DESIGNING NON-INVASIVE QUALITY CONTROL METHODS FOR PHARMACEUTICAL DRUGS

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HANİ ALAM

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Approval of the thesis:

Designing Non-Invasive Quality Control Methods for Pharmaceutical Drugs

submitted by **HANİ ALAM** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** i**n Biotechnology, Middle East Technical University** by,

Prof. Dr. Naci Emre ALTUN
Dean, **Graduate School of Natural and Applied Sciences** _____

Prof. Dr. Yeşim SOYER KÜÇÜKŞENEL
Head of the Department, **Biotechnology** _____

Prof. Dr. Halil Mecit ÖZTOP
Supervisor, **Biotechnology, METU** _____

Assoc. Prof. Dr. Emin Burçin ÖZVURAL
Co-supervisor, Food Engineering, Çankiri Karatekin University _____

Examining Committee Members:

Prof. Dr. Alev BAYINDIRLI
Food Engineering, METU _____

Prof. Dr. Halil Mecit ÖZTOP
Supervisor, Biotechnology, METU _____

Assoc. Prof. Dr. Cem YAMALI
Basic Pharmaceutical Sciences, Çukurova University _____

Prof. Dr. Yeşim Soyer KÜÇÜKŞENEL
Biotechnology, METU _____

Assist. Prof. Dr. Umay Merve GÜVEN
Pharmaceutical Technology, Çukurova University _____

Date: 11.03.2024

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 materials and results that are not original to this work.

Name Last name: Hani Alam

Signature:

ABSTRACT

Designing Non-Invasive Quality Control Methods for Pharmaceutical Drugs

Alam, Hani

Doctor of Philosophy, Biotechnology

Supervisor: Prof. Dr. Halil Mecit ÖZTOP

Co-supervisor: Assoc. Prof. Dr. Emin Burçin ÖZVURAL

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The quality of pharmaceutical products is a critical aspect of the pharmaceutical industry that attempts to ensure that they meet the required safety and efficacy standards. Quality control ensures that these products are safe and meet the requirements of the regulators and government agencies. The main objective of quality control is to identify and quantify active substances and track impurities using analytical techniques, such as high-performance liquid chromatography (HPLC).

However, many of these techniques are time consuming, destructive to samples, damaging to the environment, and expensive to operate and maintain. This study aims to use alternative methods for quality control that are easy to operate, affordable, eco-friendly, and non-destructive to samples.

This study examined near infrared spectroscopy (NIRS) and time-domain nuclear magnetic resonance (TD-NMR) in order to produce non-destructive methods for three different quality control issues.

The first part of this study focused on measuring the mixture ratios of two different insulin drugs and succeeded by integrating two different TD-NMR techniques. The

second part measured the degradation of aspirin to salicylic acid using NIRS and TD-NMR. The third part involved the use of bovine serum albumin as a model drug for biopharmaceuticals. Later, glycation was applied and measured using different TD-NMR techniques to be compared with browning measurements and the O-phthalaldehyde (OPA) method, which are two parameters used to measure glycation.

This dissertation has demonstrated that TD-NMR and NIRS are valuable and useful methods for various pharmaceutical quality control procedures as more affordable, eco-friendly, and user-friendly alternatives to the current destructive chemical and chromatographic techniques.

Keywords: Aspirin, Insulin mixtures, Glycation, Time-Domain Nuclear Magnetic Resonance (TD-NMR), Near Infrared Spectrometry (NIRS), Pharmaceutical Quality Control

ÖZ

Farmasötik İlaçlar İçin Non-invasif Kalite Kontrol Yöntemlerinin Tasarlanması

Alam, Hani
Doktora, Biyoteknoloji
Tez Yöneticisi: Prof. Dr. Halil Mecit ÖZTOP
Ortak Tez Danışmanı: Doç. Dr. Emin Burçin ÖZVURAL

Mart 2024, 190 Sayfa

Farmasötik ürünlerin kalitesi, ilaç endüstrisinin güvenlik ve etki standartlarını karşılaması açısından çok kritiktir. Kalite kontrol, bu ürünlerin güvenli olmasını sağlayarak düzenleyicilerin ve devlet kurumlarının gereksinimlerini karşılar. Kalite kontrolün temel amacı, yüksek performanslı sıvı kromatografisi (HPLC) gibi analitik teknikler kullanarak aktif maddeleri tanımlamak, miktarlarını belirlemek ve safsızlıklarını ölçmektir.

Ancak bu tekniklerin çoğu zaman alan, numunelere zarar veren, çevreye zararlı ve işletme ve bakımı pahalı olan tekniklerdir. Bu çalışma, kalite kontrolü için kullanımı kolay, uygun fiyatlı, çevre dostu ve numunelere zarar vermeyen alternatif yöntemler kullanmayı amaçlamaktadır.

Bu çalışmada, üç farklı kalite kontrol sorunu için noninvasif teknikler üretmek amacıyla Yakın-kızılötesi spektroskopisi (NIRS) ve zamansal alanda nükleer manyetik rezonans (TD-NMR) kullanılmıştır.

Bu çalışmanın ilk bölümünde iki farklı insülin ilacının karışım oranlarını ölçülmeye çalışılmış ve bunda iki farklı TD-NMR tekniğini entegre ederek başarılı olunmuştur. İkinci bölümünde NIRS ve TD-NMR kullanılarak aspirinin salisilik aside bozunması ölçülmüştür. Üçüncü bölümünde, biyofarmasötikler için model ilaç olarak sığır serum albümini kullanıldı. Daha sonra glikasyon yapıldı ve glikasyonu ölçmek için kullanılan esmerleşme ölçümleri ve O-ftalaldehit (OPA) yöntemi ile karşılaştırılmak üzere farklı TD-NMR teknikleri kullanılarak ölçüldü.

Bu tez, TD-NMR ve NIRS'in çeşitli farmasötik kalite kontrol prosedürleri için mevcut kimyasal ve kromatografik tekniklere göre daha uygun fiyatlı, çevre dostu ve kullanıcı dostu alternatifler olarak kullanışlı yöntemler olduğunu göstermiştir.

Anahtar Kelimeler: Aspirin, İnsülin karışımları, Glikasyon, Zaman Alanı Nükleer Manyetik Rezonans (TD-NMR), Yakın Kızılötesi Spektroskopisi (NIRS), Farmasötik kalite kontrolu

To my father

May God bless his soul

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2D-ILT	Two-Dimensional Inverse Laplace Transform
2D-NMR	Two-Dimensional Nuclear Magnetic Resonance
AD	Anno Domini
AGE	Advanced Glycation End Products
API	Active Pharmaceutical Ingredients
BSA	Bovine Serum Albumin
c	Speed of Light
COX	Cyclooxygenase Enzymes
CPMG	Carr-Purcell-Meiboom-Gill
CWFP-T1	Continuous Wave Free Precession with low flip angle
E	Energy of Light
EMA	European Medicines Agency
f	Frequency of Light
FDA	Food and Drug Administration
FFC	Fast Field Cycling
GC	Gas Chromatography
GDP	Good Distribution Practice
GMP	Good Manufacturing Practice
h	Planck Constant
HF	High-Field
HF-NMR	High-Field Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
ICH	The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
IR	Infrared

IR-CPMG	Inversion Recovery Carr-Purcell-Meiboom-Gill
ISO	International Standard Organization
LC-MS	Liquid Chromatography-Mass Spectrometry
LED	Light Emitting Diode
LF	Low-Field
LF-NMR	Low-Field Nuclear Magnetic Resonance
M	Magnetization Amplitude
M_0	Magnetization Amplitude at Equilibrium
mAb	Monoclonal Antibody
MLR	Multiple Linear Regression
MRI	Magnetic Resonance Imaging
NIR	Near Infrared
NIR-CI	Near-Infrared Spectroscopy Chemical Imaging
NIRS	Near Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
NPH	Neutral Protamine Hagedorn
NSAID	Nonsteroidal Anti-Inflammatory Drug
OPA	O-phthalaldehyde
PD	Pharmacodynamics
PFG	Pulse Field Gradient
PK	Pharmacokinetics
PLSR	Partial Least Squares Regression
QA	Quality Assurance
QC	Quality Control
R^2	Correlation Coefficient
R^2 adj	Adjusted Correlation Coefficient
RF	Radio Frequency

RH	Relative Humidity
RMSE	Root Mean Square Error
SEC	Standard Error Calibration
SEP	Standard Error Prediction
SR	Saturation Recovery
t	Echo Time
T	Tesla
T_1	Longitudinal Magnetization Relaxation Time
T1D	Type 1 Diabetes
T_2	Transverse Magnetization Relaxation Time
T2D	Type 2 Diabetes
TD-NMR	Time Domain Nuclear Magnetic Resonance
TITCK	Turkish Medicines and Medical Devices Agency
TR	Repetition Time
UHPLC	Ultra-High Performance Liquid Chromatography
WHO	World Health Organization
X^*	Normalized Scaled Value of The Data
$X_1 \ldots X_n$	Independent Variables
X_{min}	Minimum Value of The Trained Group
X_{max}	Maximum Value of The Trained Group
XRD	X-Ray Diffraction
y	Dependent Variable
β_0	Parameter When All Independent Variables Are Equal to Zero
$\beta_1 \ldots \beta_n$	Parameters of The Independent Variables
τ	Recovery Time
λ	Wavelength of Light

CHAPTER 1

INTRODUCTION

1.1 Pharmaceutical Quality Control

The pharmaceutical sector is subject to strict regulations because patients and customers often cannot detect quality issues and problems in pharmaceutical products unless they experience serious ill effects or fatalities after consumption. Various defects in the quality of pharmaceutical products may exhibit and occur, including inferior lower yield of the manufacturing process, or potential damage on the pharmaceutical´s therapeutic efficiency. In addition, there is often a lack of understanding when it comes to technological deliveries and scaling up of the product. Moreover, there is a lack of understanding and a lack of examination for the causes of the manufacture's failures and misfunctions. Although the pharmaceutical industry is seen as profit-orientated, it is mistrusted by the public. There is a common belief that all pharmaceuticals approved and regulated by governments are safe, effective and meet high-quality standards. This drives manufacturers to follow regulations and quality standards issued by government agencies (Djuris et al., 2024; Poonia et al., 2023; Yu & Kopcha, 2017).

Quality assurance (QA) is the general term for ensuring high-quality pharmaceutical products, which could be stated as the total cumulative approaches to guarantee the pharmaceutical products to achieve the designed high standards for their planed and proposed usage. Quality assurance is a systemic procedure which is operated to assert and guarantee that pharmaceutical products in the market are harmless, risk free, and comply with the expected criteria based on the laws and regulations. These include all the operations from the raw materials toward the production and

distributions of end products (Cardinal, 2001; Tefera Mekasha et al., 2020; Yu, 2008).

Quality control (QC) is described as the main organized structure that provide and retain the intended quality standards in supplied products and services by cautious provisions, usage of suitable tools and apparatus, regular examinations, and the restorations as needed (Gee, 2022).

According to the International Standard Organization (ISO), quality assurance handles the actions accomplished by laboratories in order to maintain and deliver faith that products live up to the intended and required standards. However, quality control includes the separate examinations methods and techniques which satisfy the required standards(Wulandari et al., 2022).

To ensure the preservation of high quality, effective pharmaceutical products, governments and agencies work on guidelines, practices such as Good Manufacturing Practice (GMP) and Good Distribution Practice (GDP), and testing, including import testing and in-process control testing. GDP represents the minimum standards of quality and integrity needed for medicines and pharmaceuticals that manufacturers and pharmaceutical traders must provide and secure during the supply chain. As an example, these standards should be maintained when medications are stored to be always in the right environments and surroundings, including handling, storage, and transportation (*Good Distribution Practice | European Medicines Agency*, 2021). GMP represents the minimum requirements that pharmaceutical companies must guarantee during drug manufacturing, assembly, and production, ensuring high quality production and maintaining intended use (Good Manufacturing Practice, European Medicines Agency, 2021). Import testing describes how governments and official organizations would review imported drugs to ensure their qualification. For example, imported drugs must be kept from being adulterated or misbranded. Imported drugs must be safe for human consumption and appropriately effective for the intended use (FDA, 2020).

These governmental offices and agencies include the Turkish Medicines and Medical Devices Agency (TITCK) in Turkey, Food and Drug Administration (FDA)

in the USA, as well as organizations that ensure harmony and similarities of standards between different countries, such as ICH (The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use), EMA (European Medicines Agency) in European countries, and World Health Organization (WHO) by the United Nations. These organizations and agencies make it easier and more efficient for drugs to be transported between countries with confidence and agreement in safety standards and quality control protocols between different countries. It also eliminates delays and indifferences between countries while preserving the health security and quality standards that protect public health. (Haleem et al., 2015).

1.2 Traditional Quality Control Methods

Quality control has one major important goal, which is to determine and quantitate drug substances (active pharmaceutical ingredients, excipients and impurities) in addition to search for contaminations. All these are utilized by using various analytical methods, among them separation and spectroscopic methods. European Pharmacopeias (EU) and US Pharmacopeia (USP) consider liquid chromatography to be the main prevalent analytical procedure for quality control examinations. This description can be extended to the majority of pharmaceutical sections which includes Gas Chromatography, High Pressure Liquid Chromatography, and Ultra High Pressure Liquid Chromatography, (whether or not Mass Spectrometry detectors are bounded to) as the most regularly chosen methods in various pharmaceutical processes (Dispas et al., 2022; Mattrey et al., 2017).

Gas chromatography (GC) is an analytical tool that is used to separate compounds in complex mixtures on the basis of their polarity. Separation is achieved only for volatile compounds, or that can be made volatile on derivatization. (C. Moldoveanu & David, 2019). Gas chromatography has many applications in the pharmaceutical industry, such as impurity profiling, chemical separation, and chemical and metabolomics analysis (D'Atri et al., 2019; Jwaili & Jwaili, 2019). It can also be

used for enantiomer separation between different drugs or to study drug purity (Carrão et al., 2020; Matamoros et al., 2009; Ribeiro et al., 2021; Xie et al., 2020).

High performance liquid chromatography (HPLC) is a modern application of liquid chromatography and a primary analytical tool in chemical and pharmaceutical analyses. HPLC can be used for analytical validation to calibrate the active pharmaceutical ingredients, adulterated ingredients, excipients, and degraded impurities. However, HPLC has limitations, such as being a laborious and time-consuming measurement. The HPLC procedure is executed by different interactions between the separatory column as a stationary phase and various solvents in the mobile phase. Consequently, the samples should be dissolved in the mobile phase, making HPLC a destructive method. Moreover, organic solvents are widely used as mobile phases, making HPLC a costly procedure that is harmful to the ecosystem because organic waste requires appropriate waste clearance (Chew et al., 2021; Yabré et al., 2018).

The ultra-high performance liquid chromatography (UHPLC) system is superior to the HPLC system because the UHPLC system operates at high pressures of up to 1000 bar or more, whereas the HPLC system can operate only at pressures of up to 400 bar. In addition, the UHPLC system consumes smaller amounts of solvent and requires a reduced amount of analytic time than HPLC. Applying smaller particle size results in improved peak resolution, quicker analysis, and more defined and elevated peaks. Although UHPLC has become more robust, including a prolonged column lifetime and enhanced software capabilities, it still presents challenges that may limit its attractiveness to a broader user base (Rodriguez-Aller et al., 2013). These problems include the association of UHPLC with organic solvents, their toxic nature, and their harmful effects on the environment (Narwate et al., 2014; Rathod et al., 2019). Additionally, there are several other shortcomings, such as frictional heating effects, narrow analyte peaks, and column blockage (López-Ruiz et al., 2019).

Chromatographic devices are complicated and although they have seen technological leaps and advancements, they still have a major drawback as they need experienced and especially educated workers. In addition, these devices still need regular care and sustainability operations, which in turn demands continuous training and updating of the personnel. Most importantly, sample preparation is a crucial step in regards for gas chromatography. Especially because GC is a method with drawbacks such as being lengthy prolonged, time wasting, tedious and high susceptibility to errors. A large portion of the time assigned for analysis is wasted to sample gathering and sample preparations (Dugheri et al., 2020). The majority of these validation methods are uneconomic with their costly reagents and pricey operations. As over 70% of the pharmaceutical regulatory systems in some regions are inadequate in ensuring the intended pharmaceutical standards (Tchounga et al., 2023; WHO - Regional Office for Africa, 2018).

Invasive methods typically involve direct contact with or extraction of samples, which can be time consuming and may alter the sample. In contrast, non-invasive methods allow for analysis without physically disturbing the sample or preserving its integrity. These methods provide valuable insights into the chemical composition of samples without altering them. In contrast, invasive methods, such as chemical measurements, are more time consuming and can potentially impact the characteristics of the sample (Kasper et al., 2020). The limitations of these traditional invasive methods, especially for the costs, environment, and destruction of the pharmaceutical products, resulted in research for using other non-invasive analysis methods that can be used effectively without these disadvantages.

1.3 Near Infrared Spectroscopy (NIRS)

Near infrared spectroscopy (NIRS) is a high-energy vibrational spectroscopy technique performed in the wavelength range of 800–2500 nm (12500–4000 cm^{-1} wave number). Vibrations in this range are exclusive to changes in the dipole moments of the molecules. The dipole moment arises from the difference between

the electromagnetic charges and the distance between the two bonded atoms. When molecules absorb NIR light, they tend to vibrate in two approaches. The first is stretching, which changes the interatomic distance between two bonded atoms, whereas the other is bending, which changes the bond angle. Both of these are unique to different molecules. In addition, NIRS is affected by vibration frequencies that differ between chemical bonds. The remaining unabsorbed NIR light is proportional to the vibrations and is reflected toward the detector to be measured. Mainly, vibrations of –NH, –OH, –SH, and –CH bonds are detected in NIR (De Beer et al., 2011; Johnson & Naiker, 2019; Pasquini, 2018; Roggo et al., 2007). The basic principle of NIRS detection can be summarized as the change in light energy caused by its interaction with the sample. This can be expressed using the following equation:

$$E = h.f = \frac{h.c}{\lambda} \quad (1)$$

Where E is the energy of light, h is the Planck constant, f is the frequency of light, c is the speed of light, and λ is the wavelength of light (Pu et al., 2020).

The core fundamentals of NIRS are derived from the utilization of near infrared (NIR) light, which is defined as the area from the infrared (IR) spectrum starting at 650 nm toward 950 nm. Sir William Herschel was earliest to identify the IR radiation after observing a change in temperature nearby the red color while experimenting the transmission of sunlight through a variety of filters (Gomez et al., 2021; Herschel, 1800b, 1800a). Karl Norris, an agro-industrial researcher had found out the usability of the NIRS in the 1960s after implementing multiple linear regression. In 1975, Phil Williams utilized NIRS rather than the conventional Kjeldahl testing technique for measurements of proteins in wheat (Norris & Butler, 1961; Tsuchikawa et al., 2022; Williams, Phil, Marena Manley, 2019). Since then, the use of NIRS as an analytical method has increased in various sciences and industries (Eilert, 2023).

The near-infrared spectrometer consists of a light source, such as an LED, which is be the source of radiation, a monochromator that would limit the radiation to only

NIR wavelengths, a sample holder to place the sample on, and a detector to measure reflected or transmitted light (Figure 1). The detector is either a diffuse reflectance detector that is used in opaque samples, such as tablets, which would reflect only the surface, or a transmittance detector for transparent samples that has the ability to get detailed data for the whole parts of the sample (Reich, 2005). There is also a Transflectance mode, which combines both methods and is particularly appropriate for liquid samples like milk (Evangelista et al., 2021; Tsenkova et al., 2001).

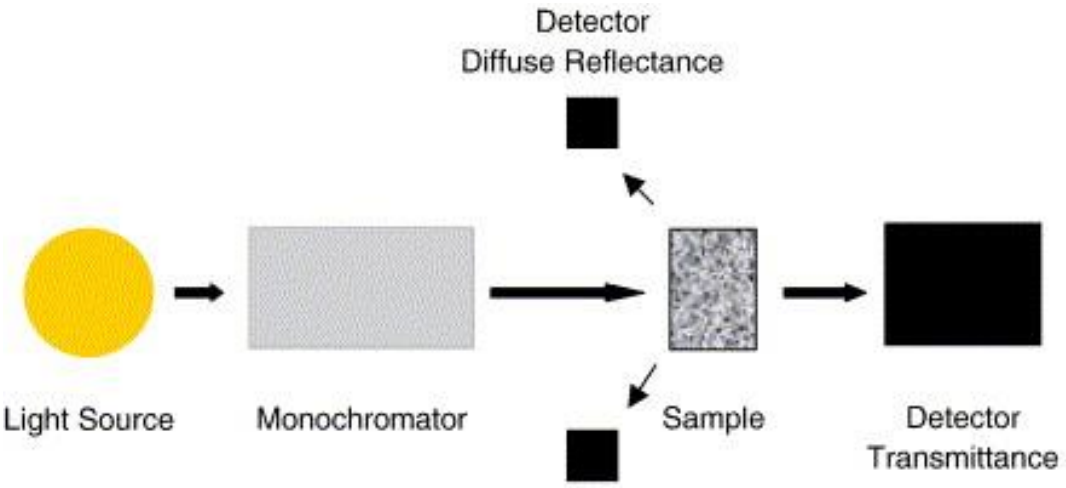

Figure 1 Basic NIR spectrometer configurations (Reich, 2005)

NIRS is a versatile analytical method which enables a simultaneous and precise measurements of several parameters at the same time with good precision with characteristics such as being rapid, nondestructive, precise, high analytical performance, and has lower costs than other laboratory analytical methods (Evangelista et al., 2021; Givens et al., 1997; Johnson, 2020; Pu et al., 2020; Yakubu et al., 2022). Furthermore, it is an environmentally friendly method (chemical-free and does not require waste disposal) (Pu et al., 2020). Although NIRS is not a primary analytical method but a secondary analytical technique as it relies on measuring reference data from a primary analytical technique in order to design a

calibration method. Yet NIRS is recognized to be similar and equally important to other primary analytical methods. Even though the predictability and measurement accuracy of NIRS relies on the predictability and measurement accuracy of the primary methods, NIRS is regarded as better in regards of reproducibility (Manley, 2014).

These characteristics have contributed to the wide availability of NIRS applications in various sciences and industries. NIRS has a long history in the food industry, including industrial on-line and in-line measurement techniques (Huang et al., 2008; Porep et al., 2015). NIRS is used in medicinal research to analyze and detect diseases in human tissues (Sakudo, 2016). NIRS has also been used in synthetic chemistry and agriculture (Jamrógiewicz, 2012). In addition, in biomedical applications, such as monitoring cerebrovascular physiology in patients with acute brain injury (Rachel Thomas, Samuel S. Shin, 2023), whereas in agriculture, NIR spectrometry has been utilized to monitor biomass conversion processes (Devos et al., 2020). In food engineering, NIRS has been applied to detect the quality of strawberries (G.Zhao et al., 2023). The use of NIRS in the pharmaceutical sector is well established, as it is included in the European Pharmacopeia and the US Pharmacopeia (Roggo et al., 2007), the European Medicine Agency (EMA, 2014), and the FDA (FDA, 2021) (Pu et al., 2020). It is used for tablets and pharmaceutical solid forms, including drug content analysis, identification, and degradation studies (Merckle & Kovar, 1998). In addition, NIRS can be used to detect drug–excipient segregation (Desai et al., 2020). NIRS can also measure the water content of pharmaceutical excipients (Luypaert et al., 2007).

While the NIRS method is powerful, it has some drawbacks, one of them is that NIRS causes the spectral data to be unified in a singular spectrum that depicts the measured sample volume without providing any data on the changes between different locations of the different sample parts. However, in order to acquire data and measure these differences is to use near-infrared spectroscopy chemical imaging (NIR-CI), an advanced NIRS method that comes with advancements in camera technologies and computers. A hyper spectral image which is built on information

and data in a three dimensional array that represents a nearinfrared chemical image (Amigo, 2010; Khorasani et al., 2016). However, NIR-CI has a primary drawback which is its high costs. Another issue is that NIR-CI is considered an immature technology that still needs to be explored and researched (Alcalà et al., 2013; Manley, 2014).

The availability of overtone and combination presets, in addition to the high magnitude of potential vibrations are making NIRS an extremely complicated data that is difficult to comprehend with its overlapping data (multicollinearity data). As a result, NIRS become challenging to represent and comprehend graphically, challenging to distinct different chemical bonds, and challenging to obtain data from the spectra. Correlations between absorption data at each wavelength and the reference value of the chemical components should be easy to predict and define. With advances in computer science and chemometrics, the relationship between NIRS measured data and reference data can be calculated to develop an NIRS measurement method (Manley, 2014).

1.4 Time Domain Nuclear Magnetic Resonance (TD-NMR)

Nuclear magnetic resonance (NMR) was first reported by Isidor Rabi in 1938, which later awarded him a Nobel prize (Rabi et al., 1938; Yao et al., 2022). Later, NMR spectroscopy in condensed matter was found in 1946. This later progressed, extended, and evolved into a cornerstone of chemical research, earning its researchers a Nobel Prize.

NMR has different functionalities which lead it to be classified into several disciplines: magnetic resonance imaging (MRI), NMR spectroscopy, NMR diffusometry, and NMR relaxometry (Yao et al., 2022). Low-Field (LF) instruments are maintained and employed with maximum strength of 2T. The increasing interest in low-field (LF) technology is due to constraints of high-field MRI and NMR instruments in industrial settings. Constraints such as the necessity of utilizing

cryogens with open magnetic fields, the necessity of strict safety regulation, and the highly expensive prices of these instruments in the commercial field (Blümich et al., 2009; Nikolskaya & Hiltunen, 2020).

Nevertheless, in certain implementations and usages, LF-NMR is known to be less precise in comparison to HF-NMR devices (Hwang, 2017; Osheter et al., 2022). Generally speaking, High field (HF) NMR spectroscopy is the desired choice over TD-NMR in the majority of applications of identifying counterfeiting food products, that is because NMR spectroscopy has the capacity to produce a characteristic spectrum of the measured sample. Additionally, TD-NMR applications does not have the capacity to measure variations derived from different chemical shifts (Esteki et al., 2018; Ezeanaka et al., 2019; Ozel & Oztop, 2021). However, these devices are costly and need momentous operative and maintenance workload, hence restricting their usages in the research and industrial sectors. Besides, LF-NMR has the capacity to operate not only in closed geometry but also in open geometry applications, particularly NMR-MOUSE (Besghini et al., 2019; Eidmann et al., 1996).

Recently, a middle-resolution benchtop NMR spectrometer utilizing permanent magnets with strength between 1 to 2.5 T, has been introduced and utilized in order to acquire data on the spectrum parameters such as chemical shifts parameters. Nonetheless, they are restricted to liquids or solutions with a diameter of 5 mm, which restricts their applications in the noninvasive analysis of many solids, such as intact food products or tablets (Moraes & Colnago, 2022).

Magnetic resonance imaging (MRI) is a very well known analytical method in the medical field, which is generally implemented to study and evaluate human organic organs and tissues for clinical diagnostic and treatment applications. Additionally, it can be used to characterize many biological and non-biological systems. However, In NMR relaxometry experiment are different than MRI since the received signal represents the whole sample, while spatial information is only available in MRI (D'Avila et al., 2005; Kirtil & Oztop, 2015).

TD-NMR is a Low-filed NMR analytical method that is increasing in popularity because it is quick, easy to operate, and offers high instrumental stability. TD-NMR uses small magnets that can generate low magnetic field ranging from 20 toward 60 MHz, it has smaller and compact size that can be used over a bench or be mobilized. Moreover, it can be effortlessly employed easily used directly on intact samples to measure proton spins and proton matrix relationships (Blümich, 2019; Kirtil, Cikrikci, et al., 2017b; Osheter et al., 2022; Rudszuck et al., 2019).

While TD-NMR can theoretically measure all different atoms and their nuclear spins, only ½ spins that are extensively occupy the measured sample with a satisfactory signal to noise ratio. Another limitation is that these spins should obtain high gyromagnetism with high Larmor frequencies at low fields of magnetism. Therefore, there are only two atoms, Hydrogen-1 and Fluorine-19, can fulfil all these restrictions (Besghini et al., 2019).

The NMR properties originate from the angular and magnetic momenta of the nucleus. A precession movement of the nucleus occurs under an external magnetic field toward specific atoms (such as hydrogen or carbon). Due to its superior magnetic momentum, hydrogen protons exhibit the uppermost sensitivity to NMR devices. Furthermore, hydrogen is incomparable available atom in the human, animal, and plant cells, in addition to many products such as foods or liquid drugs. That is why TD-NMR is usually applied to H^+ (Balthazar et al., 2021a; Fan & Zhang, 2019).

In the TD-NMR method, the sample is inserted within a large static magnetic field (B_0) (**Hata! Başvuru kaynağı bulunamadı.**a). Then, the magnetic field forces protons to be aligned in the Z direction of the applied magnetic field, making both transverse magnetization and magnetization at the XY plane equal to zero (**Hata! Başvuru kaynağı bulunamadı.**a, and 2b). Later, the sample will be excited with a radio frequency (RF) pulse, moving the net magnetization from the Z-direction into the XY plane (**Hata! Başvuru kaynağı bulunamadı.**c). Thus, the longitudinal magnetization and net magnetization in the Z-direction become equal to zero. When

the RF pulse is removed, longitudinal (T_1) and transverse (T_2) magnetization relaxation occurs as protons return to the Z-direction (Kirtil & Oztop, 2015; J.Li et al., 2021). T_1 and T_2 relaxation times represents the time needed for the magnetization vector to exponentially change until the equilibrium state is achieved (Osheter et al., 2022).

Initially TD-NMR devices were commercially employed in food quality control applications in order to measure the oil ratio of intact oilseed. With the beginning of this millennia modern usages of TD-NMR began to surface coinciding with the introduction of adaptable pulse programs and better friendlier user interfaces of computer programs. Since then, TD-NMR applications are increasing with their usages and applications especially because of their lower cost, smaller sizes, reduced weights and portability (Blümich, 2019; Colnago et al., 2021; Todt et al., 2001).

Due to variations in the magnetic field, it is not possible to obtain detailed structural information about the chemical composition of a sample. Nonetheless, the fading and decline of the TD-NMR signals, that occur after implementing a radio frequency (RF) pulse on the sample, can produce significant information that can be used in various applications. These data can be built in the form of longitudinal (T_1) and transverse (T_2) relaxation times (**Hata! Başvuru kaynağı bulunamadı.**D). These can be used to obtain more information about the nature, size, and physical states of molecules and their interactions with surrounding molecules, as well as the physicochemical characteristics of the surroundings (Blümich, 2016; Colnago et al., 2021; Mas Garcia et al., 2021; Riley et al., 2022).

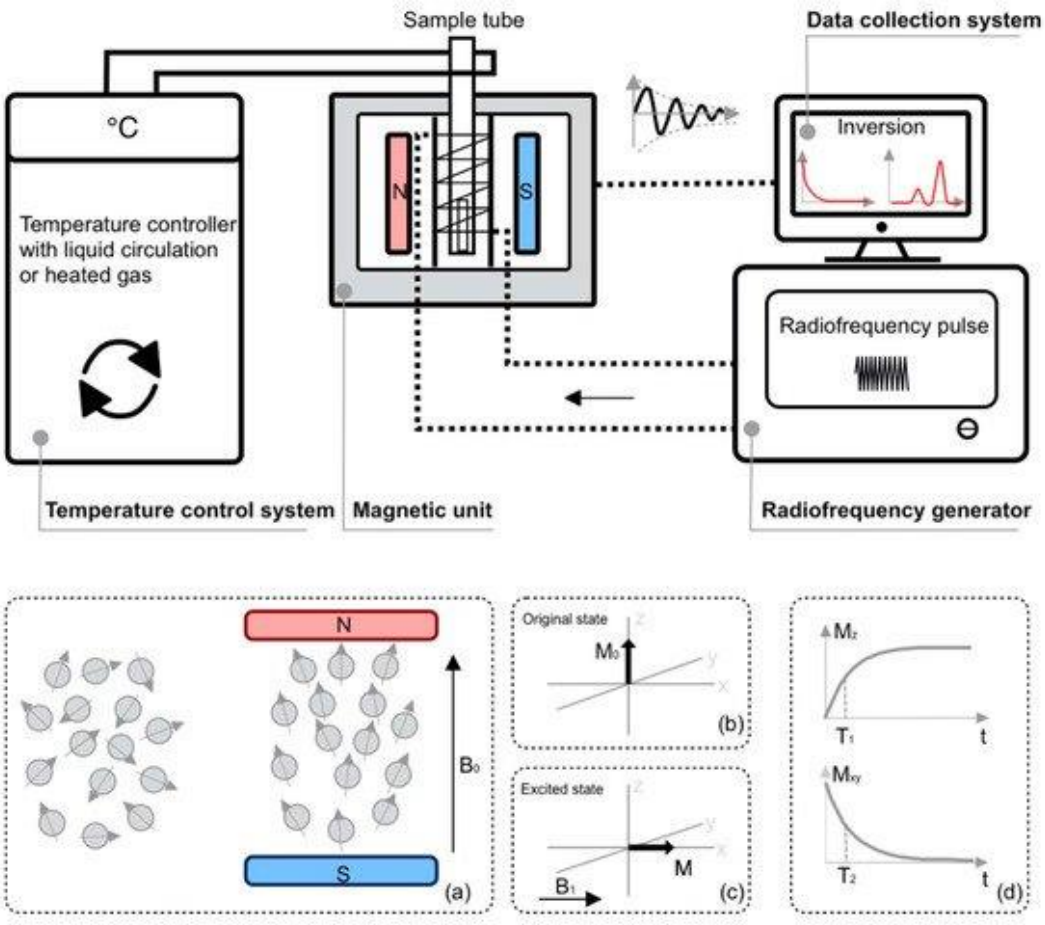

Figure 2 Representative diagram of a TD-NMR device consists of four main parts: a magnetic unit, a radiofrequency source, a temperature regulating unit, and a data processing unit. (a–d) present the standard NMR working concepts (J. Li et al., 2021).

In addition to the advantages mentioned earlier in TD-NMR, this method uses an inexpensive benchtop analytical system owing to permanent magnet technology. TD-NMR does not require any sample preparation, which makes it a non-destructive and non-invasive procedure. Additionally, TD-NMR can be used for through-package analysis. All of these traits have resulted in the increasing popularity of TD-NMR in research and industry (Santos et al., 2016).

There are advanced TD-NMR techniques that uses extra apparatuses, most knowns are the fast field cycling (FFC) and the pulse field gradient (PFG). In the FFC system, measurements of T1 are produced depending on the changes of the magnetic field

strength unlike most TD-NMR devices that have a static magnetic field strength. The PFG on the other hand is a method which measures the diffusion of molecules. A gradient radio frequency pulses can generate NMR signals that display the molecular diffusion of the sample (Marques et al., 2018; Steele et al., 2016).

TD-NMR has diverse applications as it has been extensively studied in food sciences and industry (Ozel & Oztop, 2021), agriculture (Colnago et al., 2021), building materials (Nagel et al., 2021), and medical sciences (Dupré et al., 2019), polymer sciences such as measuring and studying rubber since the 1960s (Miyaji et al., 2020). TD-NMR studies are also increasing in the pharmaceutical sciences, considering the ability of TD-NMR to detect the physicochemical properties of pharmaceuticals (Ohgi et al., 2021). Some of these studies focused on the crystallization of carbamazepine and indomethacin in powdered drugs (Okada et al., 2019), measuring water ratios in pharmaceutical forms such as wet granules (Ohgi et al., 2021), quantifying the water content in a lyophilized monoclonal antibody (mAb), spotting counterfeit Viagra found in markets from the original Viagra (Wilczyński et al., 2017), and identifying altered counterfeited antibodies in biological pharmaceuticals (Akhunzada et al., 2021).

1.5 Chemometrics

The earliest studies that describe statistical relationships with chemical changes can go back to the early 1900s and 1920s (Esbensen & Geladi, 1990). However, with the advancements of modern computers and its availability for smaller scales in universities leads to advancement studies and rise of chemometric studies in the 1960s such as work that was published by Malinowski, Jurs, and Massart (Brereton, 2014; Jurs et al., 1969; Massart et al., 1972; Weiner et al., 1970). In the 1970s the term “kemometrik” in Swedish which translate to chemometrics was first mentioned by Svante Wold, who later built the International Chemometric Society with the help of Bruce.R. Kowalski. These led to an increasing number of published works and attention toward chemometrics (Brereton, 2014; Kowalski et al., 1987; Wold, 1972).

Chemical theories in general make idealized concepts and rules which need idealized environments and conditions. This idealization has become a problem in descriptive and practical terms as real-life experiments and tests are far away from ideal conditions. Classical linear regression analytics are based on different presumptions that mirror idealized conditions and parameters which limit the analysis. However, chemometrics relies on fewer presumptions and involves extracting and representing data in a more realistic way. In addition to putting the idealized relationships that define the chemical and physical properties of any tests. They try to understand other empirical relationships and unknown patterns (Brereton et al., 2018; Wold, 1995).

Chemometrics can be defined as the use of mathematical and statistical approaches to analyze, examine and understand the experimental data in a broader and further manner than the classic univariant methods. Chemometrics offers an effective quantitative understanding on the resulted experimental data. At the same time it provides a comprehensive identification of trends and understand chemical relationships which were previously ignored. Chemometric can also be defined as a developing field of study that aims to gather and extract effective and valuable data from analytical methods (Inobeme et al., 2022).

Most researchers utilize univariate model analysis to express and calibrate their measurements by using univariate statistical methods such as analysis of variance (ANOVA) or t-tests for normally distributed data. However, the progress and improvements with computer science helped to ease the usage of more complex multivariate statistical methods. This helped the analysis of more complex and enormous data to be analyzed especially those of newer analytical devices that produce such a huge number of data and doesn´t rely on a single instrumental signal. For example, data collected from spectroscopy where data are collected from signals that are widespread over many different wavelengths (Brown & Steven Brown, 2017; Gómez-Caravaca et al., 2016; Harynuk et al., 2012).

Chemometrics is used in a variety of devices and tools especially for non-invasive and non-destructive devices that can identify and quantify contents of the sample.

Devices such as ultraviolet–visible spectroscopy, mid-infrared spectroscopy, near-infrared (NIR) spectroscopy, Raman spectroscopy, terahertz spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, laser-induced breakdown spectroscopy. The usage of chemometric analysis with these devices provide to be beneficial for various applications in agricultural sciences, food engineering, pharmaceutical industry, petrochemical analysis, environmental and medical studies (Afsah-Hejri et al., 2020; T. Chen et al., 2020; Kaavya et al., 2020; Tahir et al., 2022; H. P. Wang et al., 2022).

Chemometrics has various applications and implementations in the pharmaceutical industry including QC and process analysis. Some of these implementations were successful enough to become included in regulatory guidelines of regulation authorities. For instance, the European Pharmacopoeia (EP) and similarly the United States Pharmacopoeia (USP) have issued specific chapters on chemometrics. In addition, the Food and Drugs Administration (FDA) has given instructions for chemometrics usages. While ICH has aggregate the chemometrics usages in specific chapters (Vignaduzzo et al., 2020).

One of the main aspects of chemometric application is the preprocessing step. It has a crucial role as it eliminates many additional extra factors that cause changes in the measurement values outside of the intended samples. These artifacts include rough surfaces, optic effects, detector noise, baseline corrections, temperature and particle size (Gendrin et al., 2008).

Validation is an essential component in chemometrics as it can provide reliability and demonstrates the quality of the applied statistical methods. It can either be presented graphically to give a clear illustration of the overall procedure or can be measured numerically with statistical methods to show the quality of the applied method. Validation should either be done using a cross validation set of data or an external set of data that was not included during the preparation of the chemometric method (Anderssen et al., 2006; Brereton et al., 2018).

1.6 Novel Quality Control Methods

In this study, the focus will be on solving three different quality control issues by using non-invasive measuring techniques.

1.6.1 Quality issue of Insulin

Diabetes is an extremely popular and globularly expanding disorder in a rapid fashion. It is estimated increase in numbers to reach around 700 million adults' patient by 2045. Diabetes is a chronic metabolic disorder of the endocrine system that can be described with unnaturally high amount of glucose in the blood, which could be caused from total or proportional insulin shortage. These can appear when β-cell dysfunction, insulin resistance in tissues, or both. Although diabetes is generally categorized as an autoimmune class that starts with childhood (type 1 diabetes, T1D) and a non-autoimmune class that usually starts at adulthood (type 2 diabetes, T2D), there are additional distinct types of diabetes. These includes monogenic diabetes such as, maturity-onset diabetes of the young or neonatal diabetes, gestational diabetes in pregnant women, and potentially an autoimmune class that can develop in adults at later stages of life (Ahlqvist et al., 2018; N.H. Cho et al., 2018; Cole & Florez, 2020; Udler et al., 2018).

The typical symptoms of diabetes, such as excessive weight loss, excessive thirst, and excessive urination, were first mentioned in medicinal history 3500 years ago by the ancient Egyptians, while the word "diabetes" which comes from Greek was first mentioned by Aretaeus of Cappadocia around the 1st century AD. The sweetness of urine was discovered by Thomas Willis in the 17th century, leading to the addition of "mellitus" to the name of the disease (Mekala & Bertoni, 2020). The highest prevalence of diabetes is found in few Pacific Island nations including of adults in the Marshall Islands with 30.5%, in Mauritius with 22%, and in Papua-New Guinea with 17.7%, then succeeded in countries of the Middle East particularly Saudi Arabia, Egypt, and United Arab Emirates where around 18% of adults are affected.

Turkey has over 10 million diabetic patients (Mekala & Bertoni, 2020; Ministry of Family and Social Services, 2019).

In 1889, Minkowski and von Mering noted that the complete removal of pancreas causes the production of severe diabetes. In 1922, the first human experiments involved the administration of the pancreatic isolate and reversing the symptoms of diabetes. In May 1922, the active component was named insulin. Commercial sales started a year later as a treatment for diabetes, which led to the reward for this research and experiment with the Nobel Prize in the same year (Mekala & Bertoni, 2020; Wilcox, 2005).

Insulin is a dipeptide hormone comprised of A and B chains connected together by a disulfide bond and contains 51 amino acids. Insulin is released from the β cells of the pancreatic islets of Langerhans. Insulin is an effectual anabolic compound that advances cellular absorption, storage, and production of nutrients while obstructing nutrient breakdown and release into the bloodstream. Insulin performs other tasks such as inducing mobility of nutrients toward cells, regulating metabolic enzyme activity, checking the transcription of metabolic genes, regulates cellular growth and differentiation, and govern its own removal from the cell, all that via activating its receptor (Saltiel, 2021; Wilcox, 2005).

Beside the tasks and functions of insulin in diabetes, recent research demonstrated other functions and activities of insulin in different organs which includes the brain, heart, kidney, bone, skin, and hair follicles. Insulin assists the development of bones, reduces inflammation caused by osteoporosis, function on the central nervous system, and acts on pro-atherogenic and anti-atherogenic tasks in the cardiovascular system (Rahman et al., 2021; Rorsman & Braun, 2013).

The treatment of diabetes consists of antidiabetic drugs and gene therapy. Insulin drug is considered the primary therapy for type 1 diabetes, and their use is expanding in type 2 diabetes. People with diabetes have to use insulin shots to regulate glucose levels in their bodies. Insulin has five major types, which are Rapid-acting insulin, Regular or short-acting insulin (e.g., Humulin R), Intermediate-acting insulin (e.g.,

Humulin N), Long-acting insulin, and Ultra long-acting insulin (Swain et al., 2022). These types differ in their chemical formulas and physical structure, inducing varying pharmacokinetics with different absorption rates and duration of actions (Mathieu et al., 2017)

The timing of insulin action depends on each insulin and formulation's unique pharmacokinetics (PK) and pharmacodynamics (PD). The term "pharmacokinetics" denotes the time frame of insulin circulation levels after subcutaneous injection or other delivery methods. To maintain normal blood glucose levels without risking the patient, insulins must be given with knowledge of their PK and PD effects. Both PK and PD are influenced by insulin injection parameters and physiological factors such as workout, body heat, and insulin sensitivity. In order to administrate an insulin regimen, it is of high importance to have a good and clear understanding and knowledge of PK and PD characteristics of different insulins and their different formulations and related physiological factors. Baseline insulin, which is available in intermediate- and long-acting forms, is often the initial treatment for type 2 diabetes. Protamine, a positively charged protein, precipitates insulin hexamers, resulting in a suspension formulation. Injections of protamine/insulin crystals limit insulin hexamers' dissociation, thereby reducing insulin monomers' absorption into circulation (Hirsch et al., 2020).

