.07
Fat	0.20	0.15

Table A.2.3. Linear Discriminant Analysis: Country versus Glycerol, Lactose, Fat

Linear Method for Response: Country
Predictors: Glycerol, Lactose, Fat

Groups

Group	France	Turkey
Count	24	12

Summary of Classification

	True Group	
Put into Group	France	Turkey
France	19	3
Turkey	5	9
Total N	24	12
N correct	19	9
Proportion	0.792	0.750

Correct Classifications

N	Correct	Proportion
36	28	0.778

Squared Distance Between Groups

	France	Turkey
France	0.00000	2.37455
Turkey	2.37455	0.00000

Linear Discriminant Function for Groups

	France	Turkey
Constant	-5.1932	-4.8386
Glycerol	2.5528	-3.1594
Lactose	0.1725	0.0372
Fat	0.1442	0.2775

Summary of Misclassified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
7**	France	Turkey	France	2.8655	0.276
			Turkey	0.9352	0.724
17**	France	Turkey	France	3.380	0.439
			Turkey	2.889	0.561
20**	France	Turkey	France	1.309	0.495
			Turkey	1.269	0.505
23**	Turkey	France	France	0.4039	0.784
			Turkey	2.9833	0.216
24**	Turkey	France	France	1.261	0.833
			Turkey	4.478	0.167
27**	Turkey	France	France	0.4789	0.657
			Turkey	1.7766	0.343
31**	France	Turkey	France	6.437	0.235
			Turkey	4.081	0.765
32**	France	Turkey	France	9.190	0.053
			Turkey	3.408	0.947

Table A.2.4. Quadratic Discriminant Analysis: Country versus Glycerol, Lactose, Fat

Quadratic Method for Response: Country
Predictors: Glycerol, Lactose, Fat

Groups

Group	France	Turkey
Count	24	12

Summary of Classification

	True Group	
Put into Group	France	Turkey
France	21	2
Turkey	3	10
Total N	24	12
N correct	21	10
Proportion	0.875	0.833

Correct Classifications

N	Correct	Proportion
36	31	0.861

From Generalized Squared Distance to Group

Group	France	Turkey
France	5.173	13.640
Turkey	7.918	3.642

Summary of Misclassified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
7**	France	Turkey	France	9.291	0.405
			Turkey	8.520	0.595
23**	Turkey	France	France	5.499	0.553
			Turkey	5.925	0.447
24**	Turkey	France	France	6.313	0.809
			Turkey	9.198	0.191
31**	France	Turkey	France	15.370	0.008
			Turkey	5.809	0.992
32**	France	Turkey	France	16.213	0.006
			Turkey	6.050	0.994

Table A.2.5. Linear Discriminant Analysis: Processing versus Glycerol, Lactose, Fat

Linear Method for Response: Processing
Predictors: Glycerol, Lactose, Fat

Groups

Group	Pasteurized	UHT
Count	8	28

Summary of Classification

	True Group	
Put into Group	Pasteurized	UHT
Pasteurized	7	16
UHT	1	12
Total N	8	28
N correct	7	12
Proportion	0.875	0.429

Correct Classifications

N	Correct	Proportion
36	19	0.528

Squared Distance Between Groups

	Pasteurized	UHT
Pasteurized	0.000000	0.442739
UHT	0.442739	0.000000

Linear Discriminant Function for Groups

	Pasteurized	UHT
Constant	-6.2900	-4.6377
Glycerol	-0.4960	-0.0159
Lactose	0.2024	0.1153
Fat	0.2135	0.2041

