

**HYDROTHERMAL LIQUEFACTION OF CHLORELLA PYRENOIDOSA AND
SPIRULINA PLATENSIS: NMR ANALYSIS OF BIO-CRUDE OIL COMPOSITION AND
MOLECULAR DYNAMICS**

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Bachelor of Science, University of Tehran, 2023

A Thesis Submitted
in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

In

BIOCHEMISTRY

Department of Chemistry and Biochemistry

University of Lethbridge

LETHBRIDGE, ALBERTA, CANADA

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DEDICATION

To my Dearest Family who have always supported me through scientific journeys!

ABSTRACT

The increasing global demand for sustainable energy sources has directed significant research toward biofuels derived from renewable biomass. This thesis investigates the potential of phytoplankton as a biofuel feedstock, focusing on its conversion into energy-dense biocrude via hydrothermal liquefaction (HTL). The study explores the influence of varying temperatures and pressures on the yield and chemical changes of the resulting products. Nuclear Magnetic Resonance (NMR) spectroscopy was employed to investigate the transformation of different organic functional groups—such as aliphatics, aromatics, and oxygen- or nitrogen-containing compounds—within the algal biomass during the HTL process.

By tracking these changes, NMR enabled a deeper understanding of the chemical pathways involved, allowing for optimization of HTL conditions to improve biocrude yield while minimizing undesirable oxygenated by-products. The findings contribute to the broader understanding of biomass conversion chemistry and highlight the value of advanced NMR techniques in monitoring and guiding the production of cleaner, more efficient algal biofuels.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Paul Hazendonk, whose guidance, patience, and support have been instrumental throughout the course of this research. His mentorship in Nuclear Magnetic Resonance (NMR) has not only broadened my technical knowledge but has also inspired me to grow as a scientist. I am especially thankful for his unwavering encouragement, both in times of success and during the more challenging moments of this journey.

I am also sincerely grateful to Dr. Peter Dibble for his valuable insights and continued support with the interpretation of my results and the progression of my project. His expertise and constructive feedback have been essential in refining the direction of my work.

My appreciation also extends to Dr. Matthew Bogard, whose guidance and knowledge have greatly contributed to my understanding of phytoplankton and its broad potential as a sustainable resource. His input has significantly enriched the biological perspective of my research.

I would also like to extend my sincere appreciation to Vincent Weiler for his invaluable technical assistance throughout my research. His help with experimental setups, equipment connections, troubleshooting, and finding practical solutions whenever a piece of the experimental setup and NMR experiment was missing was greatly appreciated and contributed significantly to the progress of this work.

I am deeply grateful to Dan Ferguson for generously sharing his knowledge and experience throughout the development and operation of the hydrothermal liquefaction system. Working with him provided me with the opportunity to learn not only about HTL experimentation, but also about the electronics, instrumentation, and physical principles involved in the system.

I would also like to sincerely thank Tony Montana, Craig Ellis, and Carl Holland for their valuable contributions to this project. Their willingness to share their expertise, resources, and time was greatly appreciated and contributed meaningfully to the completion of this research.

Beyond the academic sphere, I would like to express my heartfelt thanks to my sister, whose emotional support and encouragement have been a constant source of strength throughout my graduate studies. I am profoundly grateful to my parents for their unconditional love, kindness, and enduring belief in me. Their support and pride in my accomplishments have been the foundation upon which this journey was built. Finally, I would like to thank Barbara, whose dedication to bringing joy and cheer into my life is beyond what any human could achieve. I feel incredibly lucky to be this wonderful poodle's aunt.

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LIST OF ABBREVIATIONS

1-D — One-dimensional
2-D — Two-dimensional
AD — Anaerobic digestion
 B_0 — External magnetic field
 $CDCl_3$ — Deuterated chloroform
 CH_2Cl_2 — Dichloromethane
CMP-NMR — Comprehensive Multiphase Nuclear Magnetic Resonance
 CO_2 — Carbon dioxide
COSY — Correlation Spectroscopy
CPMG — Carr–Purcell–Meiboom–Gill
DEPT — Distortionless Enhancement by Polarization Transfer
DOSY — Diffusion-Ordered Spectroscopy
DP — Direct polarization
DPMAS — Direct Polarization Magic Angle Spinning
FID — Free Induction Decay
FFA — Free fatty acid
FTICR-MS — Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
FTIR — Fourier Transform Infrared Spectroscopy
GC–MS — Gas Chromatography–Mass Spectrometry
HMBC — Heteronuclear Multiple Bond Correlation
HSQC — Heteronuclear Single Quantum Coherence
HTC — Hydrothermal carbonization
HTL — Hydrothermal liquefaction
LCA — Life cycle assessment
LM — Levenberg–Marquardt
MAS — Magic Angle Spinning
MHz — Megahertz
NMR — Nuclear Magnetic Resonance
NS — Number of scans
PAH — Polycyclic aromatic hydrocarbon
ppm — Parts per million
PPE — Personal protective equipment
RF — Radiofrequency
SCWG — Supercritical water gasification
TAG — Triacylglycerol
 T_1 — Longitudinal relaxation time
 T_2 — Transverse relaxation time
TD — Time domain
TMS — Tetramethylsilane

CHAPTER 1: INTRODUCTION

1.1 Global Energy Landscape

The global population growth and increasing energy demands present a pressing challenge for humanity, with energy requirements projected to rise by 45% between 2020 and 2050.¹ Over the last century, fossil fuels have been the major energy source, underpinning most economic activities worldwide. Over the last century, fossil fuels have served as the major energy source, supporting most economic activities worldwide. Their continued consumption, however, is associated with significant drawbacks, including resource depletion, environmental pollution, and the growing threat of climate change.² These concerns highlight the urgent need for sustainable, renewable energy solutions. This upcoming scarcity threatens energy security worldwide, potentially leading to geopolitical conflicts and economic instability.²

The unsustainable nature of fossil fuel consumption is compounded by their finite reserves, which are being rapidly depleted. According to Gholizade (2023), current extraction rates suggest that easily accessible reserves could be exhausted within a few decades, emphasizing the need for alternative, renewable energy sources.³ The high costs and environmental impacts associated with fossil fuels underscore the importance of developing sustainable energy solutions to meet rising global demands without compromising ecological integrity.^{3,4} In response to these challenges, the scientific community has emphasized the urgency of transitioning to renewable energy sources, such as solar, wind, hydro, geothermal, and bioenergy.⁴

Among these, biofuels derived from biological materials have gained attention due to their potential for carbon neutrality and compatibility with existing fuel infrastructure.^{2,4} Specifically, phytoplankton-based biofuels offer a promising pathway owing to their high biomass productivity, ability to grow in non-arable land using wastewater or saline water, and high lipid content suitable for biodiesel production.^{4,5} The urgency of shifting toward phytoplankton biofuels is reinforced by the limitations of current biofuel production methods. For example, conventional biodiesel production from crops like soy and palm oil involves significant land, water, and fertilizer inputs, with associated environmental costs that threaten biodiversity and exacerbate climate change.^{2,4} Conversely, phytoplankton-based systems can produce high yields with minimal land use, and innovative processing techniques such as hydrothermal liquefaction and supercritical fluid extraction are being developed to convert phytoplankton biomass into energy-dense fuels efficiently.⁴ These developments are critical to overcoming economic and technical barriers to commercial-scale phytoplankton biofuel production.

The development of phytoplankton as a renewable energy resource represents a critical step toward reducing dependence on fossil fuels, preventing environmental pollution.⁴ Their cultivation can be integrated with carbon capture technologies, further enhancing their role in achieving a low-carbon economy.² As the global community seeks to meet the rising energy demands of a growing population sustainably, investing in phytoplankton-based biofuels and other renewable energy solutions emerges as an essential strategy to ensure ecological and economic resilience.^{4,5} Moreover, Connelly *et al.* (2015) emphasize that life cycle assessments (LCA) of phytoplankton biofuel systems consistently reveal significant environmental benefits, such as reductions in greenhouse gas emissions

and water use, when compared to fossil fuels and bioenergy crops. These findings support the argument that algae and cyanobacteria biofuels are a necessary and strategic component of a sustainable energy future.⁶

1.2 Phytoplankton as a Promising Biofuel Resource

Algae species and cyanobacteria have emerged as one of the most advantageous and versatile biofuel feedstocks, offering a combination of biological efficiency, environmental sustainability, and adaptability unmatched by terrestrial crops. Unlike initial biofuels made from food crops, which intensify land and water competition and require complex preprocessing of lignocellulosic biomass, phytoplankton can grow in diverse environments, including non-arable lands and wastewater, and thrive under saline conditions unsuitable for agriculture. This intrinsic flexibility removes pressure from agricultural systems and allows for the development of integrated production schemes that combine energy generation with waste remediation and carbon capture.^{2,7}

One of the defining advantages of phytoplankton lies in their extraordinary photosynthetic capacity and growth kinetics. Phytoplankton can double their biomass in as little as 24 hours under optimal conditions and often achieve photosynthetic efficiencies far exceeding those of terrestrial plants. This rapid biomass accumulation, combined with high areal productivity, enables significantly greater biofuel yields per hectare compared to traditional oil crops such as soybean or palm. Moreover, the cultivation of phytoplankton can be fine-tuned through environmental conditions such as light intensity, nutrient availability, and temperature, allowing for targeted manipulation of their biochemical profile. Depending on cultivation parameters and species selection, phytoplankton can

accumulate up to 70% of their dry weight as lipids—ideal for biodiesel or hydrothermal liquefaction (HTL)—while also offering substantial protein and carbohydrate fractions for conversion into alternative fuels or value-added co-products.⁸

In addition to their productivity, phytoplankton contribute positively to environmental stewardship. Their ability to sequester CO₂ during photosynthesis—sometimes directly from flue gases—supports global efforts to mitigate greenhouse gas emissions. These environmental services, when combined with energy generation, strengthen the role of phytoplankton in closed-loop and circular bioeconomy workflows. Phytoplankton can capture significant amounts of carbon dioxide through photosynthesis, with some species capable of fixing up to 1.83 kg of CO₂ per kilogram of phytoplankton biomass produced. Phytoplankton have demonstrated the ability to grow under elevated CO₂ concentrations, making them suitable for integration with industrial flue-gas carbon capture systems. Additionally, they can be cultivated using wastewater streams, enabling simultaneous nutrient remediation through the removal of nitrogen and phosphorus.^{5,9}

From a technological standpoint, phytoplanktons are also compatible with modern biorefinery concepts. Their biochemical diversity allows for the co-extraction of fuels, pigments, antioxidants, proteins, and other high-value compounds, enhancing the overall economic feasibility of phytoplankton-based systems. These co-products can offset production costs and open new avenues for market integration beyond the energy sector, including pharmaceuticals, cosmetics, and animal feed. Furthermore, their compatibility with thermochemical conversion techniques—particularly HTL—positions phytoplanktons as a feedstock capable of producing high-quality biocrude directly from wet biomass, avoiding the energy-intensive drying steps required for other technologies.