Some patients can use two different insulin types at different times and can mix two types of insulin inside a single syringe for single use, such as insulin NPH (insulin neutral protamine hagedorn) with regular insulin or with insulin lispro, a process referred to as “free mixing” (Eau Claire, 2023; Hirsch et al., 2020). Some patients use premixed insulin mixtures to control their blood glucose levels at a given time. Premixed insulin mixtures include Humulin 30/70 (30% intermediate-acting insulin NPH ratio to 70% regular insulin ratio), Humulin 50/50, Humulin 70/30, Humalog Mix 25, Novolin 50/50 (Brunetti, 2022). It is crucial for patients to administer exact amounts of insulins, as miscalculations of insulin drugs or between doses of mixed insulins can lead to reduced effects of the drug or serious complications such as

hypoglycemia that can end up in the emergency departments of hospitals and can be fatal (Geller et al., 2021).

There are quality control methods to overcome these errors, but they are mostly performed only for selected batches after the production lines (Carter et al., 2016). They are invasive and destroy the drugs, making them unusable. Therefore, there is an emerging need for a quick and non-destructive method to detect quantification errors. Time domain nuclear magnetic resonance (TD-NMR) is an analytical method that is becoming increasingly popular because it is quick, easy to operate, and offers high instrumental stability. This is based on continuous measurements of protonation in the sample. TD-NMR is capable of observing molecular dynamics in simple and complex environments (Balthazar et al., 2021b; Kiselev, 2019; J. Li et al., 2021). It is used in various branches of scientific research and industries, such as food (Kirtil et al., 2017), pharmaceuticals (Kuentz et al., 2006), botany (Kovrlija et al., 2020), agriculture (Colnago et al., 2021), and polymer studies (Besghini et al., 2019). TD-NMR incorporates several measurements, such as T1 longitudinal relaxation times, T2 transverse relaxation times, and T1T2 maps (Baran et al., 2023; Ozel & Oztop, 2021), which are bidimensional NMR maps generated by a correlation between T1 and T2 relaxation times. T2 relaxation times allow the characterization of nuclei by their states, such as bound water, free water, and the exchange between these two states (Santos et al., 2016), which is of immense importance when measuring a mixture of insulin drugs with different solubilities. T1T2 maps separate physical and chemical influences by distinguishing overlapping data in the T2 relaxation times (Li et al., 2021).

In this study, insulin drug mixtures were analyzed using two different TD-NMR techniques, which are T2 relaxation and T1T2 maps. Additionally, multiple linear regression (MLR) was applied to integrate the linear regressions of the two methods. The final aim is to design a novel, non-destructive, affordable, user-friendly and precise quantification method for mixed insulin drugs using TD-NMR techniques.

1.6.2 Quality issue of Aspirin

Aspirin is considered to be one of the oldest drugs known to men. It was obtained from willow bark for over millennia until Felix Hoffmann succeeded in producing acetylsalicylic acid at Bayer to proceed and secure a patent in 1899 (Sneader, 2000). The ancient Sumerians and Egyptians consumed willow bark for its medicinal values even though they were unaware of its content of salicin (Figure 3) which is the active ingredient that gives willow bark its medicinal values and was the cornerstone in the development of aspirin. Willow bark continued to be consumed during the ancient Greece era for its reducing pain characteristics. Back then it was suggested by Hippocrates for the purpose of reducing pain during childbirth, and it was mentioned during the Roman era since it was suggested by Pliny the Elder (Desborough & Keeling, 2017). In 1828, Johann Buchner processed willow bark to obtain yellow crystals which he called Salicin, named after the plant genus of the willow tree, Salix. Salicin was the active compound that gives willow bark its medicinal properties. Later, the procedure was taken another step by Pierre-Joseph Leroux. To be refined one more time by Raffaele Piria were the product called salicylic acid (Figure 3) was introduced with a higher activity (Desborough & Keeling, 2017; Leroux, 1830; R Piria, 1838; Schindler, 1978).

Aspirin became widely accessible and considered an over-the-counter drug, a drug that can be taken without a prescription. Its analgesic, antipyretic, and anti-inflammatory properties originate from aspirin being part of a group known as nonsteroidal anti-inflammatory drugs (NSAID). NSAIDs are a class of drugs that reduces pain, decreases fever, and prevents blood clots (Tolba, 2017). They are chemically and physiologically distinct from steroids (Buer, 2014). They account for 5% of the entire prescribed medicines worldwide. The irreversible acetylating

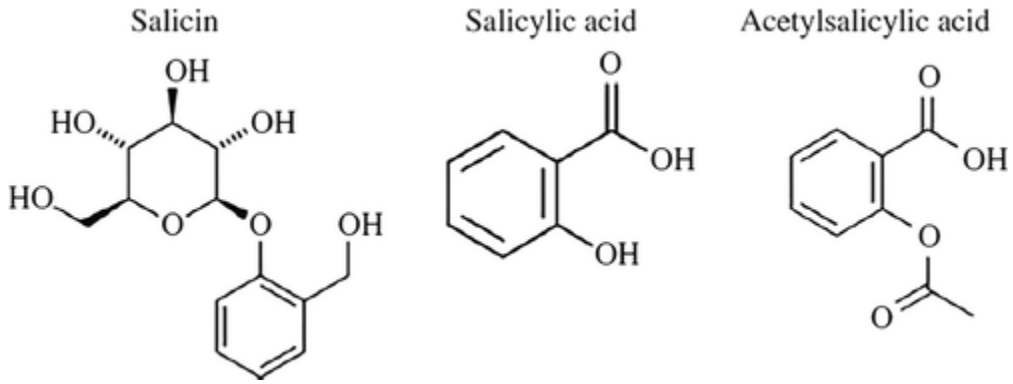

Figure 3 Chemical Structure of Salicilin, Salicylic acid and Acetyl salicylic acid (Desborough & Keeling, 2017b)

property of aspirin distinguishes it from other NSAIDs, such as diclofenac and naproxen. This irreversible acetylation forms a covalent bond between aspirin's acetyl group and the serine residue of cyclooxygenase's active site in platelets, which is irreversible. Consequently, it inhibits platelet function for more than a week. Making aspirin a suitable antiplatelet medicine with a crucial role in the prophylaxis of thrombosis (blood clots) (Santos-Gallego & Badimon, 2021; Tóth et al., 2013). In addition, NSAIDs are known to inhibit cyclooxygenase enzymes (COX), which are responsible for the synthesis of prostanoids, which in turn intermediate inflammation, pain, and fever. While most NSAIDs inhibit these enzymes reversibly, high doses of aspirin irreversibly inhibit them, and this irreversible inhibition of COX enzymes by aspirin leads to a longer half-life; in other words, aspirin has a longer duration of action than other NSAIDs (Seliger et al., 2018; Tolba, 2017).

Aspirin contains many additional pharmacological properties, making it an option for various therapies (Willetts & Foley, 2020). In addition to its treatment of pain, fever, and inflammation, aspirin is used as a prophylactic agent for the primary and secondary prevention of cardiovascular diseases (Dasa et al., 2021). Aspirin's antithrombotic properties reduce blood clots and the risk of stroke and myocardial infarction (Fiala & Pasic, 2020). Furthermore, aspirin can be used to control heart rate variability and cardiac autonomic activity (Minhas et al., 2021). It is anticipated that aspirin has an antiangiogenic characteristic that causes downscale cancer risks.

Due to this, in recent years, studies have shown that aspirin can be used as a prophylactic agent for cancer (Cao et al., 2016). For example, the risks of cancers in the GIT, such as esophageal, stomach, and colorectal cancers, were reduced by over 25% compared to those in a control group. (Bosetti et al., 2020; Xie et al., 2021).

The use of low dose aspirin in patients at risk for coronary heart disease is considered an economical approach, as it can result in reduced costs for patients, insurance, and national health services (Lamotte, Piñol, et al., 2006; Tsutani et al., 2007; Zhong et al., 2021). The cost reduction also benefits patients, resulting in savings of up to €797 per patient in Spain and €889 per patient in Japan(Lamotte, Annemans, et al., 2006; Lamotte, Piñol, et al., 2006). The European Heart Network has estimated that cardiovascular diseases cost the EU economy more than €200 billion annually (Timmis et al., 2022). Heart disease costs the United States approximately $239.9 billion annually from 2018 to 2019. This includes the costs of healthcare services, medicines, and productivity lost related to death (Tsao et al., 2023). Additionally, the economic significance of aspirin extends to its role in precision medicine and pharmacogenomics. Studies have developed cost-effective approaches to genotyping related to aspirin pharmacogenomics, providing valuable tools for personalized medicine and efficient drug delivery (Li, 2024). This emphasizes aspirin's potential to optimize treatment strategies, leading to better health outcomes and cost savings (C.Y. Li et al., 2024).

Contrastingly to aspirin's advantages and positive characteristics, aspirin has disadvantages and drawbacks. One of its primary issues is its damage to the gastrointestinal tract (Kedir et al., 2021), which led manufacturers to resolve this issue by producing enteric-coated aspirin tablets that do not dissolve in the stomach but dissolve in the intestine or for doctors to prescribe reduced doses of aspirin, especially for the prevention of cardiovascular diseases in which small amounts are adequate (Kedir et al., 2021), or for aspirin to be prescribed with proton pump inhibitor drugs to substitute the increased acidity of the stomach caused by aspirin (Tosetti & Nanni, 2017). A second issue is that aspirin drugs may interact with or interfere with other medications, such as warfarin or NSAIDs. This results in

complications with clinical management, including increasing complexity in prescribing medications as a means to avoid adverse drug reactions and changes in medicinal effectiveness (Alqahtani & Jamali, 2018; Hurlen et al., 2000; Palleria et al., 2013).

Regarding its physicochemical properties, aspirin has one substantial issue. It is hygroscopic, which means that it is affected by the humidity of its surroundings. This issue causes aspirin to degrade into salicylic acid and acetic acid (Figure 4). Due to this matter, the wet granulation technique cannot produce aspirin tablets (an inexpensive technique that uses water for tablet production). For that, slugging is the method employed in aspirins production (Avbunudiogba, 2013).

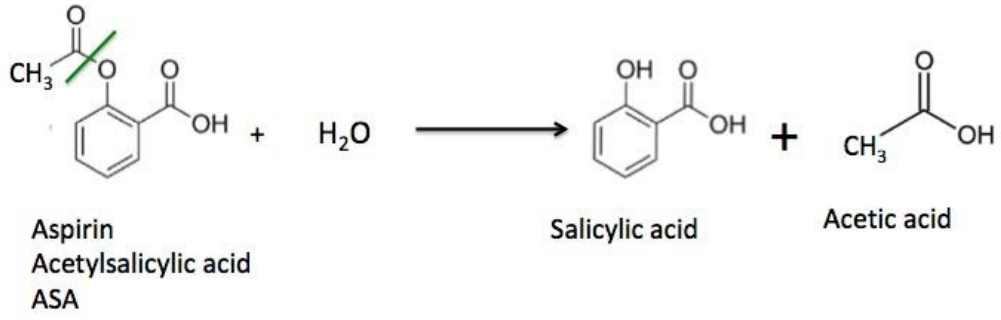

Figure 4 Aspirin degradation under humid conditions (Josh Bloom, 2018)

Temperature is another parameter that catalyzes aspirin degradation to salicylic acid and acetic acid (Al-Maydama et al., 2018). Temperature catalyzes humidity degradation, and this process is related to humidity values. The more heat is applied, the more degradation it causes (Li et al., 2008). Aspirin is recommended to be around room temperature, while the relative humidity should be no more than 55% (Yamazaki et al., 2010).

The salicylic acid formed from aspirin degradation is considered to be toxic in excessive amounts. The British Pharmacopoeia (BP) reported that the salicylic acid content within an aspirin tablet is restricted to 3% (Raimi-Abraham et al., 2017). This kind of degradation is escalated upon stress environments caused by elevated temperatures and humidity (El- Banna et al., 1978; Waterman et al., 2007). The long

term increase in salicylic acid uptake in patients leads to chronic intoxication, which is termed therapeutic salicylate poisoning. It displays central nervous system symptoms such as hyperventilation and memory deficit. It may also cause cardiac arrest or severe brain damage, which would lead to death (Kamal et al., 2020; Pearlman & Gambhir, 2009).

As a consequence of the importance of preventing excessive salicylic acid in aspirin pharmaceuticals, and with the intention of lowering the cost and using a more environmentally friendly and non-destructive method for detecting salicylic acid, this study suggests TD-NMR accompanied by NIRS as an alternative measurement to HPLC for determining unsuitable degraded aspirin pharmaceuticals caused by humidity and temperature, resulting in excessive salicylic acid production.

1.6.3 Glycation as a quality issue

Glycation is a chemical reaction occurring in biopharmaceuticals. This reaction occurs between a protein's primary amine molecule and a reduced saccharide's aldehyde unit. It usually happens on lysine residues and, to a lesser degree, on arginine residues and N-terminal amino acids. Glycation can affect the functionality and reliability of biopharmaceuticals. (Fischer et al., 2008; Gstöttner et al., 2020; Kennedy et al., 2008; Wei et al., 2017)

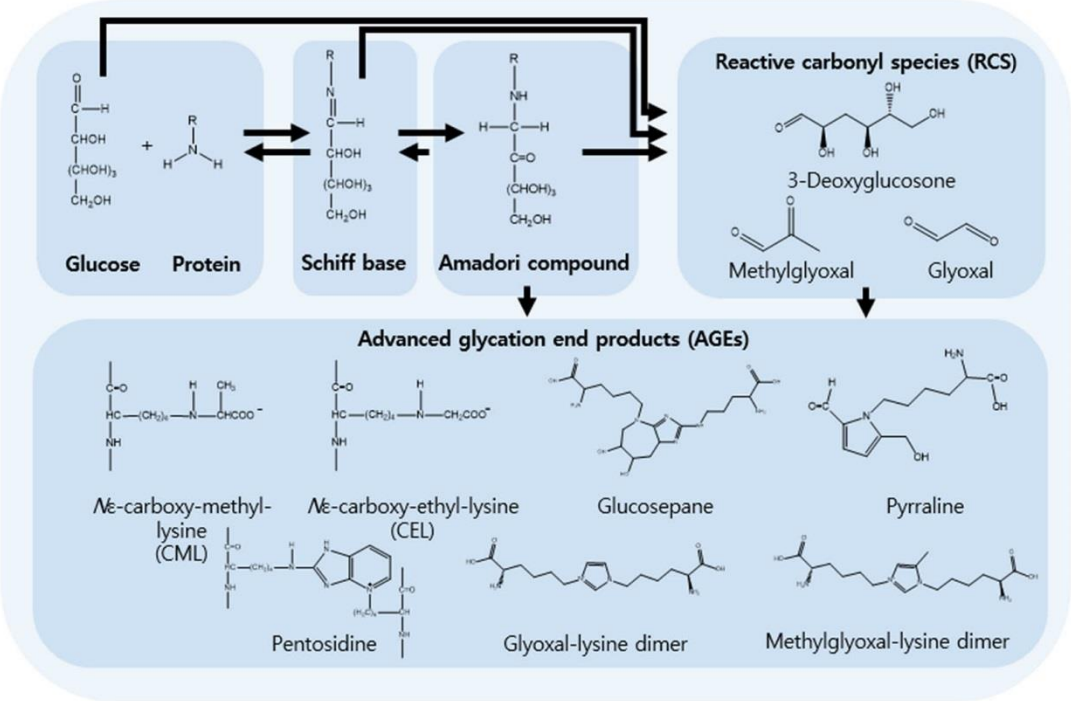

Figure 5 Glycation reaction and its followed reactions and products (S. Cho et al., 2022)

When a sugar molecule or saccharide interacts with a protein molecule (Figure 5), a complex compound known as a Schiff base is formed. This reaction occurs when the sugar's active carbonyl group combines with the protein's nucleophilic free amino group, creating a C=N double bond. Following this initial reaction, the Schiff base undergoes additional transformations such as dehydration, rearrangement, cyclization, oxidation, and another round of dehydration. These processes produce advanced glycation end products (AGEs), which are more stable compounds. (Chan-Sik Kim, Sok Park, 2017; S. Cho et al., 2022).

In 1912, Louis-Camille Maillard found that when amino acids and sugars are mixed together and heated, they turn intensely dark. This type of interaction between amino acids and saccharides is referred to as the Maillard reaction. (Hellwig et al., 2014; LC Millard, 1912). In 1985, the Nomenclature Committee of the International Union of Biochemistry and the International Union of Pure and Applied Chemistry proposed using the term glycation to refer to all reactions that join a sugar to a protein

or peptide, regardless of whether an enzyme is involved. Later, it was clarified that there is a distinctive difference between protein modifications in consequence of saccharides. As non-enzymatic modifications produce glycated proteins while enzymatic modifications produce glycoproteins (Lis & Sharon, 1993; Sharon, 1986). Glycation is a process in which saccharides change proteins non-enzymatically. This is known as selective adoption. It is important to distinguish between glycation adduct residues and the binding of free adducts since one of them creates a covalent bond and the other does not. (Agalou et al., 2005; Rabbani et al., 2007; Rabbani & Thornalley, 2012)

In recent decades, biopharmaceuticals have experienced a notable surge due to the remarkable progress made in recombinant DNA technology. Among the various types of biopharmaceuticals, monoclonal antibodies (mAbs) stand out as a prominent category, with their efficacy in treating a diverse range of ailments, including cancer, asthma, central nervous system disorders, infectious diseases, and cardiovascular diseases (Fekete et al., 2012; Le Basle et al., 2020). Over 125 antibodies have been approved and commercialized, with some of them being among the top-selling pharmaceuticals. Sugars or polyols are added to lyophilized powders to create a stable, glassy, and amorphous matrix to preserve the antibodies. Among the sugars used in antibody compositions, the non-reducing disaccharides sucrose and trehalose are recognized for their ability to stabilize antibodies and proteins (Le Basle et al., 2020; Strickley & Lambert, 2021; Sudrik et al., 2019).

During fermentation or upstream production of antibodies, glycation reactions can occur because of the presence of sugars, particularly glucose, which are used as an energy source. The reactivity of these reactions depends on the antibodies involved. The rate and extent of glycation can be influenced by factors such as temperature, pH, duration, and ionic strength, which should be maintained at physiological levels (Leblanc et al., 2016; Quan et al., 2008a; Wei et al., 2017; Yuk et al., 2011).

During the fermentation process, monoclonal antibodies are subject to glycation, which occurs as the mAb-producing cells utilize glucose as a source of energy. The

amount of sugar provided during mammalian cell culture significantly impacts glycation levels. Factors such as temperature, pH, duration, and ionic strength, which are maintained at physiological levels, can influence the rate and extent of glycation (Quan et al., 2008a; Wei et al., 2017; Yuk et al., 2011). Studies have shown that the glycation is negligible when monoclonal antibodies are stored long-term at 2–8 °C with sucrose. However, when stored at ambient temperature with dextrose, glycation occurs at significant levels (Fischer et al., 2008; Le Basle et al., 2020; Sreedhara et al., 2012).

Supercooling and storage temperatures can influence glycation. For instance, room temperature and 60% relative humidity can lead to glycation of lyophilized products, whereas 5°C does not generate glycation. No glycation was observed in liquid antibody products stored at 4° C, but considerable glycation occurred at 37° C even when sucrose, a non-reducing monosaccharide, was used instead of glucose (Awotwe-Otoo et al., 2015; Gadgil et al., 2007).

Glycated products can be detected by liquid chromatography-mass spectrometry (LC-MS) to measure the overall glycation levels or peptide mapping for individual site glycation results. Boronate affinity chromatography can be used to assess glycation and monitor overall glycation levels (Lhota et al., 2021; Quan et al., 2008b; van Schaick et al., 2023). The O-phthalaldehyde (OPA) technique can be used to measure glycated compounds. (Ertugrul et al., 2021; Fischer et al., 2008; Mo et al., 2018). However, these methods are invasive and destructive to the samples; therefore, they can only be used for selective samples instead of a non-invasive method that can be used on all samples. In this part of the study, the focus will be on using TD-NMR as a non-invasive method for the detection of glycation in pharmaceutical products by using glucose and bovine serum albumin as a model and then surrounding the model with accelerated conditions to provide glycation. This glycation was examined by TD-NMR and compared to the OPA method.

1.7 Objectives of the Study

The objective of this study is to use Time Domain NMR and Near infrared Spectroscopy as non-invasive, non-destructive, affordable, user friendly, and precise quality control methods as an alternative to the current chemical quality control methods that destroy the samples, have costs for chemical materials, need trained labor, and cannot measure all samples but can only be used on a portion of the samples.

Specific objectives

- Preparing different mixture ratios of two different insulin drugs that can represent any insulin mixing ratio.
- Analyzing these mixtures with T2 relaxation is necessary to understand the relationship between changing the mixing ratio of insulin mixtures and the mobility of water, as well as the interactions between water and insulin mixtures.
- Analyzing these mixtures with 2D-NMR to generate T1T2 maps of mixture ratios to better understand different proton domains and how they differ with changing the mixing ratios.
- Finding the relationship between different quantities of insulin mixing ratios on the one hand and TD-NMR results of T2 relaxation and T1T2 maps on the other hand.
- Integrating these methods using statistical or chemometric methods such as multiple line regression.
- Validating the final method using data outside the training data.
- To produce a non-invasive technique which can serve as a means of a quality control method to quantify and measure the mixing ratio of two different insulin drugs in order to ensure the safety of mixing ratios and avoid miscalculations that lead to health issues such as hypoglycemia.
- Preparing degraded aspirin tablets by placing them in stressful environments with elevated temperature and humidity.

- Measuring them by infrared spectroscopy and obtaining spectroscopy data.
- Use chemometrics to analyze these data.
- Development of a calibration method for measuring aspirin degradation using NIRS.
- Validate the data using a validation set of degraded aspirin tablets.
- Measure aspirin tablets by TD-NMR and compare the results to XRD crystallization and Karl-Fischer titration moisture content.
- Preparing samples with different glycation ratios.
- Samples contain model drug formulation of BSA and glucose.
- Placing these samples in an accelerated environment to promote glycation.
- Measuring the browning of these samples as a glycation measurement.
- Measuring the samples with the OPA method to ensure and quantify their glycation.
- Measuring the samples with T1 relaxation to understand the relationship between glycation and hydration.
- Measuring the samples with T2 relaxation to understand the relationship between molecules and water during glycation reaction.
- Measuring the samples with T1T2 maps relaxation to understand the relationship between glycation and different proton pool domains.
- Comparing the OPA method with other methods is necessary to find the most suitable non-invasive method that can be a substitute for the OPA method.
- Ensuring that TD-NMR can be used as a substitute for the OPA method.
- Being able to monitor glycation using TD-NMR as a quality control method.

CHAPTER 2

MATERIALS AND METHODS

2.1 Part 1. Quality Issue of Insulin

2.1.1 Sample Preparation

Humulin R® U-100 (Eli Lilly & Company, Indianapolis, IN, USA) and Humulin N® U-100 (Eli Lilly & Company, Indianapolis, IN, USA) were purchased from a local pharmacy. Humulin R® U-100 contains 100 units/ml, which is equivalent to 3.5 mg/ml of human insulin (rDNA origin), 16 mg/ml of glycerin, 2.5 mg/ml of meta-cresol, and 0.015 mg/ml of endogenous zinc. Humulin N® U-100 contains 100 units/ml, which is equivalent to 3.5 mg/ml of human insulin, 0.35 mg/ml of protamine sulfate, 16 mg/ml of glycerin, 3.78 mg/ml of dibasic sodium phosphate, 1.6 mg/ml of meta-cresol, 0.65 mg/ml of phenol and 0.025 mg/ml zinc ion.

Table 1 Mixture ratios of Humulin N (insulin-NPH) and Humulin R (regular insulin)

Mixing Ratios	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Humulin N	0%	5%	10%	15%	20%	25%	30%
Humulin R	100%	95%	90%	85%	80%	75%	70%
Mixing Ratios	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Humulin N	35%	40%	45%	50%	55%	60%	65%
Humulin R	65%	60%	55%	50%	45%	40%	35%
Mixing Ratios	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Humulin N	70%	75%	80%	85%	90%	95%	100%
Humulin R	30%	25%	20%	15%	10%	5%	0%

The samples were prepared by gently shaking the Humulin N and Humulin R bottles upside down (Turkish Medicines and Medical Devices Agency (TICK), 2023) and then transferring the solvents from both bottles to be mixed in different ratios starting from 0:100 (Humulin N: Humulin R) toward 100:0 (Humulin N: Humulin R), as depicted in Table 1. Each mixing ratio contained three samples with 0.5 ml of exact quantities from different Humulin N and Humulin R bottles. Finally, 63 samples were prepared from 20 Humulin N and Humulin R bottles. Humulin N is a suspension that tends to precipitate over time. As a means of guaranteeing the homogeneity of the samples and uniformity of the mixing state, each sample was subjected to vortexing for five seconds, followed by TD-NMR measurement precisely 15 seconds after that. Subsequently, samples were stored in refrigerators at 4° C until further analysis.

2.1.2 Benchtop TD-NMR Instrument

The experimental setup was performed by a benchtop NMR system (Pure Devices GmbH, Germany) operating at a 1H frequency of 24.15 MHz. The system was set up with a radio frequency coil measuring 10 mm, corresponding to the cylinder where the samples were placed inside 10 mm wide tubes. The device temperature was set to 28° C for all experiments. The samples were equilibrated to this temperature prior to measurements.

2.1.3 TD-NMR experiment parameters

2.1.3.1 Hydration measurements by T2 Relaxation Sequences

The T_2 relaxation times of the insulin mixtures were determined using the Carr-Purcell-Meiboom-Gill (CPMG) sequence (Figure 6). The CPMG signal is assembled by employing a 90° RF pulse following numerous 180° RF pulses separated by an equal number of times, known as the repetition time. With every single 180° pulse,

the signal intensity of protons declines depending on the inhomogeneous magnetic field of the sample (Carr & Purcell, 1954; Meiboom & Gill, 1958). The number of echoes is 12,500, the echo time interval is 10 ms, the repetition time (TR) is 14000 ms, and the number of scans is 2. The received NMR signals were evaluated using MATLAB software (The MathWorks Inc, 2023) and fitted to mono-exponential models to acquire T_2 relaxation times.

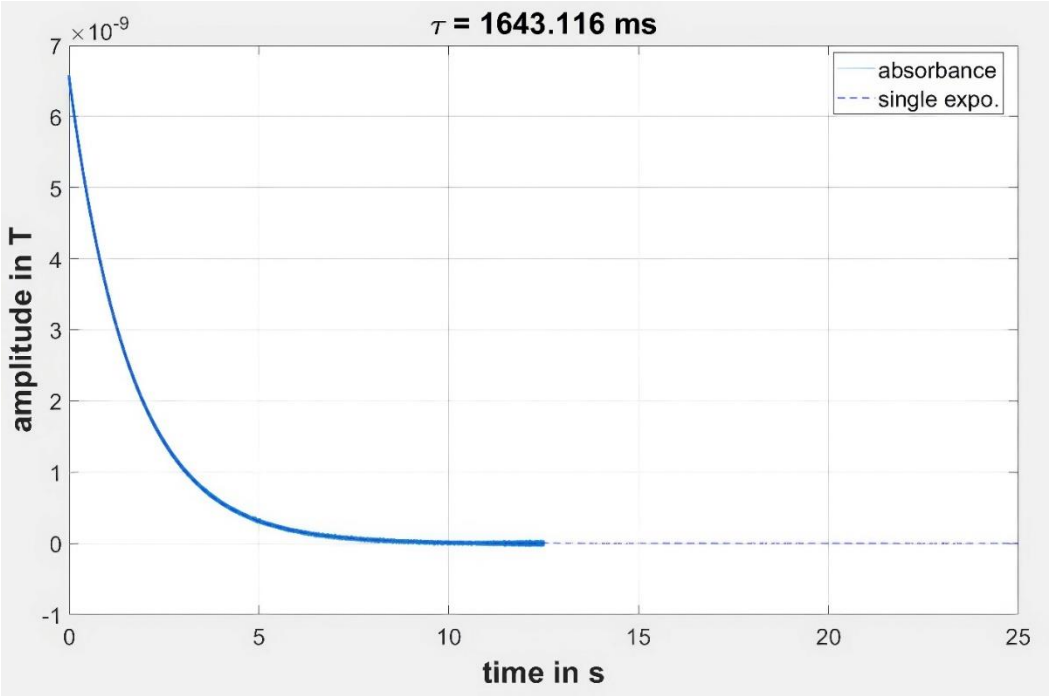

Figure 6 A representative exponential curve of spin-spin transverse T2 relaxation for insulin mixture of Humulin N 35: Humulin R 65

2.1.3.2 Proton domains by T1T2 Maps

Correlation experiments between T_1 and T_2 achieved bidimensional T_1T_2 maps Figure 8) in all samples to better understand proton interactions and demonstrate protonation differences between samples of different mixture ratios. T_1T_2 maps were acquired by applying the IR-CPMG (Inversion Recovery Carr-Purcell-Meiboom-Gill) pulse sequence to the samples. IR-CPMG consists of an inversion recovery

sequence (T_1), followed by a CPMG sequence (T_2). The sequence is reeated at different inversion recovery delays and captures a sequence of echoes for each delay (Panattoni et al., 2021). The signal amplitude data for the T_1T_2 maps were estimated using the following equation:

$$M(\tau, t) = M_0 \left(1 - e^{-\tau/T_1}\right) e^{-t/T_2} \quad (2)$$

where M is the magnetization amplitude at a given time, M_0 is the magnetization amplitude at equilibrium, τ is the recovery time, t is the echo time multiplied by the number of echoes, T_1 is the longitudinal relaxation time, and T_2 is the transverse relaxation time (Du et al., 2020). The number of echoes was set to 250, while the echo time was equal to 15 ms, with 10 points for inversion recovery. The collected IR-CPMG data were converted to T_1T_2 maps using a 2D inverse Laplace transform (2D-ILT) using in-house MATLAB codes. The cumulative signal amplitudes of certain proton pools were collected from the maps. The computational tool used was MATLAB software (The MathWorks Inc, 2023).

2.1.4 Data Analysis Procedure

This research used chemometric methods to quantitatively distinguish between different ratios of insulin drug mixtures by finding the relationship between them and their corresponding T_1T_2 maps and T_2 values. This relationship should provide a prediction method to determine an unknown mixture ratio using its corresponding T_2 value and T_1T_2 map.

The first step in the analysis was to remove outliers. Three samples were removed from the dataset because they were recognized as outliers after an intuitive assessment of the data as a whole. The remaining datasets (60 samples) were randomly divided into two groups using MATLAB code: training set (42 samples, 70%) and validation set (18 samples, 30%).

Data preprocessing involved scaling T_2 relaxation times and data from the signal amplitudes of the T_1T_2 maps to establish an equitable model for training and validation. Scaling was also done to ensure that data from T_2 values and data from T_1T_2 maps are comparable and equivalent to each other, protecting the more minor scale data from being dominated by the more extensive data (Sharma et al., 2023). Therefore, the two datasets were modified to be within the same range. Scaling was done by Min-Max normalization, which transforms the data to be in the range between zero and one and has the following equation:

$$X^* = \frac{X - X_{min}}{X_{max} - X_{min}} \tag{3}$$

where X^* is the normalized scaled value of the data, X is the original value, X_{min} is the minimum value of the trained group, and X_{max} is the maximum value of the trained group (Wang et al., 2018). The minimum and maximum values were selected only from the training group to ensure consistency in the parameters for the prediction method.

The data were processed using a multiple linear regression model (MLR). MLR is a standard and straightforward calibration method that quantifies the relationship between the linear regression of a dependent variable (mixture ratio) and one or more independent variables (T_2 relaxation times). The MLR follows the following equation:

$$y = \beta_0 + \beta_1 x_1 + \cdots + \beta_n x_n + \epsilon \tag{4}$$

Where y is the dependent variable (here, the concentration), $X_1 \ldots X_n$ represent the independent variables (T_2 relaxation times and the T_1T_2 signal amplitude), $\beta_1 \ldots \beta_n$ are the regression coefficients or the parameters of the independent variables for the equation, β_0 is the parameter when all independent variables are equal to zero, and ε is the error (Mihaela Florea et al., 2016; Uyanık & Güler, 2013).

The precision and predictability of the model were evaluated using the correlation coefficient (R^2), adjusted correlation coefficient (R^2 adj), and root mean square error

(RMSE). MATLAB (The MathWorks Inc, 2023) was used as the primary computational tool for all the data analyses.

2.2 Part 2. Quality Issue of Aspirin

2.2.1 Samples

Acetylsalicylic acid and salicylic acid were purchased from Sigma-Aldrich Chemical Co. (Saint Louis,MO, USA) Aspirin drugs were purchased from a local pharmacy in different forms with different concentrations and excipients, as stated in Table 2 and Table 3.

Table 2 Aspirin drugs with their concentrations, different API (Active Pharmaceutical Ingredients), forms, and names of manufactures

Name	Aspirin content	Pharmaceutical Form	Manufacture
Aspirin 100	100 mg	Tablet	Bayer
Aspirin 500	500 mg	Tablet	Bayer
Coraspin 100	100 mg	Coated Tablet	Bayer
Coraspin 300	300 mg	Coated Tablet	Bayer
Ecopirin Pro 81	81 mg	Coated Tablet	Abdi Ibrahim
Ecopirin 300	300 mg	Coated Tablet	Abdi Ibrahim

Table 3 Excipients of each aspirin drug

Name	Excipients
Aspirin 100	Cellulose - Maize - Starch - L-Rotlack - Saccharine
Aspirin 500	Cellulose – Maize
Coraspin 100	Bayer - Cellulose - Maize - Starch - Eudragit L30D - Triethyl Citrate - Talc
Coraspin 300	Bayer - Cellulose - Maize - Starch - Eudragit L30D - Triethyl Citrate
Ecopirin Pro 81	Microcrystalline Cellulose (112) - Starch - Talc - Eudragit L30D - Triethyl Citrate - Croscarmellose Sodium - E110
Ecopirin Pro 150	Microcrystalline Cellulose (112) - Starch - Talc - Eudragit L30D - Triethyl Citrate - Croscarmellose Sodium - E110
Ecopirin Pro 300	Microcrystalline Cellulose (112) - Starch - Talc - Eudragit L30D - Triethyl Citrate - Croscarmellose Sodium - E110
Ecopirin 100	Starch - Talc - Eudragit L30D - Triethyl Citrate - Croscarmellose Sodium - E110

2.2.2 Treatment of Stress Conditions

Moisture treatments were carried out for the degradation study of acetylsalicylic acid. To measure the relative humidity (RH) effect on acetylsalicylic acid degradation, aspirin drugs were subjected to different humidity conditions. This can be achieved by placing aspirin in a closed desiccator alongside the saturated salt solutions. Each saturated salt solution provides a different relative humidity (RH) value.

Temperature is another parameter that affects the damage and degradation of aspirin pharmaceuticals toward salicylic acid and acetic acid. Since elevated temperatures catalyze aspirin degradation by humidity, higher temperatures with higher humidity would create a more stressful environment that facilitates aspirin degradation.

There were three environments for temperature and humidity stress conditions: an environment with 29% RH at 25C° which represents an acceptable safe environment from degradation, a second environment with 75% RH at 30° C which represents an environment where degradation should happen, and a third environment with 90% RH at 45° C which represent a very risky environment where an accelerated degradation should apply .

Aspirin drugs were taken from desiccators under different stress conditions at various periods, followed by further experiments.

2.2.3 Moisture content by Karl-Fischer titration

Karl Fisher titration instrument (TitraLab KF1000 Series, HACH, UK) was used to characterize water content and get a better idea of the water uptake after being admitted to different stress conditions and through various pharmaceutical forms and ingredients of aspirin drugs. The titration is established on the oxidation of sulfur dioxide by iodine in the presence of water.

2.2.4 Quantification of Acetylsalicylic acid and salicylic acid by HPLC

After stress condition treatment, the amount of acetylsalicylic acid remaining in the pharmaceutical products and the amount of salicylic acid caused by these stress conditions were examined using high performance liquid chromatography (HPLC).

The HPLC Device Pursuit C18 Colon has Microsorb MV C18 (4.6 × 250 mm, 5 mm) and UV-Vis (Prostar 330 PDA) detector. The procedure is similar to that described by Yamazaki and others (Yamazaki et al., 2010). The mobile phase was a 3:2 monobasic potassium phosphate/methanol solution with pH 2. The flow rate was 1.2 ml/min at a column temperature of 40 °C, and the wavelength was measured at 295 nm. Measurements were performed using standard and sample solutions. The standard solution used purified acetylsalicylic acid and salicylic acid. The sample

solution was prepared by dissolving 0.5 g of the powder in 10 ml of ethanol and then filled with pure water up to 50 ml.

The degradation of acetylsalicylic acid can be measured using the following equation:

$$AspirinDegradation(\%) = \frac{Salicylic\ Acid}{ASA + Salicylic\ Acid} X100 \qquad (5)$$

2.2.5 Quantity determination by NIRS

Near-Infrared spectrometry (NIRS) analysis was used to investigate aspirin degradation into salicylic acid and acetic acid. NIRS can be used to detect chemical changes in solid surfaces such as tablets.

Near-Infrared spectrometry analysis was performed using a benchtop device NIRFlex N-500 spectrometer (Büchi, Flawil, Switzerland), which measures near-infrared reflectance data points in the spectrum region of 10,000 to 4000 cm−1 (1000–2500 nm) with a resolution of 8 cm−1 (2 nm at 1582 nm) interpolated to 4 cm−1. Each sample was measured three times, and each measurement was repeated three times. NIRS analysis was conducted using the NIR Ware software (Büchi, Flawil, Switzerland).

2.2.6 Evaluation by TD-NMR

Time-domain nuclear magnetic resonance (TD-NMR) was used to analyze the degradation of acetylsalicylic acid. Spin-lattice relaxation time experiments (T_1) were carried out using a 0.5 T (20.34 MHz) NMR instrument (Spin Track, Resonance Systems GmbH, Kirchheim/Teck, Germany). A saturation recovery sequence was employed with a 400 ms relaxation period (TR), 3600 ms observation time, and one scan. Monoexponential fitting was conducted on the relaxation curves using MATLAB.

2.3 Part 3. Glycation as a Quality Issue

2.3.1 Materials

Sodium bicarbonate ($NaHCO_3$), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H2O$), o-phthaldialdehyde (OPA), sodium dodecyl sulfate (SDS), glycine, bovine serum albumin (BSA), sodium azide (NaN_3), acetonitrile, and β-mercaptoethanol (2-mercaptoethanol) were purchased from Sigma-Aldrich Chemical Co. (Saint Louis, MO, USA). Glucose was bought from Tito (Turkey), di-sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10\ H_2O$), potassium hexacyanoferrate (II) trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$), ethanol, were supplied from Merck KGaA (Darmstadt, Germany), Distilled water was obtained from 0.2 µs/cm purity mpMinipure Dest system (mpMinipure Ultrapure Water Systems, Ankara, Turkey).

2.3.2 Methods

2.3.2.1 Sample Preparation and Glycation Procedure

Bovine serum albumin (20 mg/mL) was mixed with 180 mg/ml glucose in distilled water, in addition to 0.02% (w/v) sodium azide to inhibit microbial growth. The prepared solutions were then mixed and allowed to stand overnight. These were then divided into seven samples representing different time points (0, 0.5, 1, 2, 4, 6, and 12 hours). Three replicates were performed at each point. They were placed inside a water bath at 85° C and closed tidily to avoid evaporation. They were removed according to their time points and placed in water at room temperature for 30 minutes to stop glycation. Then, the samples were kept inside a refrigerator at 4° C for further experiments, except at time point zero, when they were not placed inside the water bath but directly inside the fridge.

2.3.2.2 Quantification of free amino groups

The quantity of accessible unbonded free amino groups was examined by the OPA method with few alterations (Diab et al., 2008; Tas et al., 2021); OPA reagent was adjusted using o-phthalaldehyde (OPA), ethanol, borax buffer, β-mercaptoethanol, and Sodium Dodecyl Sulfate (SDS) solution. For the later preparation, 40 mg of the OPA reagent was dissolved in 1 ml 95% ethanol solution. After complete dissolution, 25 mL of 100 mM borax buffer (pH 9.5) was added to the solution. The reagent preparation was completed by adding 100 µL β-mercaptoethanol and 2.5 mL 20% SDS solution. Finally, the volume of the reagent was adjusted to 50 mL. After preparation of the OPA reagent, glycated bovine serum albumin (0.5 mL) was mixed with 1.5 mL of the prepared OPA reagent for 3 minutes. Then, the absorbance values were measured at 340 nm using a Microplate Reader (FlexA-200, Allsheng, Hangzhou, China).

2.3.2.3 Browning Measurements

The degree of glycation or browning was measured according to the color change that occurred with the Maillard reaction. Samples (250 µL) were measured at 420 nm (Morales & Jiménez-Pérez, 2001; Nasrollahzadeh et al., 2017)using a UV-Vis Microplate Reader (FlexA-200, Allsheng, Hangzhou, China).

2.3.2.4 TD-NMR Measurements

The experimental setup was performed by a benchtop NMR system (Pure Devices GmbH, Germany) operating at a 1H frequency of 24.15 MHz. The system was set up with a radio frequency coil measuring 10 mm, corresponding to the cylinder where the samples were placed inside 10 mm wide tubes. The device temperature was set to 28° C for all experiments. Samples were equilibrated to this temperature before the measurements.

The T1 relaxation times of the glycated samples were determined using saturation recovery (SR) sequence with the number of echoes as 2048, echo time interval as 16.7 ms, the number of time points as 20, and the number of scans as 1.

The T2 relaxation times of the glycated samples were determined using the Carr-Purcell-Meiboom-Gill (CPMG) sequence. The number of echoes is 12,500, the echo time interval is 10 ms, the repetition time (TR) is 13000 ms, and the number of scans is 2. The received NMR signals were evaluated using MATLAB software (The MathWorks Inc, 2023) and fitted to mono-exponential models to acquire T2 relaxation times.

The T1T2 correlation maps were obtained by applying the IR-CPMG (Inversion Recovery Carr-Purcell-Meiboom-Gill) pulse sequence to the samples. The number of echoes was set to 200, and the echo time was 15 ms, with 10 points for inversion recovery. The collected IR-CPMG data were converted to T_1T_2 maps using a 2D inverse Laplace transform (2D-ILT) using in-house MATLAB codes. The cumulative signal amplitudes of certain proton pools were collected from the maps. The computational tool used was MATLAB software (The MathWorks Inc, 2023).

CHAPTER 3

RESULTS & DISCUSSIONS

3.1 Part 1. Quality Issue of Insulin

Insulin mixtures with different Humulin N and Humulin R ratios were first analyzed using spin-spin transverse relaxometry (T_2) (Figure 7). Spin-spin transverse relaxation times (T_2 relaxation times) represent the mobility and interaction of water molecules with insulin and its excipients. Since there are two distinct types of insulin with slightly different excipients, and because Humulin N is a suspension, whereas the other insulin drug is a solution, the interaction and mobility with water are expected to differ. Hence, different results are to be obtained depending on the mixture ratio. T_2 Relaxation times were between 1142 and 2389 ms. T_2 relaxation times were examined to provide data on the interactions between water molecules and other drug components in the mixtures. Based on the graph, it is evident that the three samples have identical mixing ratios at each point and exhibit the same T_2 relaxation time. This observation is consistent with previous findings, in which ten samples of different insulin products were examined at the same concentrations, and consistent uniformity results were obtained (Taraban et al., 2022). However, the samples with varying mixing ratios exhibited different T2 relaxation times. This outcome was expected because an increase in molecular concentration, such as protein or polysaccharide in a solution, leads to a higher water binding capacity, consecutively lowering the amount of free water and hence reducing the T_2 relaxation time (Dekkers et al., 2016; Małyszek et al., 2021).