Summary of Misclassified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
2**	UHT	Pasteurized	Pasteurized	3.341	0.519
			UHT	3.489	0.481
3**	Pasteurized	UHT	Pasteurized	0.6995	0.491
			UHT	0.6276	0.509
8**	UHT	Pasteurized	Pasteurized	2.574	0.508
			UHT	2.638	0.492
12**	UHT	Pasteurized	Pasteurized	2.379	0.575
			UHT	2.982	0.425
13**	UHT	Pasteurized	Pasteurized	2.402	0.571
			UHT	2.975	0.429
14**	UHT	Pasteurized	Pasteurized	2.199	0.582
			UHT	2.864	0.418
18**	UHT	Pasteurized	Pasteurized	1.593	0.528
			UHT	1.813	0.472
19**	UHT	Pasteurized	Pasteurized	2.152	0.504
			UHT	2.183	0.496
21**	UHT	Pasteurized	Pasteurized	0.5106	0.541
			UHT	0.8357	0.459
23**	UHT	Pasteurized	Pasteurized	0.6768	0.503
			UHT	0.7002	0.497
24**	UHT	Pasteurized	Pasteurized	1.637	0.504
			UHT	1.671	0.496
25**	UHT	Pasteurized	Pasteurized	3.022	0.644
			UHT	4.206	0.356
26**	UHT	Pasteurized	Pasteurized	0.8504	0.539
			UHT	1.1659	0.461
27**	UHT	Pasteurized	Pasteurized	0.3814	0.512
			UHT	0.4767	0.488
28**	UHT	Pasteurized	Pasteurized	3.434	0.592
			UHT	4.179	0.408
29**	UHT	Pasteurized	Pasteurized	4.973	0.657
			UHT	6.274	0.343
30**	UHT	Pasteurized	Pasteurized	4.468	0.606
			UHT	5.333	0.394

Table A.2.6. Quadratic Discriminant Analysis: Processing versus Glycerol, Lactose, Fat

Quadratic Method for Response: Processing
Predictors: Glycerol, Lactose, Fat

Groups

Group	Pasteurized	UHT
Count	8	28

Summary of Classification

	True Group	
Put into Group	Pasteurized	UHT
Pasteurized	8	15
UHT	0	13
Total N	8	28
N correct	8	13
Proportion	1.000	0.464

Correct Classifications

N	Correct	Proportion
36	21	0.583

From Generalized Squared Distance to Group

Group	Pasteurized	UHT
Pasteurized	2.586	6.586
UHT	12.814	6.229

Summary of Misclassified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
2**	UHT	Pasteurized	Pasteurized	9.424	0.564
			UHT	9.938	0.436
5**	UHT	Pasteurized	Pasteurized	5.732	0.774
			UHT	8.191	0.226
8**	UHT	Pasteurized	Pasteurized	7.703	0.653
			UHT	8.967	0.347
12**	UHT	Pasteurized	Pasteurized	7.875	0.705
			UHT	9.620	0.295
14**	UHT	Pasteurized	Pasteurized	4.126	0.942
			UHT	9.709	0.058
15**	UHT	Pasteurized	Pasteurized	6.406	0.812
			UHT	9.329	0.188
16**	UHT	Pasteurized	Pasteurized	5.919	0.841
			UHT	9.251	0.159
18**	UHT	Pasteurized	Pasteurized	3.874	0.908
			UHT	8.449	0.092
19**	UHT	Pasteurized	Pasteurized	7.251	0.639
			UHT	8.395	0.361
20**	UHT	Pasteurized	Pasteurized	7.192	0.508
			UHT	7.252	0.492
21**	UHT	Pasteurized	Pasteurized	2.961	0.890
			UHT	7.150	0.110
22**	UHT	Pasteurized	Pasteurized	5.209	0.868
			UHT	8.967	0.132
23**	UHT	Pasteurized	Pasteurized	4.144	0.806
			UHT	6.988	0.194
24**	UHT	Pasteurized	Pasteurized	4.812	0.843
			UHT	8.167	0.157
27**	UHT	Pasteurized	Pasteurized	4.002	0.800
			UHT	6.777	0.200

Table A.2.7. Linear Discriminant Analysis: Marketing versus Glycerol, Lactose, Fat

Linear Method for Response: Marketing
Predictors: Glycerol, Lactose, Fat

Groups

Group	Conventional	Organic
Count	24	12

Summary of Classification

	True Group	
Put into Group	Conventional	Organic
Conventional	10	3
Organic	14	9
Total N	24	12
N correct	10	9
Proportion	0.417	0.750

Correct Classifications

N	Correct	Proportion
36	19	0.528

Squared Distance Between Groups

	Conventional	Organic
Conventional	0.000000	0.506005
Organic	0.506005	0.000000

Linear Discriminant Function for Groups

	Conventional	Organic
Constant	-4.5557	-6.6948
Glycerol	-0.0084	-0.1108
Lactose	0.1194	0.1937
Fat	0.2060	0.2291