Altogether, phytoplanktons present a comprehensive solution to many of the limitations inherent in earlier generations of biofuels. Their rapid growth, high lipid yield, minimal resource demands, and environmental co-benefits establish them not merely as an alternative but as a superior and strategic feedstock in the transition toward sustainable and scalable biofuel production.^{2,7}

According to Feng *et al.* (2022), algal biomass does not require pretreatment to the extent needed for lignocellulosic feedstocks. Lipids can be directly extracted and converted into biodiesel or subjected to hydrothermal liquefaction (HTL) to produce biocrude. Phytoplanktons also demonstrate fast growth rates and the ability to consume large amounts of carbon dioxide, contributing to their environmental appeal.⁴

Recent studies emphasize these benefits. For instance, a 2023 review by Gholizade *et al.* in *Energy & Fuels* highlights the advances in bio-oil upgrading technologies from algal sources, demonstrating potential for high-energy-yield fuels with improved stability and composition.³ Similarly, Kumar *et al.* (2014), in their compositional analysis of algal biomass, emphasized the diversity of biochemical constituents in various algal species, which can be exploited for tailored fuel production strategies.¹⁰

Other research has explored integrated systems where wastewater treatment and biofuel production occur simultaneously. A 2011 study by Brentner *et al.* in *Environmental Science & Technology* used a combinatorial life cycle assessment to show that phytoplankton-based systems can outperform conventional biofuel systems in terms of land use, water footprint, and greenhouse gas emissions.⁹ These benefits extend beyond energy production, contributing to waste mitigation and nutrient recovery.

1.3 Phytoplankton Biology

Phytoplankton are microscopic photosynthetic organisms that live suspended in aquatic environments, including both freshwater and marine ecosystems. They harness solar energy through oxygenic photosynthesis, converting carbon dioxide and water into organic compounds and oxygen. Compared to terrestrial crops, phytoplankton imply much higher growth rates and photosynthetic efficiency. According to Biller and Ross(2011), the maximum photosynthetic efficiency of phytoplankton can reach approximately 3–9% of solar energy conversion, while that of terrestrial plants usually remains below 1.5% under field conditions.¹¹

This higher efficiency is partly due to phytoplankton's simple cellular structure, which allows for more effective light utilization and nutrient use. Additionally, many phytoplankton species can double their biomass under optimal conditions.¹² Such rapid growth enables the generation of significantly higher biomass yields per unit area than land-based crops.

1.3.1 Biochemical Composition and Fuel Potential

The appeal of phytoplankton for biofuel production lies not only in their rapid growth but also in their rich biochemical composition. Their cellular biomass typically includes lipids, proteins, carbohydrates, and other secondary metabolites whose relative proportions can be balanced through environmental and cultivation conditions such as nutrient limitation, light intensity, or temperature. For biofuel production, the lipid fraction—comprising mainly triacylglycerols (TAGs), free fatty acids (FFAs), and phospholipids—is of primary interest due to its convertibility into biodiesel.

Lipid content in microalgae and cyanobacteria can range from 20% to over 60% of dry weight, depending on the species and growth conditions. These lipids—especially triacylglycerols (TAGs)—are prime substrates for producing biodiesel through transesterification or can be thermochemically converted into crude-like bio-oil through hydrothermal liquefaction (HTL). Species such as *Nannochloropsis sp.*, *Chlorella vulgaris*, and *Scenedesmus obliquus* are particularly noted for their high lipid productivity. For example, *Nannochloropsis gaditana* can accumulate lipids up to 50% of its dry biomass under nitrogen-starved conditions.¹³

Proteins and carbohydrates also play vital roles in HTL pathways. Carbohydrates such as starch or cellulose can enhance bio-crude yields through Maillard reactions with amino acids during liquefaction, while proteins provide a nitrogen source that can influence product quality and nitrogen-containing heterocycles in the resulting bio-oil. Liu *et al.* (2016) emphasized that while high protein content (30–60% dry weight) can be beneficial for biomass value, it may increase the nitrogen content of bio-oils, potentially complicating upgrading steps for transportation fuel applications.¹³ Other than HTL methods, carbohydrates and proteins can be utilized as other forms of energy. For instance, carbohydrates may be fermented to produce bioethanol, while proteins can be extracted for animal feed or aquaculture applications.⁴

1.3.2 Species Used in Hydrothermal Liquefaction Research

In the context of HTL, several specific microalgae species and cyanobacteria have been widely investigated due to their favorable biochemical composition and relative ease of cultivation. Among the most extensively studied is *Chlorella vulgaris*, which is known

for its balanced composition of lipids, proteins, and carbohydrates and has been repeatedly tested in HTL studies; as reported by López Barreiro *et al.* (2013), *Chlorella vulgaris* typically contains about 20–30% lipids, 40–50% proteins, and 10–20% carbohydrates, depending on the cultivation phase.¹² Another important species is *Scenedesmus obliquus*, a green microalga that also exhibits high protein and lipid content, and which Biller and Ross (2011) highlighted for its robust performance under various cultivation conditions, making it a strong candidate for advanced applications. *Nannochloropsis sp.*, which is particularly rich in lipids, has been widely considered optimal for biodiesel and HTL bio-crude production; according to Liu *et al.* (2016), this species can achieve lipid concentrations as high as 50–60% when cultivated under stress conditions such as nitrogen limitation.^{11,13} *Porphyridium cruentum*, a red microalga containing valuable polysaccharides and lipids, has also been tested in HTL research due to its thick extracellular matrix and diverse biochemical profile.¹² Collectively, these species demonstrate that the choice of phytoplankton feedstock for HTL is strongly tied to both biochemical composition and cultivation feasibility, and several strains also show adaptability to industrial wastewater or CO₂-rich environments, adding environmental remediation as an additional advantage.

1.4 Harvesting and Cell Disruption Methods

Harvesting phytoplankton is a critical and energy-intensive step in biofuel production due to the small size (typically 2–20 µm) and low concentration (0.5–5 g/L) of phytoplankton cells in culture.⁹ Common harvesting methods include centrifugation, filtration, and flocculation. Centrifugation is highly efficient, achieving up to 95% cell

recovery, but it is energy-intensive and costly for large-scale operations.⁹ Filtration, using membrane or pressure filters, is effective for larger microalgae and cyanobacteria but prone to getting mixed with smaller species.

Flocculation is a low-energy alternative that aggregates phytoplankton cells using chemical or biological agents. Aluminum sulfate and chitosan are widely used flocculants, with chitosan being particularly attractive due to its renewable sourcing. Flocculant selection depends on the phytoplankton species, and dosage optimization is necessary to minimize costs and environmental impact.⁹

Cell disruption is essential for lipid extraction, with mechanical, chemical, and enzymatic methods being the most common. Mechanical techniques, such as ultrasonication and bead milling, are effective but energy intensive. Chemical methods, including acid or alkali treatment, are cost-effective but may generate inhibitory byproducts. Supercritical fluid extraction, particularly with CO₂ or methanol, offers a cleaner alternative with high selectivity and minimal solvent residues.¹⁴

1.5 Conversion Technologies for Biofuel Production

Phytoplankton biomass can be converted into renewable fuels through several thermochemical and biological processes designed to utilize the feedstock's high lipid content and rapid growth rates. Major conversion pathways include thermolysis, pyrolysis, hydrothermal carbonization (HTC), gasification, torrefaction, combustion, anaerobic digestion (AD), and supercritical water gasification (SCWG). These technologies differ in their operating conditions, energy efficiency, and product distribution, producing liquid fuels, solid fuels, or gaseous energy carriers.⁴

Among thermochemical methods, pyrolysis is one of the most widely studied techniques, where biomass is heated at 400–600 °C in the absence of oxygen to produce bio-oil, syngas, and biochar. Variations such as microwave-enhanced pyrolysis have demonstrated improved heating efficiency and higher bio-oil yields under optimized conditions.¹⁵ Despite these advantages, the resulting bio-oil often contains significant oxygen- and nitrogen-containing compounds, requiring upgrading processes such as catalytic hydrotreatment to improve fuel quality. Other thermochemical processes include hydrothermal carbonization and torrefaction, which convert biomass into carbon-rich solid fuels with improved combustion properties, and gasification, which produces syngas through partial oxidation at temperatures above 700 °C.⁴

Biological and advanced hydrothermal methods have also been explored. Anaerobic digestion converts organic matter into methane-rich biogas through microbial processes at relatively low temperatures, although conversion rates are slower compared with thermochemical methods.¹⁶ Supercritical water gasification uses water at supercritical conditions (above 374 °C and 22 MPa) to convert wet biomass into hydrogen-rich syngas without prior drying, but high operating pressures and reactor costs remain significant challenges.¹⁷ In general, while multiple conversion technologies exist, hydrothermal liquefaction has emerged as one of the most promising approaches for producing liquid bio-crude directly from wet phytoplankton biomass.

1.6 Hydrothermal Liquefaction (HTL) in Depth

Among the various thermochemical conversion pathways available for phytoplankton biomass, hydrothermal liquefaction (HTL) has emerged as one of the most

promising techniques for producing liquid biofuels. HTL directly converts wet phytoplankton biomass into bio-crude oil under subcritical water conditions, typically at temperatures of 200–350 °C and pressures of 10–20 MPa. This process mimics the natural geological formation of crude oil but accelerates it from a timescale of millions of years to only minutes or hours. A key advantage of HTL is its ability to process feedstock in their native aqueous state, eliminating the need for energy-intensive drying while preserving much of the feedstock's inherent chemical energy.⁴ Additionally, the use of catalysts under optimized temperature and pressure conditions can enhance product yields and reduce waste formation.¹⁸

Compared with other thermochemical technologies such as thermolysis, pyrolysis, hydrothermal carbonization (HTC), and gasification, HTL offers several advantages. Pyrolysis and thermolysis typically require extensive pretreatment and produce oils with high oxygen and nitrogen contents that require further upgrading. HTC mainly generates solid hydrochar rather than liquid fuels, while gasification prioritizes gaseous products such as syngas. In contrast, HTL provides a relatively efficient route for producing high-quality bio-oil directly from wet phytoplankton biomass. The lack of a drying requirement, higher liquid fuel yields, and improved bio-crude quality make HTL particularly attractive for sustainable biofuel production.^{4,14}

In HTL processing, wet phytoplankton slurries containing approximately 15–20 % solids are heated in a closed reactor at temperatures between 250 and 350 °C and pressures of 10–20 MPa. Under these conditions, the biomass is converted into several product phases, including biocrude oil, gaseous products, solid residues, and an aqueous phase. The conversion process begins with the hydrolysis of major phytoplankton macromolecules—

carbohydrates, proteins, and lipids—into smaller molecules such as sugars, amino acids, and fatty acids. These intermediates subsequently undergo dehydration, decarboxylation, Maillard reactions, and aromatization, forming the complex mixture that constitutes biocrude oil.¹⁴

Reaction conditions strongly influence product distribution and yield. Reaction times typically range from 5 to 90 minutes, with temperature playing a critical role in determining the dominant reaction pathways. Short reaction times at higher temperatures (around 400 °C) generally favor bio-oil formation, while longer reaction times at lower temperatures (around 250 °C) tend to produce greater quantities of aqueous-phase products.¹⁹ Maintaining sufficiently high pressure is also essential to keep water in the liquid phase and to enhance its polarity and catalytic properties, which facilitate acid–base reactions and improve mass transfer within the reaction medium.¹⁴ Biomass-to-water ratios between approximately 1:5 and 1:10 are typically used to balance reactant concentration and solvent availability for efficient conversion.¹⁴