The relationship between the T_2 relaxation times and mixture ratios of different insulins can be explained by the linear regression coefficient (R^2), which was measured as ~0.85. This relationship shows a positive relationship between the T_2 relaxation times of the mixture and the Humulin R ratios. At the same time, there

was a negative relationship between the T_2 relaxation times and the Humulin N ratios in the mixture. However, this relationship is not strong enough to quantify the mixture ratios. Therefore, T_2 measurements are insufficient to be used appropriately as an alternative to chemical methods, such as spectrophotometry. The graph shows that the correlation between T_2 relaxation times and mixture ratios is disrupted when the Humulin N ratio is greater than 80%. To illustrate this, the relation between the Humulin N ratio and T_2 relaxation below 80% has a higher R^2 value of ~0.96.

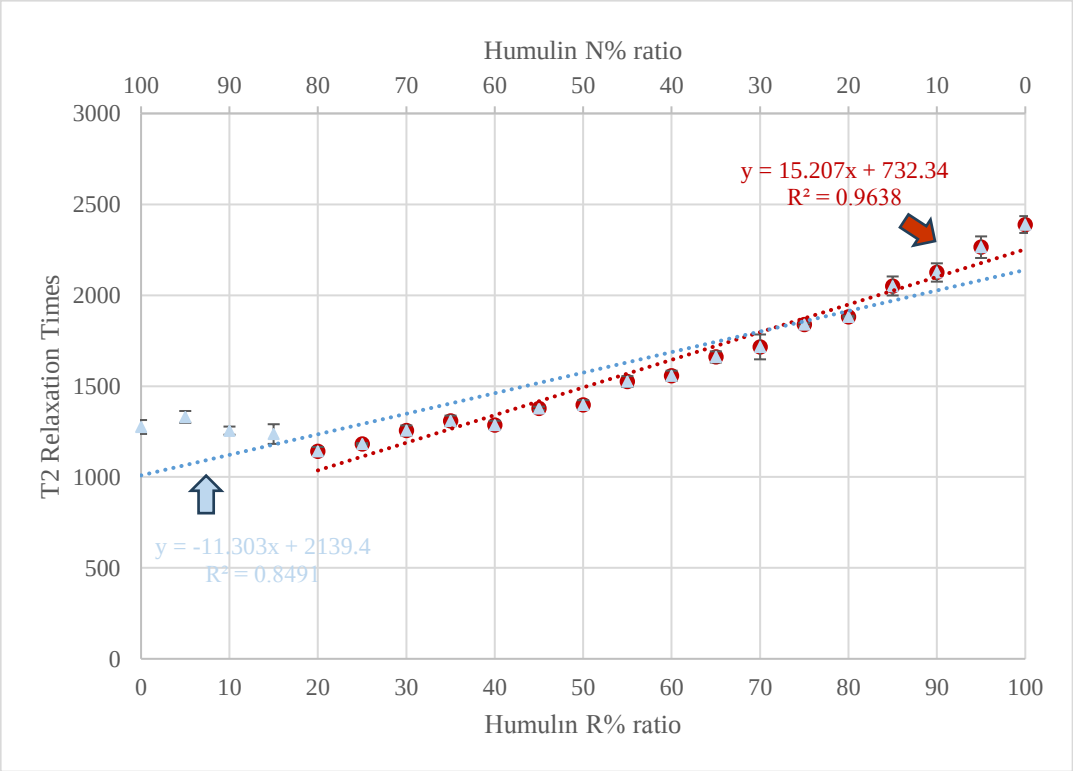

Figure 7 T2 relaxation times vs Humulin R% ratio inside Humulin R and Humulin N mixtures. The first linear trendline (▲) is between HR:HN 0:100 - 100:0, while the second linear trendline (◆) is between HR:HN 20:80 - 100:0.

When the mixture ratios are changed, the concentrations of substances from each drug change, including the concentrations of meta-cresol, phenol, zinc, and the proportion of insulin-protamine complexes to protamine-free insulins. Studies support the concept that measuring T_2 relaxation times would have a linear relationship with the concentration of a substance, such as by increasing the free base content in binary powder blends, which would cause a decrease in the T_2 values

(Chiba et al., 2022). Although Stueber and Jehle (2017) measured the concentrations of a blend of binary molecules (Pajzderska & Wąsicki, 2021; Stueber & Jehle, 2017), they presumed that TD-NMR could be used to measure a mixture of four different molecules. Other researchers measured the concentration of adulterated milk in water and found that increasing the water ratio increased the T_2 relaxation times (Santos et al., 2016). In a pharmaceutical study, Akhunzada et al. (2021) measured different antibodies at different concentrations using TD-NMR, and they succeeded in quantifying variations up to 2 mg/ml (Akhunzada et al., 2021).

However, they mixed different solutions without mixing any suspension; in addition, the substance variations in the mixtures in our study are much lower, as can be seen from the contents of each drug in the 2.1.1 Sample Preparation subsection. All of these studies either measured simple components or relied on measuring one or a few components, such as an antibiotic or a binary blind. The complex was measured by changing the amount of solvent water. However, insulin mixtures contain the same amount of solvent water and are more complex. They have different physical forms: one is suspension, and the other is a solution. Different mixture ratios contain several substances with various concentrations, which may explain why using T_2 relaxation times alone was insufficient to determine all mixture ratios.

When Humulin N and Humulin R are mixed, different physical characteristics change between different mixing ratios, and two of them can explain the change in the TD-NMR results. The first is the mobility of water; the higher the mobility, the longer the T_2 relaxation time, which illustrates the generally higher T_2 value with a decrease in the Humulin N suspension ratio (Pocan et al., 2019). However, it still does not describe why the decline is not linear for ratios above 80% of the Humulin N ratio. The size of the molecules is another physical property that affects the T_2 relaxation results. The larger the undissolved molecules, the higher the T_2 relaxation time (Okada et al., 2021). Increasing the ratio of Humulin N would increase the suspension and macromolecules of the protamine insulin compound compared to the protamine-free insulin molecules, and the ratio of macromolecules and larger

molecules would increase, which explains why high ratios of Humulin N would disrupt the decrease in T_2 relaxation times.

When analyzing complex mixtures, T_2 relaxation can result in an oversimplification of complex molecular dynamics owing to the overlap of signals from different components, leading to misinterpretations of the results. Therefore, it is essential to investigate an alternative model that can effectively rationalize the multiexponential behavior of T_2 relaxation and provide a deeper understanding of complex systems. Previous studies have demonstrated that T_1T_2 maps can address these challenges (Colnago et al., 2021; J. Li et al., 2021). The T_1T_2 map is a bidimensional NMR system (2D-NMR) that can provide valuable information on different water domains and the distribution of protons based on their T_2 relaxation time as one dimension and the correlated T1 relaxation time as the other dimension. This method is highly efficient for distinguishing the contributions of different domains that carry hydrogen molecules (Fleury & Romero-Sarmiento, 2016).

As shown in Figure 8, the system consists of several proton pools, of which two are more present, α and ß. The ß proton pool decreases in size (Figure 8 a,b,c,d,e) as the Humulin R ratios increase and the Humulin N ratio decreases until the ß proton pool becomes unnoticeable when the Humulin R ratios reach 100% (Figure 8**Hata! Başvuru kaynağı bulunamadı.** f). The presence of this ß proton pool can account for the observed elevation of T_2 values in mixtures containing Humulin N ratios exceeding 80%. Humulin N is a suspension that undergoes precipitation of its crystallized contents, which is caused by a composite structure of insulin, zinc, and protamine, whereas Humulin R is a solution (Hirsch et al., 2020). Increasing the suspension ratio causes an increase in the concentration ratio of solids in a mixture, leading to an increase in the interaction between water and a solid surface and an

increase in its proton pool amplitude. It is typical for the T_2 relaxation of water in contact with a solid surface to be lower than that of water containing dissolved molecules (Fleury & Romero-Sarmiento, 2016), which may explain why the ß proton pool has a shorter T_2 relaxation time than the α proton pool and why it increases with the increase in Humulin N.

Figure 9 shows the total cumulative signal from the newly emerged ß proton pool (T_1 ~ 1600 ms, T_2 ~ 200 ms) for each sample against the mixture ratios of Humulin N and Humulin R. It is noticeable that the decrease in the amplitude signal of the proton pool can be related to the reduction in the Humulin N ratio. However, with a correlation coefficient of 0.6914, this measurement method could not be used as a

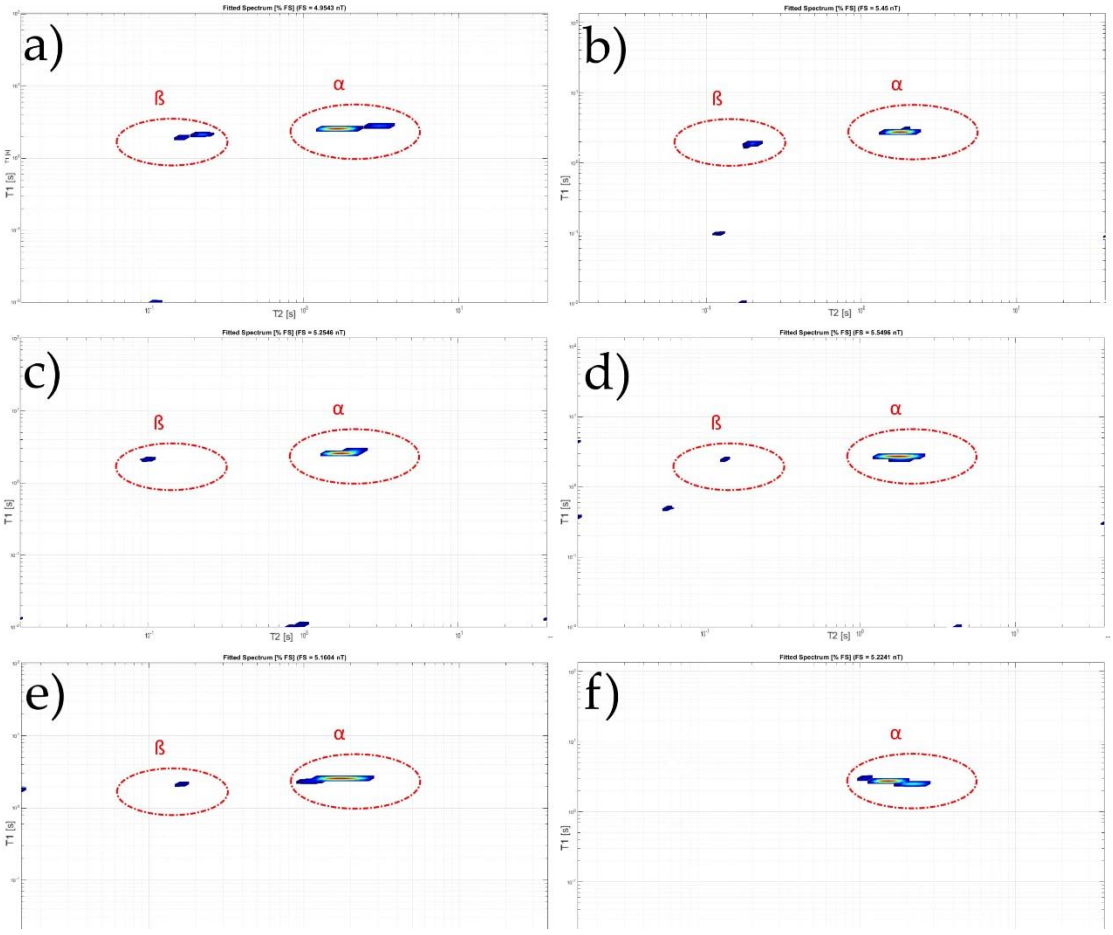

Figure 8 T1T2 maps of different insulin mixture ratios, a) HN: HR 100:00 b) HN:HR 80:20 c) HN: HR 60:40 d) HN: HR 40:60 e) HN: HR 20:80 f) HN: HR 0:100. α and ß are two main proton pools.

reliable quantitative method. When the Humulin N ratio is below 30%, the proton pools are difficult to distinguish from each other or from noise and cannot be detected in some mixtures. On the contrary, when the Humulin N ratio is greater than 70%, the signals of the proton pools can be seen to have a highly positive relationship with the mixture ratios with a correlation coefficient R^2 of 0.988. These indicate that measuring T1T2 maps of mixture solutions and extracting proton pool signals can only be helpful for mixtures with a high Humulin N ratio, which is insufficient for practical applications. The stable increase in the signal from the ß proton pool only with high Humulin N ratios can be attributed to the partial dissolution of solid molecules in the unsaturated Humulin R solution during mixing. This process reduces the number of undissolved particles in the mixture, thereby decreasing the signal from the immobilized water. However, after saturation, the proton pool increased steadily. The locations of the two main proton pools α (T_1~2500 ms, T_2~1700 ms, T_1/T_2 ~1.5) and ß (T_1~2100 ms, T_2~200 ms, T_1/T_2~10) in the map are consistent with other studies, which have reported similar results for the proton pools of water and undissolved solids, respectively (Baran et al., 2023; Fleury & Romero-Sarmiento, 2016; S. Wang et al., 2020). In mixtures with high Humulin N ratios, each of the two major proton pools is divided into two separate proton pools, four in total. This division is caused by a better separation between the supernatant and precipitation phases, which is attributed to the different signals from the bulk water and undissolved particles. Otherwise, in smaller Humulin N ratios, these proton pools overlapped and merged together. Other proton pools from T_1T_2 maps are considered artifacts or noise because of their small size and because none are presented in a stable form between different measurements.

Many studies have shown that T_1T_2 maps are commonly used as identification techniques (Silletta et al., 2022). They can be used as fluid quantification methods to measure migrated water (Zheng et al., 2017) or to determine the amount of starch, proteins, or pentosans in the water (Serial et al., 2016). T_1T_2 Maps were used to determine the ratios of pine resin to water in the wood (Xin et al., 2020). These results demonstrate the benefits of T_1T_2 maps in differentiating between solids and liquids,

as well as between different liquid phases. Nevertheless, T_1T_2 maps do not distinguish much between the same liquid phase but with varying dissolution rates. Such is the case with insulin mixtures with lower Humulin N ratios.

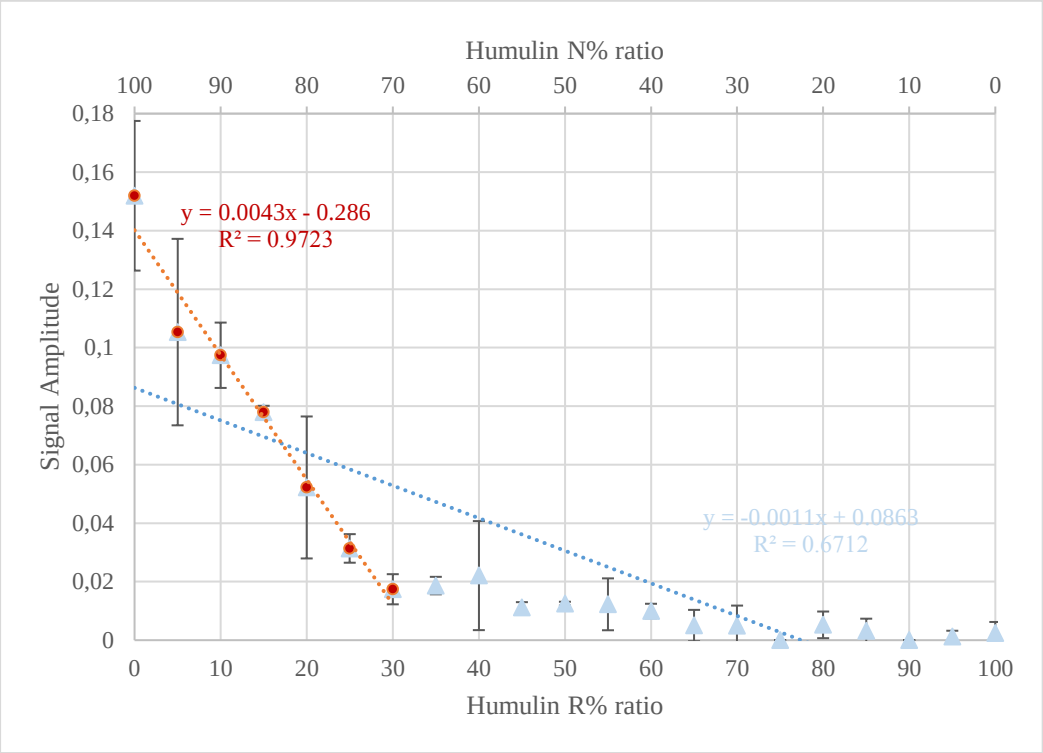

Figure 9 Signal amplitude of ß proton pool compared to different mixture ratios of Humulin N and Humulin R. The first trendline (●) is between HR:HN 0:100 – HR:HN 20:80, while the second trendline (▲) is between HR:HN 0:100 – HR:HN 100:0.

The measurement of insulin mixtures of Humulin N and Humulin R in different ratios showed opposite results depending on the measurements. Although T_2 relaxation showed a high correlation with most of the mixture ratios, it showed a poor correlation when the Humulin N ratio was high. In contrast, T_1T_2 maps showed a high correlation only for mixtures with high Humulin N ratios. These two methods can be integrated using MLR to establish a link between the two measurement methods and the mixture ratios. MLR is a statistical technique that can model the linear relationship between the dependent variable (mixture ratio) and the independent variables (T_2 relaxation and proton pool signal) (Maulud & Mohsin Abdulazeez, 2020).

The two datasets were divided into training and validation groups and then scaled by Min-Max normalization. Subsequently, an MLR model was established to provide a linear regression model by integrating the two methods (Figure 10). The model is considered accurate as it showed high accuracy measurements: RMSE = 4.57, R^2 = 0.976, and adjusted R^2 = 0.975.

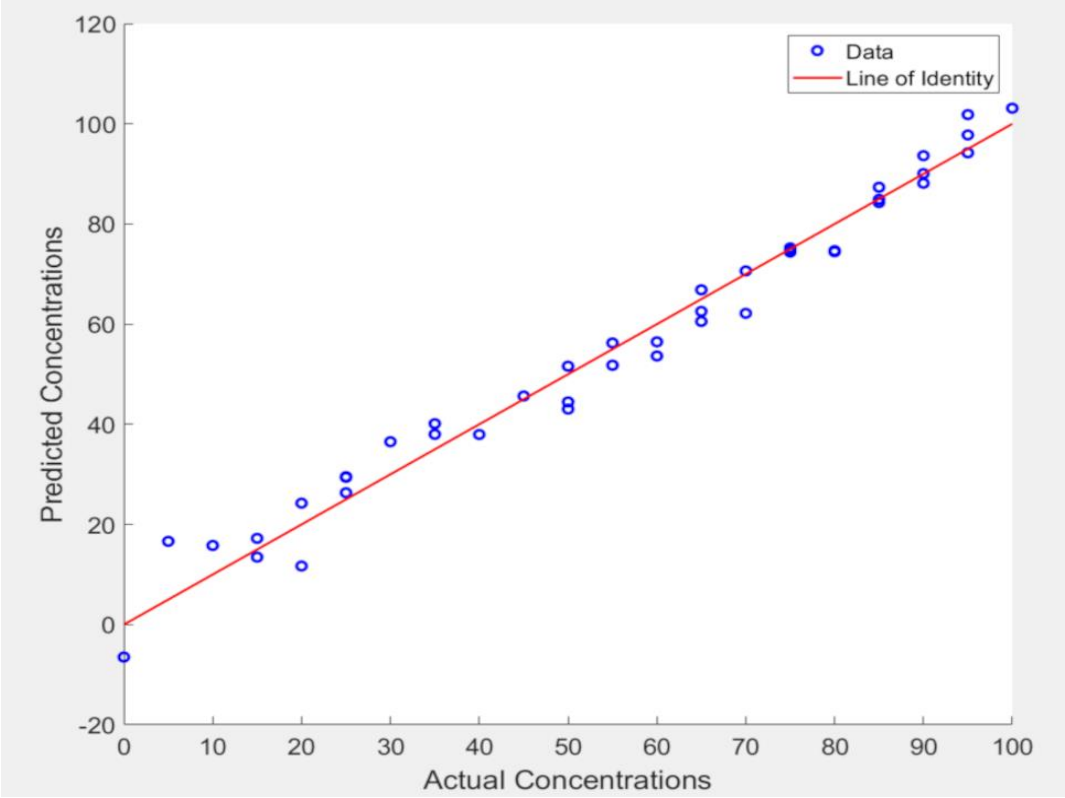

Figure 10 Actual vs. Predicted Concentrations, after applying Multiple Linear Regression (MLR) on the training group.

All these results demonstrate that the new model fits well with the training dataset. The data was validated using the validation group (Figure 11). The validation group showed high accuracy measurements with RMSE = 6.2561, R^2 = 0.9581, and Adjusted R^2 = 0.9525. This confirms that the method is suitable for measuring mixture ratios.

This integrated TD-NMR method requires a few minutes to detect the mixing ratio in each sample, making it a quick and desirable approach. It is an intuitive and user-friendly method (Yildiz et al., 2018) that nurses and physicians in hospitals and

healthcare centers can easily apply. Furthermore, it is non-invasive and non-destructive, which allows its application to all insulin drugs mixed before administration to the patient without damaging or impacting the mixture. Moreover, it can be automated (Fenerick et al., 2022). The aforementioned characteristics cause this integrated method to avoid a remarkable quality control issue (Carter et al., 2016), as it can be performed by manufacturers or regulatory agencies for every mixed drug produced by manufacturers or every product in a batch instead of measuring only a small quantity of each batch, whether used for high production by manufacturers or quality control regulators. All these benefits make the integrated method of this study a suitable alternative or an excellent complement to current quality control methods, such as HPLC, that lack these characteristics and benefits.

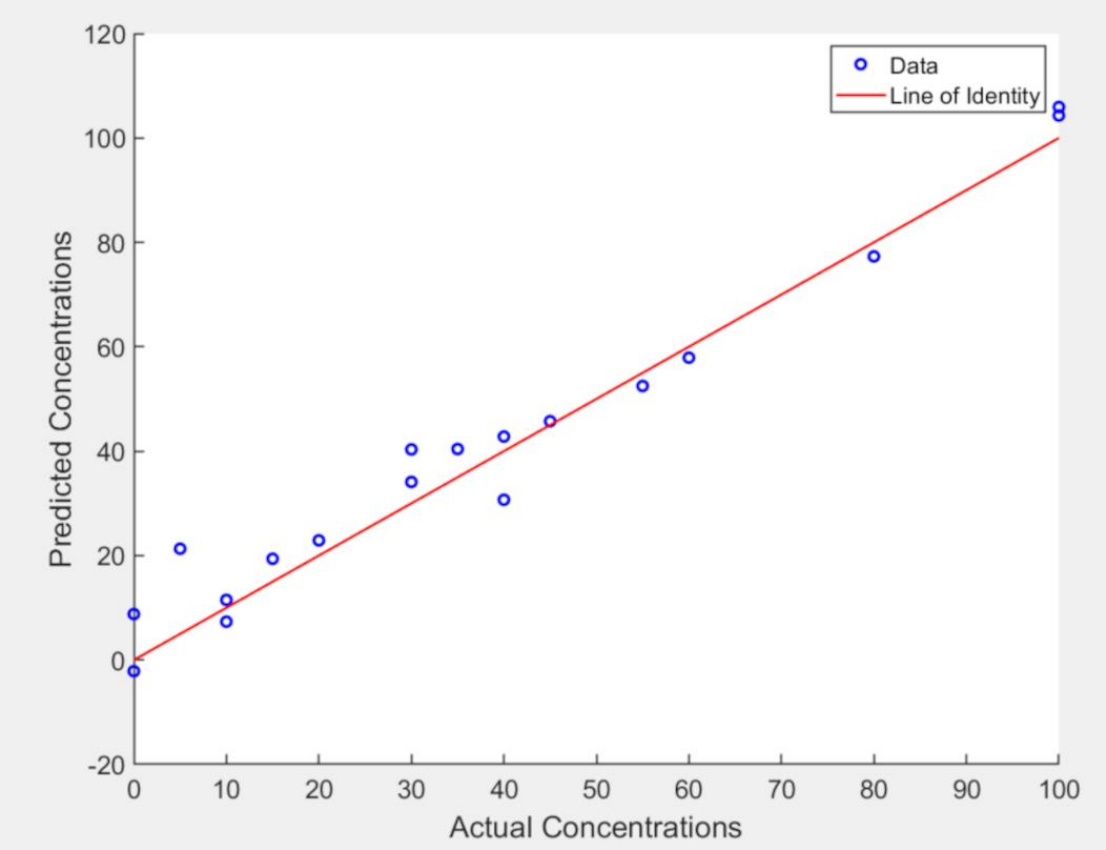

Figure 11 Actual vs. Predicted Concentrations, after applying the Multiple Linear Regression MLR to the validation group.

One limitation of this study is that neither insulin bottles nor insulin mixtures were measured to ensure that all components between bottles were the same. However, such a change in different bottles from different batches is improbable (Taraban et al., 2022). In addition, TD-NMR can be used to measure bottles before mixing to ensure uniformity. Although this study focused on two different insulin mixtures without measuring other insulin mixtures, such as insulin lispro, it is highly implied that this measurement approach should also be applicable to other insulin mixtures.

The combination of medications and solvents for suspensions and solutions is common in the pharmaceutical industry. Therefore, this integrated TD-NMR method can be used to quantify other mixed pharmaceutical drugs in addition to mixed insulins. Combination strategies of different antibiotics can be used for bactericidal synergism, combat drug resistant microbial pathogens, and suppress resistance development (P. H. Chen et al., 2022; Xu et al., 2018). Pediatricians and pharmacists can dilute an adult suspension drug with a solution to achieve the correct and appropriate dosage for a child (O'Hara, 2016). This is needed due to the lack of suitable drug preparations and because the proper dose of a drug is correlated with the age and weight of the child (Belayneh et al., 2020). This integrated TD-NMR method shows promise for measuring mixed or combination drugs to minimize medication errors and reduce hospital admissions and therapeutic complications (Kelly et al., 2020). The results demonstrate that by merging or joining two different TD-NMR methods, the shortcomings of each of these TD-NMR methods can be compensated for and solved. If there is a change in protonation and if a TD-NMR method has a partially stable correlation with this change, then the measurement and integration of other TD-NMR methods should be considered in future studies. Furthermore, measuring other pulse sequences, such as 2D-CPMG–CWFP–T1 (Two dimension- CPMG - Continuous Wave Free Precession with low flip angle), instead of IR-CPMG, may yield different results because it allows a higher resolution in the direct dimension T_1 but a lower resolution in the indirect dimension T_2 (Monaretto et al., 2020).

Since this study presented a TD-NMR technique that measures preparations of different mixing ratios of suspensions and solutions, then this technique should be considered for measuring similar mixture preparations outside the pharmaceutical industry, including the energy industry where nanoparticle suspensions can be processed by solution mixing (Mohanty et al., 2021), chemical engineering, for example, suspension polymerization is applied by adding suspension or solution drops (Hashim & Brooks, 2004), and food engineering, such as measuring the ratio of suspended particles, which is essential for the stability of drinks (Muhammad et al., 2021). It is also important to consider that this study provides a TD-NMR technique to measure the changing solubility of solid molecules and the increasing amount of undissolved particles (or the precipitated environment); therefore, this method has the potential to be applied to study precipitation reactions, reactions that cause the formation of solid particles in solvents (Karpinski & Wey, 2002) in chemical and pharmaceutical research.

3.2 Part 2. Quality Issue of Aspirin

3.2.1 Crystallization detection by XRD

The study incorporated XRD measurements to determine the proportional ratio of crystalline and amorphous components in aspirin tablets prior and after putting them under stress conditions of humidity and temperature. From Table 4 and Figure 12, we can see that the degree of crystallinity for Coraspin 100, Coraspin 300, and Ecopirin 300 increased, whereas the crystallinity ratio for Ecopirin Pro decreased, and Aspirin 100 and Aspirin 500 did not change significantly.

3.2.2 Quantification by NIRS

Partial Least Squares Regression (PLSR) was carried out using the whole NIR spectra to determine the correlation between the NIR values of tablets and HPLC

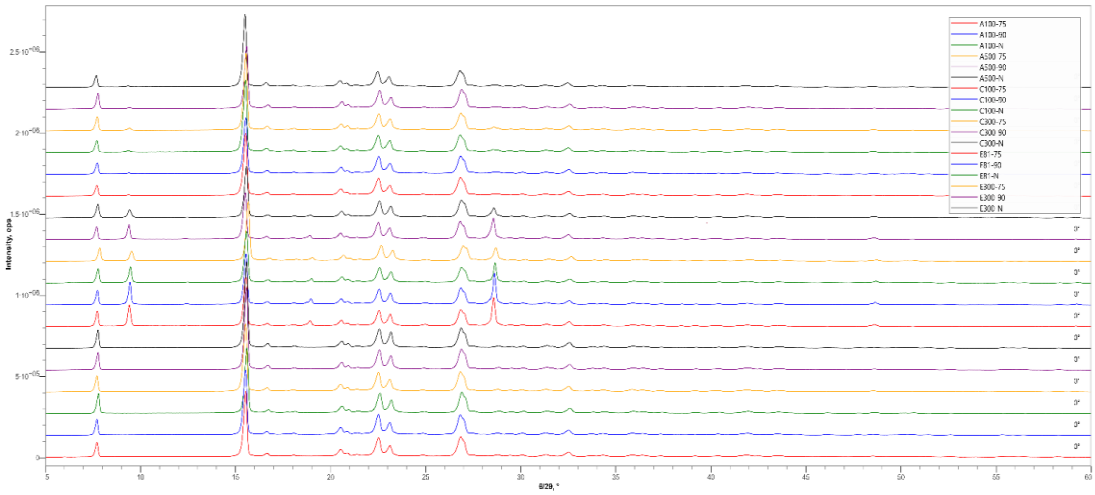

Figure 12 XRD spectra for aspirin tablets before and after being under stress conditions of humidity and temperature.

values of the same samples. A total of 1500 spectra were collected (three spectra for each sample) and used to develop a calibration model corresponding to 100 samples of aspirin tablets of each kind (500 samples in total). These samples were analyzed using HPLC to form a standard reference for calibration. NIRCal software performed wavelength selection according to a repeated method, combining all spectral data and preprocessing to acquire the best combination for calibration.

Table 4 Crystallinity of tablets under different environments

	Asp 100 before	**Asp 100 75% - 30°**	**Asp 100 90% - 45°**	**Asp 500 before**	**Asp 500 75% - 30°**	**Asp 500 90% - 45°**
Crystallinity rate	57.3%	57.7%	57.4%	41.5%	39.5%	40.7%
	Cor 100 before	**Cor 100 75% - 30°**	**Cor 100 90% - 45°**	**Cor 300 before**	**Cor 300 75% - 30°**	**Cor 300 90% - 45°**
Crystallinity rate	40.1%	41.3%	41.7%	39.3%	40.3%	40.5%
	Eco Pro before	**Eco Pro 75% - 30°**	**Eco Pro 90% - 45°**	**Eco 300 before**	**Eco 300 75% - 30°**	**Eco 300 90% - 45°**
Crystallinity rate	39.3%	37.4%	36.8%	38%	38.5%	38.7%

Partial Least Squares Regression (PLSR) was implemented using the whole NIR spectra to determine the correlation between the NIR values of tablets and HPLC values of the same samples. A total of 1500 spectra were collected (three spectra for each sample) and used to develop a calibration model corresponding to 100 samples of aspirin tablets of each type (500 samples in total). These samples were analyzed using HPLC to form a standard reference for calibration. NIRCal software performed wavelength selection according to a repeated method, combining all spectral data and preprocessing to acquire the best combination for calibration.

Table 5 shows number of principal components and measurements quality parameters of NIRS for each different aspirin tablets.

	Aspirin 100	**Aspirin 500**	**Coraspin 100**	**Coraspin 300**
Principal Component (PC)	5	4	4	5
Q-value	0.71	0.74	0.80	0.76
Standard Error Prediction (SEP)	0.35	0.15	0.35	0.27
Standard Error Calibration (SEC)	0.33	0.13	0.33	0.25
Consistency SEC/SEP	94%	86%	94%	93%
PRESS	4.4	2	7	4

The Q-value represented the calibration rate and was calculated using NIRCal software, integrating different statistical measurement measures (SEC, SEP, and regression coefficients). The calibration rating ranged between 0 (incompetent) and 1 (perfect). At the same time, the Q-value should be above 0.60 to be considered

trustworthy for measurements. From Table 5, we notice that 4 or 5 principal components were needed to obtain a good measurement parameter. This also shows that calibration error ratio is small while the correlations are high. Figures 13 – 16 shows differences between original spectra and the spectra after applying preprocessing and PLSR. However, while these results show that size of tablets is not an issue toward measuring tablets and while film coated tablets can also be measured and calibrated. The sugar coated Ecopirin tablets failed to give a good results of calibration measurements indicating that the thin film layer can let NIRS to pass through it while the sugar coated layer doesn´t by blocking NIR.

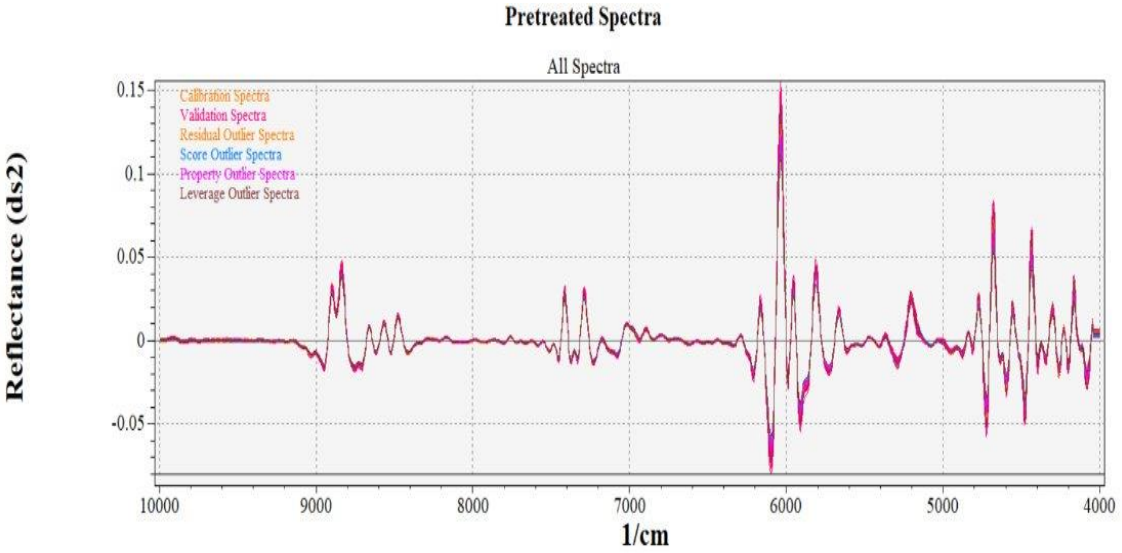

Figure 13 Differences between original spectra, preprocessed spectra, and processed spectra by PSLR for Apsirin 100 tablets.

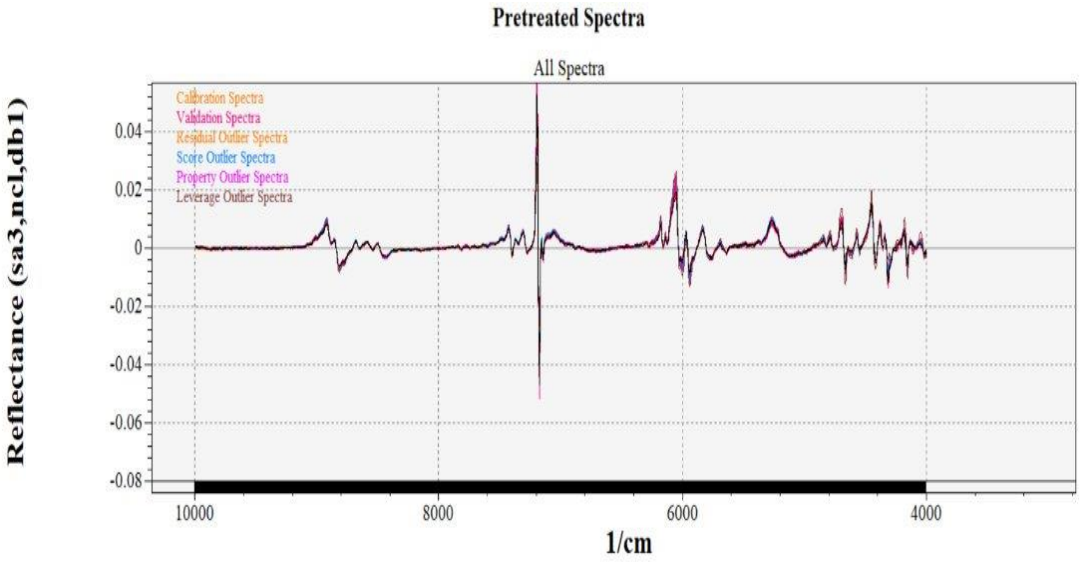

Figure 14 Differences between original spectra, preprocessed spectra, and processed spectra by PSLR for coraspin 100 tablets.

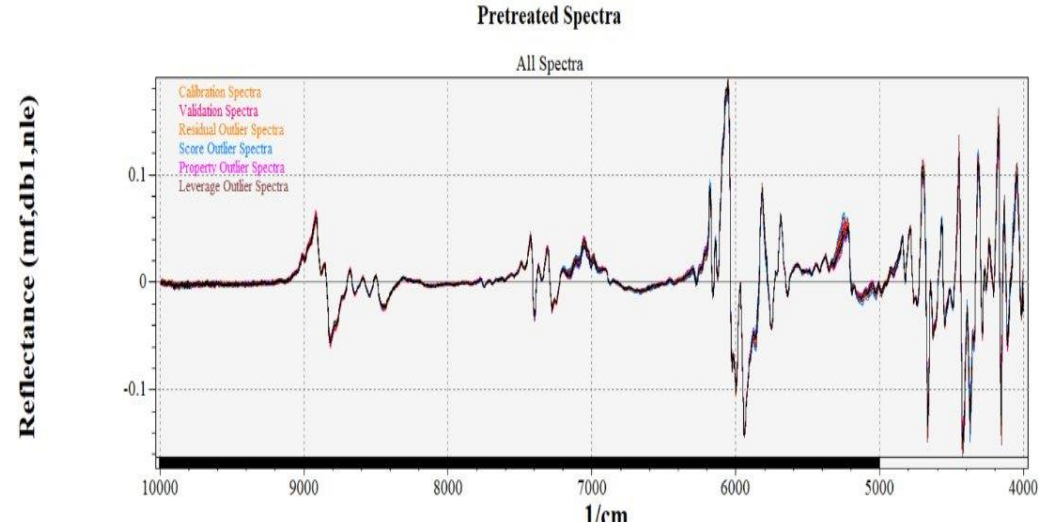

Figure 15Differences between original spectra, preprocessed spectra, and processed spectra by PSLR for Apsirin 500 tablets.

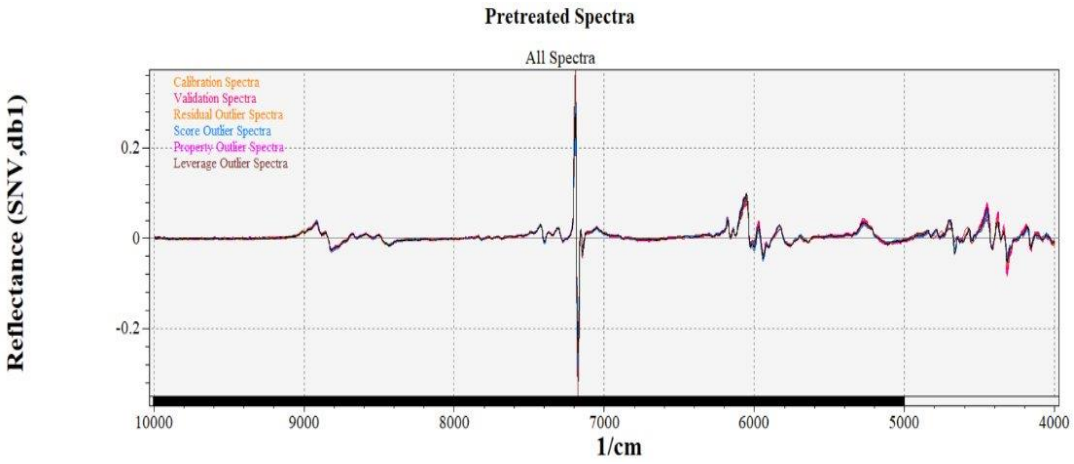

Figure 16 Differences between original spectra, preprocessed spectra, and processed spectra by PSLR for Coraspin300 tablets.

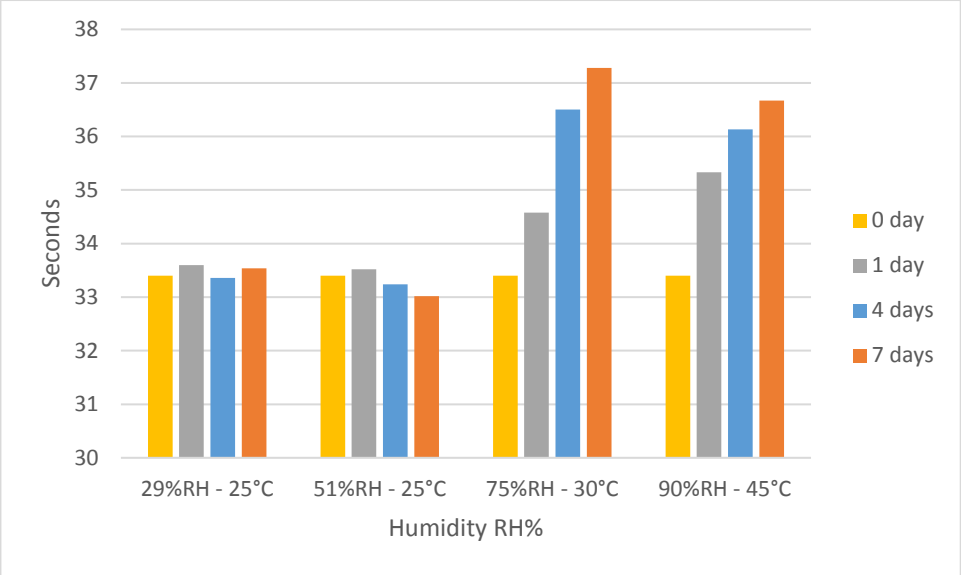

Figure 17 T1 relaxation times of coraspin 100 during a week under different environments.

3.2.3 Protonation measurement by TD-NMR

NMR relaxometry is an easy to use, fast, and non-destructive analytical method used in this study to investigate changes in protons in aspirin tablets. The relaxation

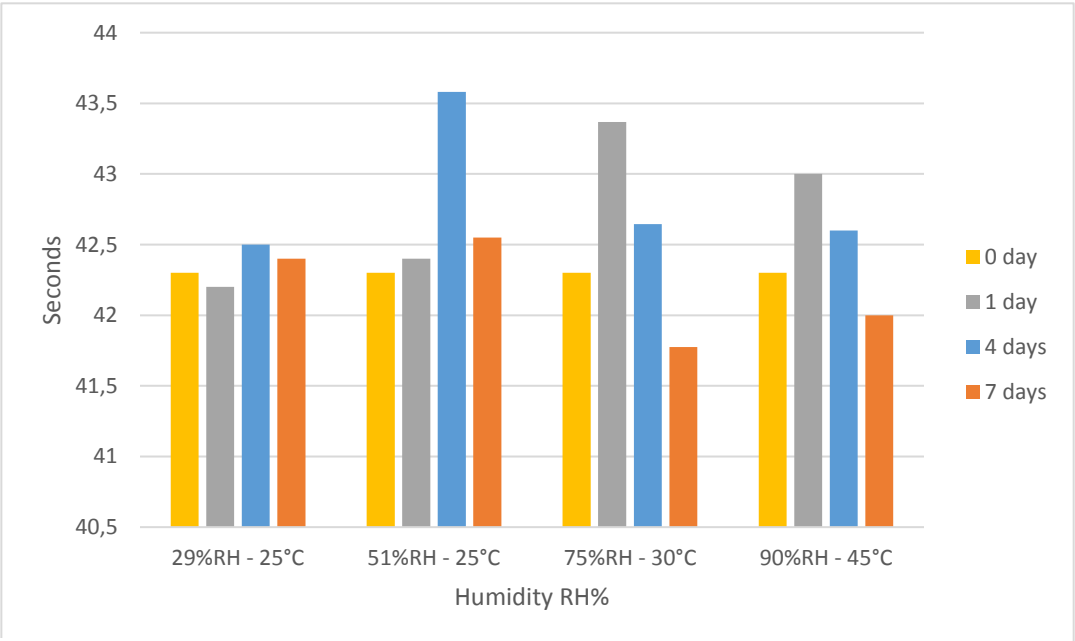

Figure 18 T1 relaxation times of coraspin 100 during a week under different environments.

times of aspirin tablets were measured under different humidity and temperature environments, as shown in Figures 17, 18, and 19. The T1 data were analyzed using MATLAB (MathWorks, Inc., Natick, Massachusetts, United States) by fitting the data to a mono-exponential model.