Summary of Misclassified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
1**	Conventional	Organic	Conventional	3.411	0.495
			Organic	3.369	0.505
2**	Conventional	Organic	Conventional	3.844	0.390
			Organic	2.948	0.610
3**	Conventional	Organic	Conventional	0.7990	0.460
			Organic	0.4749	0.540
4**	Conventional	Organic	Conventional	2.192	0.379
			Organic	1.207	0.621
9**	Organic	Conventional	Conventional	3.386	0.563
			Organic	3.889	0.437
11**	Conventional	Organic	Conventional	4.501	0.475
			Organic	4.299	0.525
12**	Conventional	Organic	Conventional	2.916	0.479
			Organic	2.746	0.521
14**	Conventional	Organic	Conventional	3.324	0.329
			Organic	1.902	0.671
15**	Organic	Conventional	Conventional	2.472	0.571
			Organic	3.041	0.429
18**	Organic	Conventional	Conventional	1.731	0.523
			Organic	1.911	0.477
23**	Conventional	Organic	Conventional	0.8827	0.449
			Organic	0.4711	0.551
24**	Conventional	Organic	Conventional	1.942	0.421
			Organic	1.306	0.579
25**	Conventional	Organic	Conventional	4.330	0.369
			Organic	3.258	0.631
26**	Conventional	Organic	Conventional	1.2382	0.460
			Organic	0.9167	0.540
27**	Conventional	Organic	Conventional	0.6524	0.445
			Organic	0.2131	0.555
28**	Conventional	Organic	Conventional	4.264	0.418
			Organic	3.604	0.582
30**	Conventional	Organic	Conventional	5.408	0.410
			Organic	4.683	0.590

Table A.2.8. Quadratic Discriminant Analysis: Marketing versus Glycerol, Lactose, Fat

Quadratic Method for Response: Marketing
Predictors: Glycerol, Lactose, Fat

Groups

Group	Conventional	Organic
Count	24	12

Summary of Classification

	True Group	
Put into Group	Conventional	Organic
Conventional	6	0
Organic	18	12
Total N	24	12
N correct	6	12
Proportion	0.250	1.000

Correct Classifications

N	Correct	Proportion
36	18	0.500

From Generalized Squared Distance to Group

Group	Conventional	Organic
Conventional	6.314	12.250
Organic	6.698	3.288

Summary of Misclassified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
1**	Conventional	Organic	Conventional	9.385	0.313
			Organic	7.816	0.687
2**	Conventional	Organic	Conventional	9.963	0.275
			Organic	8.027	0.725
3**	Conventional	Organic	Conventional	7.129	0.208
			Organic	4.460	0.792
4**	Conventional	Organic	Conventional	8.462	0.193
			Organic	5.597	0.807
8**	Conventional	Organic	Conventional	9.146	0.152
			Organic	5.707	0.848
10**	Conventional	Organic	Conventional	8.834	0.120
			Organic	4.852	0.880
11**	Conventional	Organic	Conventional	11.142	0.172
			Organic	8.003	0.828
12**	Conventional	Organic	Conventional	9.506	0.266
			Organic	7.478	0.734
14**	Conventional	Organic	Conventional	10.179	0.094
			Organic	5.638	0.906
16**	Conventional	Organic	Conventional	9.235	0.146
			Organic	5.700	0.854
19**	Conventional	Organic	Conventional	8.580	0.166
			Organic	5.352	0.834
23**	Conventional	Organic	Conventional	7.157	0.170
			Organic	3.990	0.830
24**	Conventional	Organic	Conventional	8.307	0.140
			Organic	4.683	0.860
25**	Conventional	Organic	Conventional	10.168	0.228
			Organic	7.725	0.772
26**	Conventional	Organic	Conventional	7.452	0.334
			Organic	6.074	0.666
27**	Conventional	Organic	Conventional	6.996	0.175
			Organic	3.894	0.825
28**	Conventional	Organic	Conventional	10.268	0.336
			Organic	8.903	0.664
30**	Conventional	Organic	Conventional	11.30	0.358
			Organic	10.13	0.642

Table A.2.9. Linear Discriminant Analysis: Milk Type versus Glycerol, Lactose, Fat