Numerous studies demonstrate that HTL can produce bio-oils with favorable fuel properties.¹⁴ Two-step HTL processing has also been investigated as a strategy to improve conversion efficiency. In the case of *Enteromorpha clathrata*, a staged HTL approach increased oil yield and reduced solid residue formation by limiting prolonged exposure to high temperatures that can cause secondary decomposition reactions.²⁰

Solvents play critical roles in determining HTL reaction efficiency and product distribution. Water serves as the primary solvent in HTL systems, but the addition of co-solvents such as ethanol can enhance yields and selectivity. Ethanol promotes esterification reactions and has been shown to significantly increase oil yield when used in two-step HTL

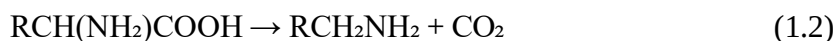
systems; for example, increasing ethanol concentration from 0 % to 50 % substantially improved oil production in HTL of *Enteromorpha clathrata*.^{20,14} The choice of catalyst can also shift product distributions; acid catalysts tend to promote aromatics formation through decarboxylation reactions, whereas alkaline catalysts favor hydrolysis and ester formation pathways.¹⁴

The chemical transformation pathways of individual components play an important role in controlling the composition of HTL products. During HTL, carbohydrates are first hydrolyzed into smaller sugar units, which can then undergo dehydration, fragmentation, and cyclization reactions to form organic acids, furans, and aromatic compounds. For example, glucose produced from carbohydrate hydrolysis dehydrate to form 5-hydroxymethylfurfural (HMF), as shown in the simplified reaction:



Further decomposition of sugar-derived intermediates can generate small organic acids such as acetic acid and formic acid, while subsequent cyclization and condensation reactions may contribute to the formation of aromatic structures in the bio-oil.¹⁴

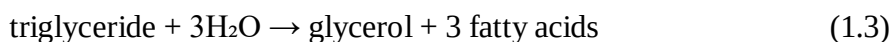
Proteins follow a different conversion pathway, beginning with hydrolysis into amino acids, followed by deamination, decarboxylation, and Maillard-type reactions with sugar-derived compounds. A simplified example is the decarboxylation of an amino acid to form an amine and carbon dioxide:



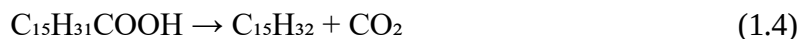
Amino acids can also undergo deamination, producing ammonia and organic acids or carbonyl-containing intermediates. These nitrogen-containing intermediates react further to form heterocyclic compounds such as pyrroles, pyridines, pyrazines, and indoles,

which increase the nitrogen content of the resulting bio-oil and can also promote char formation through Maillard polymerization reactions.²⁰

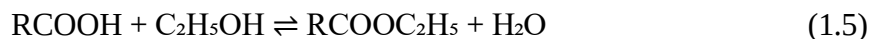
Lipids mainly undergo hydrolysis of triglycerides into free fatty acids and glycerol. A simplified hydrolysis reaction can be represented as:



The fatty acids may then undergo decarboxylation to produce long-chain hydrocarbons. For example, palmitic acid can decarboxylate to form pentadecane and carbon dioxide:



In alcohol-containing systems, fatty acids also undergo esterification, such as:



These equations form fatty acid ethyl esters that can improve the fuel-like character and calorific value of the oil.

Two-step HTL strategies can further enhance these reaction pathways by first generating intermediate compounds at lower temperatures and subsequently promoting aromatization and hydrocarbon formation at higher temperatures, ultimately increasing oil yield while reducing char formation.²⁰ In addition to biomass conversion, HTL has also been explored in broader environmental applications. Recent studies report that HTL can be applied to the conversion of plastic waste into valuable chemicals and fuel-like products, highlighting the versatility of hydrothermal processing technologies beyond biofuel production.²¹

1.7 Nuclear Magnetic Resonance (NMR) in HTL Bio-Oil Analysis

Analytical characterization of bio-oils produced by hydrothermal liquefaction (HTL) is fundamental for evaluating product quality and optimizing process parameters.²² Nevertheless, HTL bio-oils present significant analytical challenges due to their complex, multiphase nature and diverse chemical composition. Typical HTL products consist of an aqueous phase, gaseous fraction, solid residues, and a viscous biocrude containing both hydrophilic and hydrophobic components.²² The presence of water and suspended solids can interfere with direct analytical measurements and reduce quantification accuracy. In addition, the high viscosity and tendency of HTL bio-oil to undergo phase separation complicate sample handling and preparation for conventional instrumental analysis.²²

Traditional analytical methods such as gas chromatography–mass spectrometry (GC-MS) and Fourier-transform infrared spectroscopy (FTIR) provide useful information but have important limitations when applied to HTL bio-oils. GC-MS is widely used to profile volatile and semi-volatile compounds; however, its applicability is restricted to thermally stable and sufficiently volatile molecules capable of eluting from the chromatographic column.²³ Many heavy fractions and oxygenated oligomers present in HTL oils remain non-volatile under standard GC conditions, resulting in incomplete chromatographic representation of high-molecular-weight components.²³ Furthermore, analysis of aqueous HTL process waters often requires derivatization steps, such as methyl chloroformate treatment, to detect polar compounds, which can introduce additional processing steps and potential artifacts.²³ FTIR spectroscopy provides rapid functional-group identification, but severe spectral band overlap in complex mixtures can obscure subtle structural differences and limit reliable compound identification.²²

Nuclear Magnetic Resonance (NMR) spectroscopy overcomes many of these limitations by enabling direct and non-destructive observation of molecular nuclei in intact bio-oil samples.²⁴ Comprehensive multiphase NMR (CMP-NMR) can differentiate liquid, gel, and solid phases within heterogeneous systems, allowing the chemical and physical properties of HTL products to be examined without extensive fractionation.²⁴ The high chemical-shift dispersion of ^{13}C NMR enables resolution of overlapping functional groups, facilitating identification of aliphatic chains, aromatic structures, and oxygen-containing heterocycles in complex mixtures.²⁴ Unlike GC-MS, NMR does not require volatility or derivatization, allowing detection of the entire molecular-weight range present in bio-oils, including non-volatile oligomers and macromolecules.²⁴

NMR spectroscopy probes the magnetic behavior of atomic nuclei in an external magnetic field to provide information about molecular structure and dynamics. The resonance frequency of a nucleus is determined by the Larmor relationship ($\omega_0 = \gamma B_0$), and local electronic shielding produces chemical shifts (δ) that distinguish different carbon environments, such as aliphatic (0–50 ppm), O-alkyl (50–100 ppm), aromatic (100–160 ppm), and carbonyl groups (160–220 ppm) in ^{13}C spectra.²⁵ Solid-state techniques such as magic-angle spinning (MAS) reduce anisotropic interactions and improve spectral resolution in heterogeneous materials, while cross-polarization (CP) enhances signal intensity by transferring polarization from abundant ^1H nuclei. Direct polarization (DP) methods allow quantitative measurement of carbon functional groups within complex matrices.^{25,26} These techniques enable detailed characterization of bio-oil residues derived from HTL, which often contain both mobile aliphatic chains and rigid aromatic structures

²⁷.

Both solution-state and solid-state NMR experiments are widely applied in biofuel research. One-dimensional NMR spectra provide the fundamental chemical-shift fingerprint of a sample, while two-dimensional experiments such as HSQC and COSY correlate nuclei across two frequency dimensions, allowing overlapping signals to be resolved and molecular connectivity to be established.²⁸ In addition, relaxation measurements provide information about molecular mobility and aggregation. The spin-lattice relaxation time (T_1) reflects interactions with the surrounding molecular environment, whereas the spin-spin relaxation time (T_2) reflects local dipolar interactions and molecular motion on micro- to millisecond timescales.²⁶ Studies of oil-sand asphaltenes, for example, revealed biexponential T_2 decay corresponding to rigid aromatic cores and more mobile aliphatic regions, consistent with hierarchical aggregation models.^{26,28} These approaches allow differentiation of high-molecular-weight aromatic clusters from smaller aliphatic molecules within HTL bio-oil systems.

Although direct NMR studies of HTL-derived bio-oils remain relatively limited, analogous studies on related carbon-rich materials demonstrate the technique's suitability for such complex systems. Solid-state ^{13}C NMR with direct polarization and magic-angle spinning (DPMAS) has been used to quantify carbon functional groups in heterogeneous organic matrices.²⁵ Romero *et al.* (2020) applied DPMAS ^{13}C NMR to biochar-manure composites, revealing significant increases in fused aromatic domains following biochar addition.²⁷ Complementary studies combining NMR with high-resolution mass spectrometry have further demonstrated the ability of these techniques to reveal molecular-level transformations during thermal processing.^{27,29} These findings highlight the value of

combining solution-state and solid-state NMR methods to resolve the structural complexity of HTL-derived bio-oils.

Overall, NMR spectroscopy provides a comprehensive analytical framework for studying HTL bio-oils. Unlike traditional chromatographic methods that focus on specific compound classes, NMR offers a holistic view of the entire sample and allows quantitative determination of functional-group distributions without prior separation.²⁵ Solid-state NMR can quantify aromatic and aliphatic carbon fractions in insoluble residues, while solution-state experiments reveal molecular connectivity within soluble fractions.²⁸ Relaxation measurements further provide insight into molecular size and aggregation behavior.²⁶ Together, these capabilities make NMR an indispensable tool for investigating the chemical composition and transformation pathways of bio-oils produced through hydrothermal liquefaction.

1.8 Research Gaps, Objectives, and Research Questions

Rapidly rising global energy demand and the environmental consequences associated with fossil fuel consumption have driven extensive research into renewable energy alternatives. First- and second-generation biofuels, derived from food crops and lignocellulosic biomass, have been widely investigated; however, these approaches remain limited by high resource requirements, land competition, and technological complexity, which restrict their sustainability and scalability.^{2,7} In contrast, third-generation biofuels derived from microalgae and cyanobacteria offer several advantages, including rapid growth rates, minimal competition with arable land, and the capacity to sequester carbon dioxide directly through photosynthesis.^{4,5}

1.8.1 Research Gaps

Despite the growing interest in phytoplankton-based biofuels, important research gaps remain in understanding how phytoplankton biomass is converted into bio-crude during HTL and how the resulting products can be effectively characterized. These gaps limit the ability to connect processing conditions with molecular composition, product quality, and overall conversion efficiency. Addressing these limitations is necessary for improving the design, optimization, and interpretation of HTL systems.

First, there is an incomplete understanding of the molecular transformations that occur during hydrothermal liquefaction (HTL). Although HTL has demonstrated strong potential for converting wet biomass into energy-dense biocrude, the specific reaction pathways through which lipids, proteins, and carbohydrates transform under varying temperature, pressure, and catalytic conditions remain poorly understood.^{18,30} Without detailed knowledge of these chemical pathways, optimization of HTL processes often relies on empirical experimentation rather than predictive understanding.