The longitudinal relaxation time (T1), which is called spin-lattice relaxation time, can be described as the time needed for the spins of the protons to release the energy that they capture from the radio frequency (RF) pulse toward the nearby lattice as a means to return to the equilibrium state (Hashemi et al., 2010).

T1 value is reliant on the mobility of water protons. hus, we can conclude that adsorbed water molecules on tablets should increase T1 values due to an increase in protons' mobility. T1 values can also be increased in relation to higher crystallinity rate and crystal sizes (Adam-Berret et al., 2009; Okur et al., 2022), and since aspirin tablets did increase their crystallinity rate during high temperature and high relative humidity conditions, then it is expected to cause an increase in T1 values.

Since it is recommended for aspirin to be around room temperature while relative humidity should be no more than 55% (Yamazaki et al., 2010), then looking at Figure 17, Figure 18 and Figure 19, we notice that there wasn't much change for

T1 values, onfirming that there wasn't degradation or crystallization during these mild conditions.

However, under harsh simulated conditions with a high relative humidity of 75% or 90%, we notice an apparent change in T1 values within a week in these environments. This increase can be explained by an increase in crystallinity, water

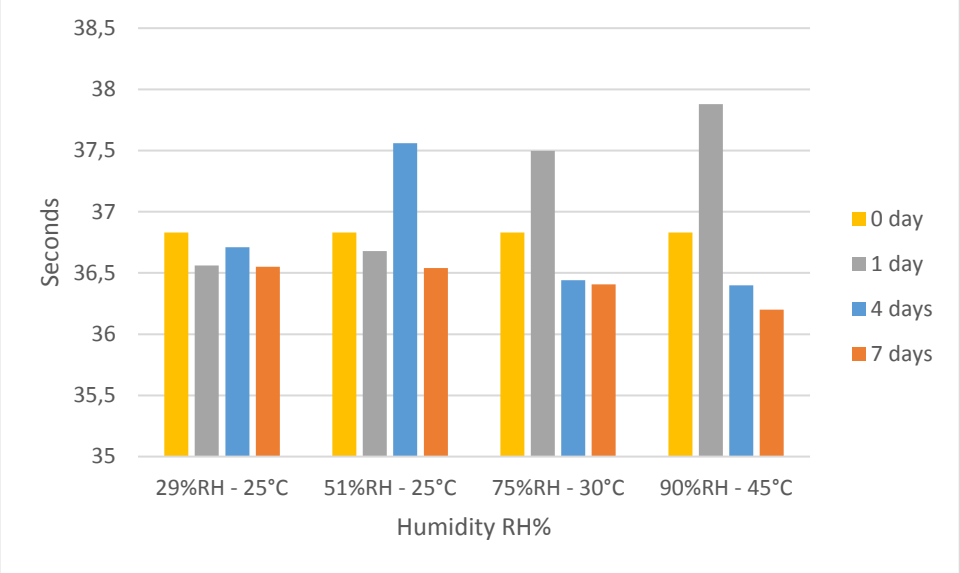

Figure 19 T1 relaxation times of Aspirin 500 during a week under different environments.

adsorption, and aspirin degradation to acetylsalicylic acid and acetic acid. The decrease can be interpreted as a cause of the hydrolysis reaction of cellulose and maize in Aspirin 100 and Aspirin 500, which leads to an increase in free hydroxide groups. Thus, there is a reduction in proton mobility in the medium despite an increase in crystallinity and aspirin degradation. Coraspin 100, on the other hand, needs an alkaline medium to degrade since it was prepared to degrade in the intestines.

3.3 Part 3. Glycation as a quality Issue

3.3.1 Free Amino Group Determination by OPA Method

Figure 20 shows a continuous decline in the free amino group during the experiment, which was expected as the elevated temperatures promote glycation reaction. Elevated temperatures can unfold proteins and expose more amino groups to the medium. Moreover, higher temperatures increase the reactivity between the aldehyde groups and amino groups (Bodiga et al., 2013; Garcia-Amezquita et al., 2014). The decrease was sharp during the first hour of the experiment, and then, the decrease in the free amino group decreased at a slower rate.

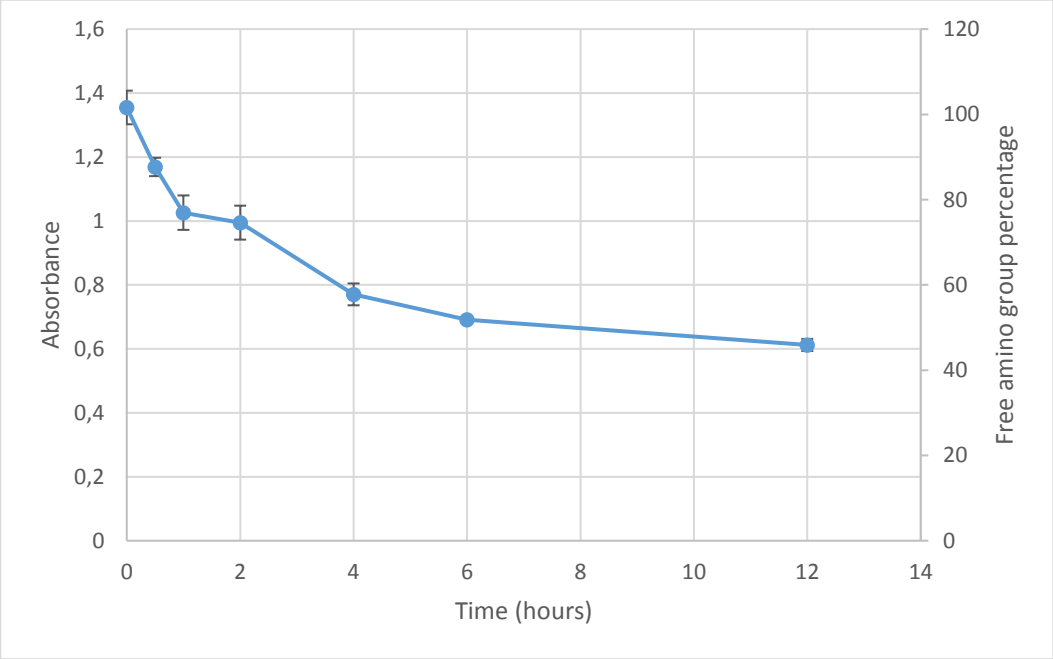

Figure 20 Free amino group determination of glycated samples by OPA Method under 12 hours of elevated temperatures.

This is because the amount of free amino groups decreased, and the glycation reaction caused an increase in the medium's acidity with the introduction of methylglyoxal and glyoxal (D.Zhao et al., 2021), which slows the glycation reaction. While buffers do help avoid pH changes, mAb pharmaceuticals do not necessarily contain buffers within them (Food and Drug Administration (FDA), 2012).

3.3.2 Browning Measurements

The color change of the samples with time was a direct and easy indication of the progress of the glycation reaction. The light absorbance at 420 nm was used to monitor the glycation reaction. Figure 21 shows that there is a general increase in the absorbance over time. Nonetheless, the first hour showed a slight decrease, and the increase seemed to stop after 6 hours. The unclear changes in the first hour can be explained as the UV-visible spectrometry may not be reliable for minor changes (Sooväli et al., 2006). In addition, UV–Vis absorbance at 420 nm is typically used to evaluate brown polymers in the final products. In the initial stages of glycation, the reaction is reversible until more advanced glycation end products are generated, which are chemically more stable (Chen et al., 2020; S. Cho et al., 2022; Yang et al., 2011; Ye et al., 2022). The browning increases discontinue after six hours, and that can either mean that the glycation has stopped, which is disproven by the previous results of the OPA method, or the generated AGE is colorless and may even dilute the brown color as the formed AGE partially causes color change while the majority of AGE including CML- modified molecules would not participate in the browning reaction (Vetter & Indurthi, 2011). However, for a better understanding of the changes between 6-12 hours, more time points should be measured in between.

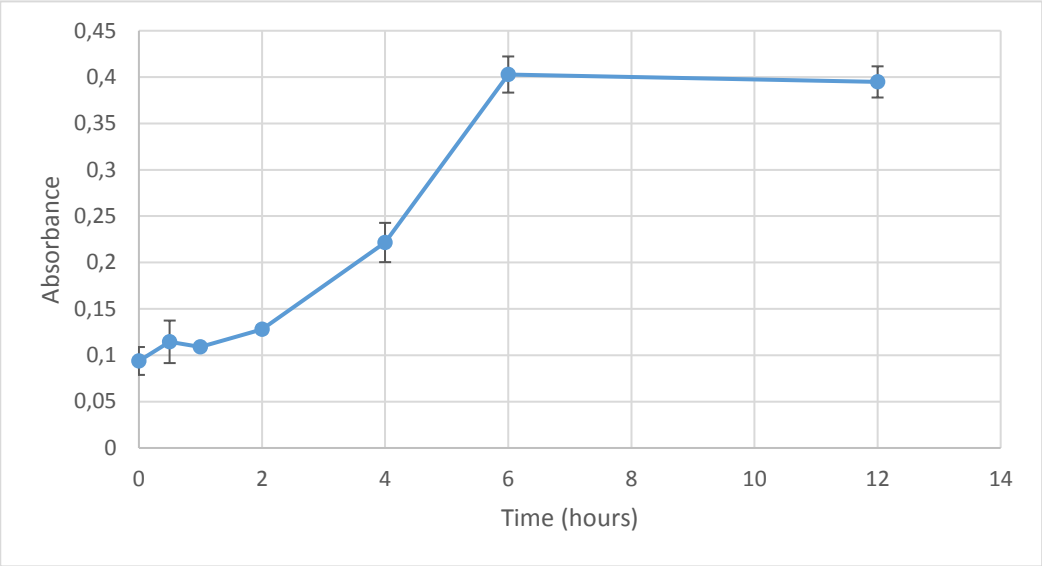

Figure 21 Browning measurements of glycated samples by OPA Method under 12 hours of elevated temperatures. Based on reading at 420 nm.

3.3.3 TD-NMR Measurements

T1 relaxation times and T2 relaxation times were measured for all Figure 22 and Figure 23. The T1 relaxation times decrease with a decrease in hydration, which the glycation reaction can cause (Davies et al., 2019; Schumacher et al., 2017).

T2 relaxation time identifies differences in the spin's mobility in a solution. It can also provide details about the water's mobility and freedom and its interaction with the surrounding molecules. In this study, The T1 and T2 relaxations of all measurements were more accurately characterized by a monoexponential pattern. As glycation progressed, T1 and T2 relaxation times decreased. T1 relaxation times are often assessed using the SR sequence, whereas T2 relaxation times are assessed using the CPMG sequence (Kirtil & Oztop, 2015; Tas et al., 2022).

TD-NMR measures the T1 and T2 relaxation signals from all protons in the sample. Non-exchangeable protons, including those from non-exchangeable CH bonds in solids, do not affect the signal because of the measurement delay restriction in low-

field NMR systems (Kirtil, Dag, et al., 2017). The change in T2 relaxation times with glycation was slower at some time points than at others, which can be explained by the various effects of glycation that can change and affect T2 relaxation times. For example, as a consequence of agglomeration and fibril formation caused by glycation, there is a decrease in the T2 relaxation points. Another explanation for the decline in T2 relaxation is the unveiling of hydrophobic amino acids by the structural changes caused by glycation (Rabbani et al., 2021). These hydrophobic compounds interact less with water and would decrease the T2 relaxation times (Suekuni & Allgeier, 2023).

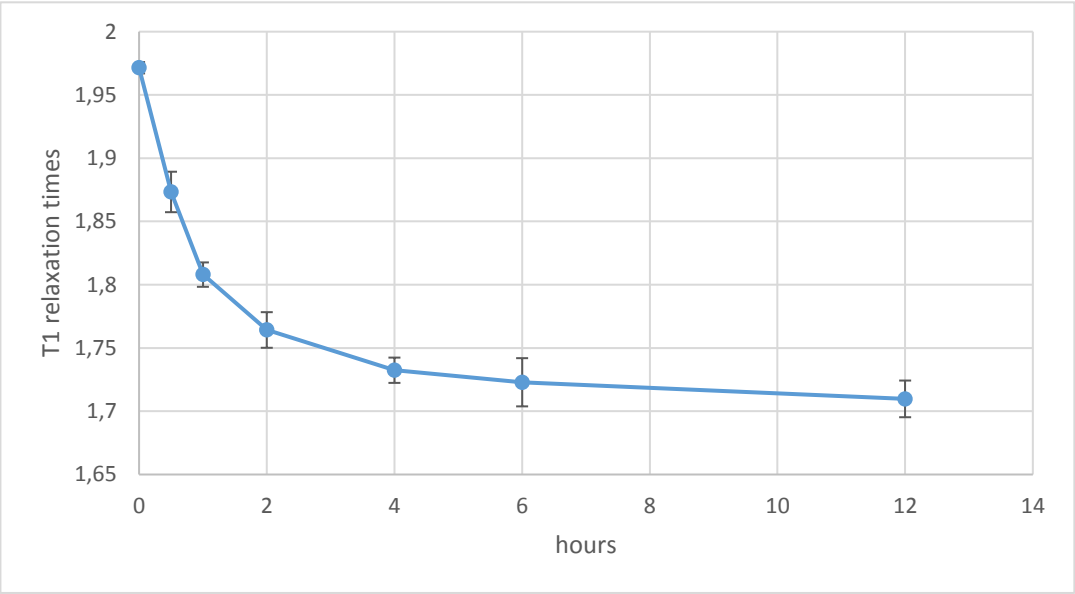

Figure 22 T1 relaxation time of BSA and Glucose mixtures between 12 hours.

The T1T2 map is a two-dimensional NMR system (2D-NMR) that offers clearer insights into various water domains and proton distributions, which relies on their T2 relaxation time in the x-axis correlated to T1 relaxation time in the Y-axis. This approach is quite practical for separating the contributions of several domains that contain hydrogen molecules with different activities (Fleury & Romero-Sarmiento, 2016)

The T1T2 maps of the glycated solutions displayed two prominent proton pools, α, and ß (Figure 24). The T1/T2 value of any proton pool can identify the molecules of a proton pool, even if its T1 and T2 values or its placement has changed. This

indicates that changes in the T1/T2 values of the proton pool change the chemical structure related to that proton pool. Measuring T1/T2 placement points for α proton pools (Figure 25) shows an increase for T1/T2, with the increase in time indicating changes in the characteristic of the proton pool and changes in the chemical structure or changes in the mobility of water accordingly(J. Li & Ma, 2022; Silletta et al., 2022), which can be explained by the glycation reaction.

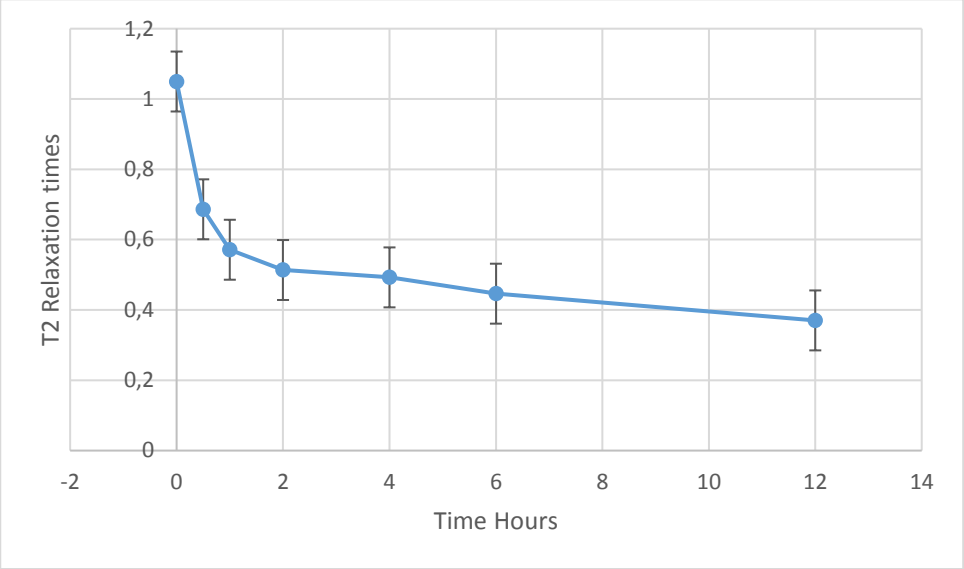

Figure 23 T2 relaxation times of BSA and Glucose mixtures between 12 hours.

This ensures that these changes in the T1, T2, and T1T2 maps change accordingly with glycation. They were correlated with the OPA method (Table 6). Because T1/T2 changes were disproportional with OPA, T2/T1 were also added to compare proportional change. The correlation was measured with r, R2, and p-value.

Table 6 shows that T2/T1 highly correlates with the OPA method with r= 0.98, R2 = 0.96, and p-value < 0.01. This demonstrates that T2/T1 should be considered as an alternative non-invasive and non-destructive method for glycation measurements.

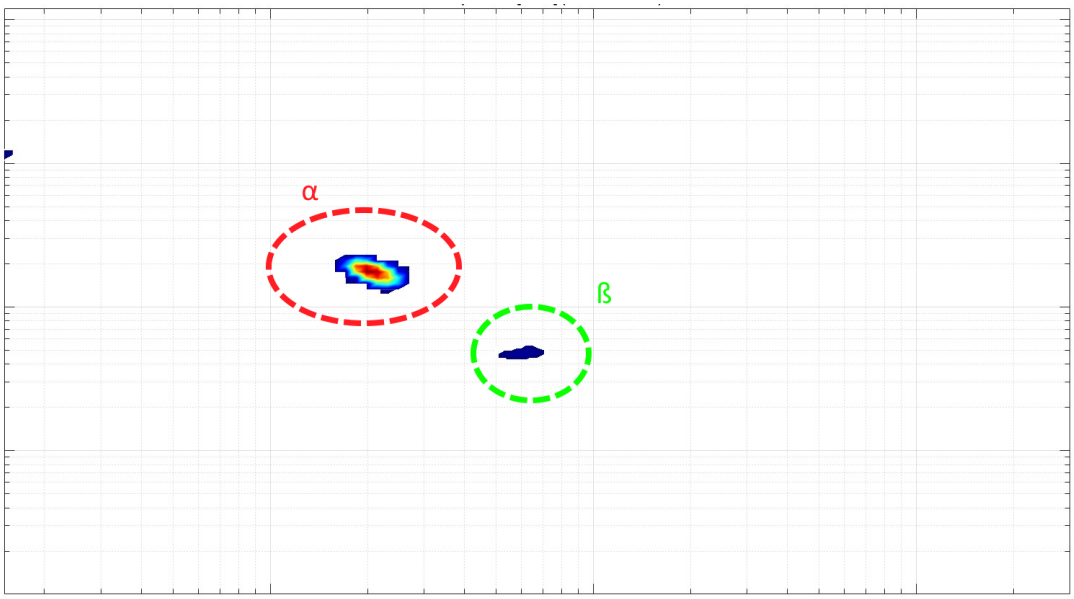

Figure 24 A representative T1T2 map for glycation.

However, this study has a few limitations, as it is done on accelerated conditions that may not resemble the bad storage conditions that may apply practically. Furthermore, it was applied to premade model samples of glucose and BSA instead of examining the theory on pharmaceuticals from the market. Unlike LC-MS, this method cannot detect amino acids that undergo glycation (Mou et al., 2022).

Table 6 Correlation between measurements of OPA method and other measurements.

	T1	**T2**	**T1/T2**	**Brown**	**T2/T1**	**1/Brown**
r	0.95	0.91	-0.93	-0.88	0.98	0.96
R2	0.91	0.83	0.87	0.78	0.96	0.92
p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

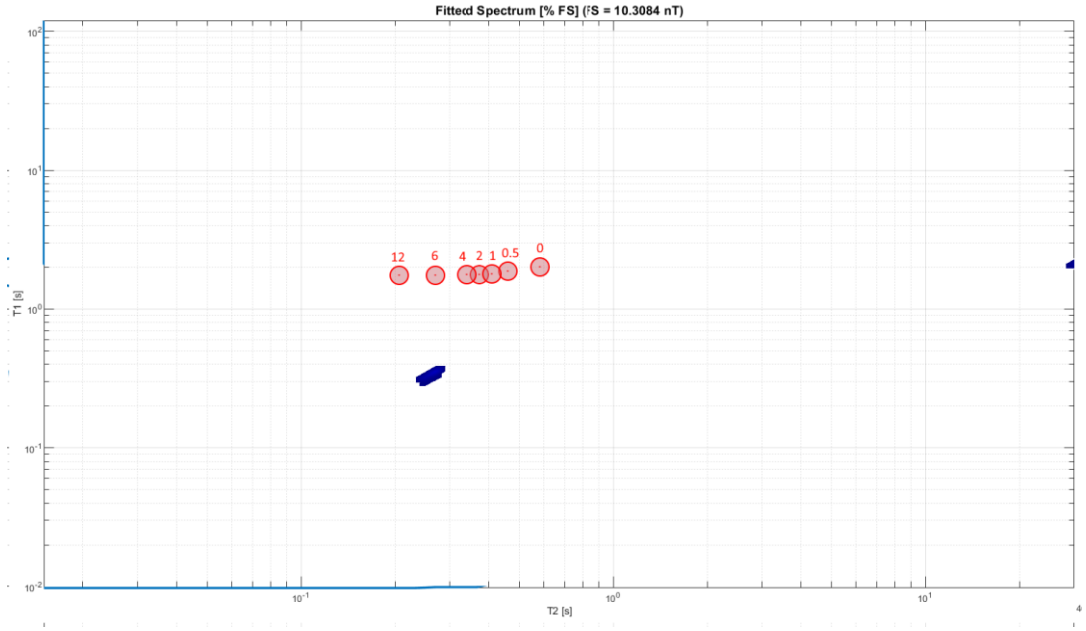

Figure 25 A representative that shows the center point of each α proton pool of each time point.

CHAPTER 4

CONCLUSIONS

- In this study, three novel pharmaceutical quality control methods were developed.
- These methods are non-invasive, non-destructive, affordable, user-friendly, and environmentally friendly.
- The first quality control method aimed to quantify the correct mixture ratio of mixed insulin drugs.
- Measurements were performed using T2 relaxation times and proton pools of the T1T2 maps.
- By integrating two TD-NMR techniques, T2 relaxation times and T1T2 maps, a new method was developed that can successfully predict the mixture ratios.
- This method proposes that merging two TD-NMR spectra can be used to compensate for each of their shortcomings.
- Studies should be conducted to examine the potential of this integration to measure other solvent combinations in the pharmaceutical and other industries.
- Aspirin tablets of varied sizes and coatings were purchased.
- They were measured using NIRS and chemometrics by applying chemometrics and then compared to HPLC to produce a calibration method.
- The calibration method successfully predicted the validation set.
- Aspirin tablets were measured with T1 relaxation times, and the crystallinity measured by XRD, and moisture content measured by Karl-Fischer titration were compared.

- Bovine serum albumin (BSA) was used as a model protein for biopharmaceuticals.
- Glycation of BSA was measured using different techniques of TD-NMR and compared with the OPA method and Browning measurement.
- Measurements of the T2/T1 value of a specific proton pool inside T1T2 maps can be used as an alternative measurement for glycation degree. It was highly fitted and correlated with the OPA method, even more than the fitting between the OPA method and the browning measurement.
- It is recommended that more studies be conducted using biopharmaceuticals from the market and different glycation preparations.

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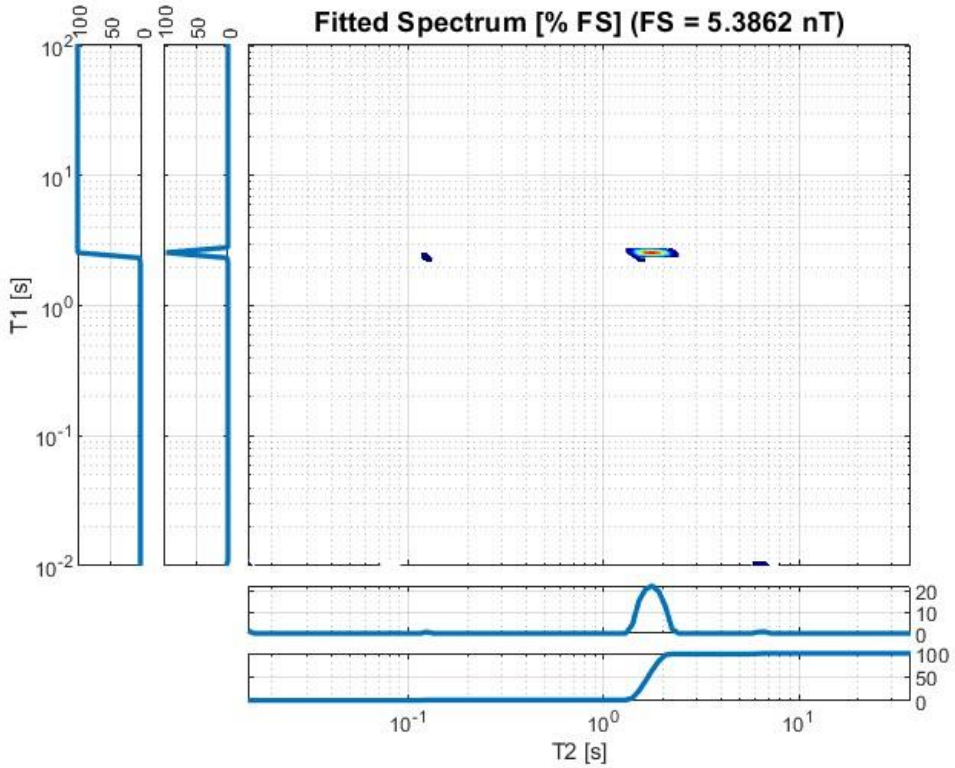
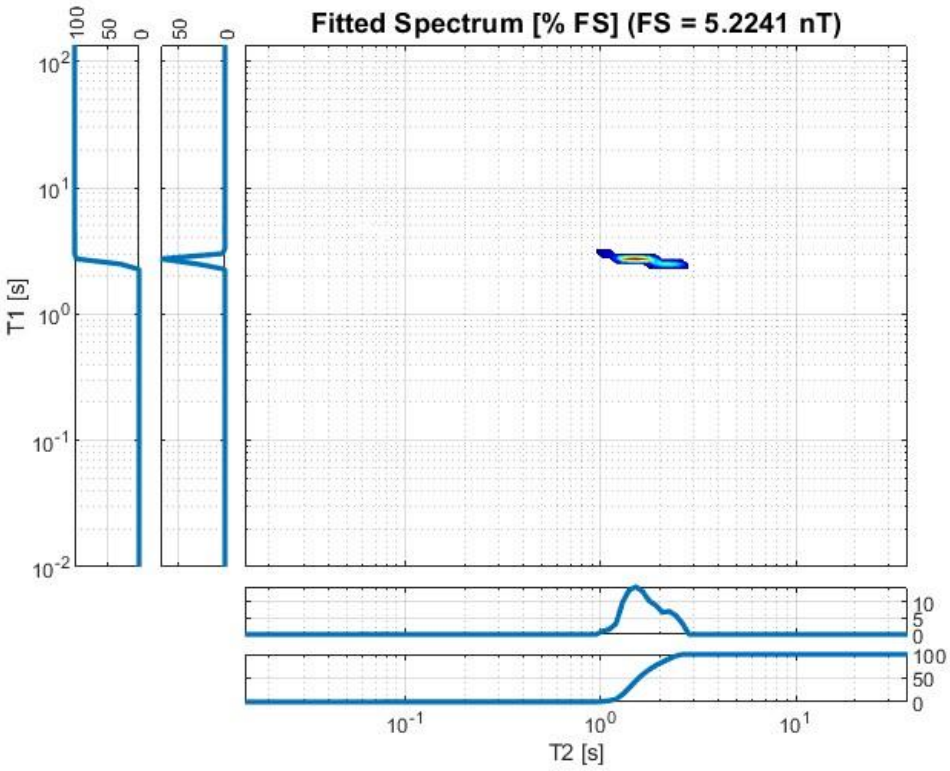
APPENDICES

A. T1T2 Maps of different insulin mixtures



T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 0:100



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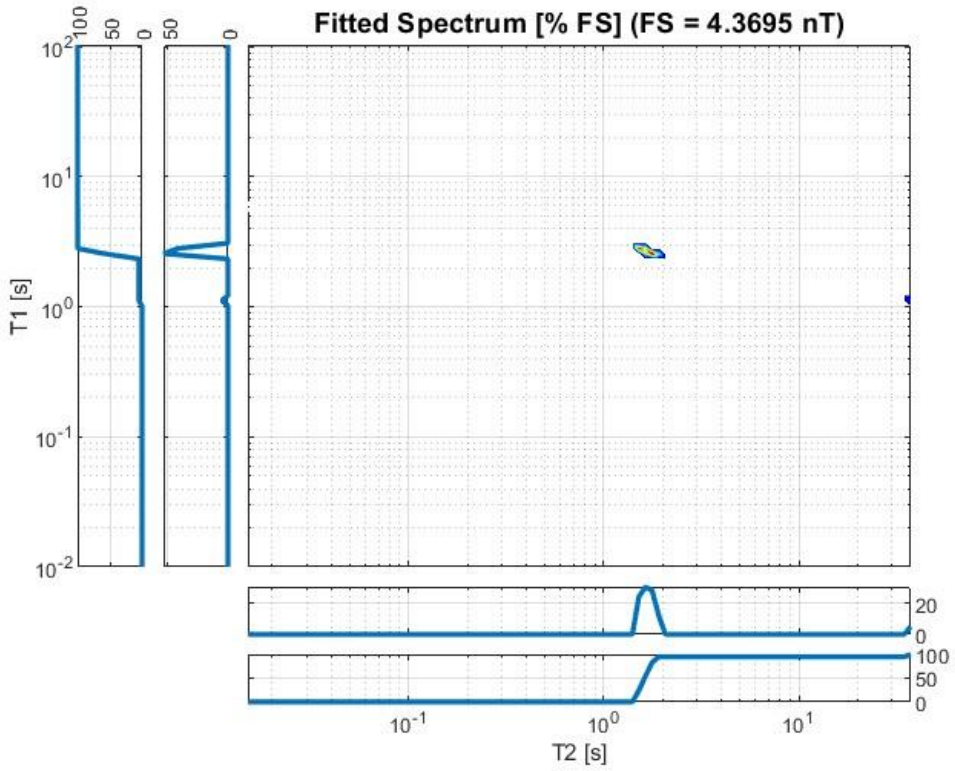

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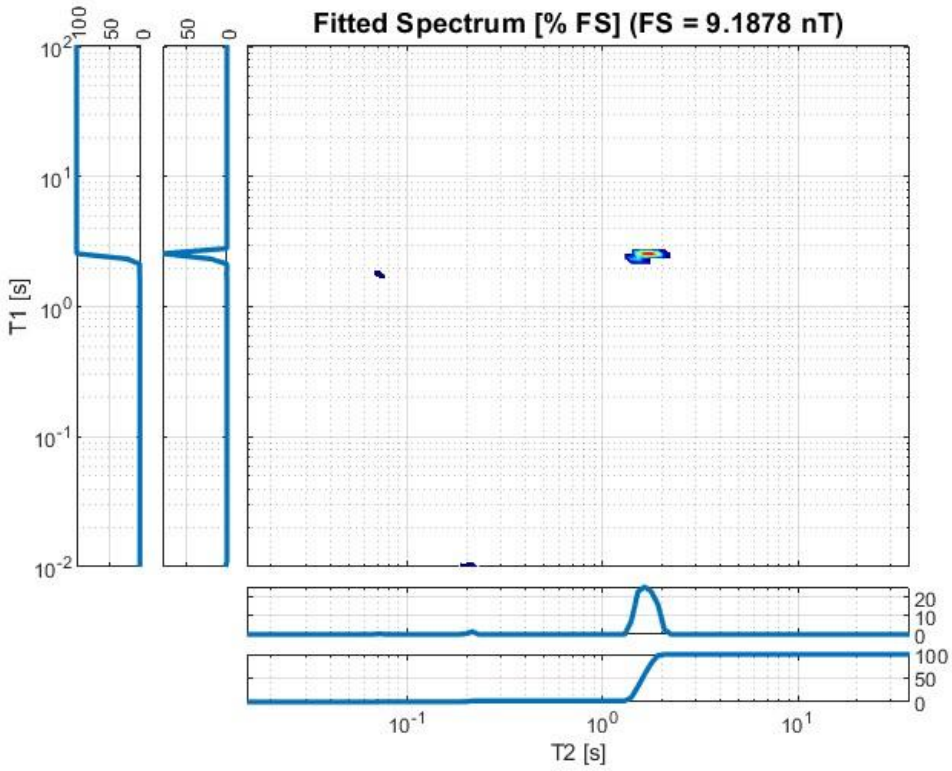

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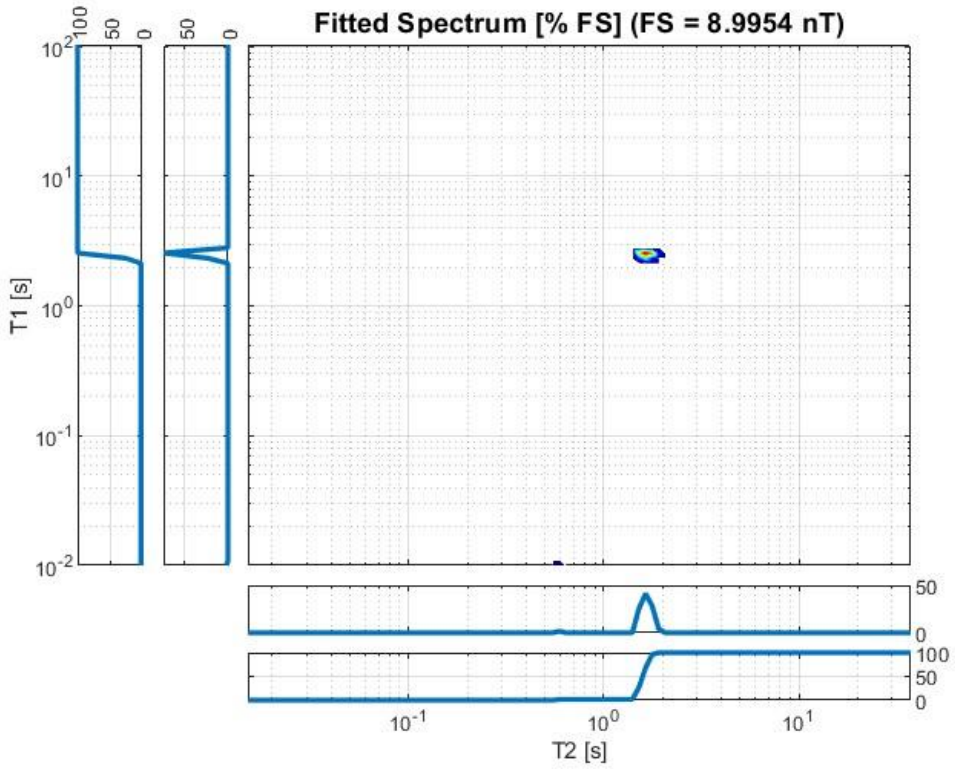

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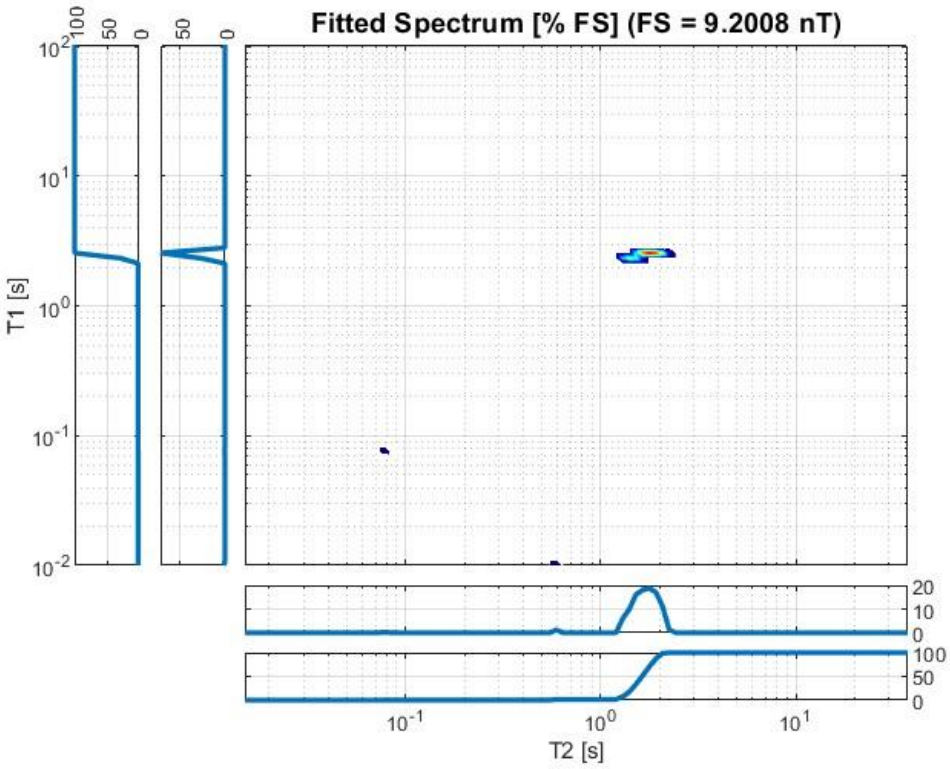

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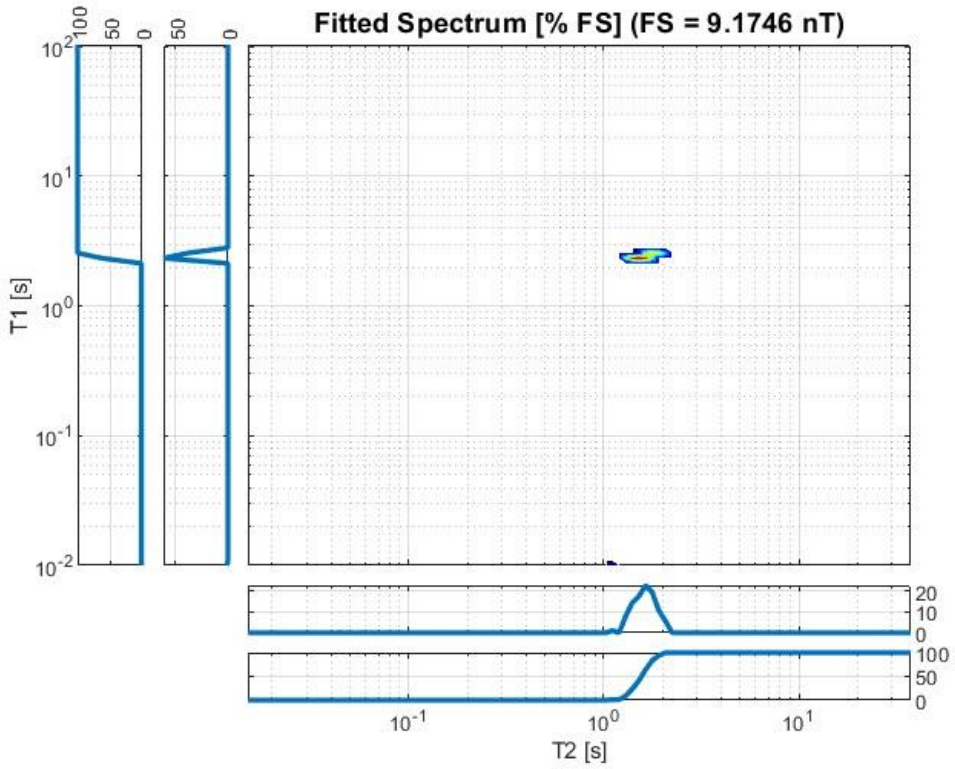

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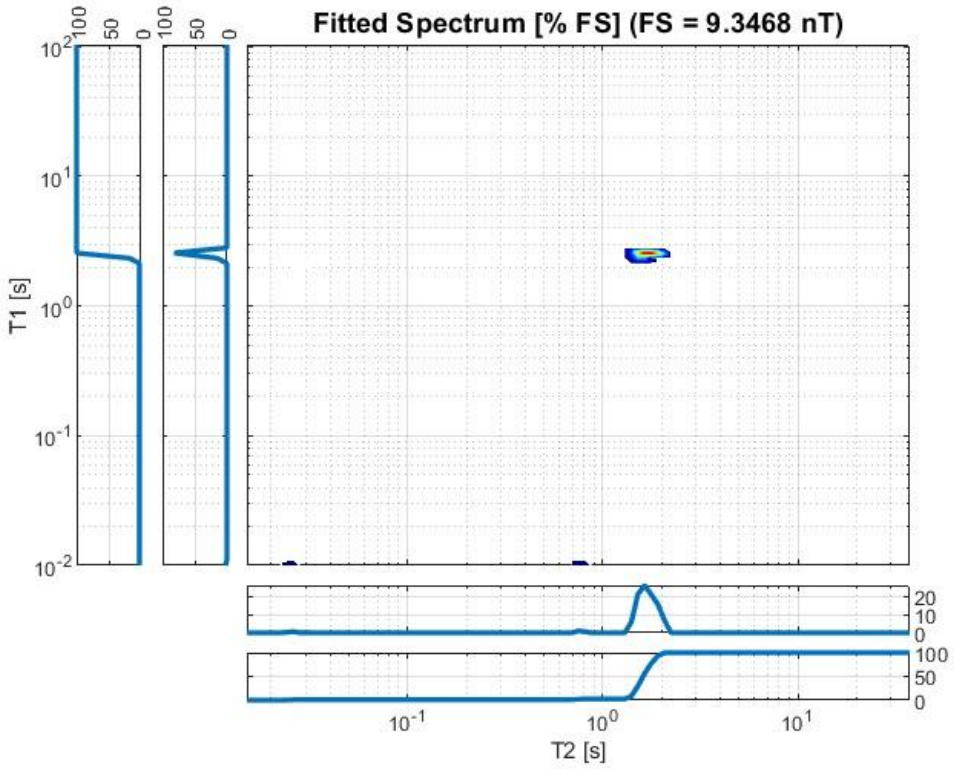

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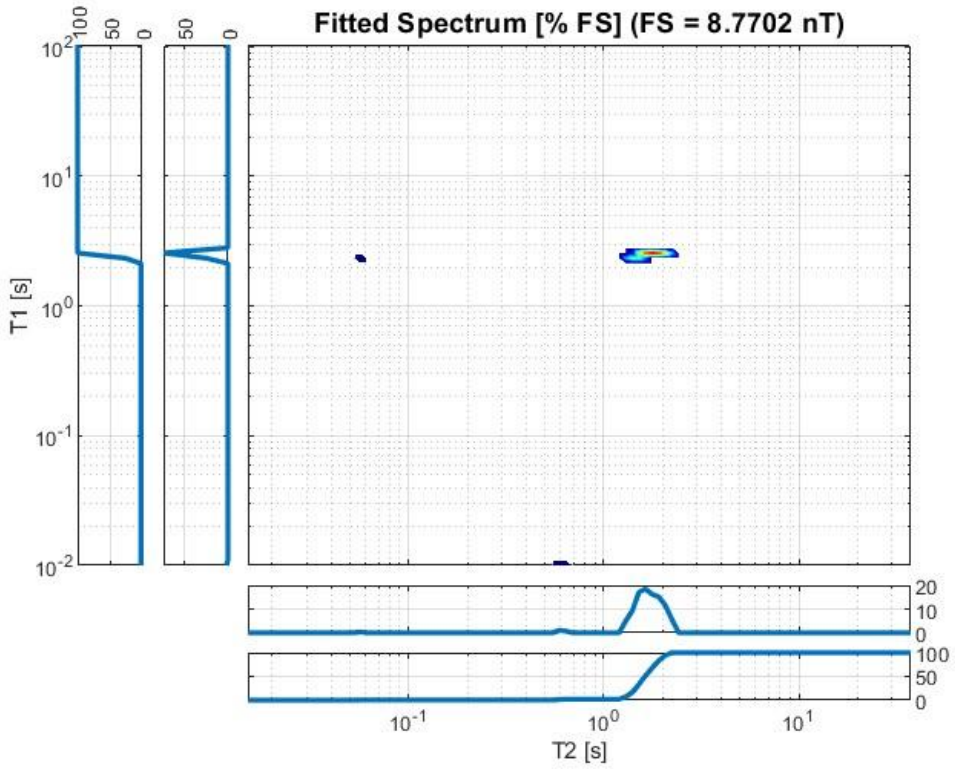