Linear Method for Response: Milk Type
Predictors: Glycerol, Lactose, Fat

Groups

Group	Cow	Goat
Count	31	5

Summary of Classification

	True Group	
Put into Group	Cow	Goat
Cow	16	1
Goat	15	4
Total N	31	5
N correct	16	4
Proportion	0.516	0.800

Correct Classifications

N	Correct	Proportion
36	20	0.556

Squared Distance Between Groups

	Cow	Goat
Cow	0.000000	0.375189
Goat	0.375189	0.000000

Linear Discriminant Function for Groups

	Cow	Goat
Constant	-4.7284	-6.7842
Glycerol	-0.0197	-0.2497
Lactose	0.1137	0.1305
Fat	0.2081	0.2555

Summary of Misclassified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
2**	Cow	Goat	Cow	3.552	0.408
			Goat	2.810	0.592
3**	Cow	Goat	Cow	0.6624	0.462
			Goat	0.3611	0.538
4**	Cow	Goat	Cow	1.882	0.395
			Goat	1.032	0.605
5**	Cow	Goat	Cow	1.763	0.430
			Goat	1.203	0.570
6**	Cow	Goat	Cow	2.926	0.331
			Goat	1.517	0.669
7**	Cow	Goat	Cow	1.6269	0.389
			Goat	0.7234	0.611
14**	Cow	Goat	Cow	2.985	0.307
			Goat	1.355	0.693
17**	Cow	Goat	Cow	3.029	0.298
			Goat	1.314	0.702
19**	Goat	Cow	Cow	2.007	0.637
			Goat	3.134	0.363
23**	Cow	Goat	Cow	0.7118	0.463
			Goat	0.4116	0.537
24**	Cow	Goat	Cow	1.744	0.420
			Goat	1.098	0.580
25**	Cow	Goat	Cow	3.965	0.404
			Goat	3.189	0.596
26**	Cow	Goat	Cow	1.0953	0.468
			Goat	0.8363	0.532
27**	Cow	Goat	Cow	0.48923	0.449
			Goat	0.08307	0.551
28**	Cow	Goat	Cow	4.062	0.416
			Goat	3.383	0.584
29**	Cow	Goat	Cow	6.034	0.380
			Goat	5.059	0.620

Table A.2.10. Quadratic Discriminant Analysis: Milk Type versus Glycerol, Lactose, Fat

Quadratic Method for Response: Milk Type
Predictors: Glycerol, Lactose, Fat

Groups

Group	Cow	Goat
Count	31	5

Summary of Classification

	True Group	
Put into Group	Cow	Goat
Cow	19	0
Goat	12	5
Total N	31	5
N correct	19	5
Proportion	0.613	1.000

Correct Classifications

N	Correct	Proportion
36	24	0.667

From Generalized Squared Distance to Group

Group	Cow	Goat
Cow	6.262	15.297
Goat	6.615	2.058

Summary of Misclassified Observations

Observation	True Group	Pred Group	Group	Squared Distance	Probability
1**	Cow	Goat	Cow	9.760	0.120
			Goat	5.770	0.880
3**	Cow	Goat	Cow	6.911	0.125
			Goat	3.020	0.875
5**	Cow	Goat	Cow	8.017	0.081
			Goat	3.155	0.919
7**	Cow	Goat	Cow	7.928	0.065
			Goat	2.605	0.935
8**	Cow	Goat	Cow	8.736	0.196
			Goat	5.917	0.804
10**	Cow	Goat	Cow	8.195	0.267
			Goat	6.177	0.733
18**	Cow	Goat	Cow	7.800	0.271
			Goat	5.820	0.729
23**	Cow	Goat	Cow	6.960	0.091
			Goat	2.366	0.909
24**	Cow	Goat	Cow	7.983	0.094
			Goat	3.453	0.906
26**	Cow	Goat	Cow	7.427	0.157
			Goat	4.064	0.843
27**	Cow	Goat	Cow	6.726	0.097
			Goat	2.257	0.903
28**	Cow	Goat	Cow	10.609	0.042
			Goat	4.354	0.958

A.3. Regression Analysis for Honey

Notation: 30_70 means, honey has been adulterated with 70% fructose syrup.