Second, the application of comprehensive analytical techniques to HTL products remains limited. Conventional analytical approaches such as gas chromatography–mass spectrometry (GC–MS) and Fourier-transform infrared spectroscopy (FTIR) are effective for identifying volatile compounds and functional groups, but they often fail to fully capture the complexity of HTL bio-oils, which contain non-volatile oligomers, nitrogen- and oxygen-rich heterocycles, and solid residues.^{22,23} While nuclear magnetic resonance (NMR) spectroscopy has been applied in related areas such as asphaltene aggregation and biochar characterization, its application to phytoplankton HTL products remains relatively limited.^{27,28}

Third, the molecular-level evolution of phytoplankton HTL products under different processing severities remains insufficiently understood. Although temperature and residence time are known to strongly influence HTL conversion, fewer studies have examined how these conditions alter the structural features of the resulting bio-crude, particularly across different phytoplankton species. In this context, comparing *Chlorella* and *Spirulina* provides an opportunity to examine how species-dependent biomass characteristics are reflected in the development of aliphatic, oxygenated, and aromatic regions during HTL. Advanced NMR techniques can therefore help clarify how increasing HTL temperature affects molecular composition, spectral complexity, and relaxation behavior in HTL-based bio-crudes.¹⁹ Addressing these knowledge gaps is essential for improving the predictability and efficiency of HTL processes.

To address these limitations, the present study applies an analytical framework based on advanced nuclear magnetic resonance (NMR) spectroscopy, including solution-state techniques. Unlike traditional analytical methods that primarily target specific compound classes, NMR provides a comprehensive profile of complex organic mixtures, enabling both qualitative structural interpretation and quantitative characterization of volatile and non-volatile fractions.

Furthermore, this research extends beyond static chemical characterization by incorporating analytical modeling strategies. These approaches allow spectral data to be interpreted in terms of underlying chemical transformation pathways occurring during HTL. Such insights contribute to a more mechanistic understanding of biomass conversion and support the development of predictive models linking feedstock composition and processing parameters to product distribution.

1.8.2 Research Objectives

The primary objective of this research is to optimize the hydrothermal liquefaction (HTL) process for biomass in order to maximize biofuel yield while improving the efficiency and sustainability of the conversion process. This objective is pursued through systematic investigation of key reaction parameters, including temperature and pressure to determine conditions that produce high-quality biofuels while minimizing waste formation.

A second objective of this study is to develop a reliable analytical methodology for characterizing the chemical transformations occurring during HTL. By employing advanced NMR-based analytical techniques, the research aims to investigate how the chemical composition of phytoplankton bio-oil evolves under different processing conditions, providing insights into the distribution of molecular fractions across the liquid, solid, and gaseous phases.

Finally, this work evaluates the broader potential of phytoplankton-derived biofuels as a sustainable renewable energy resource by considering their energy content and environmental implications. Through this analysis, the study contributes to ongoing global efforts to reduce dependence on fossil fuels, mitigate climate change, and promote sustainable bioenergy systems.

1.8.3 Research Questions

Based on the objectives outlined above, this research addresses the following key questions:

1. The HTL process involves subjecting biomass to elevated temperatures and pressures, which strongly influence reaction pathways and bio-oil formation. Variations in these parameters affect chemical transformations, bio-oil yield, and the formation of by-products. What temperature and pressure conditions optimize biofuel yield while minimizing energy consumption and waste generation?
2. How do *Chlorella pyrenoidosa* and *Spirulina platensis* differ in the molecular composition of their HTL-derived bio-oils, particularly in the aliphatic, oxygenated, olefinic, and aromatic regions?
3. Traditional analytical methods such as GC–MS provide valuable information on volatile compounds but do not fully capture the complexity of HTL-derived bio-oils. How can nuclear magnetic resonance (NMR) spectroscopy be applied to improve understanding of the chemical transformations occurring during HTL and support optimization of phytoplankton biofuel production?
4. How do T_1 and T_2 relaxation measurements contribute to understanding molecular mobility, spectral broadening, and aggregation-related behavior in phytoplankton HTL bio-oils produced under different HTL severities?

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CHAPTER 2: METHODOLOGY

2.1 Introduction to Methodology

2.1.1 Overview of Experimental Design

This research was developed to explore the potential of phytoplankton as a feedstock for renewable fuel production and to investigate the chemical transformations that occur during hydrothermal liquefaction (HTL). The overall plan combined thermochemical processing with advanced spectroscopic analysis, allowing both product yields and molecular structures to be examined.

The methodology can be divided into three main stages. First, phytoplankton biomass was prepared and conditioned for hydrothermal liquefaction experiments. This step ensured consistent feedstock quality and allowed meaningful comparisons across experimental runs. Second, the biomass underwent HTL under carefully controlled conditions of temperature and holding time. The processing parameters, namely temperature, holding time, and pressure, were systematically varied within well-defined ranges to evaluate their effects on oil yield and product distribution, including the formation of gaseous products and the water-soluble phase. Finally, the resulting products were analyzed using a combination of traditional analytical tools and Nuclear Magnetic Resonance (NMR) spectroscopy, which provided detailed molecular-level insights into the complex mixture of compounds formed.

This integrated approach was chosen to ensure that both macroscopic results (such as yield and phase distribution) and microscopic details (such as molecular transformations and relaxation behaviors) were captured. By combining multiple layers of analysis, the

design provided a comprehensive picture of the HTL process, supporting both empirical observations and mechanistic interpretations.

This chapter provides an overview of the materials, instruments, and procedures used throughout this research. It outlines how samples were prepared, how HTL experiments were carried out, and how the resulting products were processed and analyzed. The chapter also summarizes the analytical techniques, including NMR spectroscopy and data-acquisition methods—that were employed to characterize the chemical composition and relaxation behavior of the samples. Together, these sections establish the methodological foundation of the study and ensure that the experiments described in later chapters can be clearly understood and reproduced.

2.1.2 Reason Behind Chosen Techniques

Hydrothermal liquefaction (HTL) was selected as the core conversion technique because it is especially suited to phytoplankton feedstocks. Unlike pyrolysis or transesterification, HTL processes wet biomass directly, eliminating the need for energy-intensive drying. This feature is critical when dealing with feedstock, which typically contain more than 80% water. The technique also enables simultaneous conversion of lipids, proteins, and carbohydrates, providing higher overall carbon recovery compared to lipid-extraction-based routes. By adjusting temperature, pressure, and catalysts, HTL can generate energy-dense bio-oils that are closer in quality to petroleum-derived fuels.

One of the main challenges with HTL is that the bio-oil it produces is very complex. Standard techniques such as GC–MS can identify the lighter, volatile compounds, but they often miss the heavier or more polar molecules. To deal with this problem, this study uses

Nuclear Magnetic Resonance (NMR) spectroscopy as a main tool. NMR is powerful because it can characterize whole mixtures without the need for complicated separation steps. It also gives information about the types of functional groups, the carbon structure, and how different molecules interact—details that other methods cannot easily provide.

To strengthen the interpretation of HTL results, simpler model systems such as asphaltenes and crab oil samples were also examined. These materials have been studied in the context of petroleum and heavy oil research, and their well-documented spectral features make them valuable benchmarks. By comparing phytoplankton-derived bio-oils against these more established materials, it was possible to validate spectral assignments and highlight unique differences in composition and relaxation behavior.

Furthermore, relaxation-based NMR experiments (T_1 and T_2) provide dynamic information about molecular environments. These measurements are especially valuable for understanding bio-oils, which often contain asphaltene-like species, aggregated molecules, and viscous fractions. By combining HTL with advanced NMR analysis, the research goes beyond bulk yield measurements and investigates the chemistry at a more precise level, addressing an important gap in the current literature.

2.1.3 Reproducibility, Accuracy, and Safety Considerations

Ensuring reproducibility and accuracy was a key consideration throughout the methodology. For each HTL experiment, identical procedures were followed in biomass preparation, reactor loading, and post-reaction handling. Multiple replicates were performed to account for variability, and control experiments were conducted under

baseline conditions to compare with modified runs. Product yields were determined gravimetrically, and care was taken to minimize losses during separation and storage.

In NMR experiments, reproducibility was ensured through relaxation delays and acquisition parameters. Each sample was prepared with controlled concentration and solvent conditions, and experiments were repeated where necessary to confirm results. Signal processing, including baseline corrections, was carried out using standardized software routines on Mnova and Topspin to reduce operator bias.

As part of reproducibility testing, asphaltene and crab oil samples were also analyzed alongside HTL products. Their simpler and more predictable spectral patterns served as internal checks, ensuring that experimental parameters and analysis workflows were reliable before being applied to more complex phytoplankton mixtures.

Safety considerations were also central to the design. Hydrothermal liquefaction involves working at high temperatures (200-350 °C) and elevated pressures (10-20 MPa). For this reason, all experiments were performed in a sealed reactor system equipped with pressure relief valves and monitored using appropriate safety protocols. Personal protective equipment (PPE) was always used, and reactor operations were performed in accordance with institutional laboratory safety guidelines. In addition, solvents used in product recovery and NMR sample preparation, such as chloroform (CDCl_3), were handled in fume hoods with waste disposal practices.

Overall, the methodology was structured to maximize reproducibility and accuracy while minimizing risks. This ensured that the data collected were not only scientifically valid but also generated safely and responsibly.

2.2 Sample Preparation

2.2.1 Sample Selection, Preparation, and Handling

The selection of phytoplankton species and their preparation methods play a critical role in determining biomass consistency and suitability for downstream processing. Previous studies have shown that microalgae strains such as *Chlorella pyrenoidosa* and cyanobacteria like *Spirulina platensis* are typically cultivated under controlled conditions, including temperatures of 24–25 °C, light intensity of approximately 2000 lux, continuous aeration, and a 12:12 light–dark photoperiod to enhance biomass productivity and lipid accumulation.¹ Harvesting of such cultures is often energy-intensive due to the dilute nature of their suspensions, with centrifugation and flocculation being the most commonly applied techniques. While centrifugation provides high recovery efficiency, flocculation offers a lower-energy alternative, though concerns remain regarding the environmental impact of chemical additives.² Furthermore, additional cell disruption methods, including mechanical lysis, microwave-assisted extraction, and supercritical CO₂ processing, are frequently employed to access intracellular lipids, but these approaches can significantly increase overall energy demand.²

In contrast to these conventional workflows, the present study did not involve cultivation, harvesting, or cell disruption steps. Instead, pre-dried and finely powdered biomass was directly sourced from a commercial supplier (Creative Enzymes, Shirley, NY, USA), ensuring a standardized and reproducible feedstock. Two phytoplankton strains, *Chlorella pyrenoidosa* and *Spirulina platensis*, were selected based on their well-documented biochemical composition and availability in bulk quantities. According to the provided certificates of analysis, both samples exhibited high protein content, low moisture

and ash levels, and negligible concentrations of heavy metals, microbial contaminants, pesticides, and residual solvents. These characteristics confirmed the high quality and suitability of the biomass for experimental use.

Because the biomass was supplied in a pre-dried powdered form, no additional drying, harvesting, or disruption treatments were required. This eliminated the need for energy-intensive processing steps and minimized the risk of altering the chemical composition of the samples. For experimental preparation, the powders were simply dispersed in water to form homogeneous slurries, followed by gentle mixing to ensure consistency across replicates. The materials were stored in a dark, dry, and temperature-stable environment to prevent moisture uptake and oxidative degradation prior to use.