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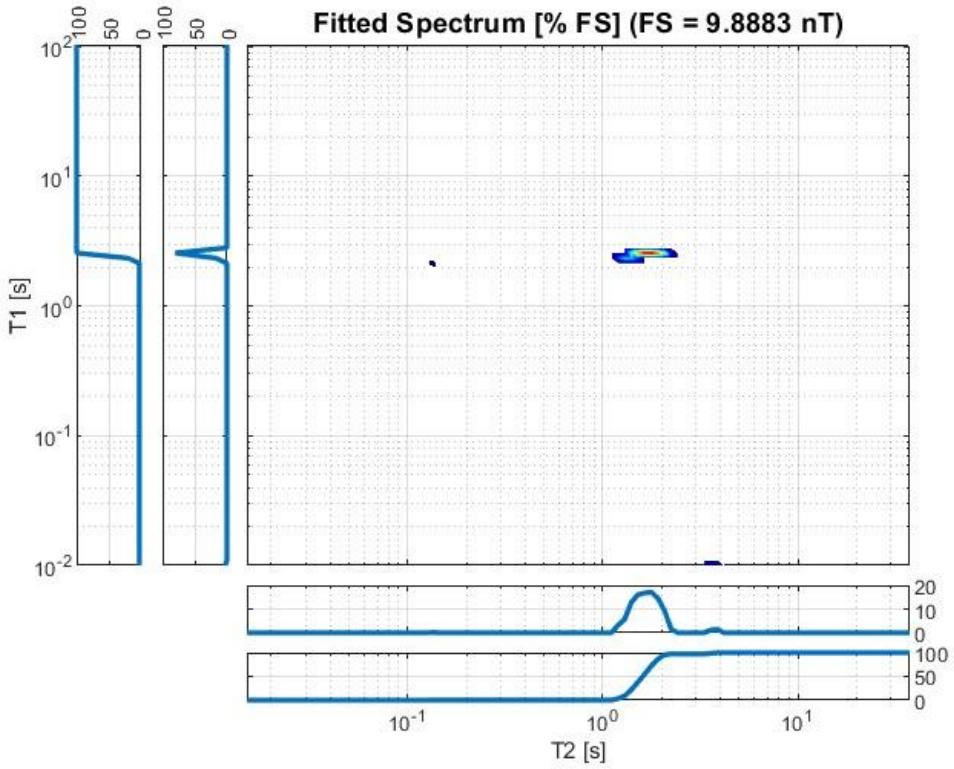

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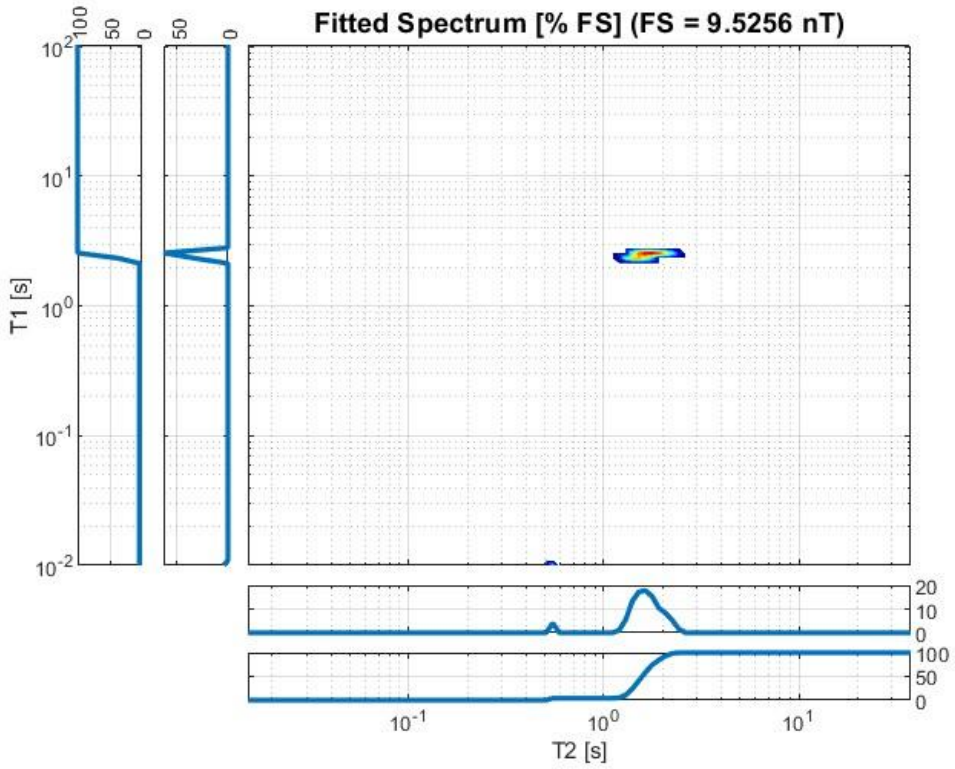

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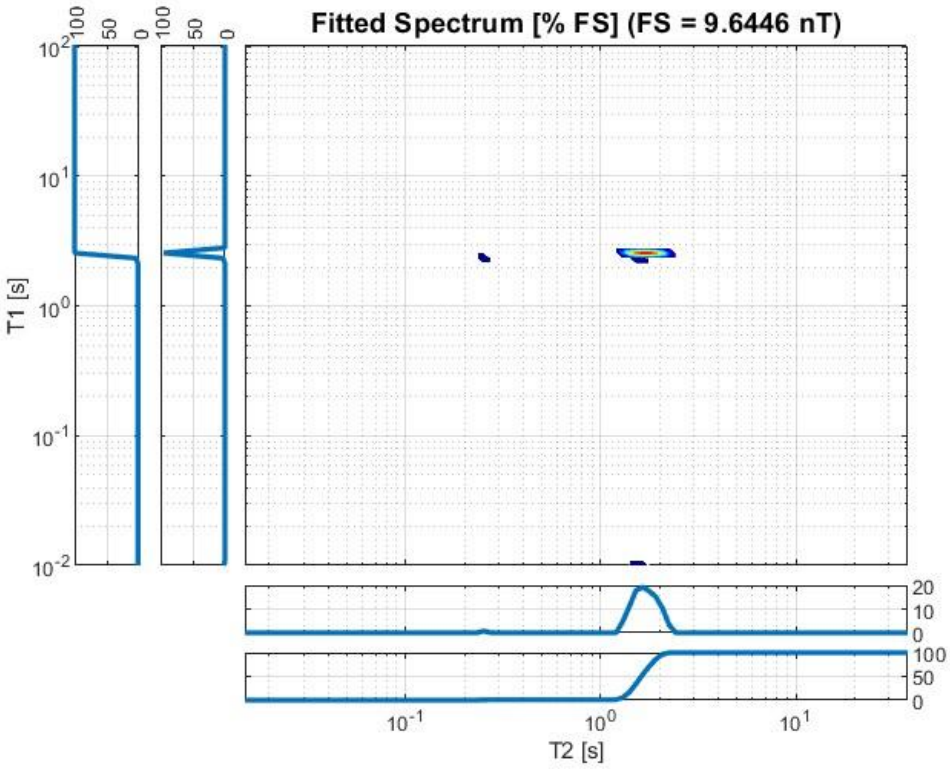
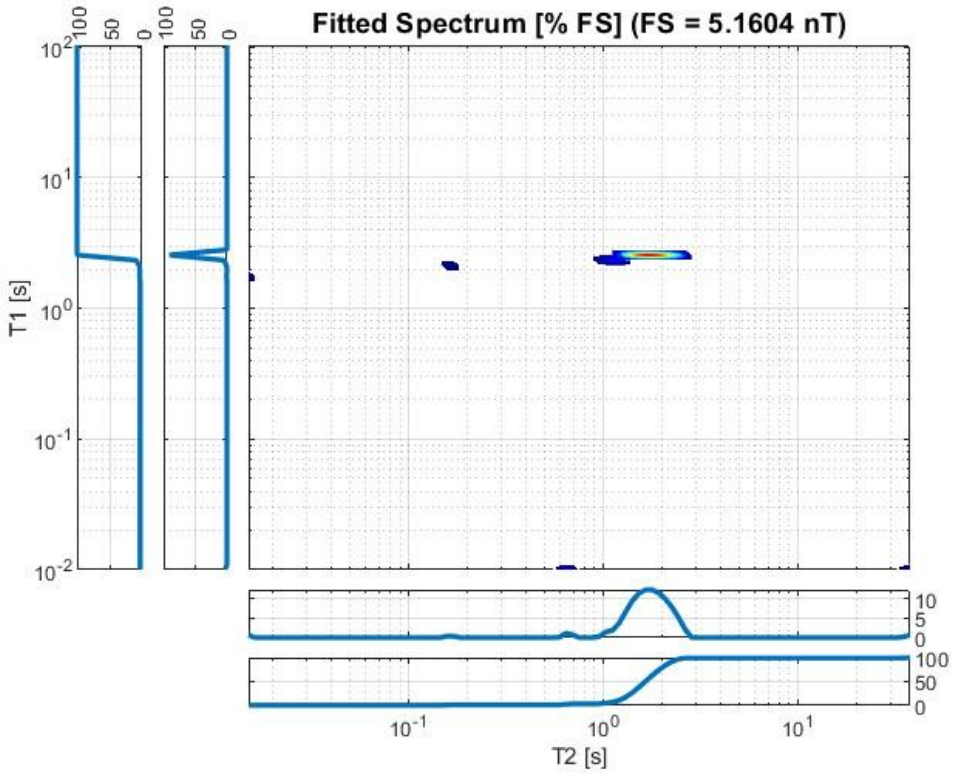
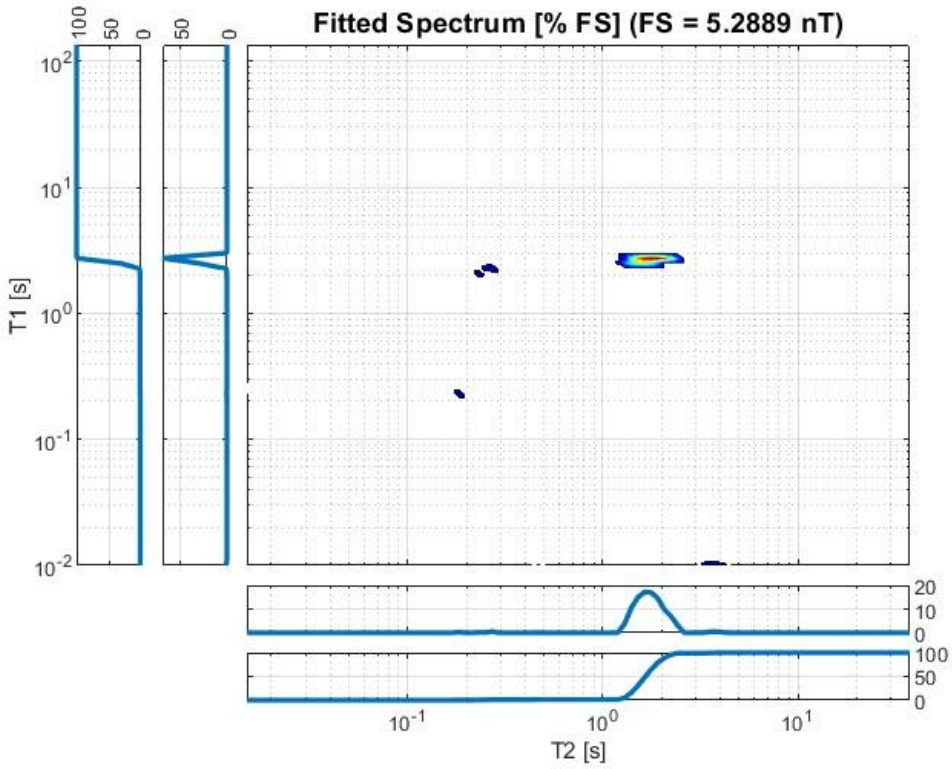


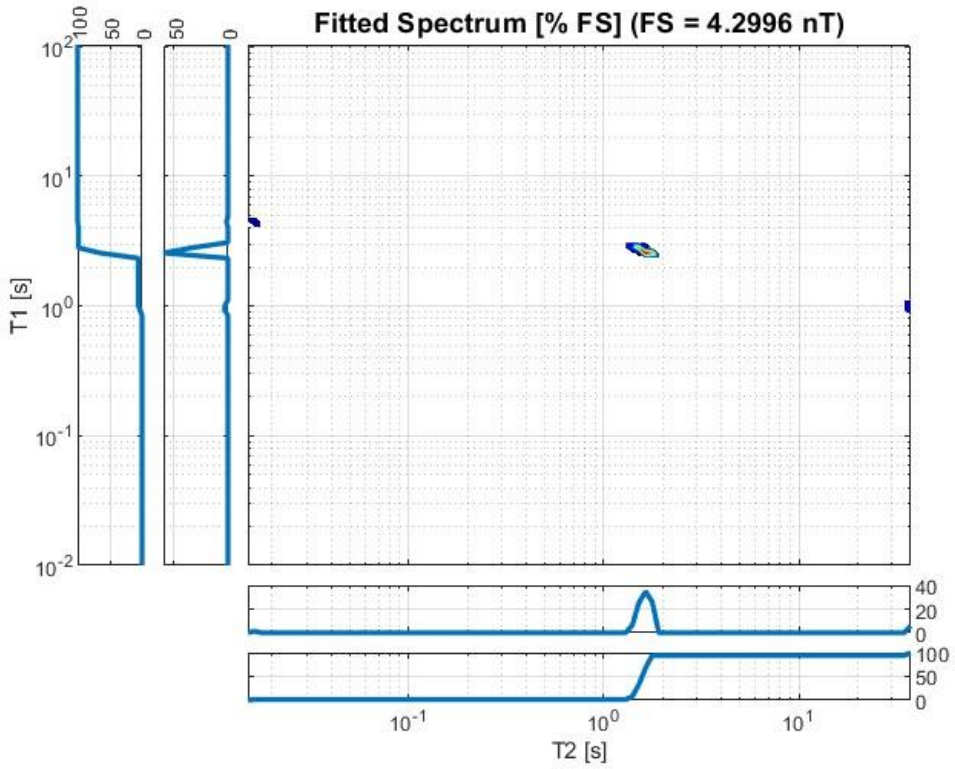
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 15:85



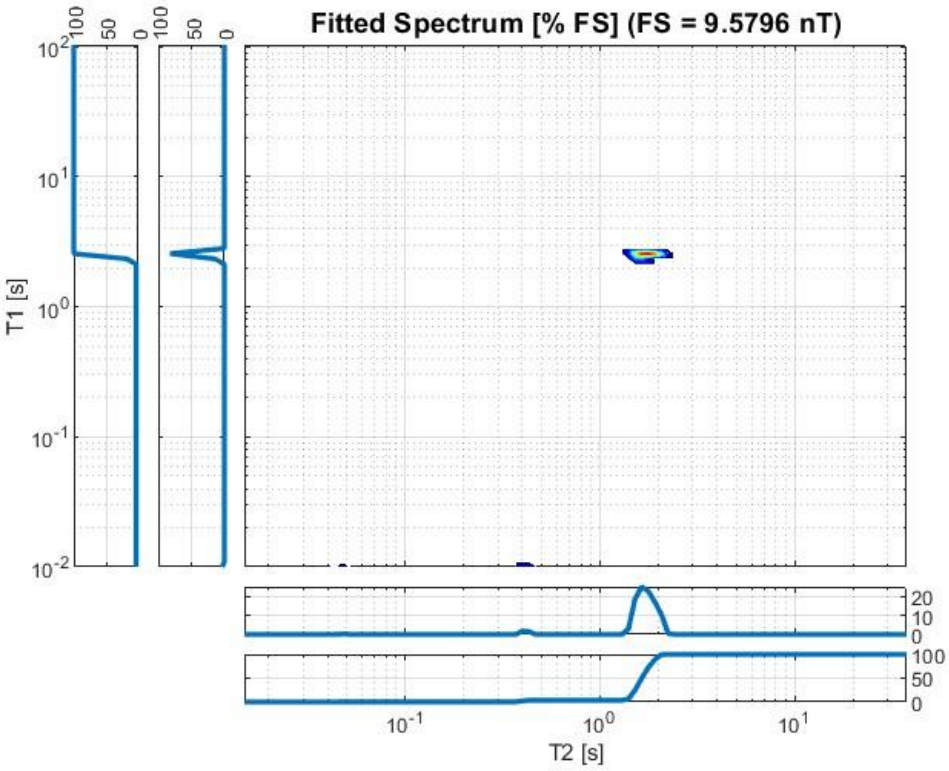
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 20:80



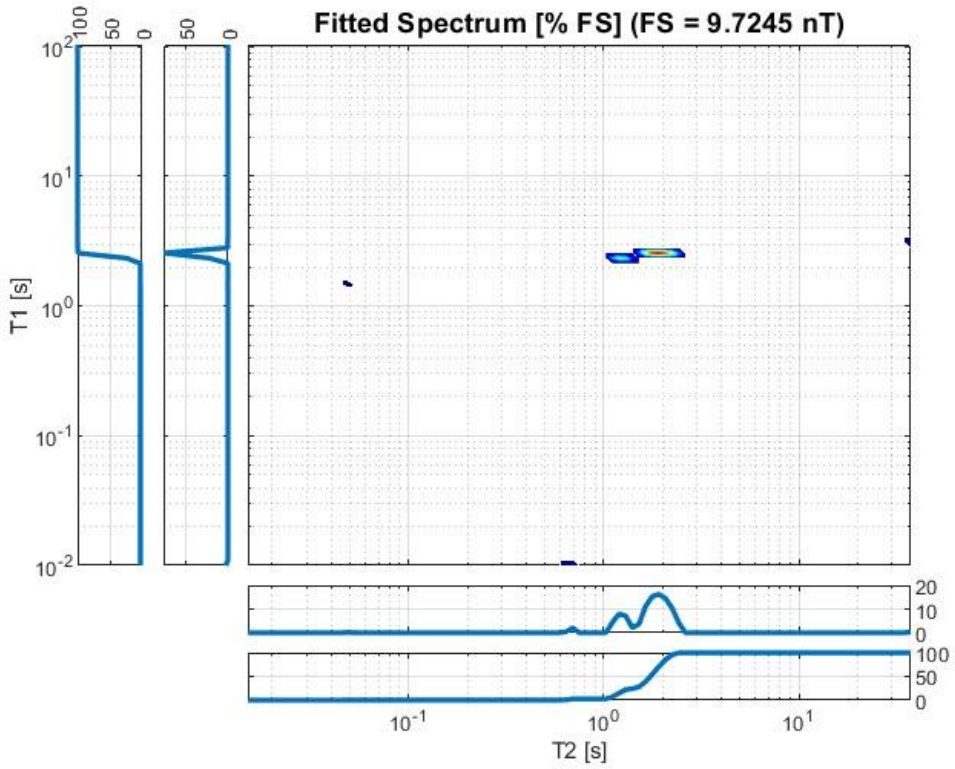
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 20:80



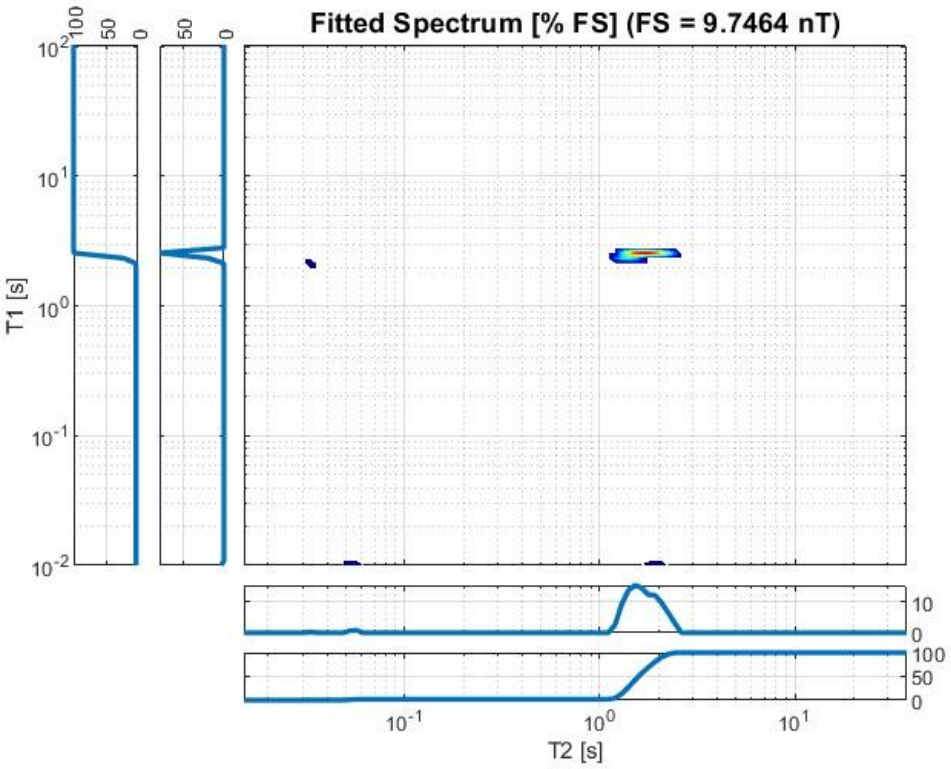
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 20:80



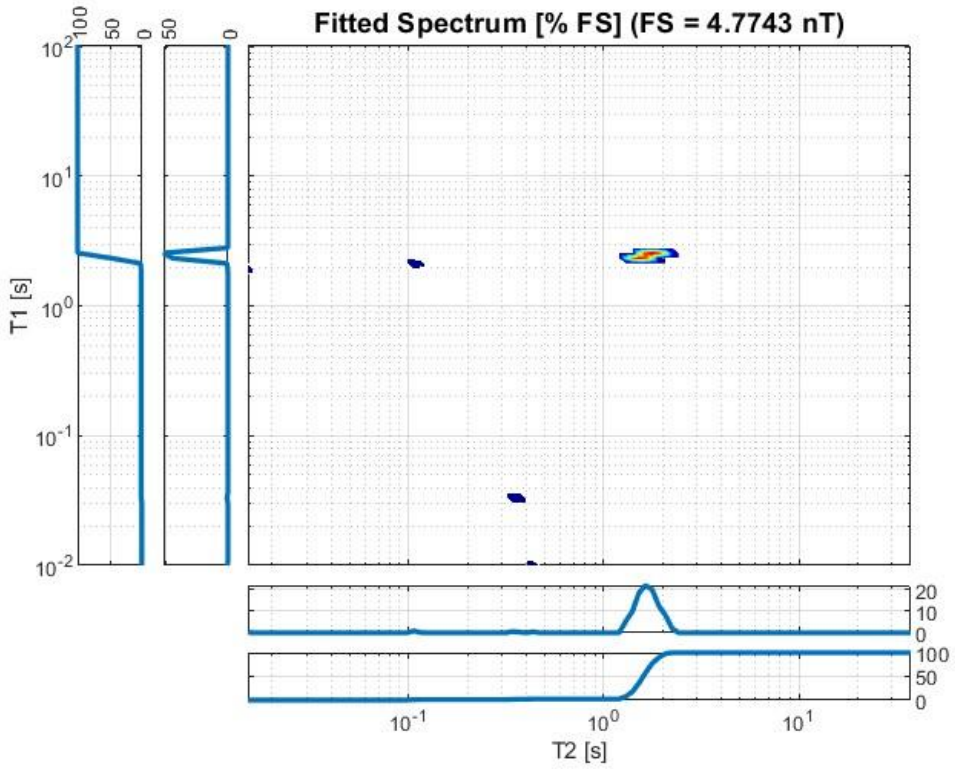
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 25:75



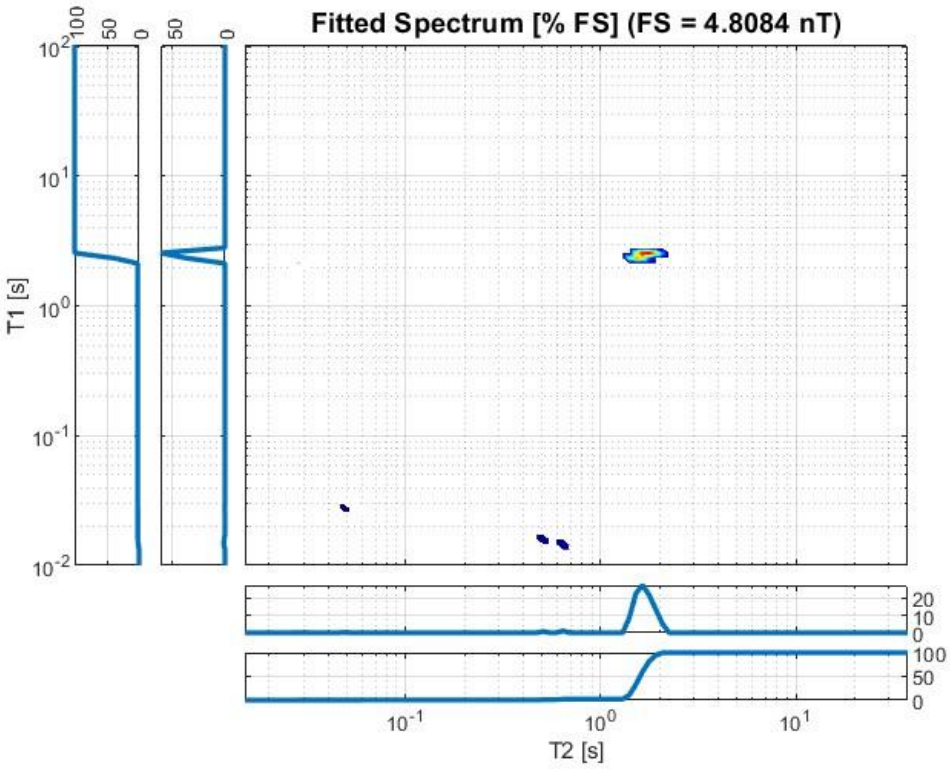
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 25:75



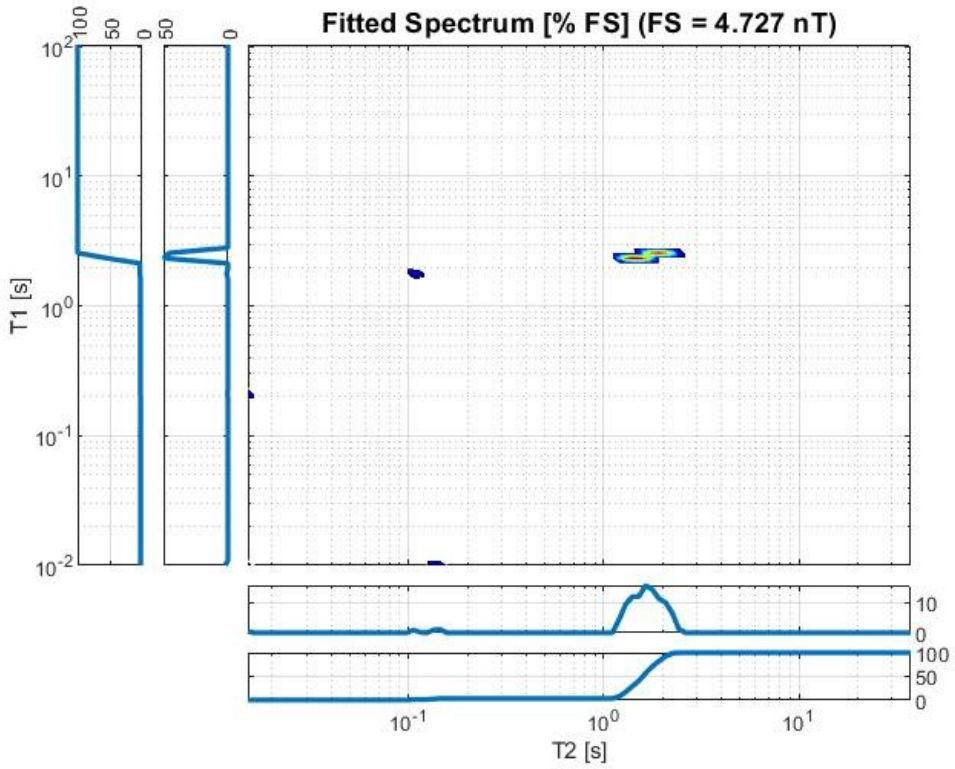
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 25:75



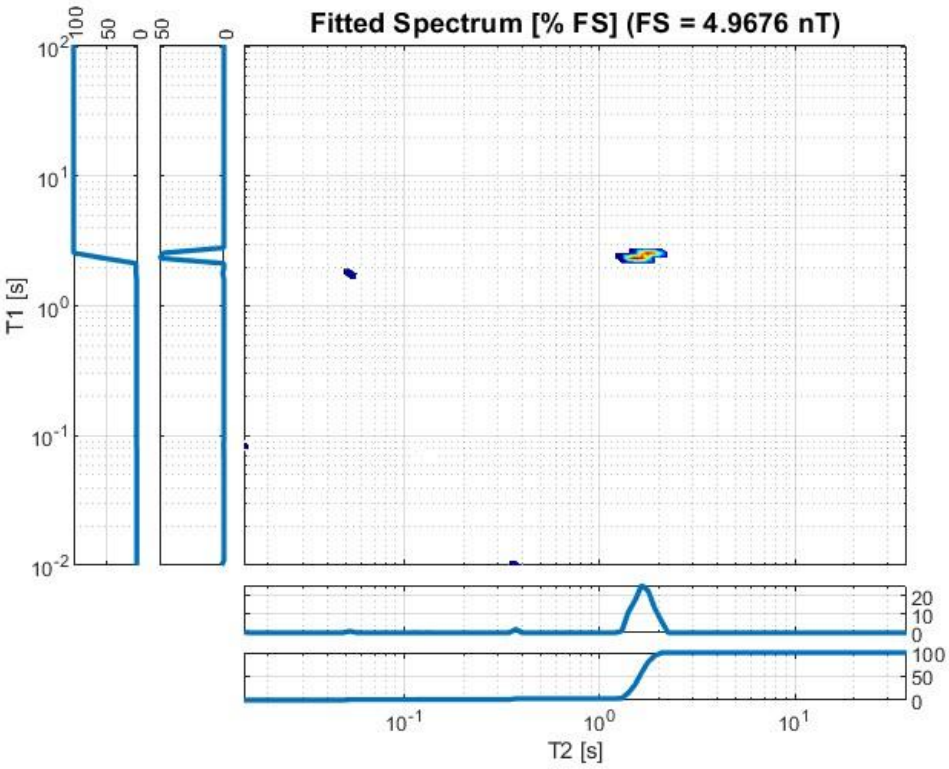
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 30:70



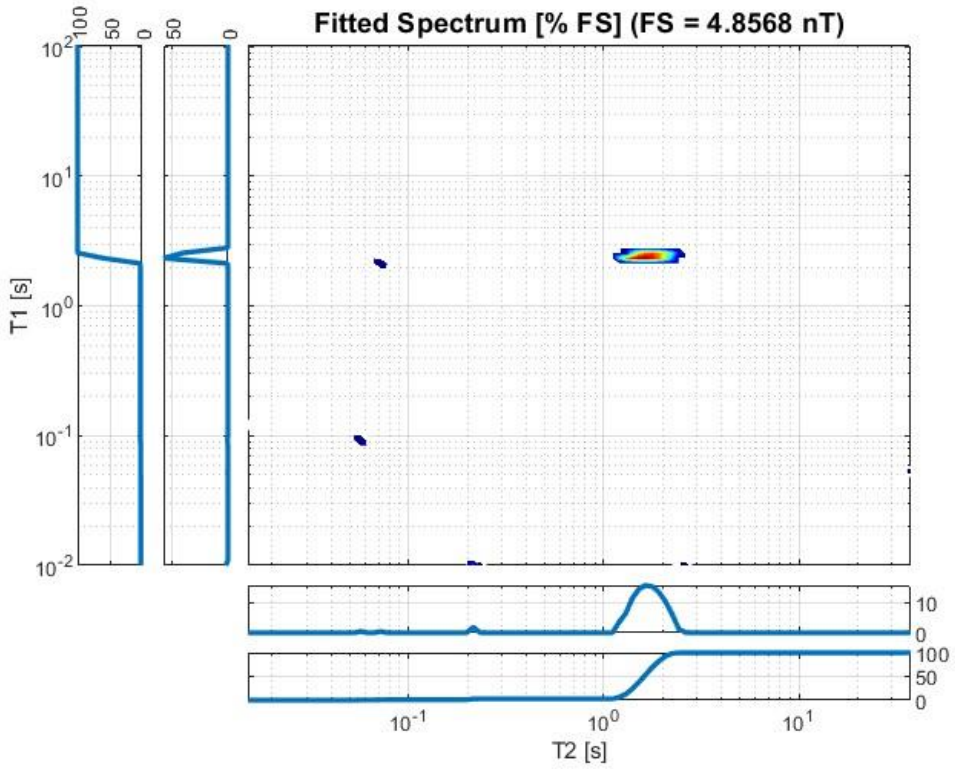
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 30:70



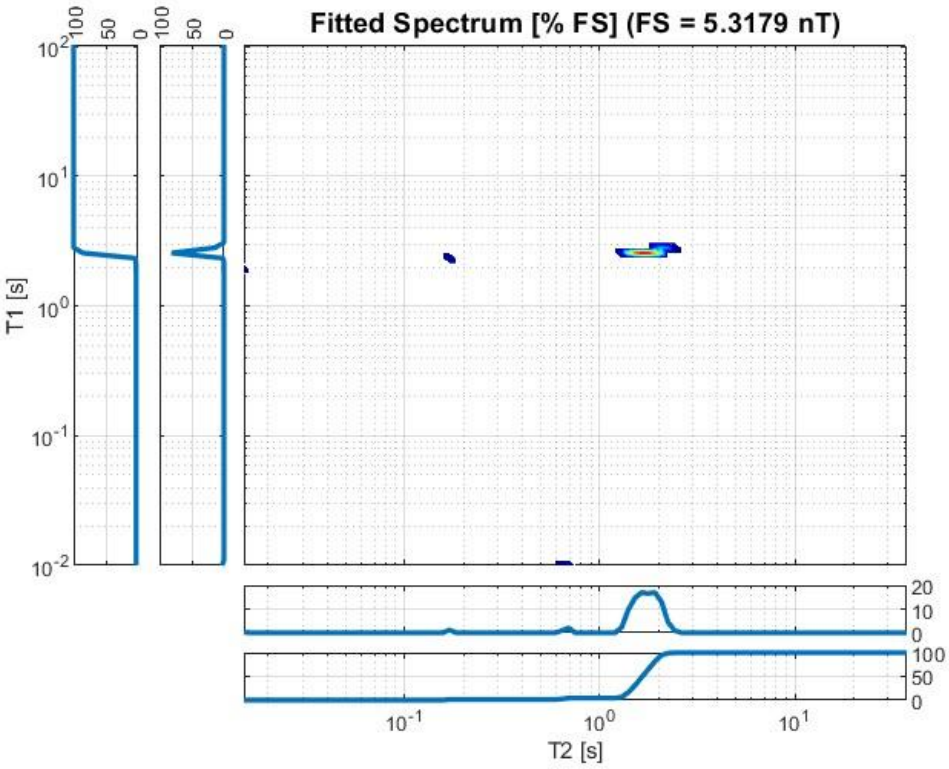
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 35:65



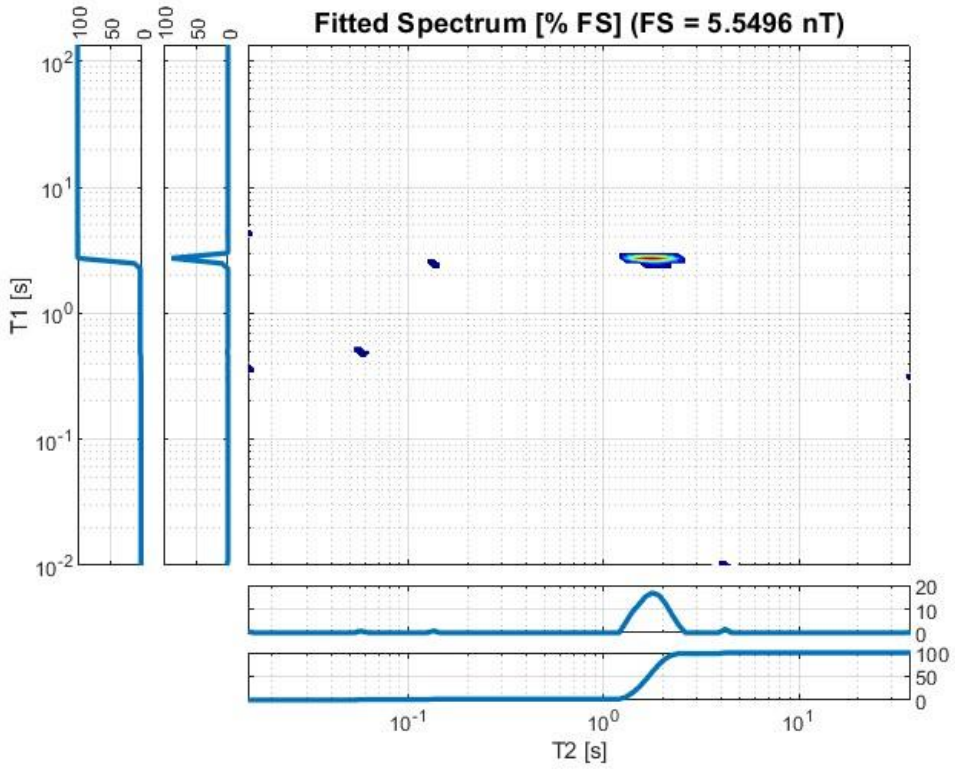
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 35:65



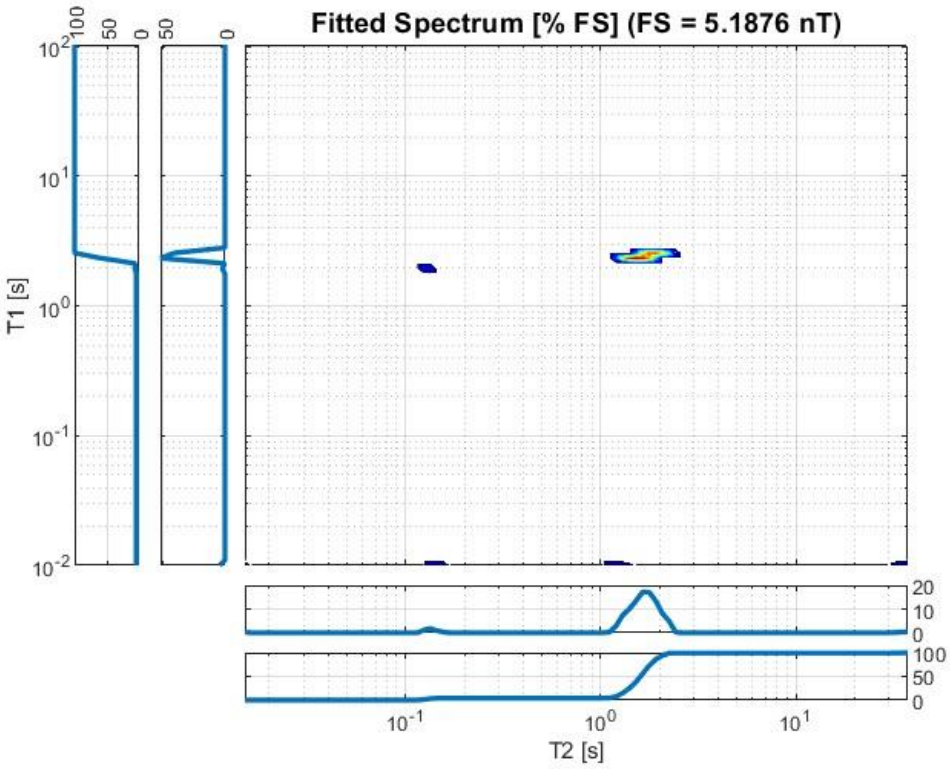
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 35:65



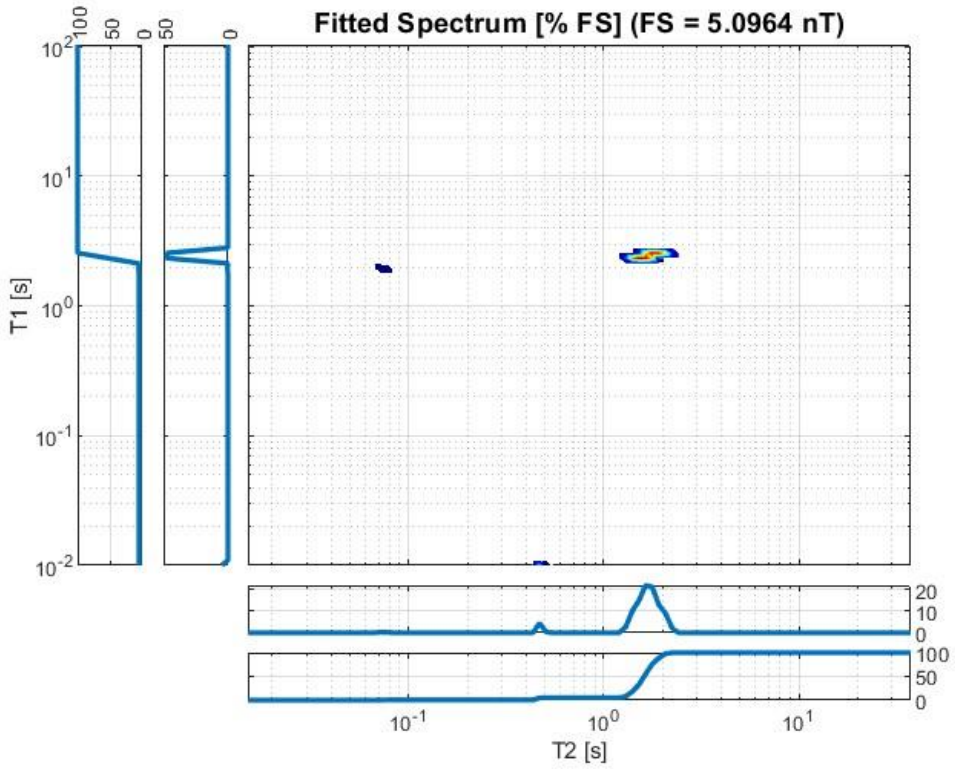
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 40:60