General Regression Analysis: T2Alpha versus Fructose syrup

```
Regression Equation

T2Alpha  =  1209.09 + 1.78587 FS


30 cases used, 9 cases contain missing values


Coefficients

Term         Coef  SE Coef        T      P
Constant  1209.09  5.29947  228.153  0.000
FS           1.79  0.08966   19.918  0.000


Summary of Model

S = 16.2312      R-Sq = 93.41%        R-Sq(adj) = 93.17%
PRESS = 8487.07  R-Sq(pred) = 92.41%


Analysis of Variance

Source         DF  Seq SS  Adj SS  Adj MS        F         P
Regression      1  104515  104515  104515  396.712  0.000000
  FS            1  104515  104515  104515  396.712  0.000000
Error          28    7377    7377     263
  Lack-of-Fit  11    2848    2848     259    0.972  0.504424
  Pure Error   17    4528    4528     266
Total          29  111891


Fits and Diagnostics for Unusual Observations


No unusual observations
```

General Regression Analysis: T2Sugar_1 versus Fructose syrup

```
Regression Equation

T2Sugar_1  =  885.871 + 0.133088 FS


36 cases used, 3 cases contain missing values
```

Coefficients

Term	Coef	SE Coef	T	P
Constant	885.871	1.15446	767.344	0.000
FS	0.133	0.01920	6.932	0.000

Summary of Model

S = 3.84727 R-Sq = 58.56% R-Sq(adj) = 57.34%
PRESS = 562.119 R-Sq(pred) = 53.71%

Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	1	711.16	711.158	711.158	48.0465	0.000000
FS	1	711.16	711.158	711.158	48.0465	0.000000
Error	34	503.25	503.250	14.801		
Lack-of-Fit	11	96.78	96.780	8.798	0.4978	0.885223
Pure Error	23	406.47	406.470	17.673		
Total	35	1214.41				

Fits and Diagnostics for Unusual Observations

No unusual observations

General Regression Analysis: T2Water_1 versus Fructose syrup

Regression Equation

T2Water_1 = 1243.1 + 5.87335 FS

36 cases used, 3 cases contain missing values

Coefficients

Term	Coef	SE Coef	T	P
Constant	1243.10	13.8915	89.4862	0.000
FS	5.87	0.2360	24.8864	0.000

Summary of Model

S = 48.4064 R-Sq = 94.80% R-Sq(adj) = 94.64%
PRESS = 89808.4 R-Sq(pred) = 94.13%

Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	1	1451215	1451215	1451215	619.335	0.000000
FS	1	1451215	1451215	1451215	619.335	0.000000
Error	34	79668	79668	2343		
Lack-of-Fit	11	37418	37418	3402	1.852	0.102691
Pure Error	23	42250	42250	1837		
Total	35	1530883				

Fits and Diagnostics for Unusual Observations

No unusual observations

General Regression Analysis: T2Bulk_1 versus Fructose syrup

Regression Equation

T2Bulk_1 = 1030.35 + 2.29558 FS

38 cases used, 1 cases contain missing values

Coefficients

Term	Coef	SE Coef	T	P
Constant	1030.35	5.88835	174.981	0.000
FS	2.30	0.09966	23.033	0.000

Summary of Model

S = 20.5663 R-Sq = 93.65% R-Sq(adj) = 93.47%
PRESS = 16998.0 R-Sq(pred) = 92.91%

Analysis of Variance

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	1	224405	224405	224405	530.542	0.000000
FS	1	224405	224405	224405	530.542	0.000000
Error	36	15227	15227	423		
Lack-of-Fit	11	4694	4694	427	1.013	0.463639
Pure Error	25	10533	10533	421		
Total	37	239632				

Fits and Diagnostics for Unusual Observations

No unusual observations

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2014-Present	Onkogen Diagnostik Sistemler	General Manager
2012-2014	H2Biyotek	Business Development Manager
2007-2012	Onkogen Diagnostik Sistemler	Sales and Marketing Manager
2001-2007	METU Food Engineering	Research Assistant

FOREIGN LANGUAGES

Native Turkish, Advanced English, Intermediate French

PUBLICATIONS

1. Soyler A., Bouillaud D., Farjon J., Giraudeau P., Oztop M.H., Potential of Benchtop Quantitative NMR for Monitoring Enzyme Catalyzed Reactions. EUROMAR, July 1-5, Nantes, France. 2018.

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HOBBIES

Table Tennis, Languages, Travel