2.2.2 Crab Oil and Asphaltene Samples

Crab oil was selected as a low-molecular weight model system to represent the more mobile, hydrocarbon-rich fraction of complex bio-oil mixtures. Its relatively sharp aliphatic resonances provided a useful reference for interpreting the aliphatic regions of the HTL bio-oils. In contrast, asphaltenes represent the aromatic and heavier fractions, consisting primarily of polyaromatic hydrocarbons with heteroatoms such as nitrogen, oxygen, and sulfur. Their structure includes fused aromatic cores with alkyl side chains, leading to strong intermolecular interactions and aggregation behavior that depends on solvent, concentration, and temperature. These properties make asphaltenes particularly suitable as analogues for the heavier, more complex components of bio-oils.³

Model samples were prepared at different concentrations and analyzed in CDCl_3 to evaluate the influence of solvent polarity and molecular environment on chemical shifts,

relaxation times, and signal intensities. These comparative studies enabled the identification of relationships between molecular group composition and relaxation behavior, providing a foundation for interpreting more complex spectra. Additionally, the use of these systems was essential for optimizing NMR acquisition parameters, as reliable T_1 and T_2 measurements depend on appropriate repetition delays and scan numbers. Once validated, these parameters were applied to phytoplankton bio-oils to ensure quantitative reliability.

The asphaltene samples used in this work were sourced from the Manifa oil field and exhibited consistent molecular structures with systematic variations in aggregation behavior. Previous studies have shown that increasing reservoir depth correlates with larger aggregate sizes, while maintaining similar aromatic compositions, making these samples suitable for validating NMR methods and relaxation analysis.³

In addition, a crab-derived bio-oil produced via slow pyrolysis at Cape Breton University was used as a marine-based model system. The pyrolysis process involved a gradual heating rate of $2\text{ }^{\circ}\text{C min}^{-1}$ to $450\text{ }^{\circ}\text{C}$, followed by a 90-minute hold, yielding a nitrogen- and oxygen-rich liquid fraction characteristic of marine biomass conversion. This sample provided a relevant reference for studying molecular transformations and optimizing NMR-based compositional analysis of complex bio-oils.

2.2.3 Solvents

Chloroform-d (CDCl_3) was selected as NMR solvents for dissolving the oil samples because both provide excellent solubility for the highly hydrophobic and chemically diverse components typically present in bio-oils and asphaltenes. The oils generated from

hydrothermal liquefaction contain a mixture of long-chain hydrocarbons, aromatic compounds, esters, heteroatom-containing groups, and heavier resinous species.⁴ Due to their non-polar to moderately non-polar character, CDCl₃ effectively dissolves these species without inducing chemical reactions or altering functional groups. Its relatively low viscosity also improves molecular tumbling, resulting in sharper lines and more reliable relaxation measurements compared with polar or highly viscous deuterated solvents. In addition, the solvents provides strong deuterium lock signals and have well-defined solvent peak, minimizing spectral interference in the chemical-shift regions where bio-oil components resonate.

CDCl₃ offers a clean reference environment for both aliphatic and aromatic resonances, while its narrow solvent peak at 7.26 ppm is easy to suppress or exclude. These properties improve shimming performance, stabilize the magnetic-field lock, and produce spectra with consistent line widths and chemical-shift positions. Because the solvents dissolve a wide range of hydrocarbon and heteroatom-containing species uniformly, they reduce variability in signal intensity, relaxation behavior, and overall spectral quality, making the NMR measurements highly reproducible across different sample batches and experimental runs.

To evaluate concentration-dependent behavior in these model systems, three sample loadings—5 mg, 15 mg, and 50 mg—were prepared for both crab oil and asphaltenes in the NMR solvent. These concentrations were selected to capture a representative range of solute–solvent interactions and to ensure that both low- and high-intensity spectral features could be observed. For the crab oil samples, lower concentrations (5 and 15 mg) provided spectra dominated by well-resolved lipid resonances with minimal viscosity effects, while

the higher concentration (50 mg) allowed assessment of peak broadening, changes in relaxation behavior, and the emergence of minor components that may only become visible at elevated sample loading. In the case of asphaltenes, these same concentrations were chosen to deliberately probe their characteristic aggregation and coagulation behavior. Asphaltenes are known to self-associate in solution, and their aggregation state is strongly dependent on concentration, solvent environment, and aromatic content. Using 5, 15, and 50 mg samples enabled systematic comparison of spectral broadening, relaxation changes, and solubility limits as the system transitioned from predominantly monomeric or nanoaggregate states toward larger associated structures. This concentration series therefore provided a controlled framework for evaluating how molecular environment, aggregation, and solvent choice influence the NMR response of both model materials, ultimately guiding parameter optimization for the more complex bio-oil samples.

2.3 NMR

2.3.1 Nuclear Magnetic Resonance

Atoms contain nuclei made up of protons and neutrons, and many of these nuclei possess an intrinsic property called spin. Nuclear spin behaves like a small magnetic moment, meaning many spinning nuclei act as tiny magnets. However, only nuclei with an odd number of protons, neutrons, or both (such as ^1H , ^{13}C , ^{15}N , ^{19}F , ^{31}P) exhibit this magnetic behavior, making them observable in NMR spectroscopy. Nuclei with a non-zero spin have an intrinsic angular momentum $\hbar I$ that is related to the spin quantum number I which is expressed by Eq 2.1:

$$\frac{\hbar}{h} = \sqrt{I(I+1)} \quad (2.1)$$

where $\hbar = h/2\pi$, h is Planck's constant, and I is the spin quantum number. The spin quantum number I can take on positive integer values (0, 1, 2..., n) or half-integer values (1/2, 3/2, ..., $n/2$). Based on classical electromagnetism, the magnetic dipole moment ($\vec{\mu}$) of a nucleus arises because of rotation associated with the spin angular momentum ($\vec{S}$) of the nucleus which is expressed by Eq:

$$\vec{\mu} = \frac{e\hbar}{2m_p} \vec{S} \quad (2.2)$$

The energy of interaction between an applied magnetic field $\vec{B}_0$ and a magnetic moment $\vec{\mu}$ can be expressed as follows:

$$E = -\vec{\mu} \cdot \vec{B}_0, \quad \vec{B}_0 = (0, 0, B_0) \quad (2.3)$$

When no magnetic field is applied, the orientation of these nuclear magnets is random, and the net magnetization of a sample is zero. Once an external magnetic field (B_0) is applied, however, the nuclear magnetic moments align either with or against this field. These two possible orientations correspond to different energy levels — a lower energy state (parallel to B_0) and a slightly higher one (antiparallel to B_0). Although the energy difference between these states is very small, it becomes measurable at high magnetic fields and forms the basis for NMR detection.

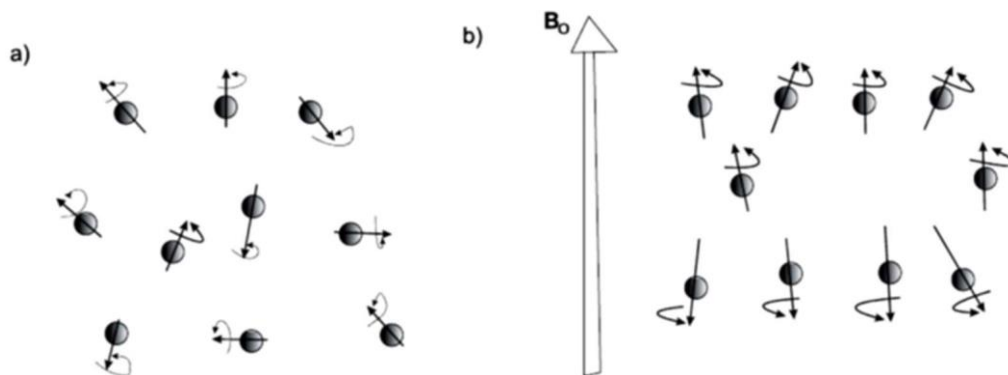


Figure 2.1 Distribution of spin magnetic moments in the a) absence and b) presence of an external magnetic field B_0 .⁵

The population of nuclei in the two states follows the Boltzmann distribution, meaning that slightly more nuclei occupy the lower-energy state. This small imbalance creates a net magnetization vector in the direction of B_0 , which can then be manipulated using radiofrequency (RF) pulses. The frequency at which a particular nucleus precesses around B_0 is called the Larmor frequency:

$$\omega_0 = \gamma B_0 \quad (2.4)$$

where γ is the gyromagnetic ratio specific to each nucleus. This frequency determines the resonance condition and explains why nuclei in different elements — or even in different chemical environments — resonate at different points in the NMR spectrum.

When an RF pulse at the Larmor frequency is applied, it tips the net magnetization away from the B_0 axis, usually into the transverse plane. After this pulse, the nuclei begin to relax back to equilibrium, generating an oscillating magnetic signal that can be detected by the receiver coil. This decaying signal is called the free induction decay (FID).

The detected time-domain signal, called the FID, is described by:

$$S(t) = S_0 \exp(-t/T_2) \exp(i\omega_0 t) \quad (2.5)$$

where S_0 is the initial signal amplitude, t is time, and T_2 is the transverse relaxation time. Applying a Fourier transform converts the FID into the frequency domain, producing the NMR spectrum whose peak width is inversely related to T_2 ($1/\pi T_2$).

By applying a Fourier transform to the FID, the data are converted from the time domain into a frequency-domain spectrum, showing peaks at positions characteristic of each nucleus and its surrounding electronic environment.³

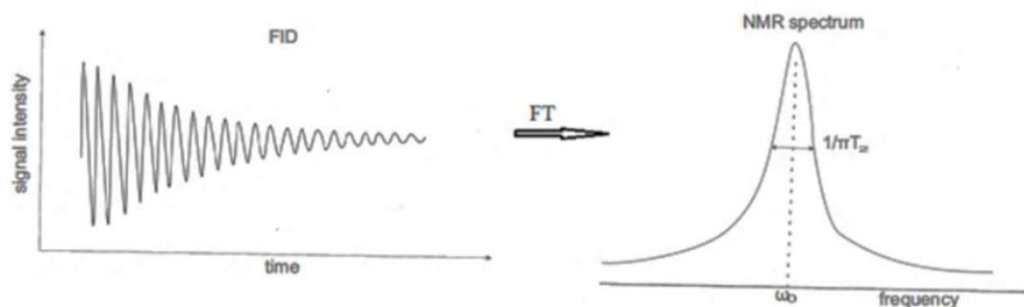


Figure 2.2 The FID is digitized, and the NMR spectrum is then generated using the Fourier Transform. $1/\pi T_2$ gives the full width at half height.⁵

The strength of the magnetic field determines the sensitivity and resolution of the experiment. Higher-field instruments (e.g., 700 MHz spectrometers) produce larger energy separations and therefore stronger signals, allowing for finer chemical-shift resolution. In NMR, these chemical shifts (reported in ppm) depend on how the electrons surrounding a

nucleus shield it from B_0 . Consequently, even small changes in molecular structure or bonding can shift the resonance frequency, enabling detailed structural interpretation.