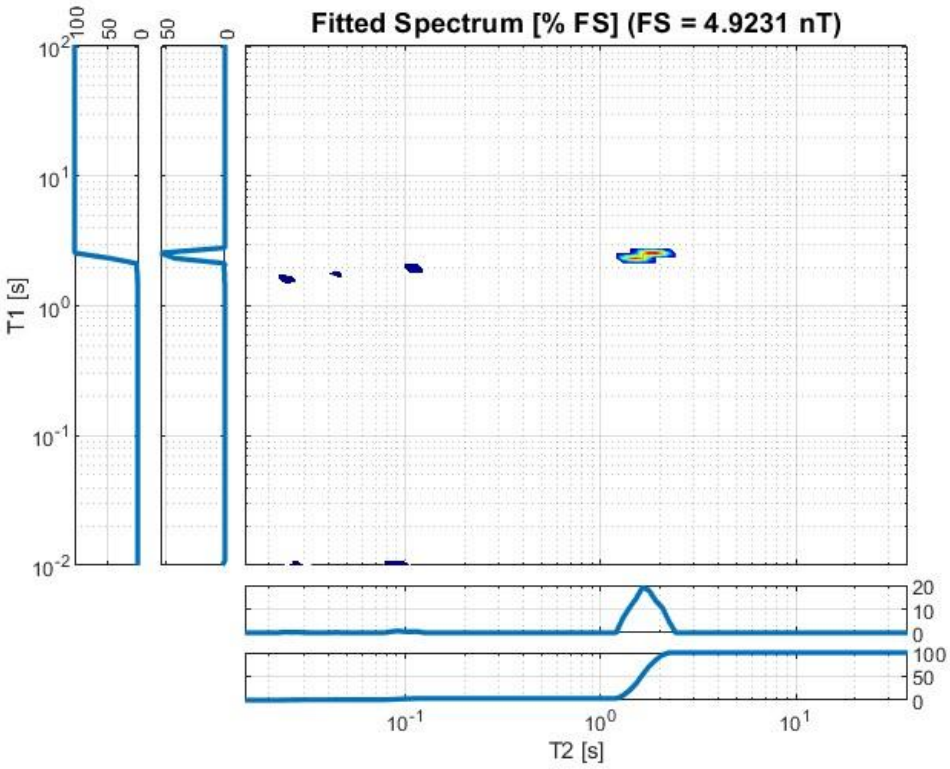
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 40:60



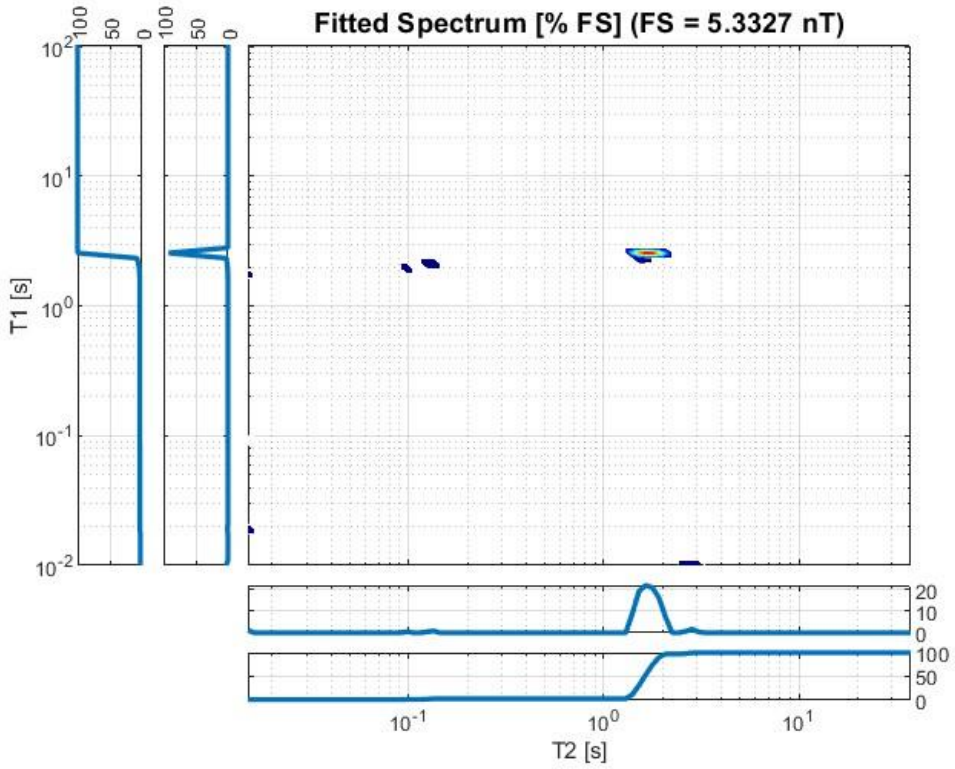
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 45:55



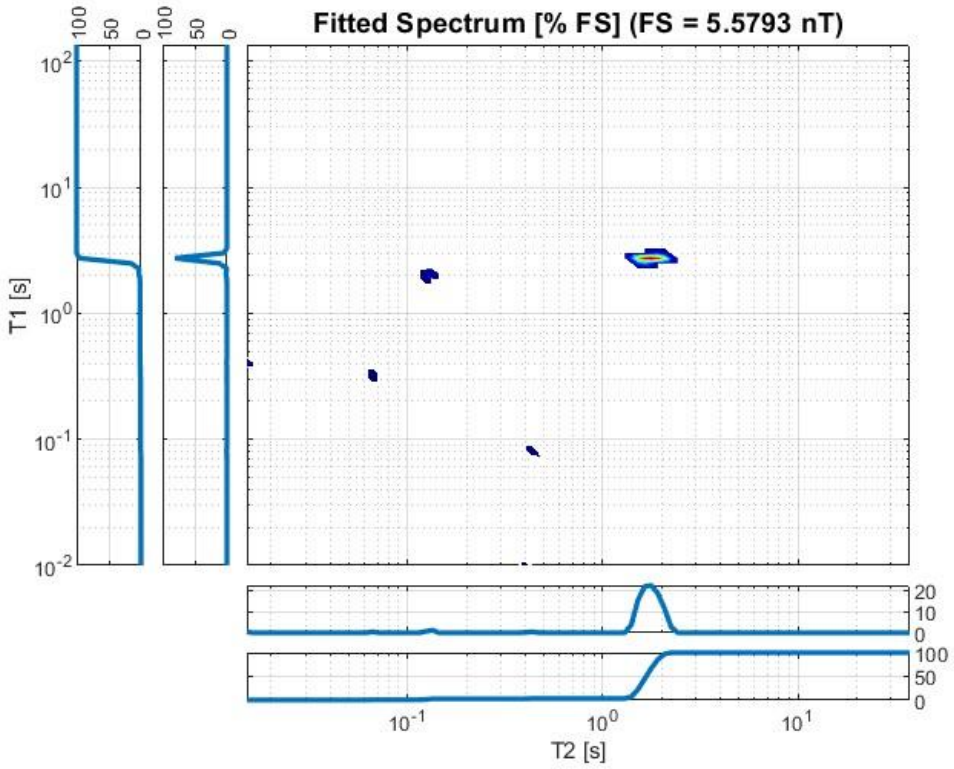
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 45:55



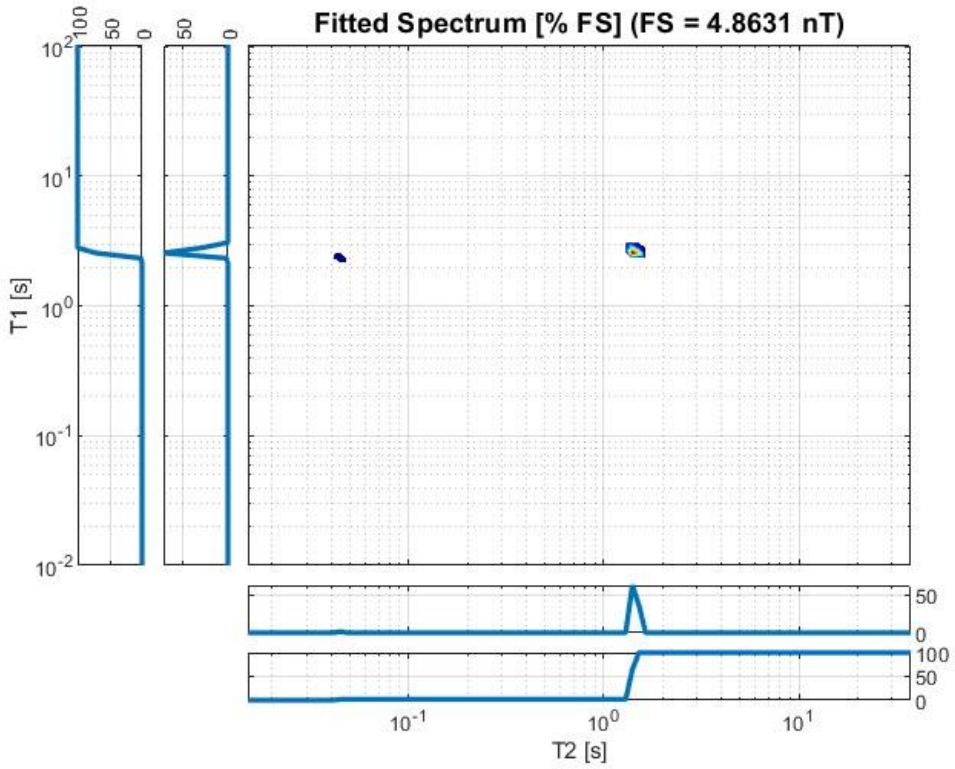
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 45:55



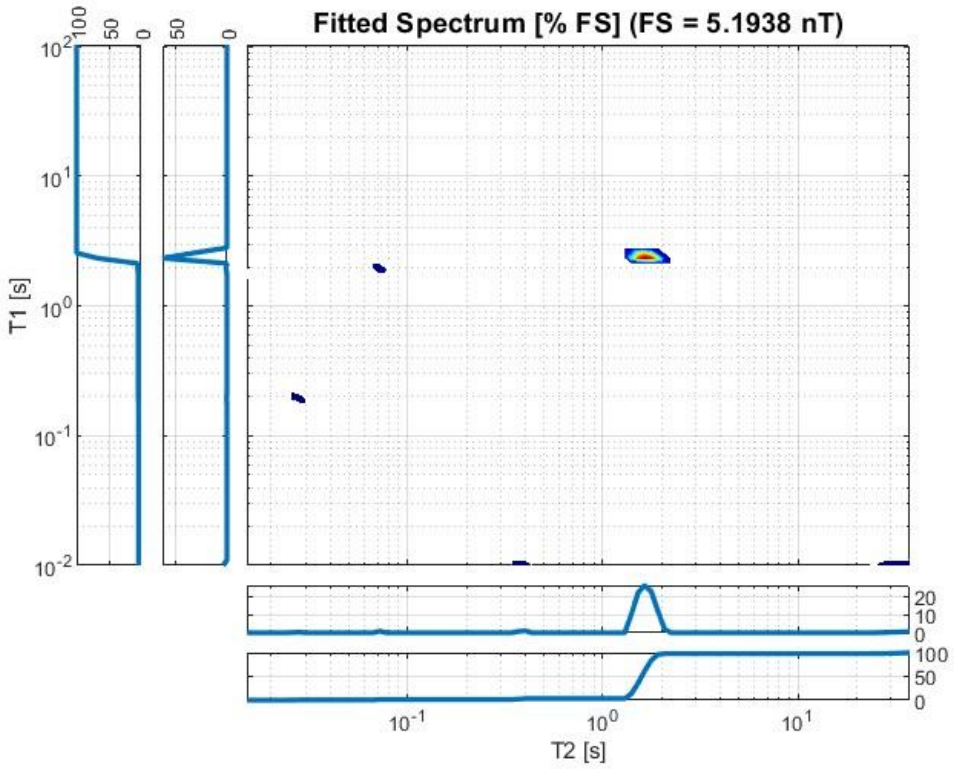
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 50:50



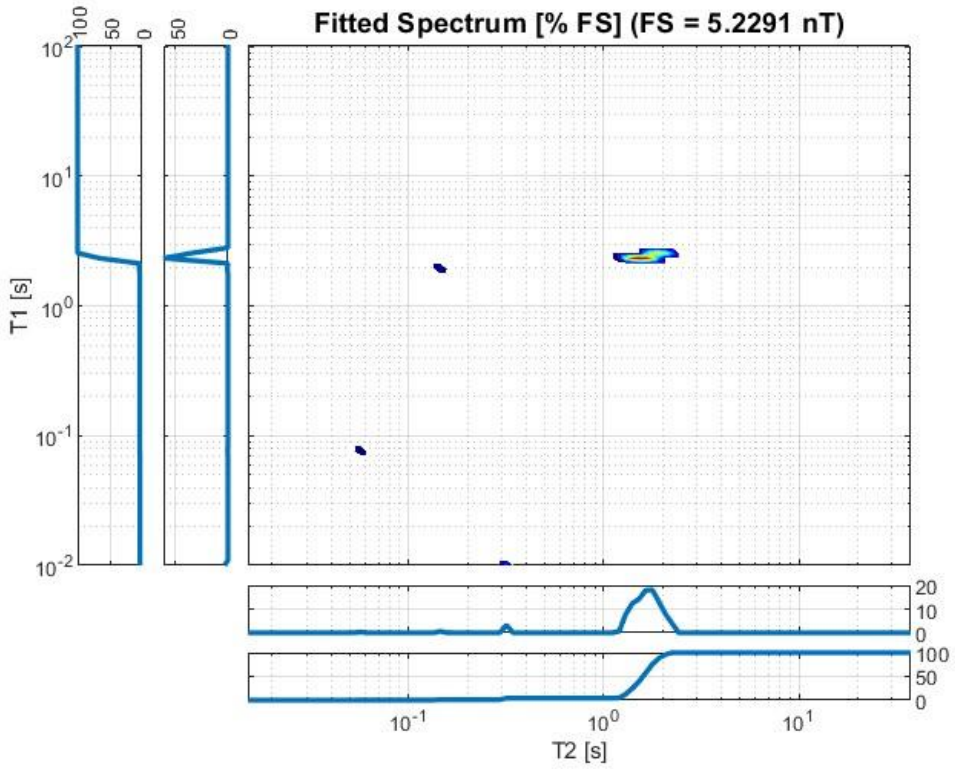
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 50:50



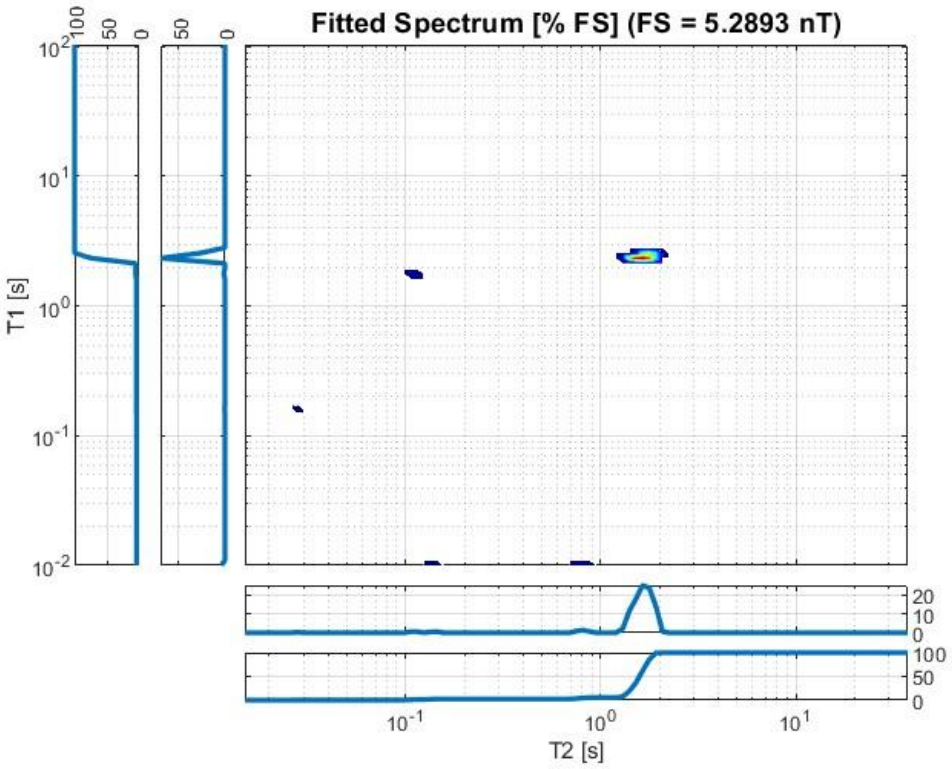
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 50:50



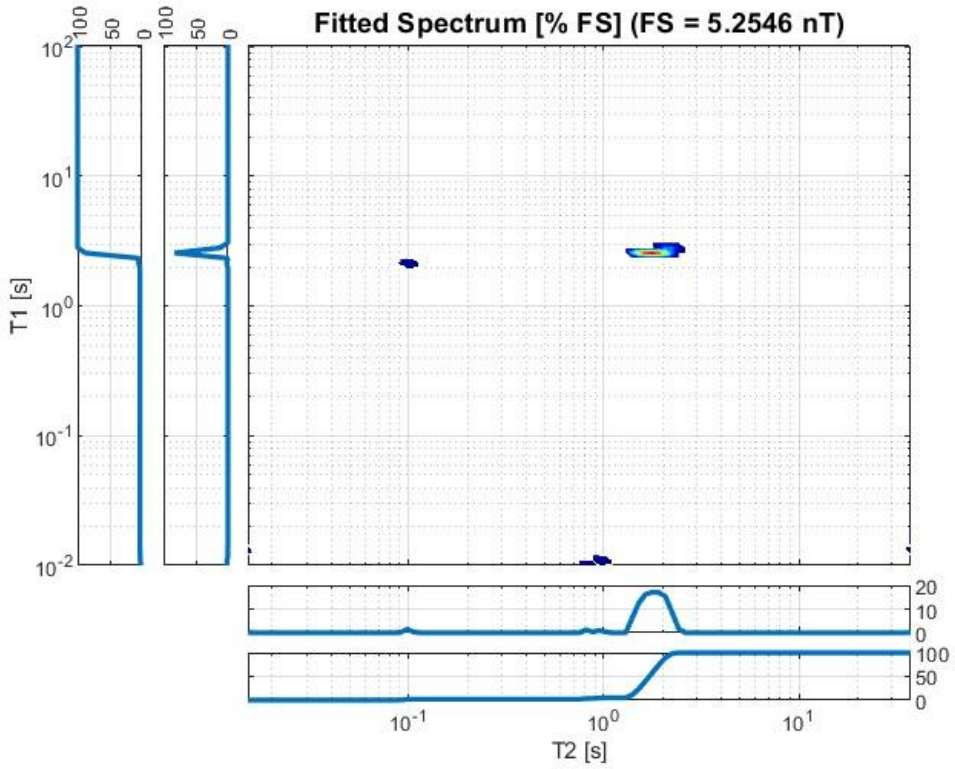
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 55:45



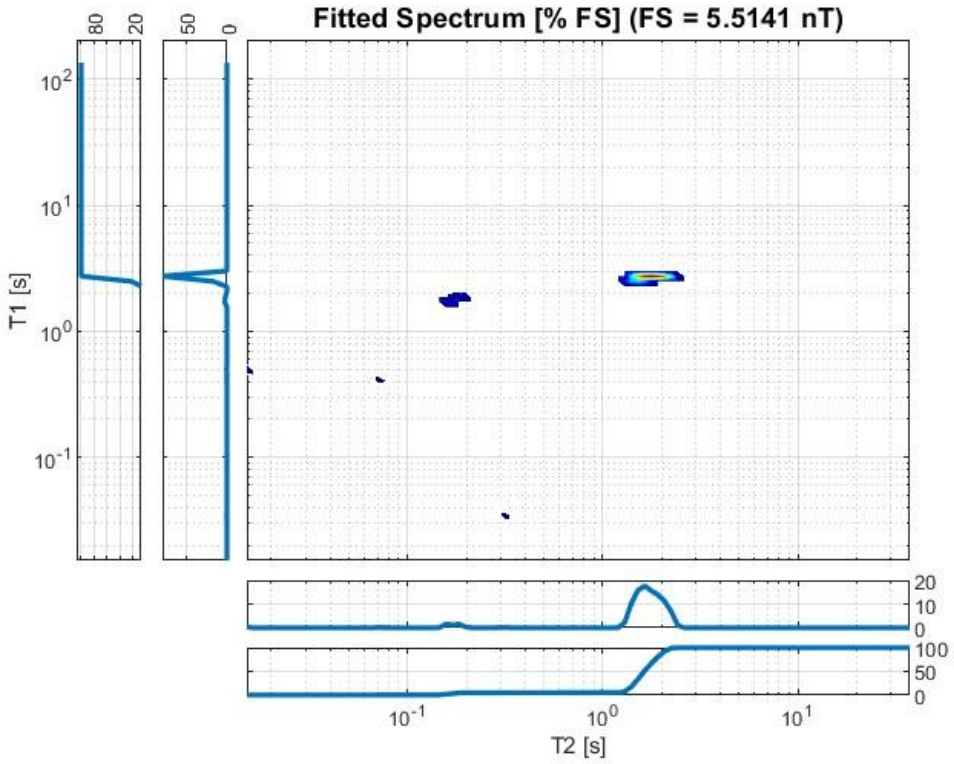
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 55:45



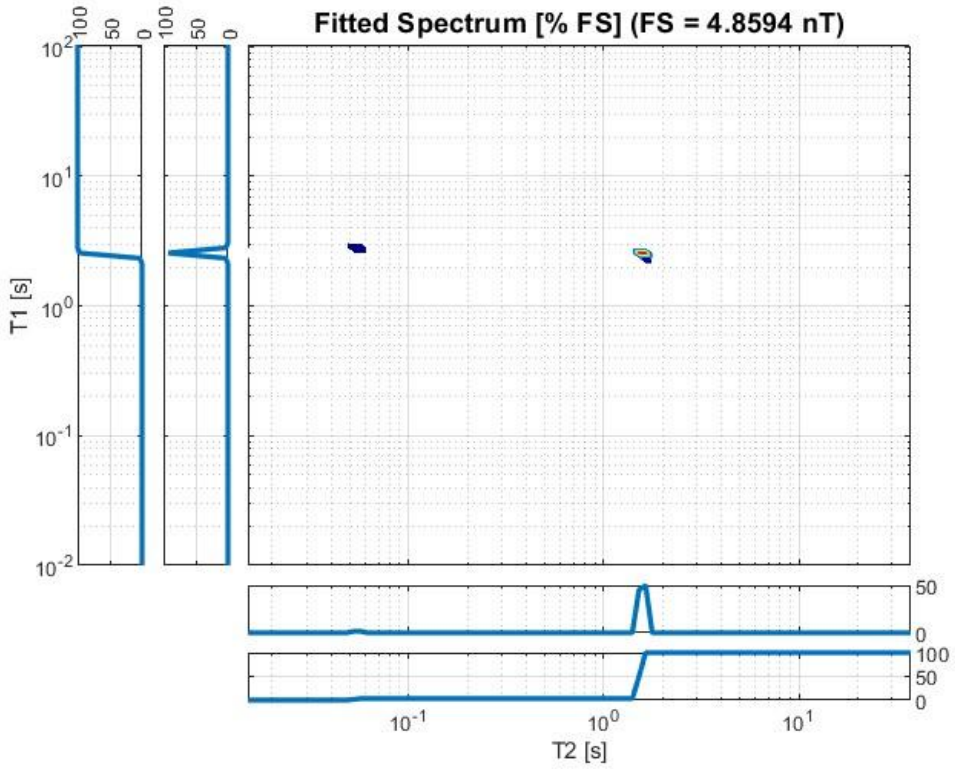
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 55:45



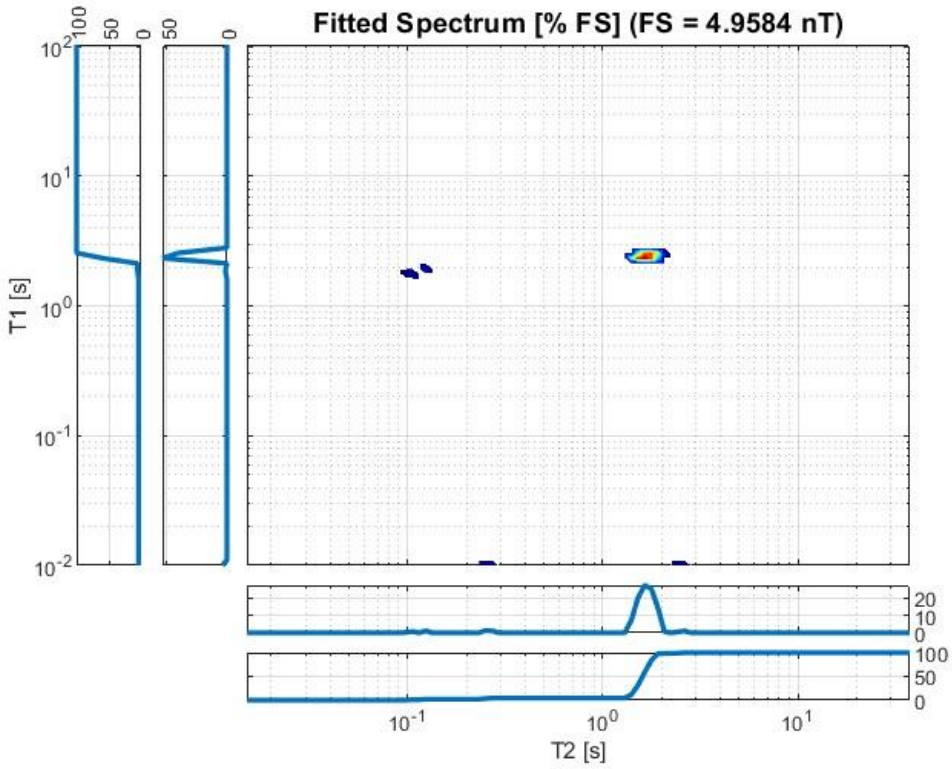
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 60:40



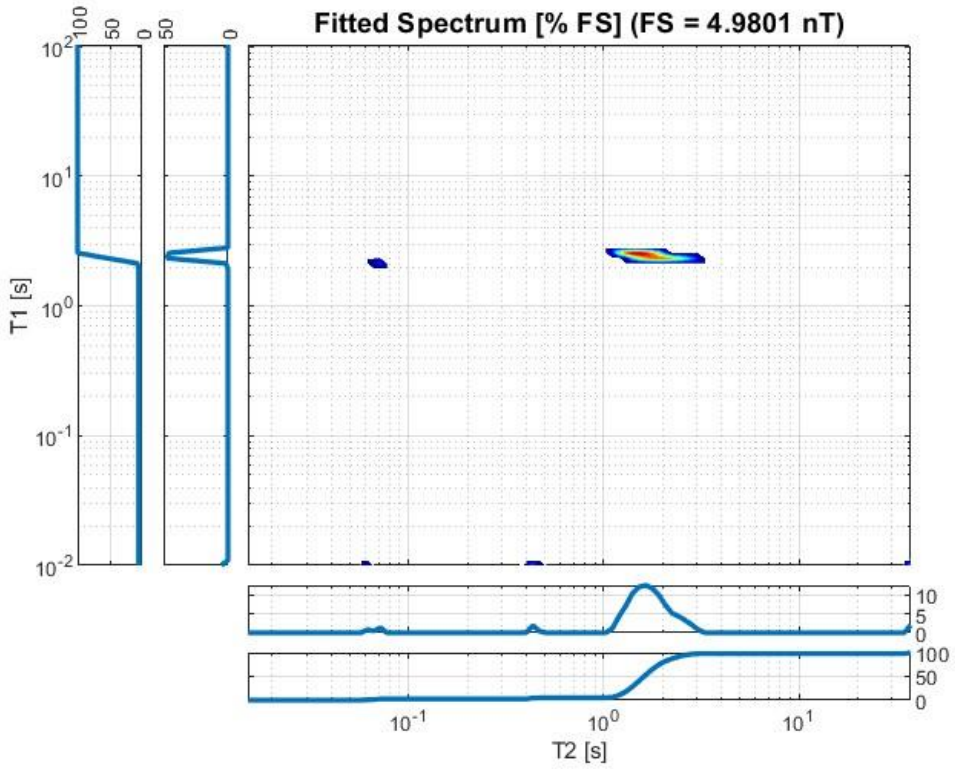
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 60:40



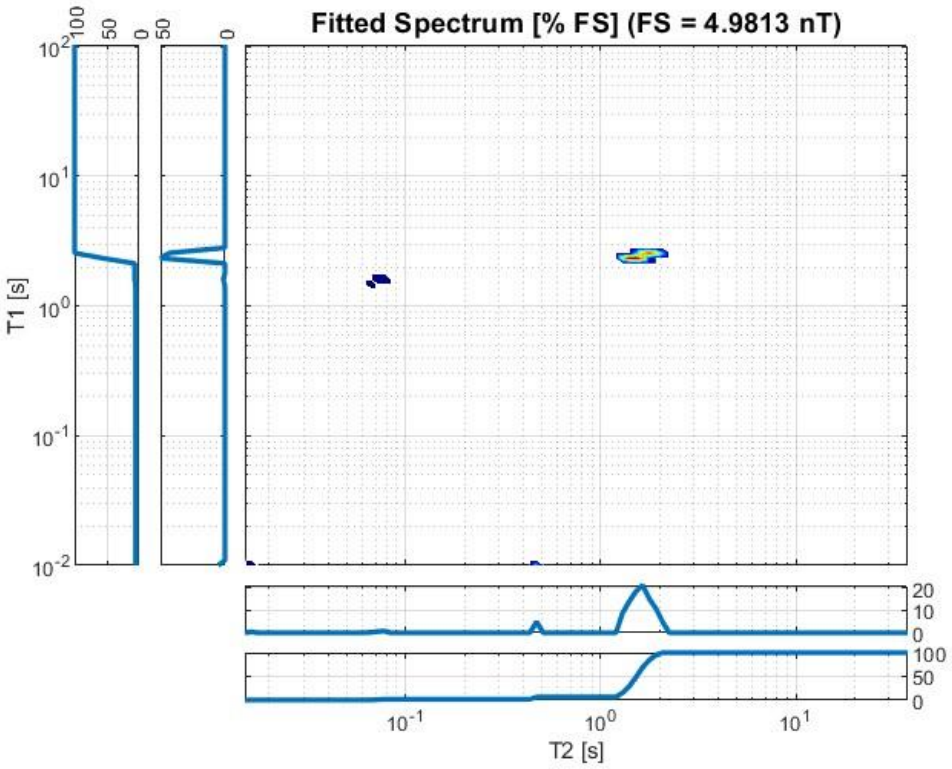
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 60:40



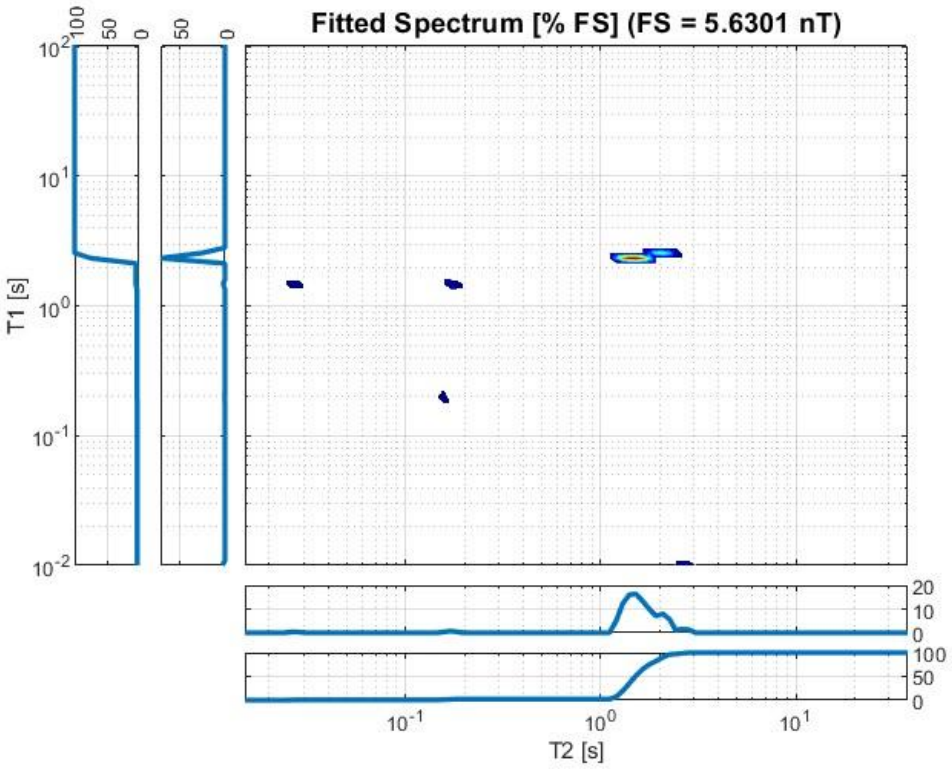
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 65:35



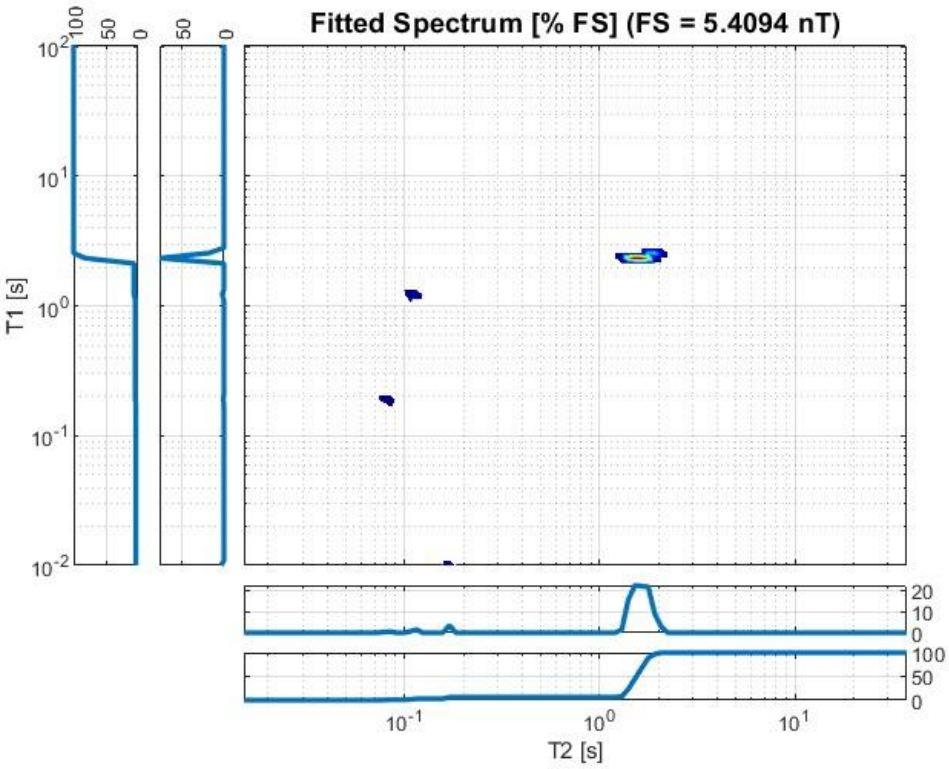
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 65:35



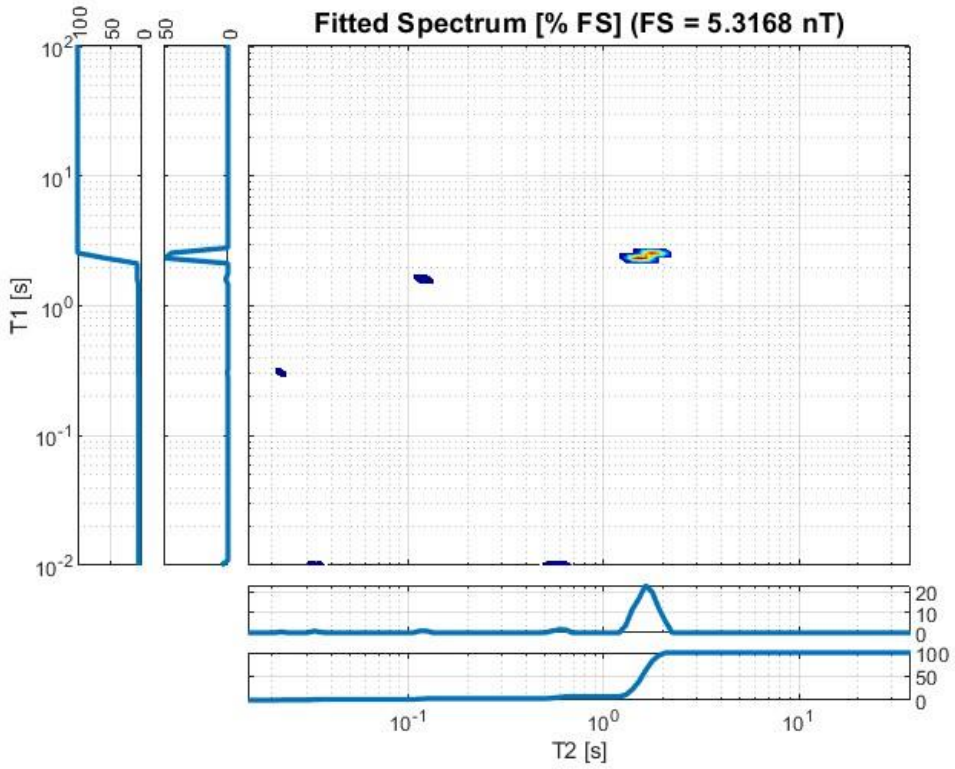
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 65:35



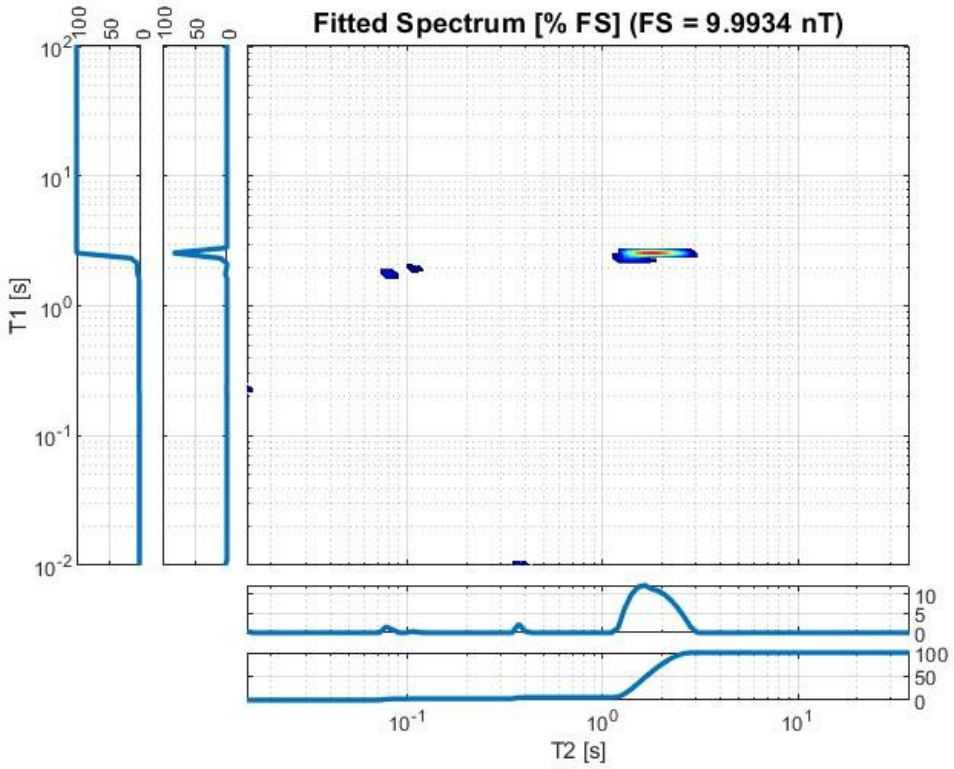
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 70:30



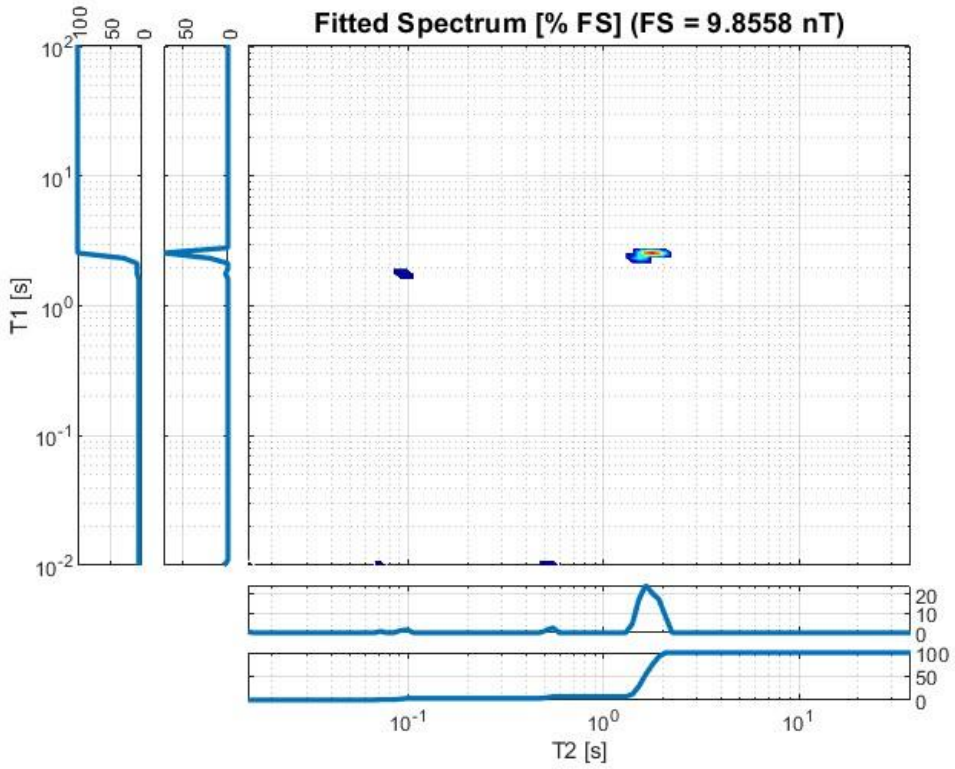
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 70:30



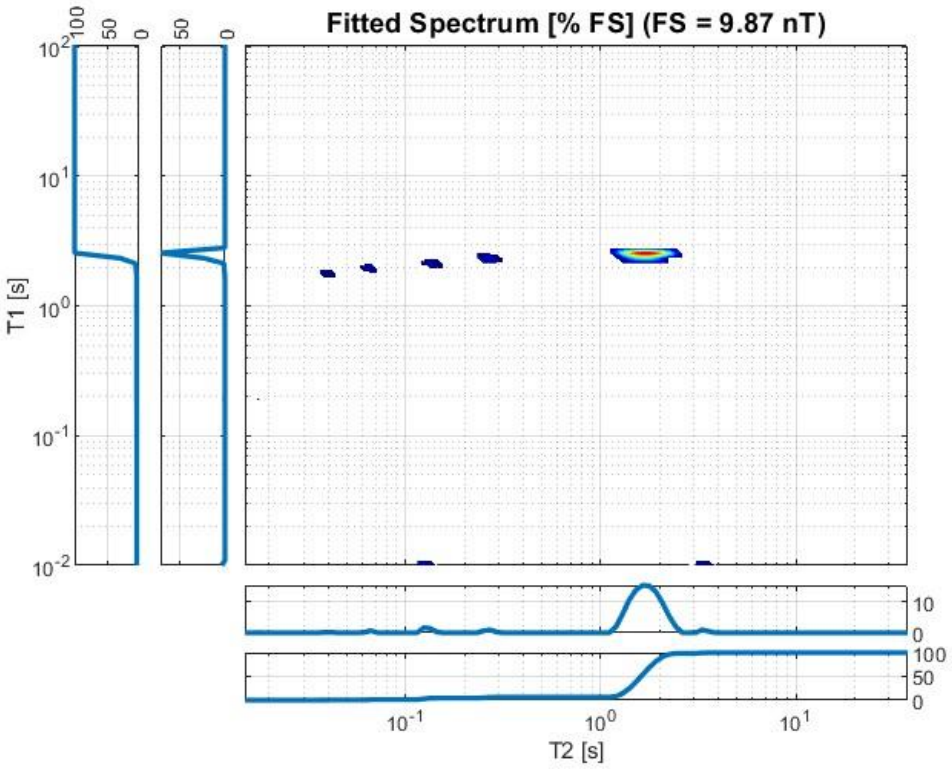
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 70:30



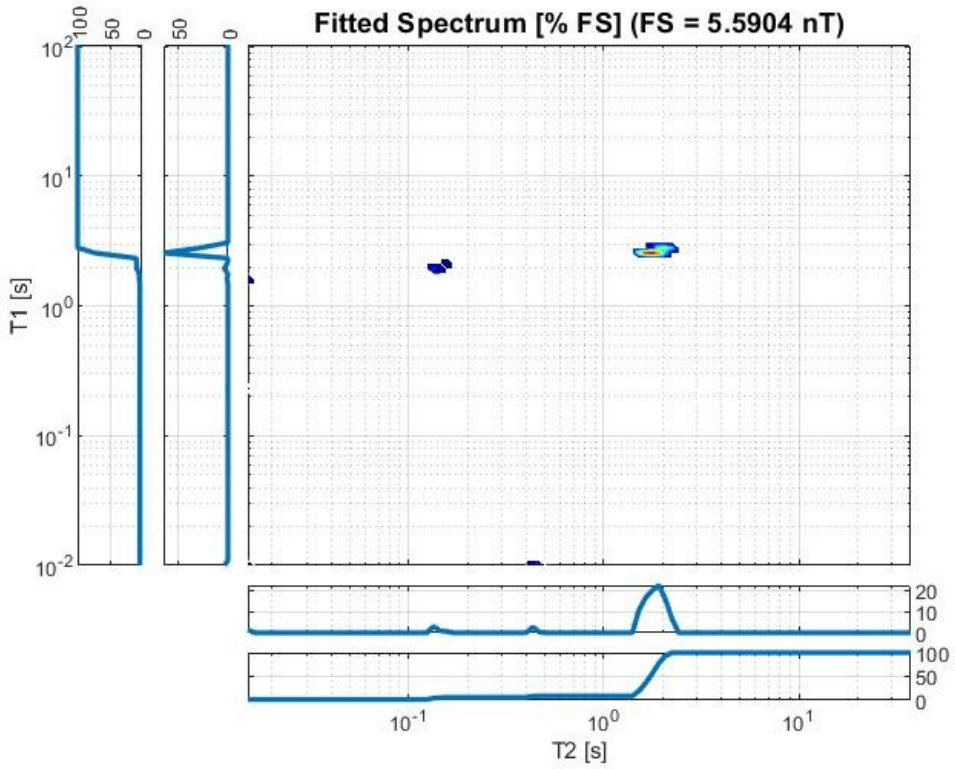
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 75:25



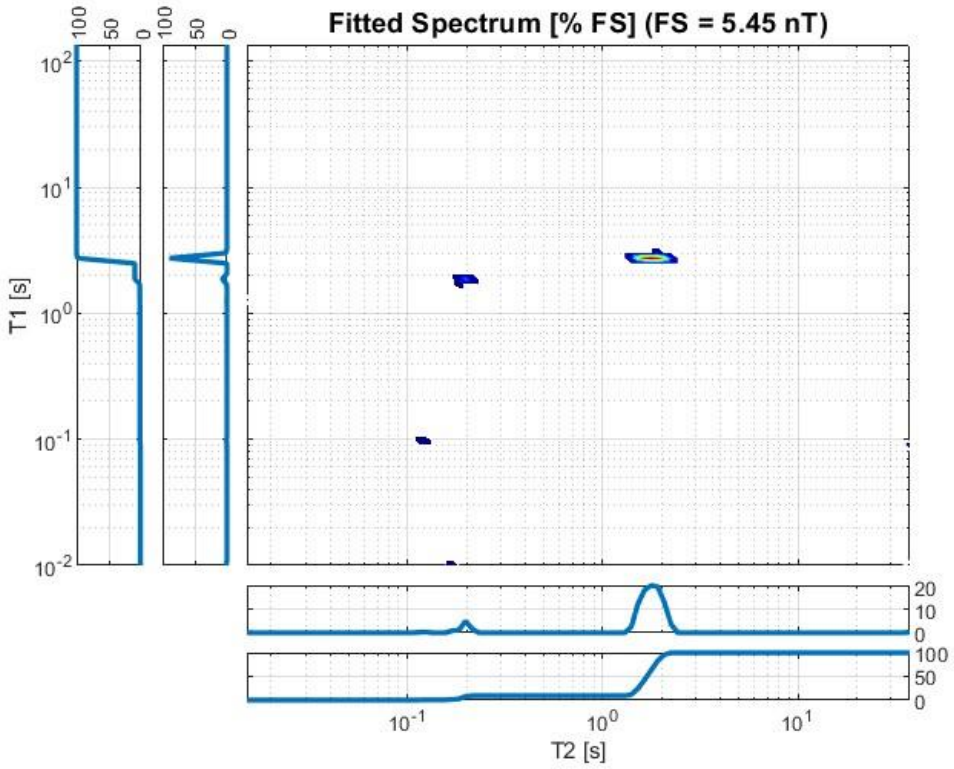
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 75:25



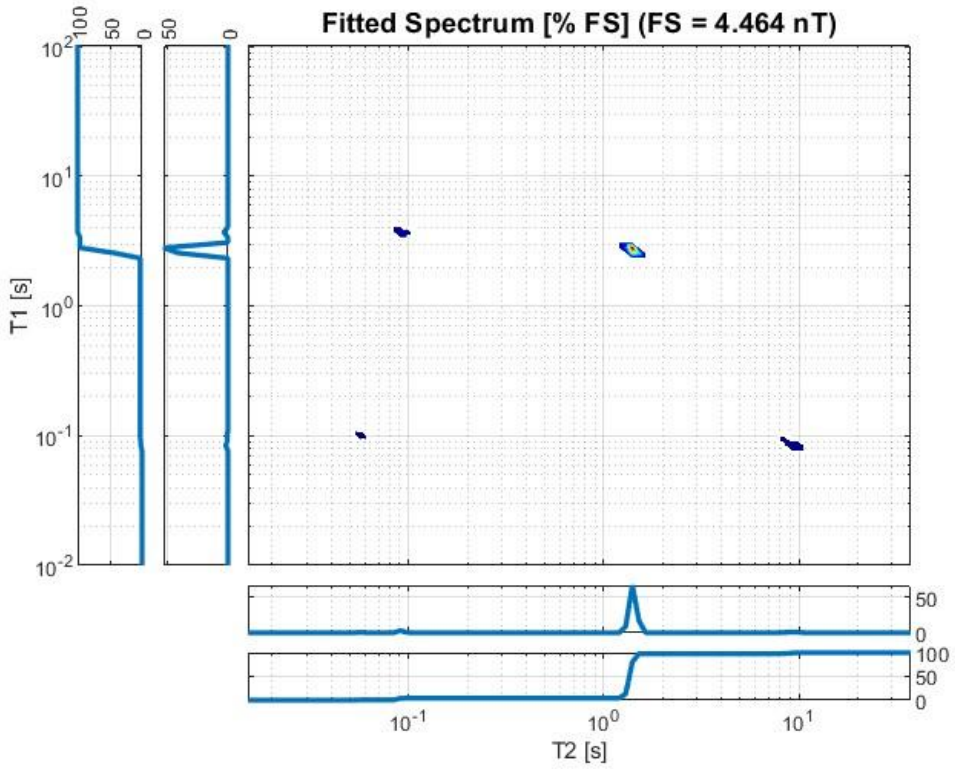
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 75:25



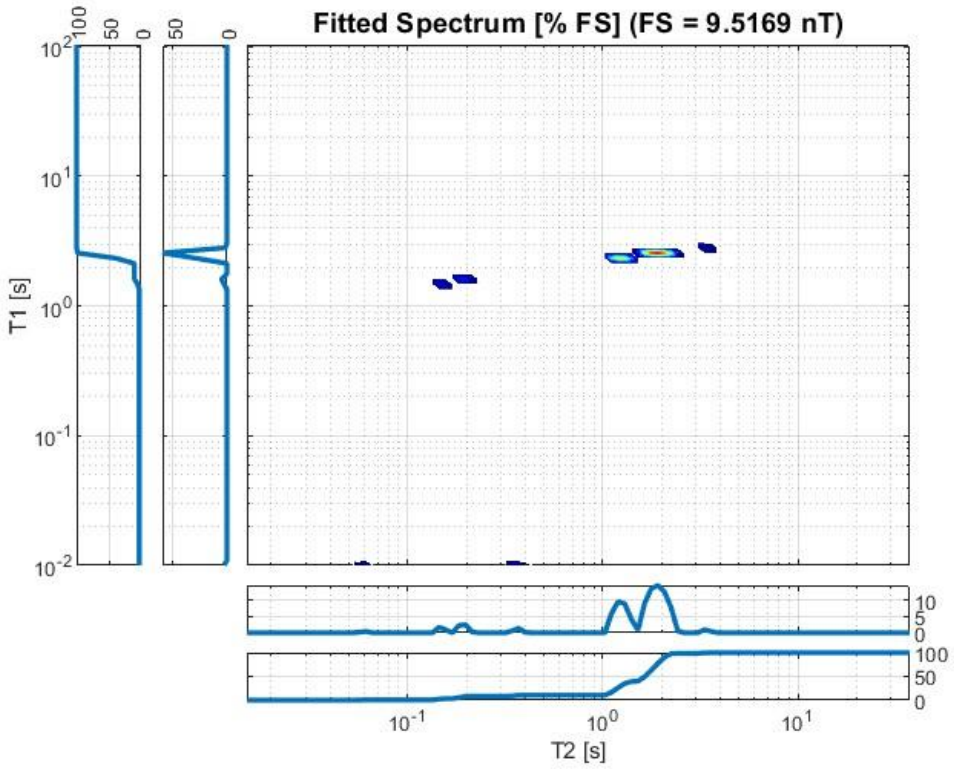
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 80:20



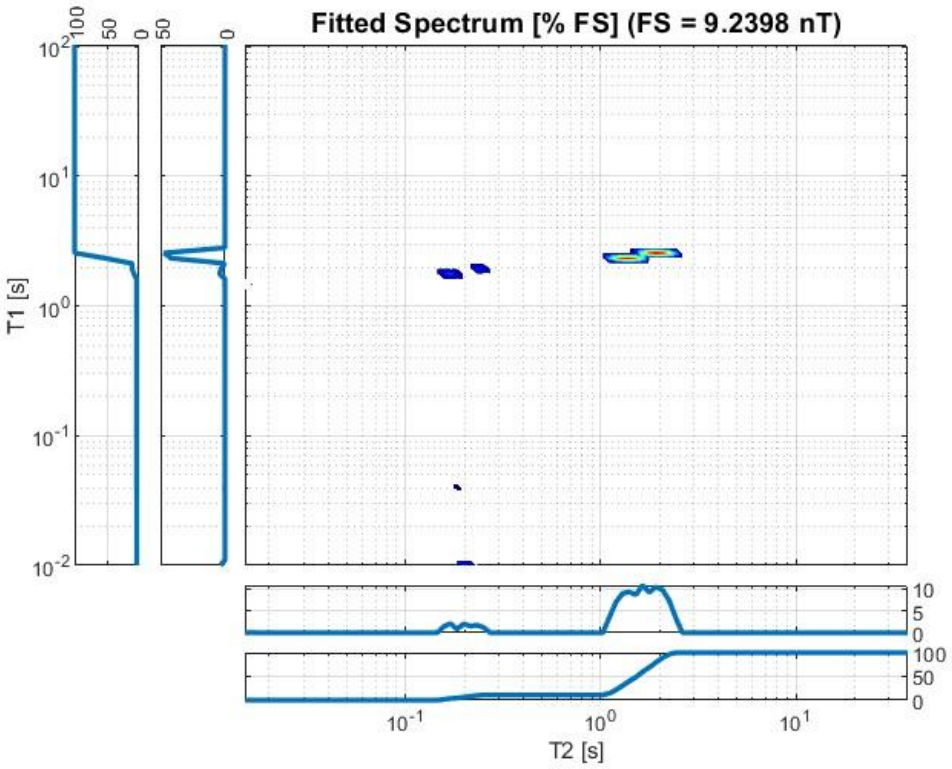
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 80:20



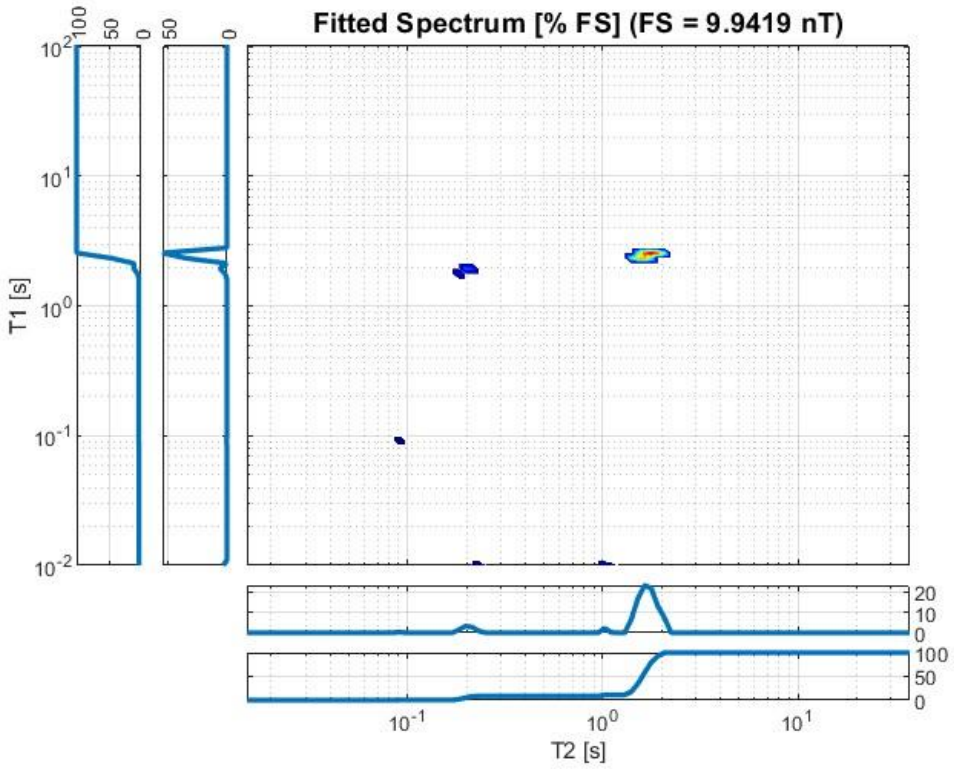
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 80:20



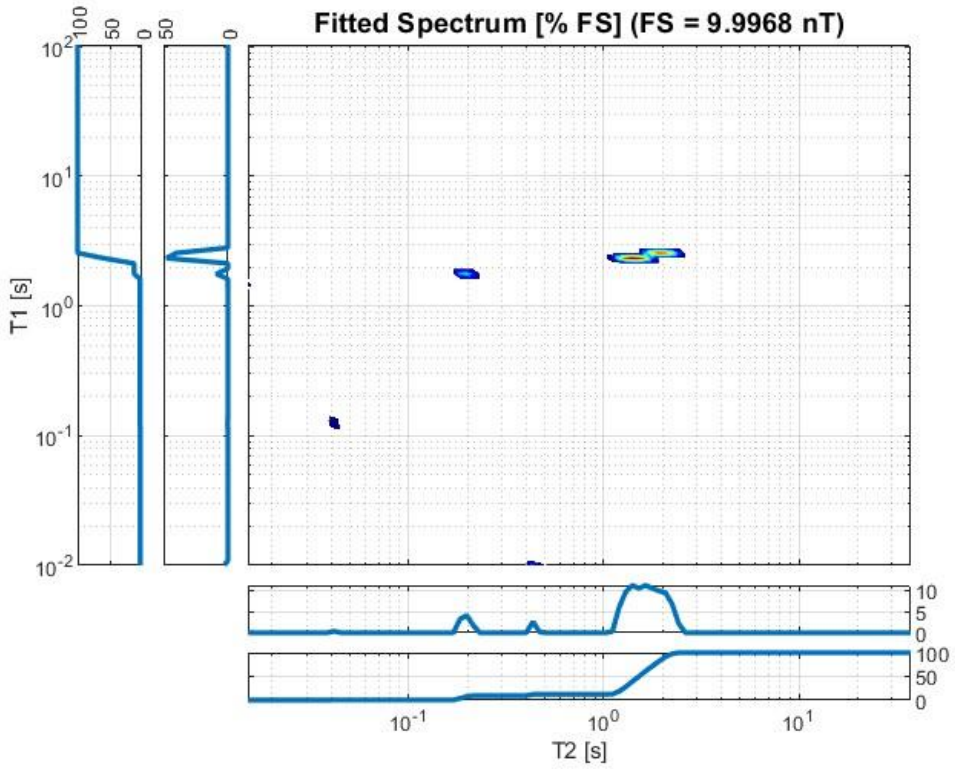
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 85:15



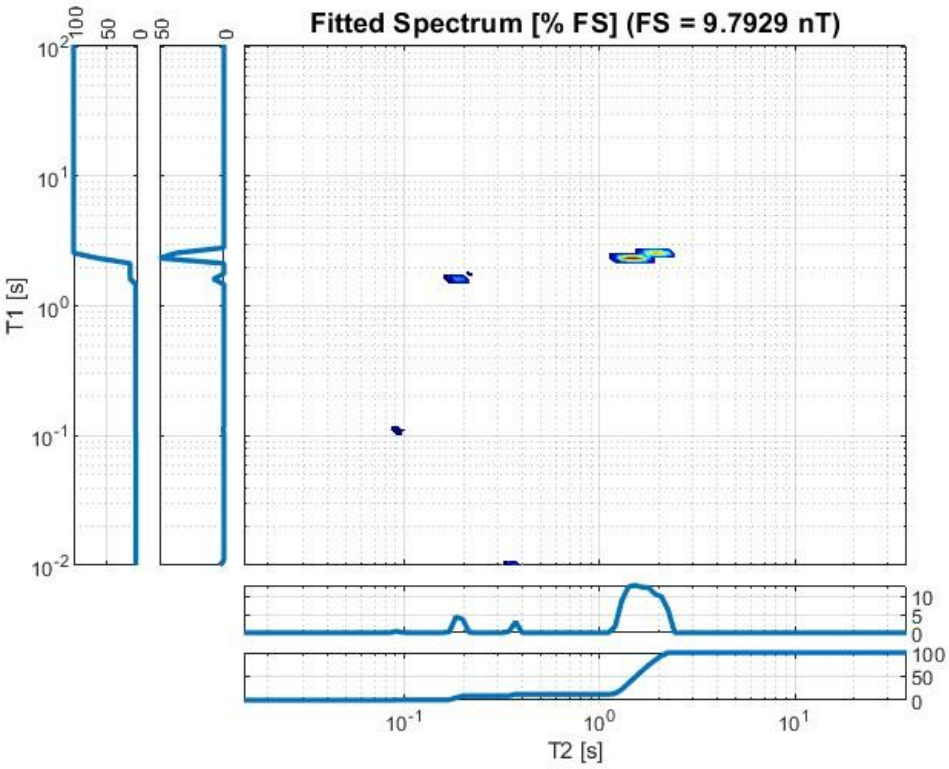
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 85:15



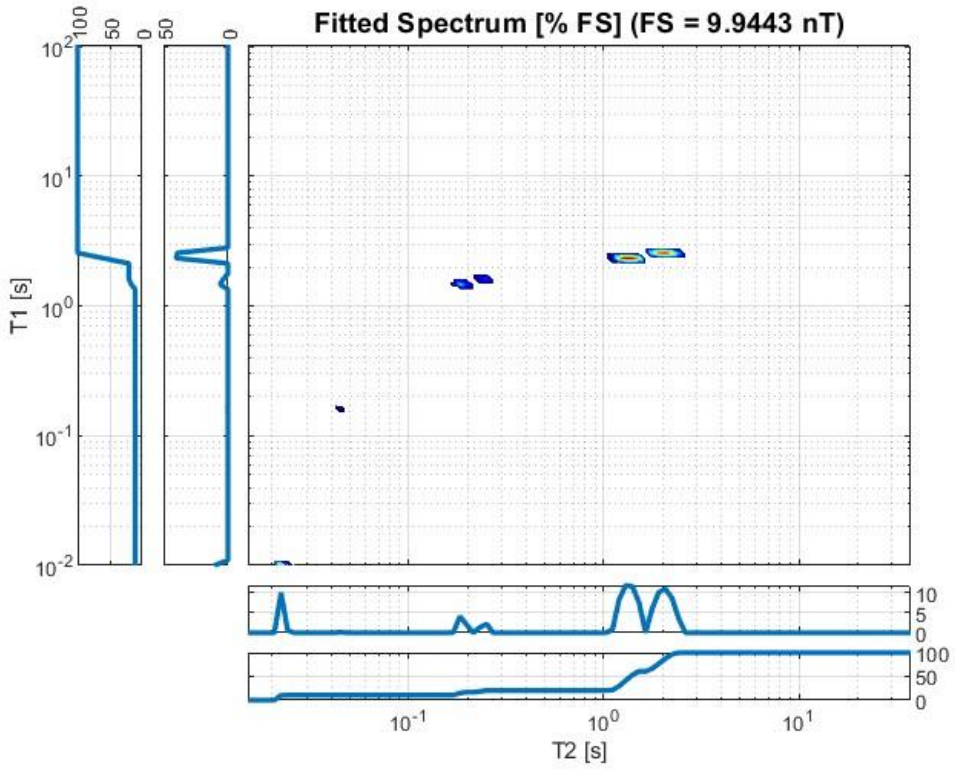
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 85:15



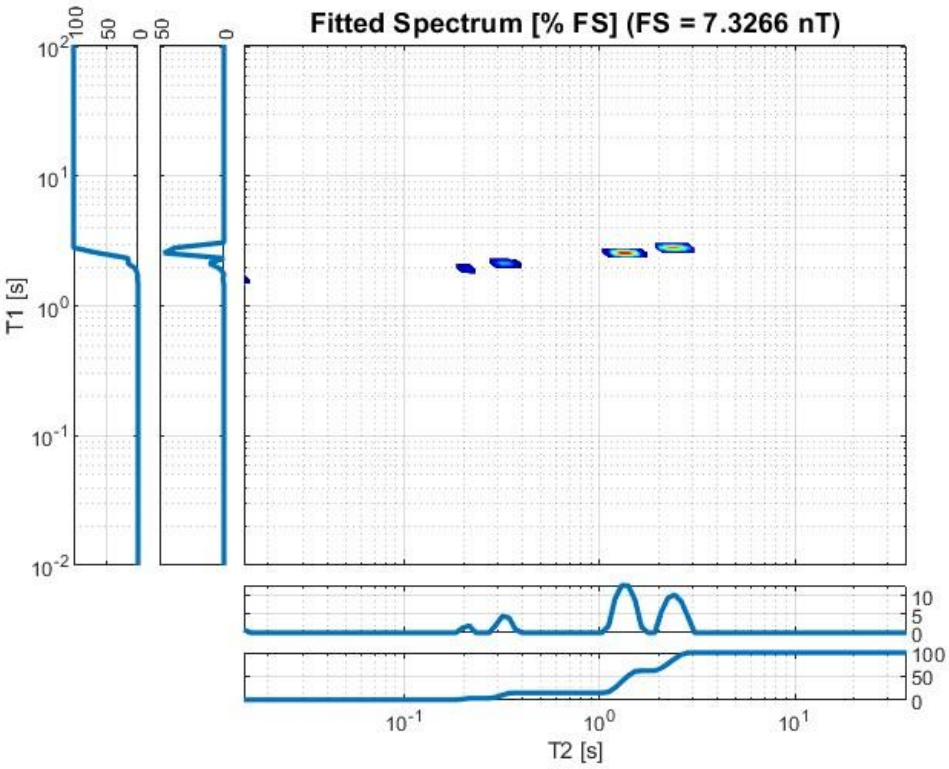
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 90:10



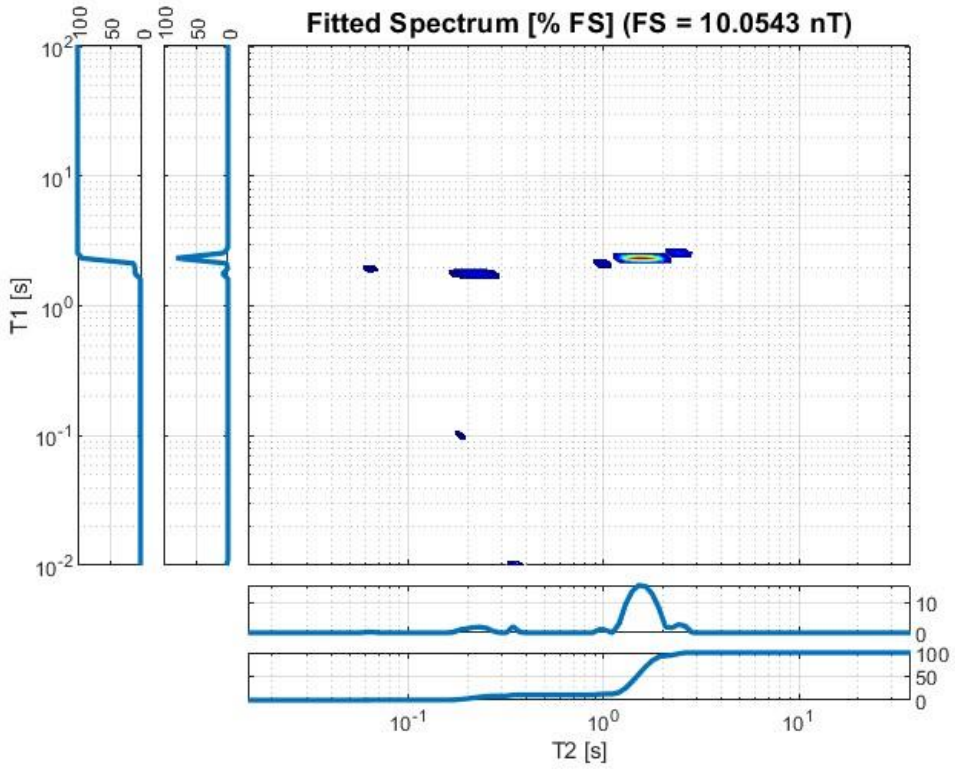
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 90:10



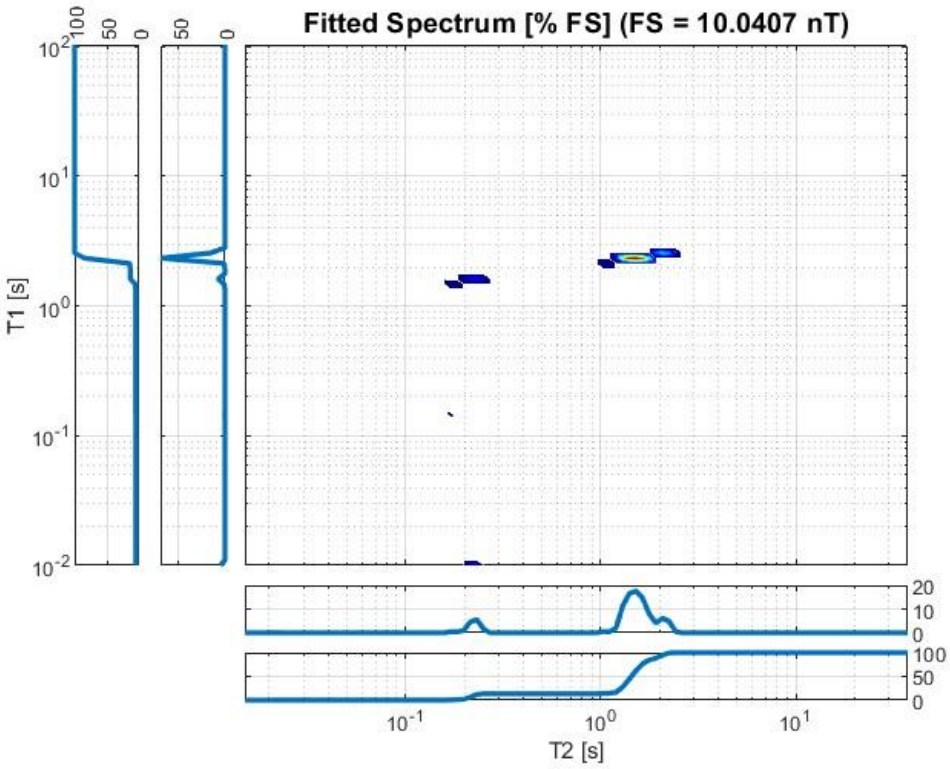
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 90:10



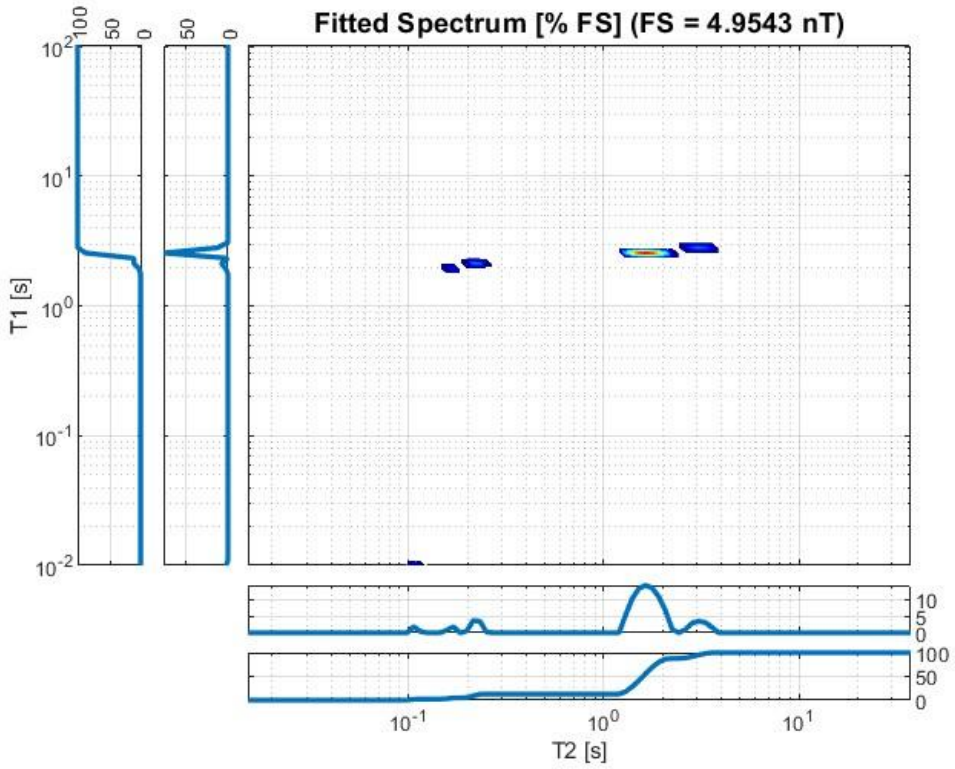
T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 95:5



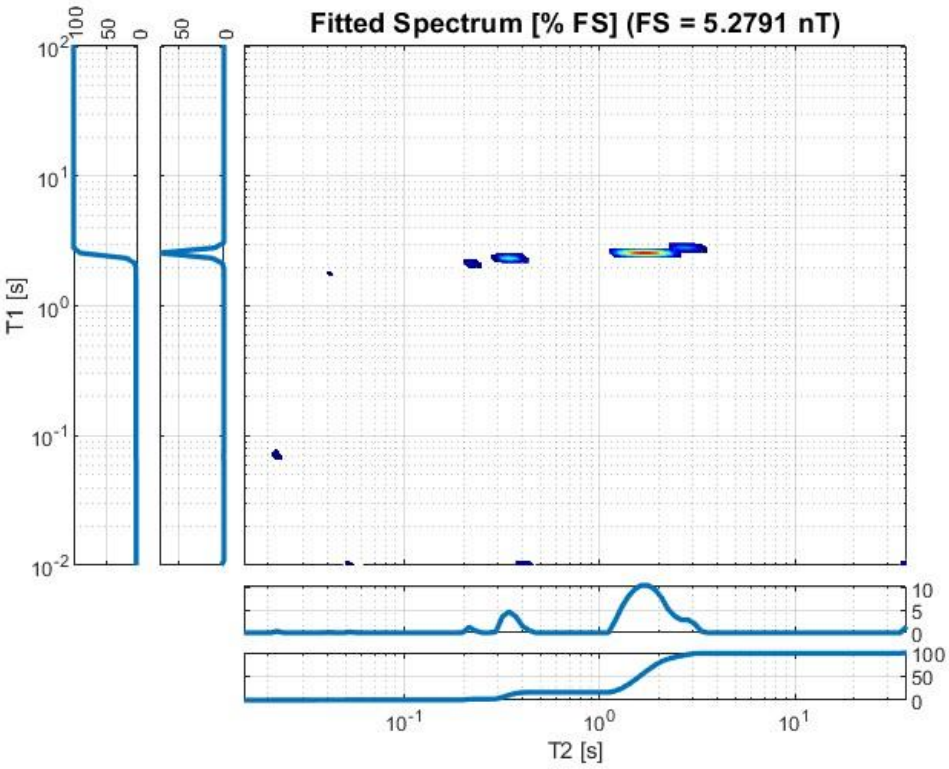
T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 95:5



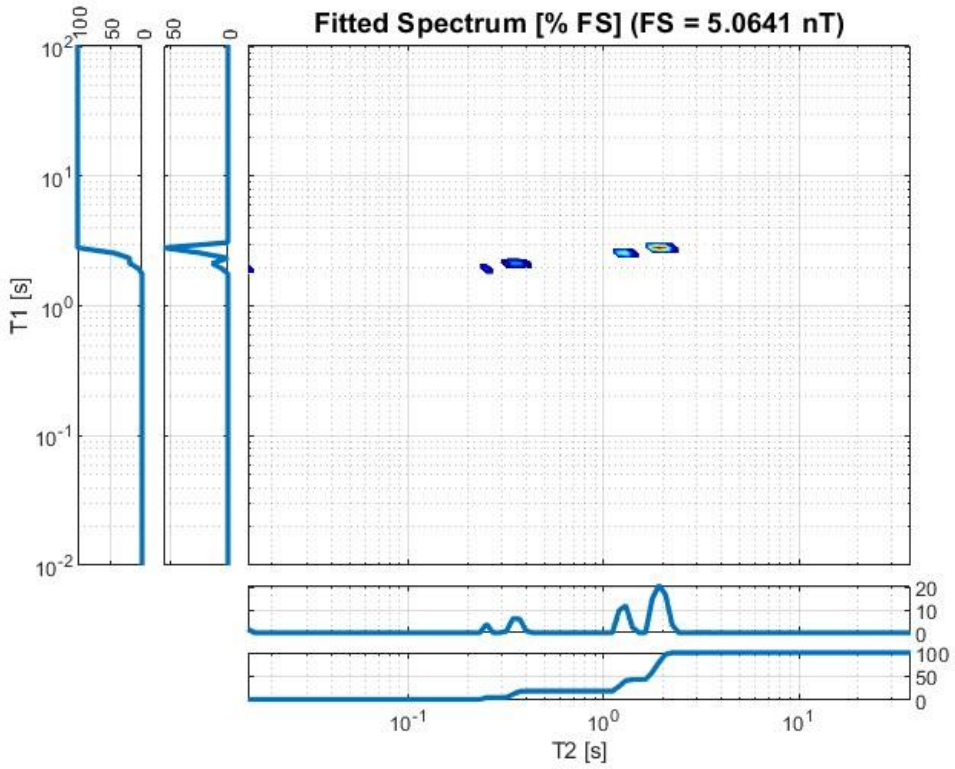
T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 95:5



T1T2 Map of 1st insulin mixture prepared as insulin NPH: insulin Regular 100:0

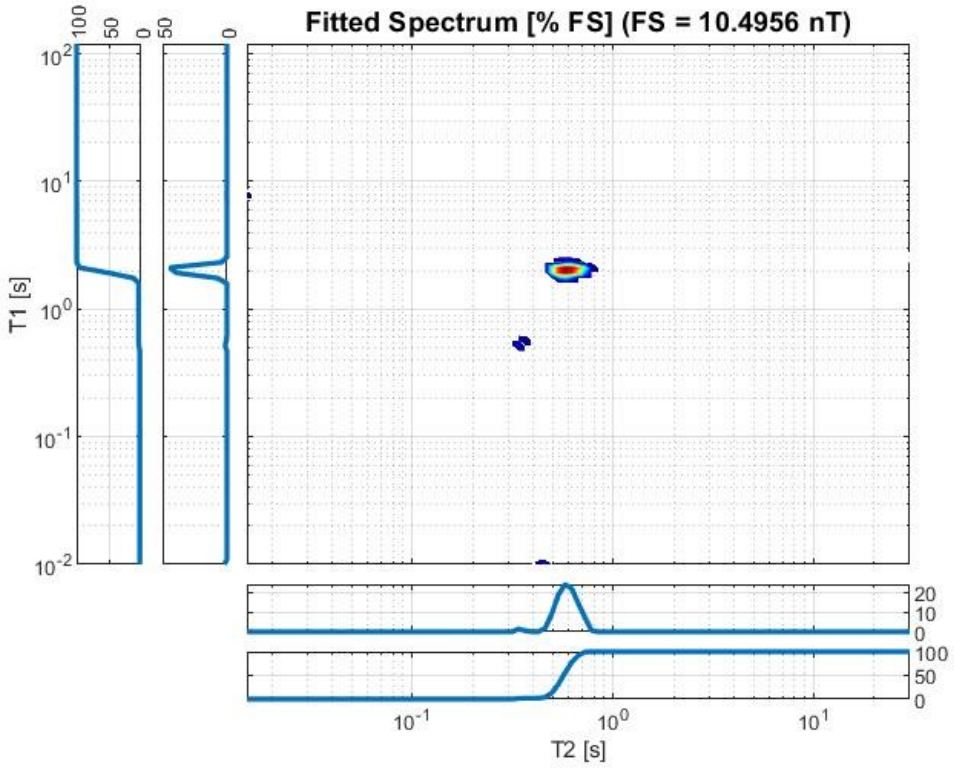


T1T2 Map of 2nd insulin mixture prepared as insulin NPH: insulin Regular 100:0

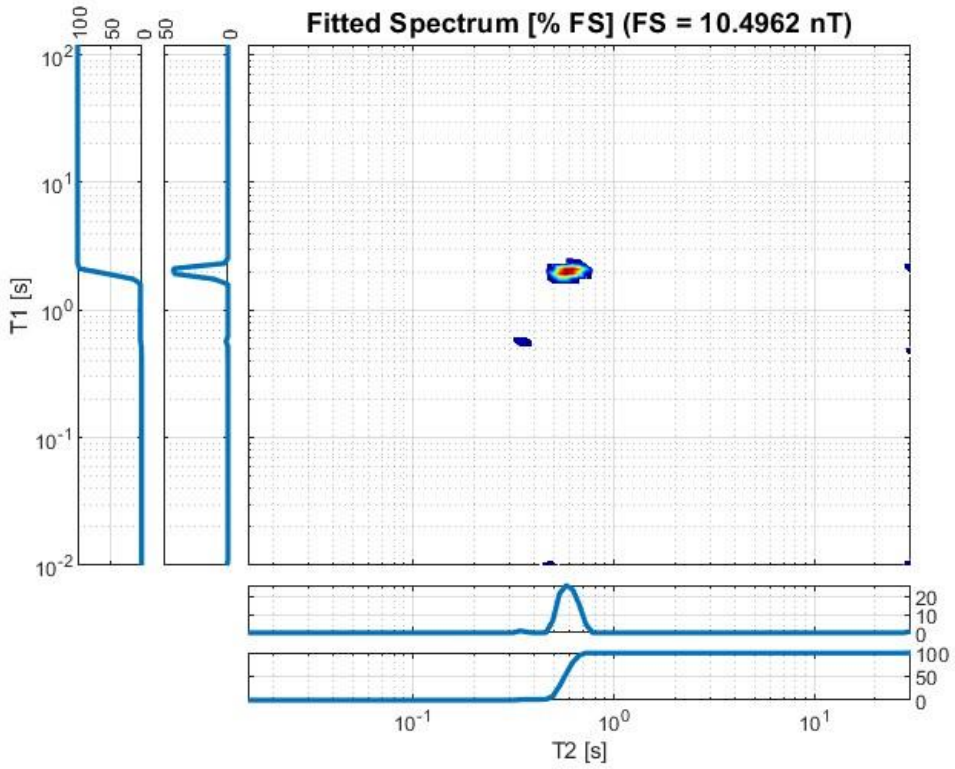


T1T2 Map of 3rd insulin mixture prepared as insulin NPH: insulin Regular 100:0

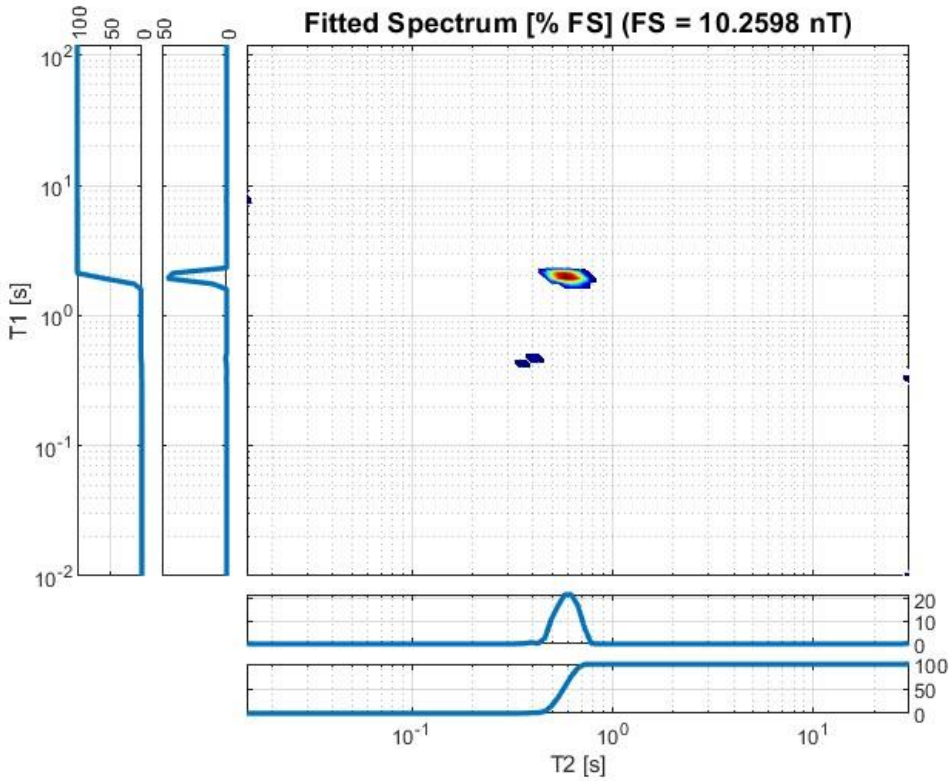
B. T1T2 Maps of glycated samples



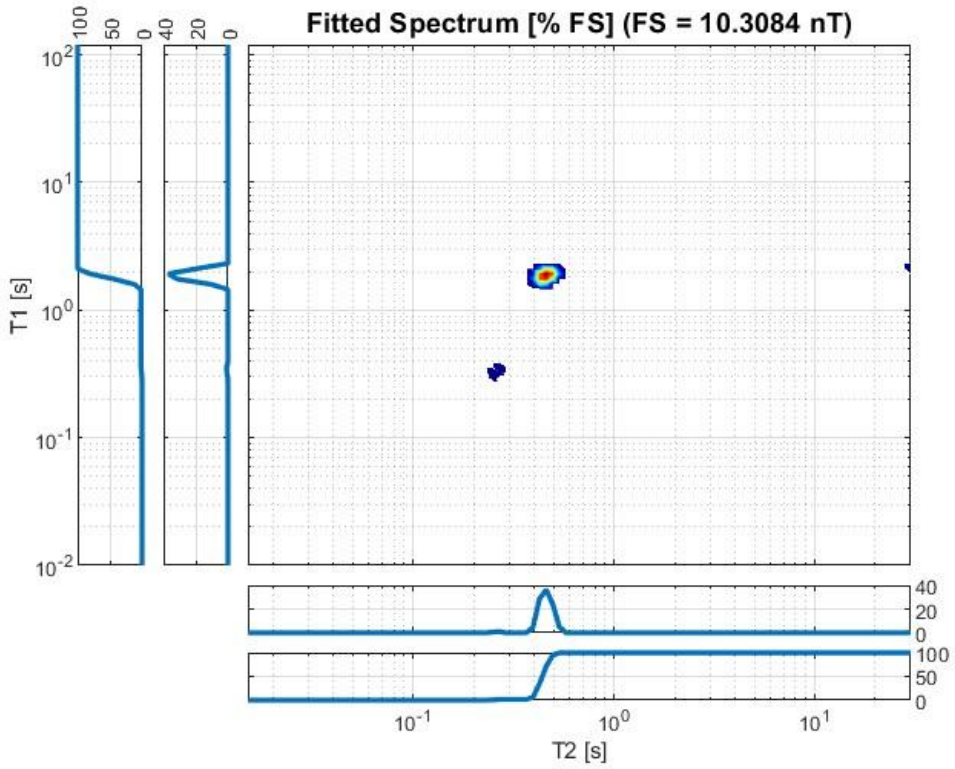
T1T2 Map of 1st replica of glycated samples at 0 hours' time point