2.3.2 Nuclear Shielding, Chemical Shift and J Coupling

When a nucleus is placed in an external magnetic field (B_0) the surrounding electrons circulate and generate small local magnetic fields that oppose the applied field. This phenomenon is known as nuclear shielding. As a result, each nucleus experiences a slightly reduced effective magnetic field (B_{eff}) described by the equation:

$$\vec{B}_{\text{eff}} = \vec{B}(1-\sigma), \quad \vec{B} = (0, 0, B_0) \quad (2.6)$$

where σ is the shielding constant. The magnitude of σ depends on the local electron density and molecular geometry. Nuclei in electron-rich environments are more shielded and therefore resonate at lower frequencies, while nuclei near electronegative atoms or within electron-withdrawing groups are deshielded and resonate at higher frequencies.

The resonance position of a nucleus relative to a standard compound such as tetramethylsilane (TMS) is expressed by the *chemical shift*, δ , given by equation 2.7 and reported in parts per million (ppm).

$$\delta = \frac{V_{\text{sample}} - V_{\text{ref}}}{V_{\text{ref}}} \times 10^6 \quad (2.7)$$

Because the difference in frequency is extremely small compared with the spectrometer frequency, the chemical shift scale conveniently allows comparison between spectra recorded at different field strengths. The chemical shift is therefore a direct reflection of the local electronic environment of each nucleus.

In addition to the effect of local electron density, the induced magnetic field can also vary due to the anisotropy of certain chemical groups. In conjugated π systems such as alkenes, alkynes, and aromatic rings, the application of B_0 causes the delocalized electrons to circulate in closed loops. The magnetic field generated by these π -electron currents opposes B_0 inside the loop and reinforces it outside. This phenomenon is known as the ring current effect. In benzene, for instance, nuclei located in the plane of the ring experience a reinforced field and become deshielded, resonating downfield near 7–8 ppm. In contrast, nuclei located above or below the aromatic plane lie within the shielding cone of the induced field and appear up field, sometimes even at negative chemical shifts. Similar anisotropic effects occur in alkyne and carbonyl systems. In an alkyne, the π -electrons create a cylindrical shielding region along the $C\equiv C$ bond, causing the acetylenic protons to appear near 2–3 ppm, while in carbonyl and alkene groups, the induced magnetic field partially deshields nearby protons, moving their resonances to higher δ values. Thus, the combined effects of electron density and magnetic anisotropy determine the exact resonance frequency of each nucleus.

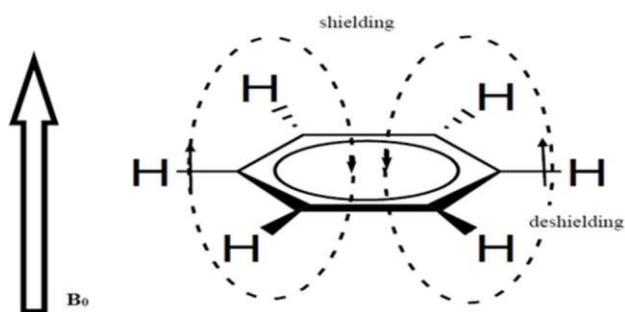


Figure 2.3 The ring current effect in benzene rings.⁵

Apart from these shifts in resonance position, nuclei also interact with one another through the bonding electrons, producing spin–spin coupling, often referred to as scalar coupling. This interaction does not arise from direct magnetic dipole interactions through space but from transmission of spin information via the bonding electrons. When one nucleus is placed in a magnetic field, its nuclear magnetic moment polarizes the electrons in the bonding orbital, and this polarization is transferred through the bond to neighboring nuclei. The mechanism, known as the Fermi contact interaction, results in a small modulation of the local magnetic field at the second nucleus, which causes its resonance to split into multiple lines. The magnitude of this splitting is characterized by the coupling constant J , measured in hertz (Hz).

The scalar coupling Hamiltonian can be expressed as $2\pi J \hat{I} \cdot \hat{S}$, where I and S are the spin angular momentum operators of the coupled nuclei. The coupling constant J depends on the number of bonds separating the nuclei, the type of bonding orbital, and the relative orientation of the atoms. One-bond couplings (^1J) are typically large, as observed in ^{13}C – ^1H or ^{15}N – ^1H pairs, while two- and three-bond couplings (^2J and ^3J) are smaller and are responsible for the multiplet patterns observed in ^1H NMR spectra. The size of ^3J couplings often follows the Karplus relationship, which correlates the coupling constant with the dihedral angle between the coupled nuclei.

Spin–spin coupling provides detailed information about the number and arrangement of neighboring atoms. For example, a proton bonded to a carbon atom bearing two equivalent neighboring protons will appear as a triplet due to the three allowed spin combinations of its neighbors. These splitting patterns reveal the number of adjacent spins

and thus the molecular connectivity. The sign and magnitude of J also depend on

hybridization and bond character; σ -bonds transmit coupling more efficiently than π -bonds, and s-character in the bond generally increases J.

In liquids, the direct dipole–dipole interaction between nuclear magnetic moments averages to zero because of rapid molecular tumbling. However, this interaction contributes to relaxation processes and to polarization transfer in more advanced multidimensional NMR experiments. The scalar coupling, being transmitted through the bonding electrons, remains unaffected by molecular motion and therefore appears as a permanent splitting pattern in the spectrum.⁶

In summary, both chemical shift and scalar coupling originate from the response of nuclear spins to their electronic and magnetic surroundings. Chemical shift reflects variations in electron density and magnetic anisotropy, while J coupling arises from the polarization of bonding electrons that communicate spin information between nuclei. Together they form the basis of structural interpretation in NMR spectroscopy, allowing the identification of functional groups, bond connectivity, and conformational details of complex molecules.⁶

2.4 Quantitative NMR Approaches

2.4.1 Principles of Quantitation

NMR is inherently quantitative because signal intensity (integrated peak area) is directly proportional to the number of spins (nuclei) contributing to the resonance:

$$S \propto N \cdot (1 - e^{-tR/T_1}) \quad (2.8)$$

where S is the measured intensity, N is the number of contributing nuclei, t_R is the relaxation delay, and T_1 is the spin-lattice relaxation time. Provided that relaxation delays are chosen long enough ($5 \times T_1$), signal areas accurately represent molar ratios.

2.4.2 External Standard Calibration

Burton, Quilliam, and Walter (2005) demonstrated the effectiveness of external standards in quantitative ^1H NMR. By sealing standards in separate but identical tubes, they achieved precision of $\sim 1\%$, overcoming limitations of internal standards when analytes are unstable, hygroscopic, or hazardous.⁷ This method is particularly relevant for toxins and metabolites that are difficult to weigh accurately.

2.4.3 Applications to Phytoplankton Biomass

Quantitative ^1H and ^{13}C NMR have been applied to estimate total lipid content in phytoplankton and to characterize fatty acid composition. For example, the ratio of methylene to olefinic proton integrals provides an estimate of unsaturation. Similarly, ^{13}C spectra allow direct calculation of chain length distributions by comparing carbonyl, methylene, and olefinic carbon peaks.⁴

In red algae, isotopic enrichment studies have provided deeper metabolic insights. Simon-Colin *et al.* (2004) used ^{13}C -enriched bicarbonate to trace carbon partitioning into osmolytes such as floridoside, showing that high salinity triggered biosynthesis from stored carbon reserves, while low salinity enhanced *de novo* synthesis.⁸ This demonstrates NMR's ability to follow carbon flow in living systems.

2.5 Magic Angle Spinning (MAS) and Solid-State Applications

2.5.1 Theory of MAS

For intact biomass or hydrothermal liquefaction residues, solution-state NMR is often insufficient due to poor solubility. Solid-state NMR, combined with magic angle spinning (MAS), addresses this by physically spinning the sample at 54.7° relative to the magnetic field. At this angle, dipolar couplings and chemical shift anisotropies are averaged, narrowing line widths and yielding spectra with resolution approaching that of liquids.⁶

2.5.2 Application to Bio-oils

MAS ^{13}C NMR has been employed to examine functional groups in HTL bio-oils. Typically, signals between 0–50 ppm represent aliphatic carbons, 110–160 ppm correspond to aromatic and olefinic carbons, and 160–190 ppm are carbonyl carbons. By quantifying these regions, one can evaluate aromaticity, oxygen content, and degree of aliphaticity, all of which are critical for predicting fuel performance.

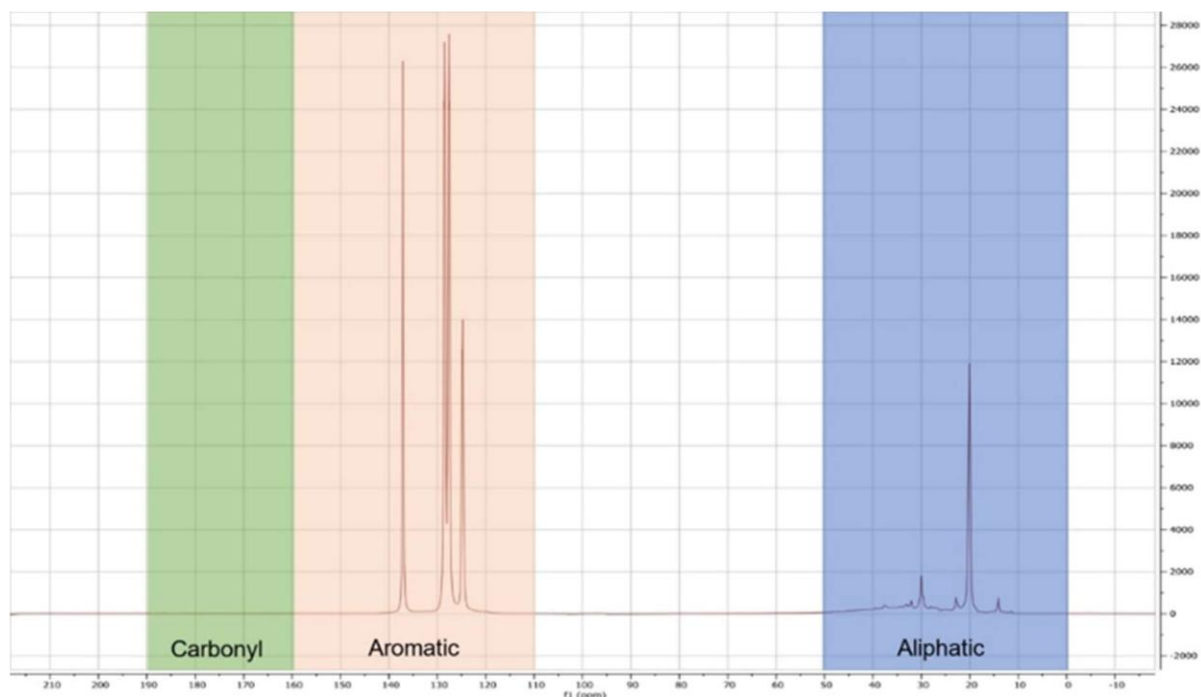


Figure 2.4 ^{13}C NMR spectrum of hydrothermal liquefaction (HTL) oil highlighting aliphatic (0–50 ppm), aromatic (110–160 ppm), and carbonyl (160–190 ppm) carbon regions. Spectrum acquired from a 70 g/L asphaltene sample in toluene using 1024 scans and a 10 s recycle delay.