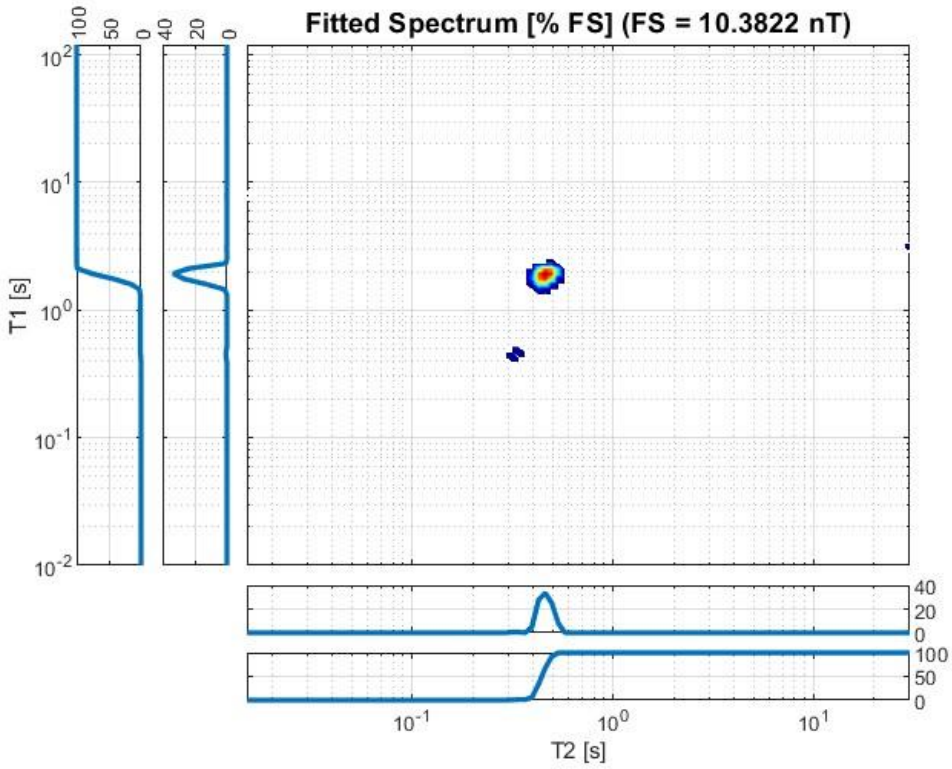
T1T2 Map of 2nd replica of glycated samples at 0 hours' time point



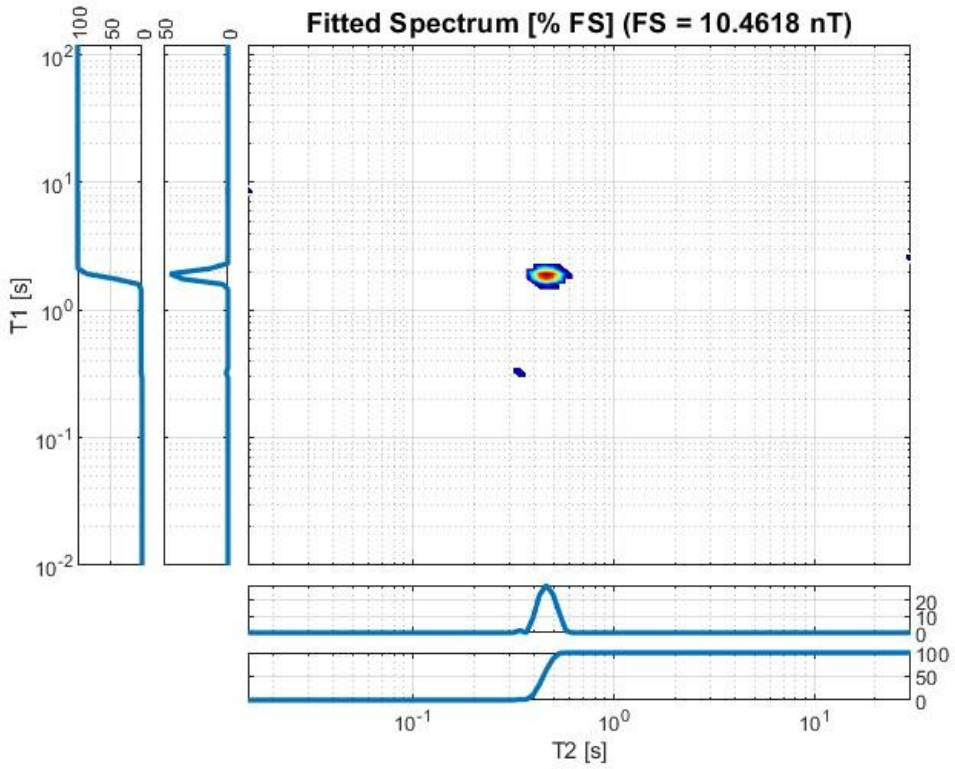
T1T2 Map of 3rd replica of glycated samples at 0 hours' time point



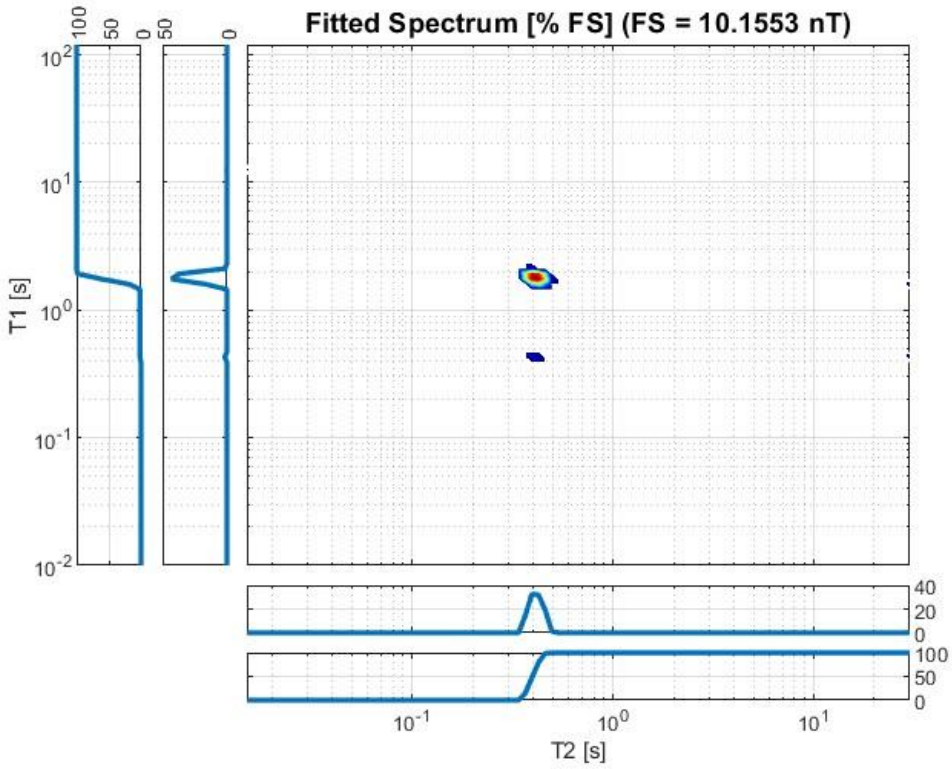
T1T2 Map of 1st replica of glycated samples at 0.5 hours' time point



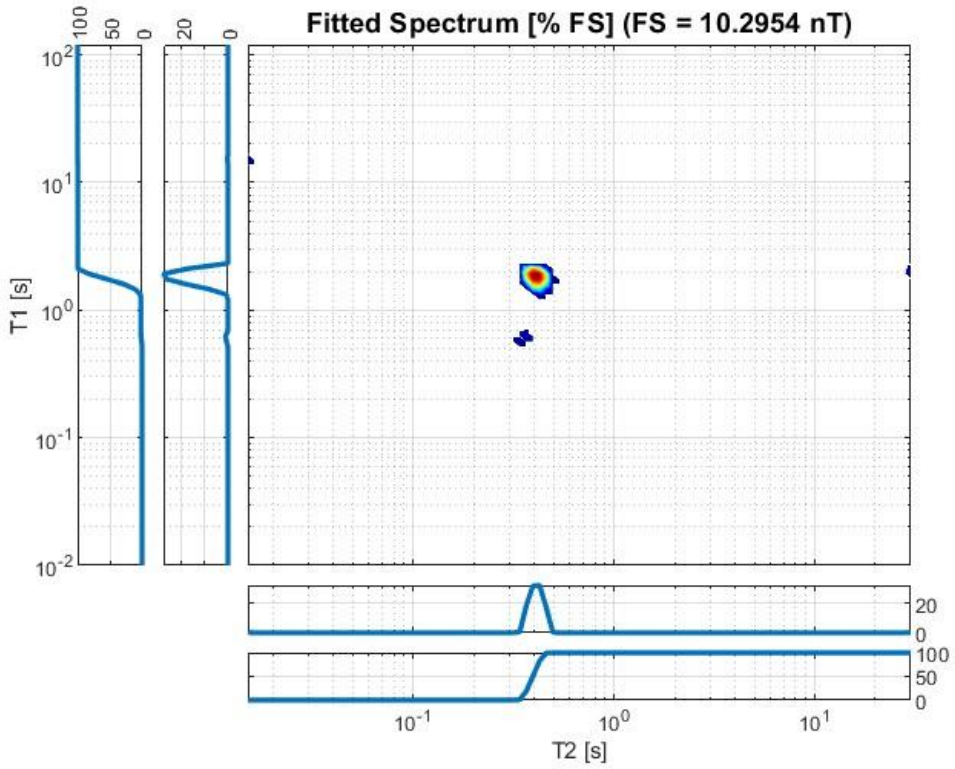
T1T2 Map of 2nd replica of glycated samples at 0.5 hours' time point



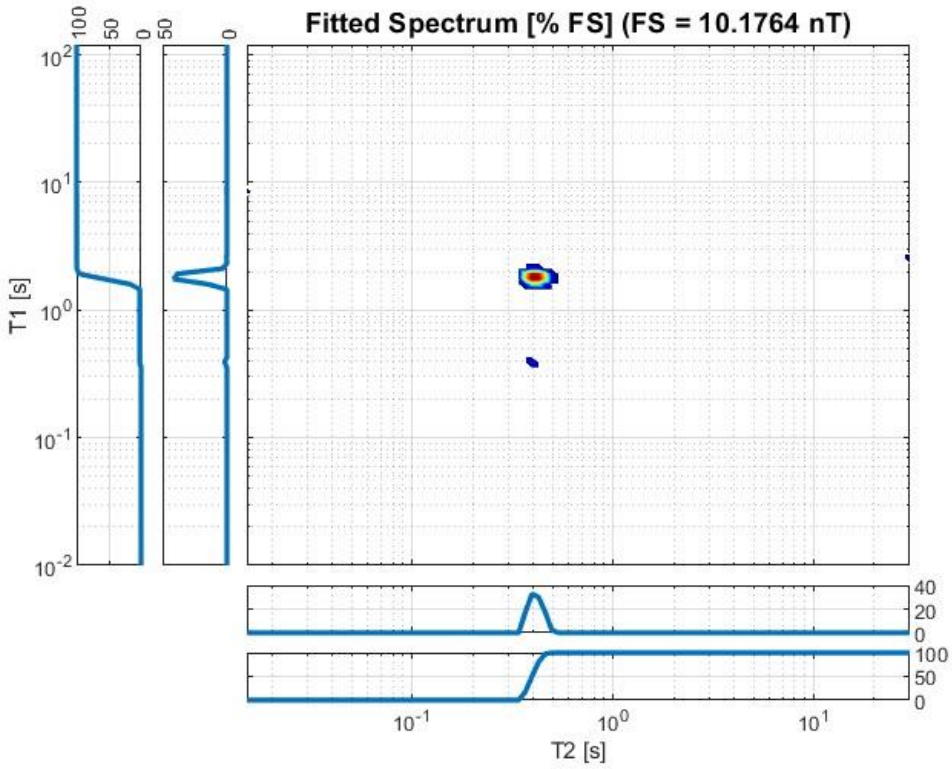
T1T2 Map of 3rd replica of glycated samples at 0.5 hours' time point



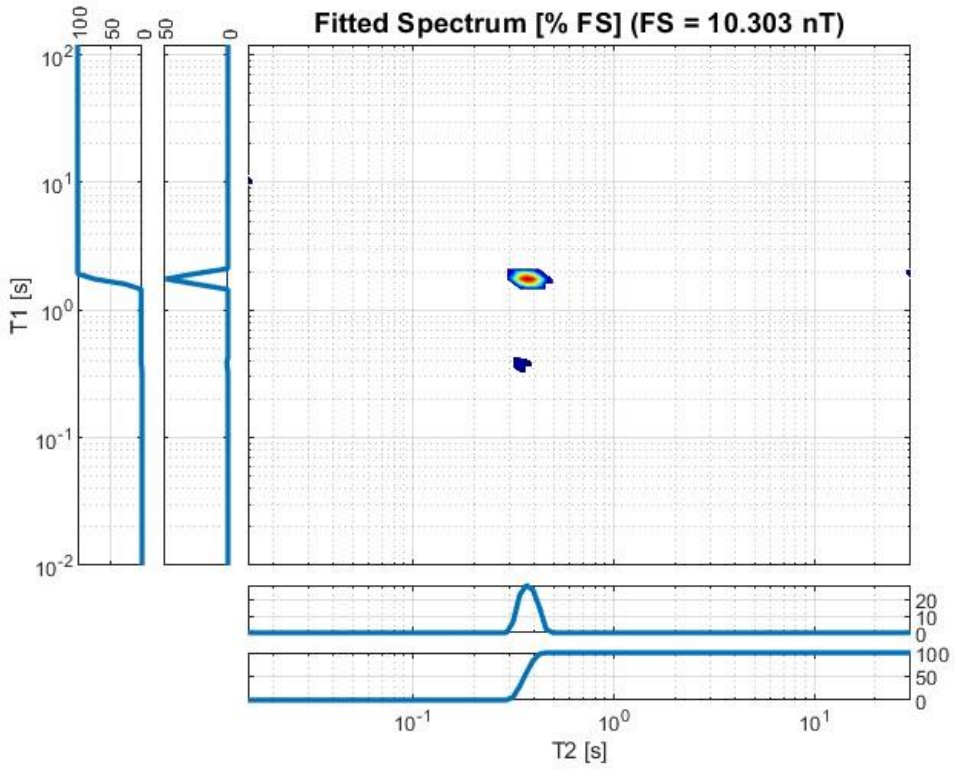
T1T2 Map of 1st replica of glycated samples at 1 hour time point



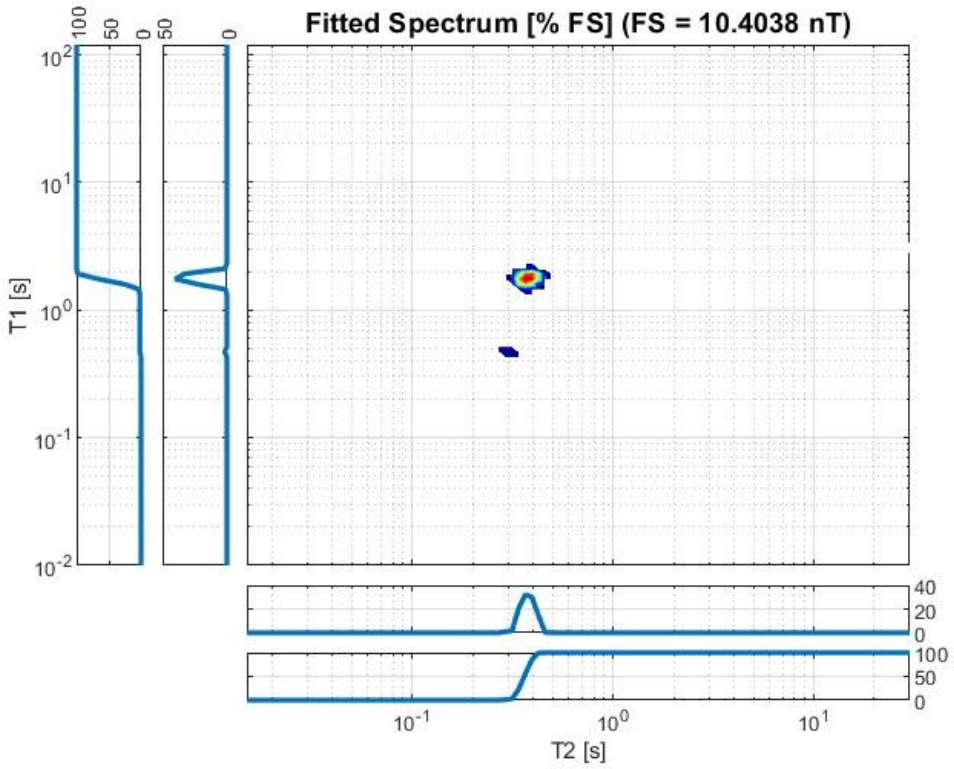
T1T2 Map of 2nd replica of glycated samples at 1 hours' time point



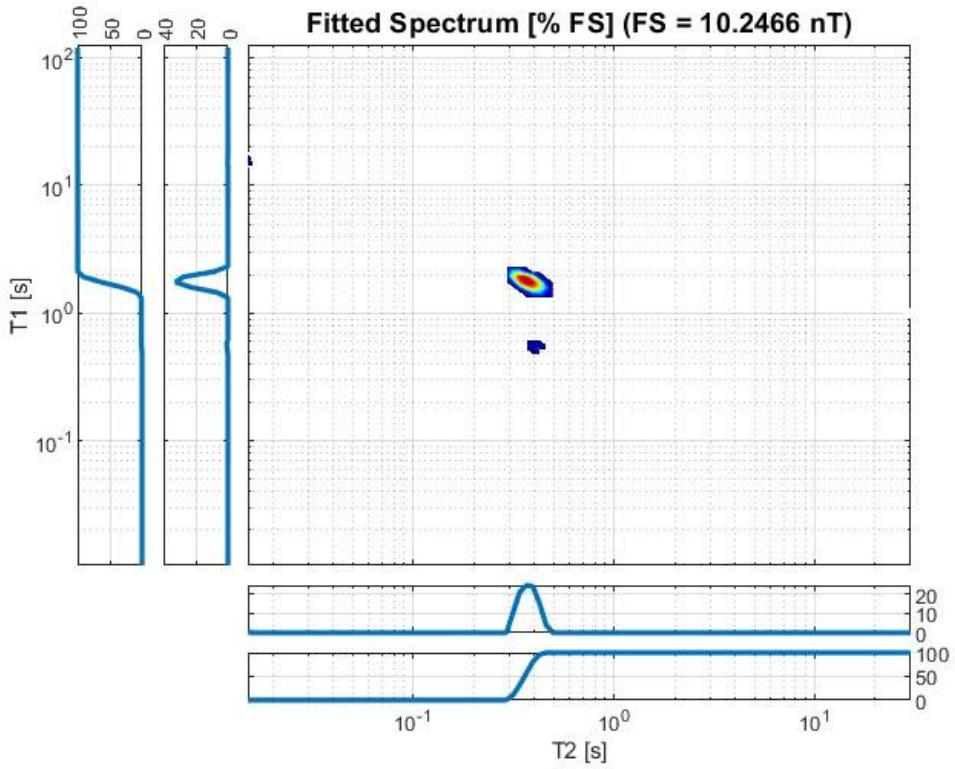
T1T2 Map of 3rd replica of glycated samples at 1 hours' time point



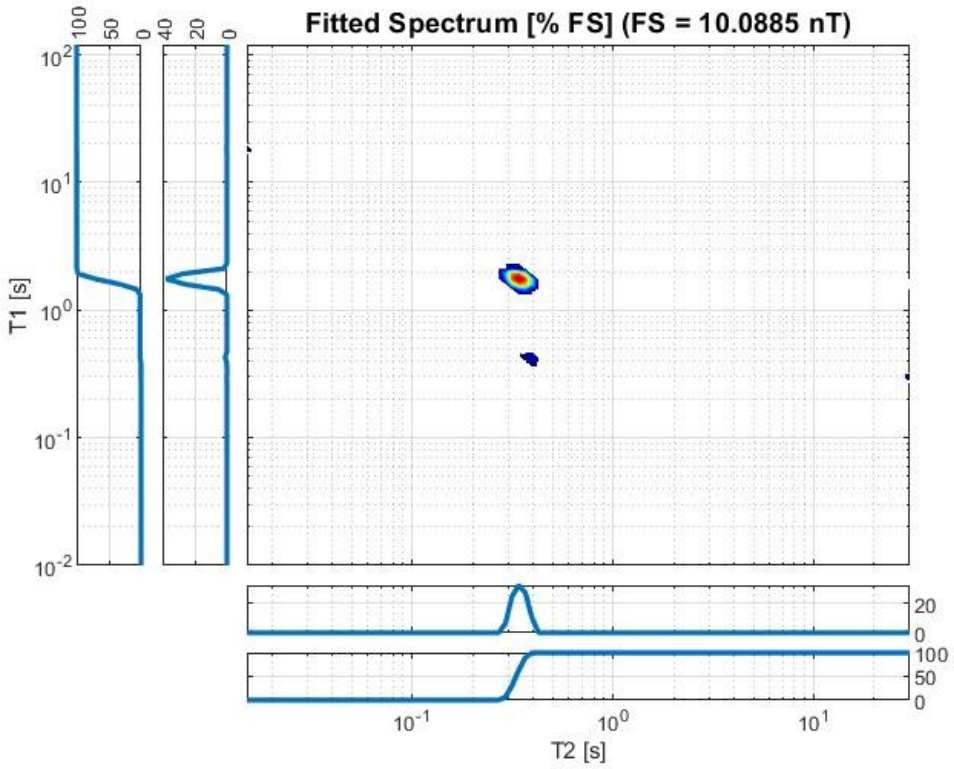
T1T2 Map of 1st replica of glycated samples at 2 hours' time point



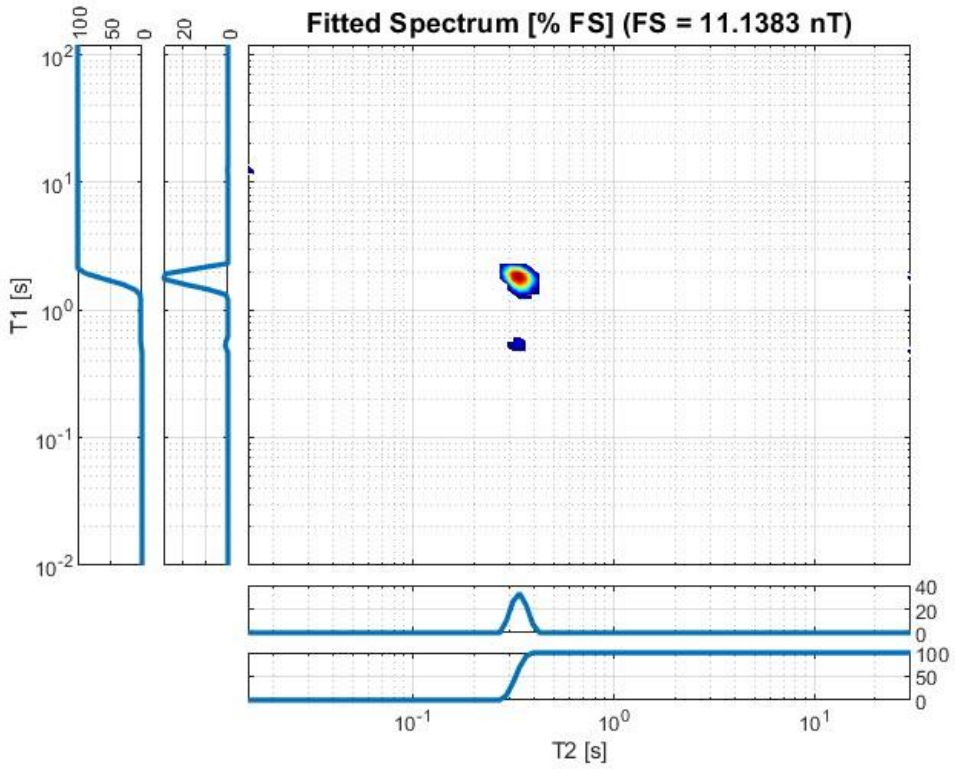
T1T2 Map of 2nd replica of glycated samples at 2 hours' time point



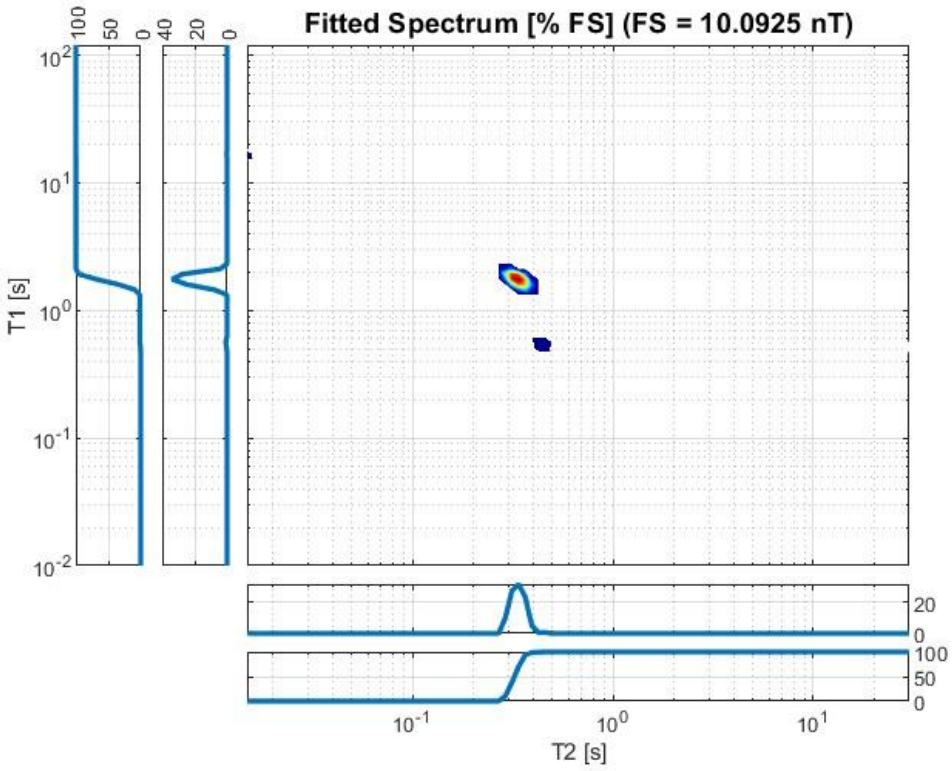
T1T2 Map of 3rd replica of glycated samples at 2 hours' time point



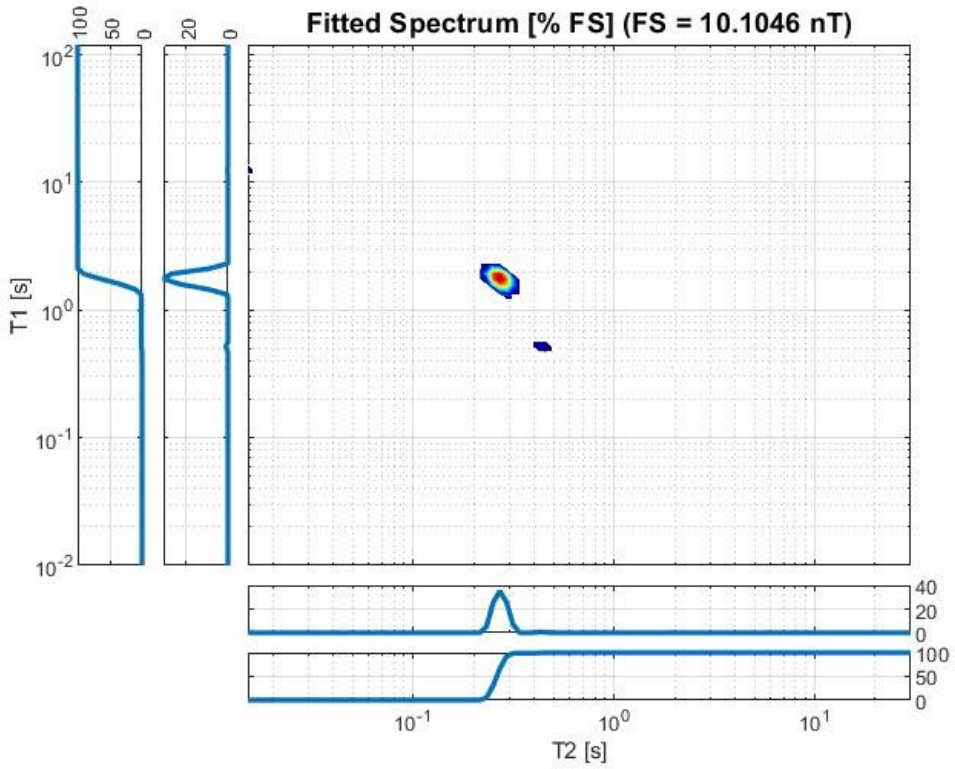
T1T2 Map of 1st replica of glycated samples at 4 hours' time point



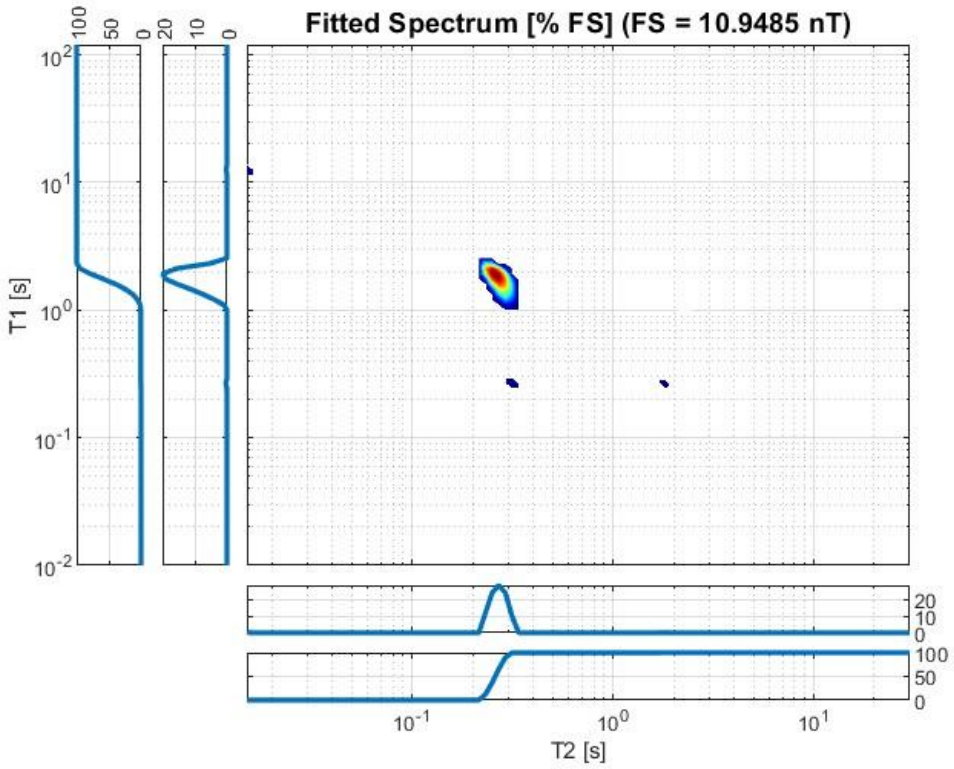
T1T2 Map of 2nd replica of glycated samples at 4 hours' time point



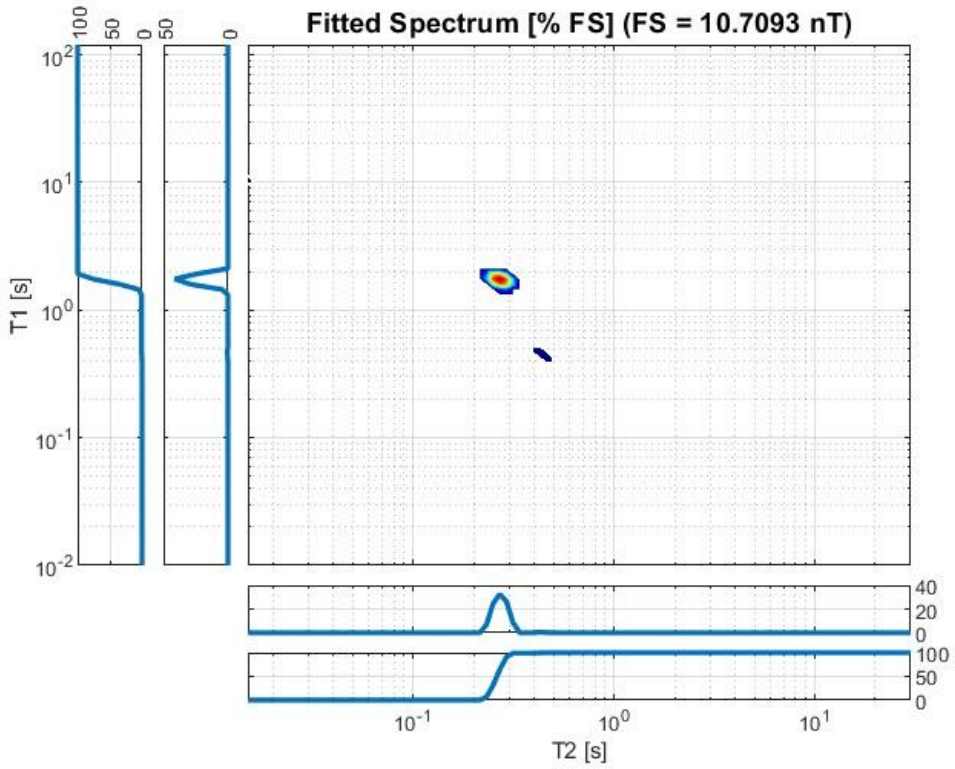
T1T2 Map of 3rd replica of glycated samples at 4 hours' time point



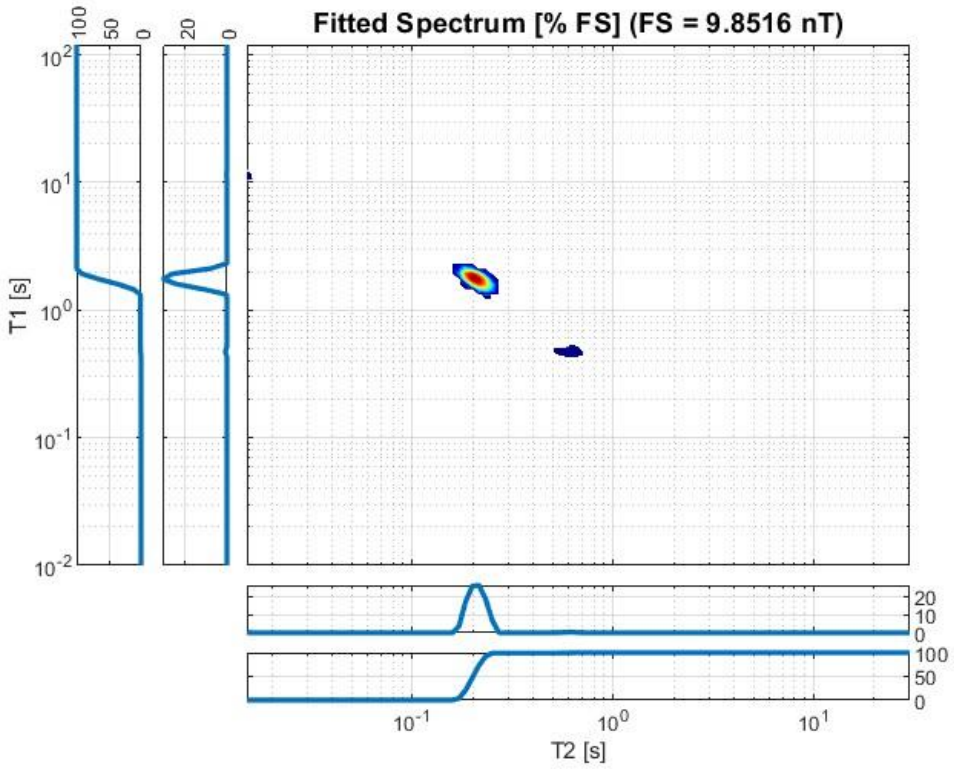
T1T2 Map of 1st replica of glycated samples at 6 hours' time point



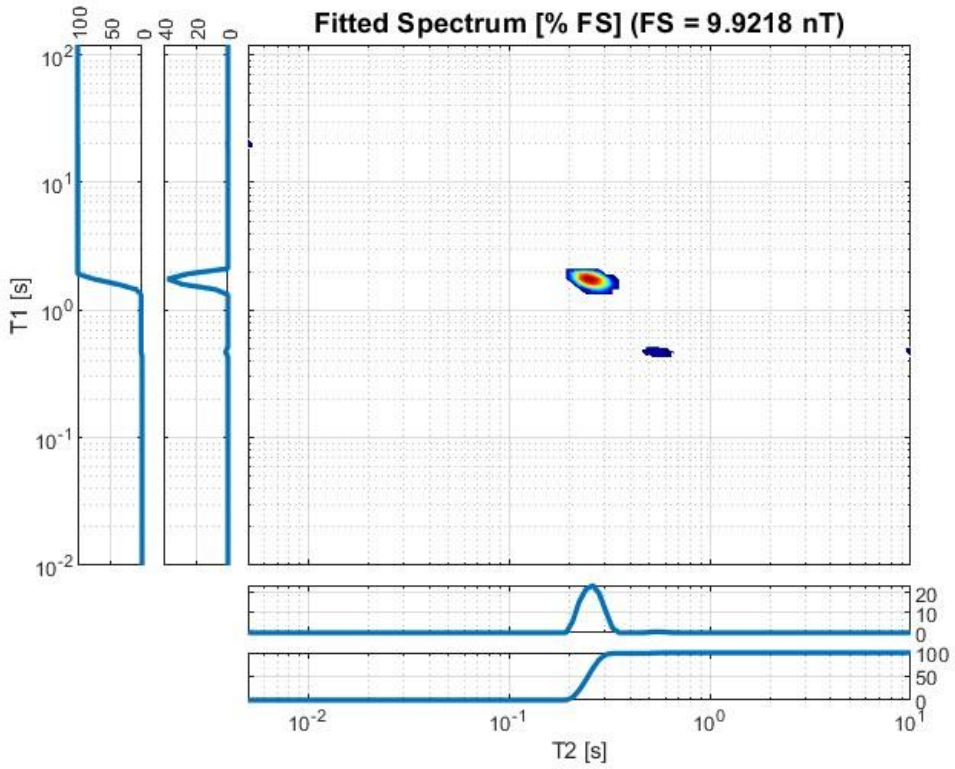
T1T2 Map of 2nd replica of glycated samples at 6 hours' time point



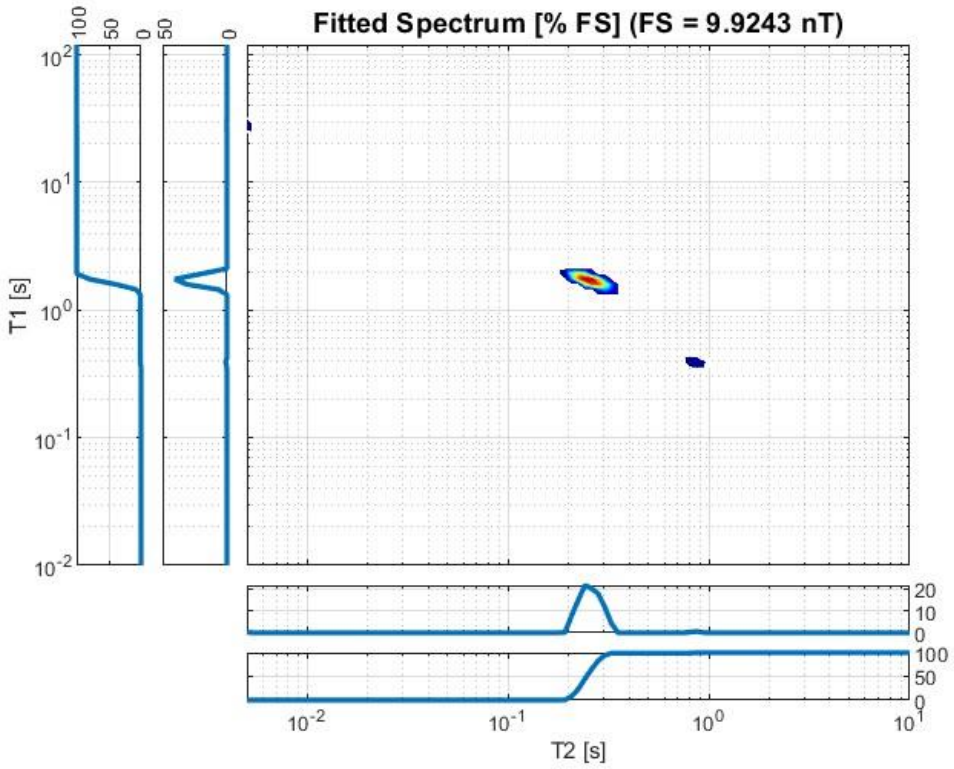
T1T2 Map of 3rd replica of glycated samples at 6 hours' time point



T1T2 Map of 1st replica of glycated samples at 12 hour time point



T1T2 Map of 2nd replica of glycated samples at 12 hours' time point



T1T2 Map of 3rd replica of glycated samples at 12 hours' time point

CURRICULUM VITAE

Surname, Name: Alam, Hani

EDUCATION

Degree	Institution	Year of Graduation
MS	METU Department of Biotechnology	2017
BS	SPU Faculty of Pharmacy	2013

FOREIGN LANGUAGES

Advanced English, Fluent Arabic, Fluent Turkish

PUBLICATIONS

1. Alam, H., Öztop, H.M
   Measuring Aspirin degradation using Near Infrared Spectroscopy and Time Domain NMR. Pharmaceutical Research (To be published)
2. Alam, H., Özeşme Taylan, G., Gezici Koç, Ö., Yamalı, C., Öztop, H.M
   Applying TD-NMR as a novel noninvasive method to detect and measure glycation in biopharmaceuticals. International Journal of Pharmaceutics (To be published).
3. Alam, H., Özeşme Taylan, G., Yamalı, C., Öztop, H.M
   Synergetic Quantification of Insulin Drug Mixtures Using Time Domain NMR (TD-NMR). Journal of Pharmaceutical and Biomedical Analysis (To be published).

4. Alissa Alam, H., Dalgıç A.D., Tezcaner A., Ozen C., Keskin D. A comparative study of monoaxial and coaxial PCL/gelatin/Poloxamer 188 scaffolds for bone tissue engineering. International Journal of Polymeric Materials and Polymeric Biomaterials, 69(6),339-350, (2019). DOI: 10.1080/00914037.2019.1581198
5. Keskin, D., Alissa Alam, H., Tezcaner, A., Dalgıç, A. D., & Özen, C., (2018). Coaxial electrospun scaffolds for dual delivery of bioagents for bone tissue engineering. 29th Annual Congress of the European Society for Biomaterials (pp.334). Maastricht, Netherlands (Poster Presentation)
6. Alissa Alam, H., Dalgıç, A. D., Tezcaner, A., Özen, C., & Keskin, D., (2017). Local Delivery of Bioactive Agents for Enhanced Bone Tissue Engineering. 22nd International Biomedical Science and Technology Symposium (Poster Presentation)