2.6 One-Dimensional NMR Experiments

2.6.1 ^1H NMR

Proton nuclear magnetic resonance (^1H NMR) spectroscopy is a fast and widely used analytical technique for elucidating molecular structures in chemical compounds, including crude oils and related products. This method detects hydrogen nuclei in different chemical environments, which resonate at characteristic frequencies within a narrow chemical shift range (0–20 ppm). The resulting chemical shifts and signal splitting patterns provide detailed information about functional groups and molecular connectivity. When experimental parameters such as recycle delays are properly optimized, signal intensities can also be used for semi-quantitative interpretation, as they reflect the relative number of

hydrogen nuclei in each chemical environment. In complex mixtures such as asphaltenes and heavy bio-oils, ^1H NMR spectra often become highly congested because of extensive peak overlap and the presence of many chemically distinct proton environments. This overlap leads to broad signals and loss of fine coupling information, thereby complicating direct spectral interpretation. In such cases, spectral deconvolution techniques are employed to resolve overlapping peaks and enable more accurate chemical assignment. One inherent limitation of ^1H NMR is that it cannot detect quaternary carbons, which necessitates complementary structural analysis using ^{13}C NMR spectroscopy.

2.6.2 Dept 135 and 90 NMR

To improve spectral interpretability, DEPT-90 and DEPT-135 experiments were performed. DEPT experiments enhance signals from protonated carbons while suppressing non-protonated carbons, allowing the spectra to be separated based on carbon protonation rather than chemical shift alone.⁶ In DEPT-90 spectra, only CH (methine) carbons are observed, while CH_2 , CH_3 , and quaternary carbons are suppressed. In DEPT-135 spectra, CH and CH_3 carbons appear as positive signals, CH_2 carbons appear as negative signals, and quaternary carbons are absent. Together, these experiments help distinguish overlapping resonances and provide clearer insight into the distribution of different carbon types within complex phytoplankton samples.

2.7 NMR Relaxation Experiments

2.7.1 Introduction to Relaxation Times

Nuclear Magnetic Resonance (NMR) relaxation processes provide essential information about molecular dynamics, molecular interactions, and local environments. Two primary relaxation times are typically analyzed in NMR studies: spin-lattice relaxation (T_1) and spin-spin relaxation (T_2).

T_1 , or longitudinal relaxation, measures the time required for nuclear magnetization to return to thermal equilibrium along the direction of the static magnetic field (B_0) after being disturbed by a radiofrequency pulse. This process involves energy exchange between the nuclear spins and their surrounding lattice. In contrast, T_2 , or transverse relaxation, reflects the dephasing of nuclear spins in the plane perpendicular to B_0 due to spin-spin interactions. While T_1 is sensitive to molecular tumbling and energy dissipation, T_2 is more influenced by local magnetic field variations and molecular rigidity.

Understanding both relaxation mechanisms is critical for interpreting signal intensity decay and optimizing pulse sequences in NMR spectroscopy. Moreover, these parameters offer quantitative insight into system behavior, especially in complex matrices such as bio-oils derived from feedstock or samples processed via hydrothermal liquefaction.^{5,6}

2.7.2 Inversion Recovery (T_1 Measurement)

2.7.2.1 Pulse Sequence

The inversion recovery (IR) technique is the most widely used method for determining T_1 values. The pulse sequence begins with a 180° pulse that inverts the magnetization vector from the $+z$ axis to $-z$. After a variable delay time (τ), a 90° pulse is applied to rotate the partially recovered magnetization into the xy -plane, where the resulting free induction decay (FID) signal is detected (Keeler, 2002). By repeating this sequence over a range of τ values, one can observe the recovery profile of the magnetization. The signal intensity as a function of delay time follows the equation:

$$M_z(\tau) = M_0(1 - 2e^{-\tau/T_1}) \quad (2.9)$$

where M_0 is the equilibrium magnetization, and $M_z(\tau)$ is the observed longitudinal magnetization at time τ . The inversion pulse flips the spins, and the relaxation back toward equilibrium is monitored by the 90° read pulse. Modern spectrometers (e.g., Bruker Avance or Agilent) often provide built-in IR pulse programs (e.g., `t1ir`) to automate these measurements.

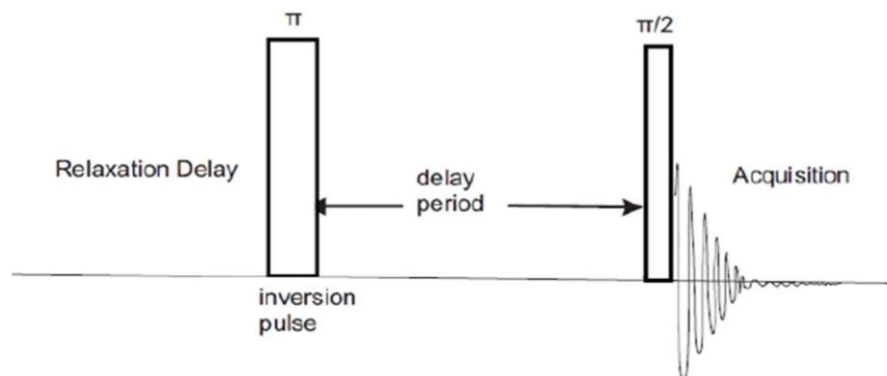


Figure 2.5 Pulse-sequence of Inversion recovery experiments.⁵

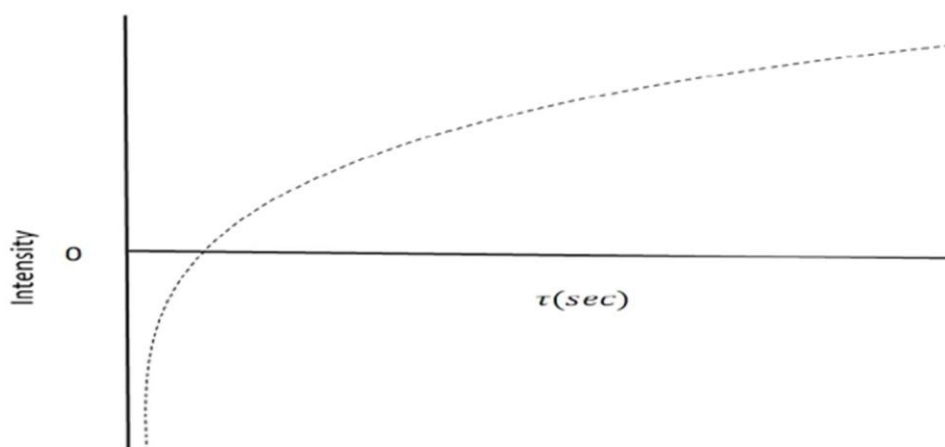


Figure 2.6 Inversion recovery curve for T₁ experiment.³

2.7.3 Carr–Purcell–Meiboom–Gill (CPMG) for T₂

2.7.3.1 Pulse Sequence

The CPMG sequence is designed to measure T₂ relaxation by suppressing the effects of magnetic field inhomogeneity. It consists of a 90° pulse followed by a series of 180° refocusing pulses spaced by a delay τ . These pulses generate a train of spin echoes whose amplitudes decay over time due to transverse relaxation:

$$M_{xy}(t) = M_0 e^{-t/T_2} \quad (2.10)$$

The advantage of the CPMG sequence over a simple spin-echo experiment lies in its ability to minimize dephasing from field imperfections by continuously refocusing the transverse magnetization.

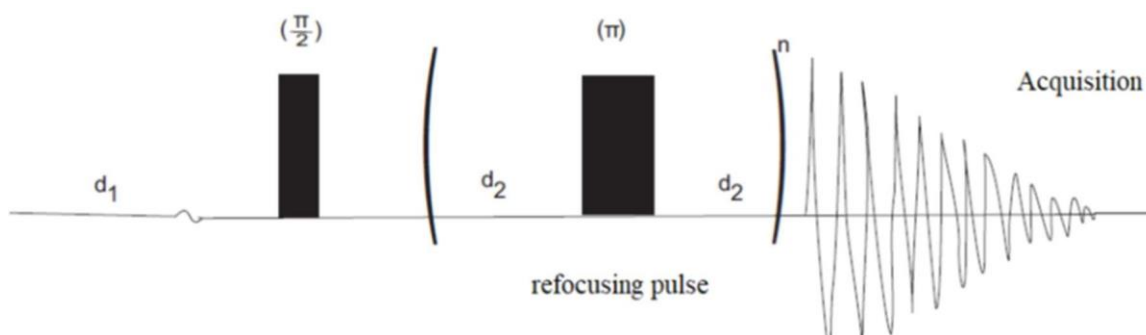


Figure 2.7 Pulse sequences of CPMG experiment.³

2.7.3.2 Echo Spacing (τ) and Its Impact

The interpulse delay τ is a crucial factor in CPMG experiments:

Short τ : Reduces the influence of diffusion in field gradients, ideal for homogeneous samples.

Long τ : May cause signal loss due to diffusion or J-coupling modulations.

An appropriate τ should be selected based on sample viscosity and expected T_2 values. For samples with short T_2 (e.g., bio-oil fractions or high-viscosity extracts), shorter τ values (0.5–2 ms) provide better decay resolution.⁹

2.8 Two-Dimensional NMR Spectroscopy: HSQC and HMBC

2.8.1 Introduction

Two-dimensional (2-D) NMR experiments are essential for resolving the overlapping resonances typically encountered in complex biomaterials such as phytoplankton biomass extracts. While one-dimensional (1-D) ^1H and ^{13}C spectra provide general information on chemical shifts and functional group distribution, the limited chemical shift dispersion of protons often leads to significant overlap, obscuring minor components or making unambiguous assignments impossible. Heteronuclear correlation experiments such as HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple Bond Correlation) overcome this by spreading resonances into two frequency dimensions, creating a spectral “map” of proton–carbon connectivities.⁶

In the context of phytoplankton-derived biofuels, these experiments are particularly powerful because they allow one to assign glycerol backbones, fatty acyl chain positions, and minor oxygenated compounds present in bio-oils or extracts. They also facilitate isotopic enrichment studies, such as those following ^{13}C flux into metabolites like floridoside.⁸

2.8.2 HSQC (Heteronuclear Single Quantum Coherence)

2.8.2.1 Pulse Sequence

The HSQC experiment correlates protons with directly attached carbons (one-bond coupling, $^1J_{\text{CH}} \approx 125\text{--}160\text{ Hz}$). The pulse sequence uses an initial 90° ^1H excitation pulse, followed by an INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) transfer period,

where polarization is transferred from protons to carbons through scalar coupling. After evolution under the carbon chemical shift during the indirect time domain (t_1), magnetization is transferred back to protons for detection in the direct dimension (t_2) (Berger & Braun, 2004). Gradient pulses are typically used for coherence selection, suppressing artifacts, and ensuring cleaner spectra.⁶ The final spectrum is a 2-D contour plot where cross-peaks indicate ^1H – ^{13}C one-bond correlations. This provides a fingerprint of hydrogen–carbon connectivity, greatly assisting in assignments of fatty acyl chains, glycerol carbons, and secondary metabolites in the extracts.⁴

2.8.2.2 Considerations for Algal Samples

Algal samples pose challenges due to broad background signals from proteins, carbohydrates, and pigments. High lipid content improves the detection of sharp methylene and olefinic resonances, but minor metabolites may require long acquisition times. Solvent choice is also critical; CDCl_3 is often used for lipids, but aqueous D_2O mixtures may be required for polar fractions, leading to lower resolution. The number of scans and points must therefore be carefully balanced against available sample and instrument time.

2.8.3 HMBC (Heteronuclear Multiple Bond Correlation)

2.8.3.1 Pulse Sequence

The HMBC experiment detects longer-range (two- and three-bond) ^1H – ^{13}C correlations. The pulse sequence is similar to HSQC but uses longer delay periods to evolve under these small couplings. A critical feature is the use of low-pass J-filters to suppress the intense one-bond correlations, which would otherwise dominate the spectrum.⁶ This selective filtering ensures that weaker long-range connectivity is observed.

2.8.3.2 Interpretation

HMBC spectra reveal connectivity across functional groups, such as glycerol methylene protons correlated with carbonyl carbons of fatty acid esters. In phytoplankton lipid extracts, HMBC can confirm ester linkages, identify unsaturated chain positions, and characterize minor oxygenated components produced during hydrothermal liquefaction. This makes it a critical tool for functional group mapping beyond what HSQC can offer.

2.8.3.3 Considerations for phytoplankton Samples

Because phytoplankton extracts often contain a mixture of neutral lipids, glycolipids, and pigments, HMBC spectra can become highly congested. The weaker nature of long-range correlations makes sensitivity a limiting factor. Thus, the number of scans per increment is particularly important, and careful optimization of pulse delays is necessary to avoid losing weak signals. Additionally, line broadening from viscous or heterogeneous samples can further obscure cross-peaks, requiring longer acquisition times and advanced processing techniques such as window functions or zero-filling.^{4,10}

2.9 Hydrothermal Liquefaction Device

Hydrothermal liquefaction (HTL) is a thermochemical process that converts wet biomass into bio-oil using water at elevated temperature and pressure. In this project, a stirred high-pressure autoclave system (CJF-1L model) was employed for biomass conversion experiments. This device has a working capacity of 1 L, a maximum temperature of 350 °C, and a pressure rating of 22 MPa. Heating is supplied by a 2.2 kW electric unit operating at 220 V, while a mechanical stirring system ensures the slurry is

kept homogeneous during reaction. The design allows uniform heating and controlled residence time, both of which are crucial for reproducible results.

The CJF-1L system simulates the geological conditions that form fossil crude oil but accelerates them dramatically, producing a crude-like product within minutes or hours rather than over millions of years.¹¹ The device's ability to maintain controlled subcritical and near-critical conditions enables efficient conversion of slurries without the need for drying, which makes it particularly suitable for research on phytoplankton-derived fuels.¹²

2.9.1 Comparison with Other Reactor Systems

Different HTL devices have been described in the literature, ranging from small-scale stainless-steel autoclaves to larger pilot reactors. For example, Wang *et al.* (2021) reported the use of a 50 mL stainless steel autoclave capable of reaching 400 °C and 60 MPa for two-step hydrothermal liquefaction of macroalgae.¹³ While these systems provide excellent control, they are limited in sample size. Guo *et al.* (2022) used micro autoclaves of similar design to co-process microalgae with plastic waste at 350 °C and 16 MPa, demonstrating flexibility in feedstock testing.¹⁴ In contrast, the CJF-1L offers a mid-range capacity with more practical yields for detailed analysis, while still being relatively safe and manageable in a laboratory setting.

2.9.2 Temperature and Pressure Control

The operation of the device is centered on maintaining conditions where water becomes a highly reactive medium. At subcritical ranges of 280 ± 40 °C, water loses much

of its polarity and its ionic product increases, which facilitates hydrolysis and depolymerization reactions.¹⁵ Under these conditions, proteins break down into amino acids and nitrogen-containing compounds, lipids are hydrolyzed to fatty acids, and carbohydrates depolymerize into smaller sugars and organic acids.¹⁶

Pressure must also be carefully controlled. The CJF-1L maintains pressures of 10–20 MPa, which are high enough to keep water in the liquid state at elevated temperatures.¹⁵ This pressurized liquid environment is essential because it prevents evaporation and allows reactions to proceed in a condensed phase. At higher pressures, solubility of gases such as carbon dioxide also increases, enhancing hydrolysis reactions. If pressure falls too low during heating, steam pockets can form, leading to non-uniform reactions and potential safety risks. For this reason, pressure gauges and alarms are always monitored closely during each run.

2.9.3 Reactor Configuration and Components

The CJF-1L hydrothermal liquefaction (HTL) reactor is constructed from stainless steel to withstand high temperatures, pressures, and corrosive byproducts. It features a cylindrical vessel with reinforced seals, pressure relief valves, an electric heating jacket for uniform heating, and a stirring mechanism to ensure homogeneous temperature distribution. Compared to smaller micro-autoclaves, which limit product recovery, and larger pilot-scale systems, which are complex and costly, the 1 L capacity of the CJF-1L provides an effective balance between analytical capability and operational practicality.^{13,14}

The reactor lid is secured using eight stainless-steel bolts tightened in a cross-pattern sequence with a calibrated torque wrench to ensure even pressure distribution and

a reliable seal. Proper torque application (70–110 ft·lbf) is essential to prevent leakage, gasket failure, or mechanical damage, particularly under operating conditions of up to 350 °C and 10 MPa. The lid is also equipped with automatic and manual pressure valves for controlled pressure release, as well as connections for cooling and inert gas flow, enhancing both safety and operational flexibility.

Temperature control is achieved using a PCE-RE100P controller, which supports both constant and programmed heating modes and includes safety alarms to prevent overheating. Temperature data from a J-type thermocouple is transmitted via a Modbus interface to a Raspberry Pi system, where it is recorded using a custom Python script. Similarly, a stainless-steel pressure transmitter monitors internal pressure (0–10 MPa) and integrates with the same data acquisition system, enabling real-time monitoring of both temperature and pressure. This integrated setup ensures precise control, reproducibility, and enhanced safety during HTL experiments.

To further improve consistency and prevent contamination, a custom stainless-steel sample container was used to hold the biomass within the reactor. This design isolates the sample from direct contact with the reactor walls and heating elements while allowing efficient heat transfer, ensuring uniform thermal exposure and improved reproducibility across experiments.

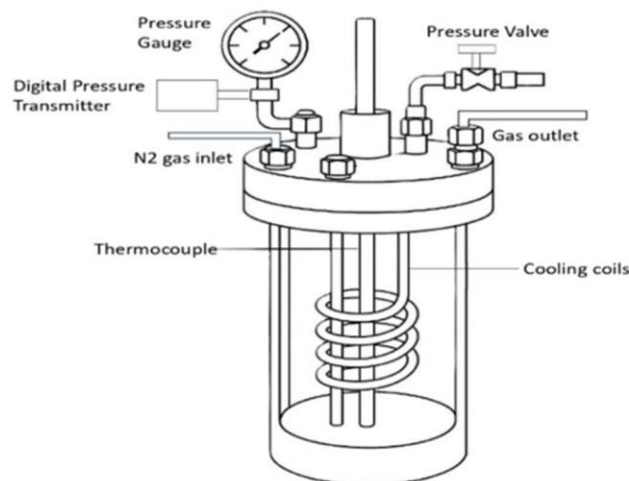


Figure 2.8 Schematic diagram of the hydrothermal liquefaction (HTL) reactor system showing the main components.

2.9.4 Controller Configuration and Data Acquisition System

The digital temperature controller used in this system was powered through an external switch, and the operation sequence was initiated by pressing the SET key on the front panel. Prior to each run, the desired set value (SV) temperature was entered through the profile control page. The controller compares this target value with the real-time process value (PV) and regulates the power accordingly. To ensure accurate thermal response, the PID (proportional–integral–derivative) parameters were reviewed before operation so the device could predict the approach to the set temperature and terminate power once equilibrium was achieved. In this setup, the controller supplied output power to the solid-state relay (SSR) until the SV was reached. For our experiments, the proportional band was set to zero, forcing the system to deliver full (100 %) power output during heating. The

device was operated in on/off control mode rather than continuous PID mode to maintain a simple and consistent heating profile.

An additional control feature available on the device was the SV deviation function, which defines the permissible difference between the set and measured temperatures. When the temperature difference ($SV - PV$) became smaller than the specified deviation value, the controller automatically cut power to minimize temperature overshoot. Up to three alarm channels could also be programmed to alert the operator in case of temperature deviation or overshoot during operation. The controller supports a maximum of eight programmable temperature segments; however, only a single segment was used per experimental run to maintain a stable, uniform heating phase. In the output configuration menu, both the minimum and maximum output limits were adjusted, and the thermocouple type and its corresponding measurement range were specified to match the reactor sensor calibration.

The Python program running on the Raspberry Pi was used to collect and record process data from the Modbus interface during each heating cycle. The script was configured to define the Modbus registers corresponding to the thermocouple and pressure transmitter, allowing the system to accurately access the real-time temperature and pressure readings inside the reactor. Through a continuous loop structure, the program repeatedly read the defined registers at predetermined time intervals and simultaneously retrieved the temperature, pressure, duty cycle, and the exact time of each data point. These measurements were continuously displayed and recorded during the heating process, providing a synchronized and automated data acquisition routine. The corresponding data points and the graphs are presented in Appendix A.

At the end of each experimental run—or when the process was manually stopped—the program automatically saved all collected data into a text file, ensuring that the full time-resolved dataset was securely stored for later analysis. This setup provided a reliable and consistent record of the reactor’s thermal and pressure evolution over time, enabling detailed correlation between the heating profile, set temperature, duty cycle behavior, and the resulting pressure response of the hydrothermal liquefaction (HTL) system. By integrating Modbus communication with a Python-based data logging routine, the system ensured precise synchronization between the controller and sensors, improving both the accuracy and reproducibility of process monitoring during high-temperature and high-pressure operations.

2.9.5 Safety and Sampling Considerations

Hydrothermal liquefaction (HTL) operates under elevated temperature and pressure conditions, making safety a critical aspect of reactor operation. The CJF-1L reactor is equipped with built-in safety features, including pressure relief valves, reinforced walls, and automatic shut-off systems to mitigate risks associated with over-pressurization. However, safe operation also depends on strict adherence to laboratory protocols. Heat-resistant insulated gloves must be used when handling the reactor, as standard nitrile or latex gloves are insufficient for high-temperature surfaces.

All experiments were conducted within a certified chemical fume hood or a well-ventilated laboratory environment to safely manage gaseous by-products. The gases generated during HTL, primarily water vapor, carbon dioxide (CO₂), and light hydrocarbons, may pose risks such as asphyxiation or flammability if accumulated. To

minimize these hazards, vented gases were directed through a hose into the fume hood exhaust, ensuring safe removal from the workspace and maintaining air quality.

After each reaction, the reactor was allowed to cool to near room temperature prior to gradual depressurization to prevent sudden gas release or liquid discharge. The resulting products were then separated into oil, aqueous, gas, and solid phases, with gaseous products vented through the hood and other fractions collected for further analysis.¹¹ These procedures minimize risks associated with burns, toxic exposure, and high-pressure release.

The stainless-steel reactor, with an internal volume of approximately 1 L, was typically loaded with ~650 mL of deionized water and the sample contained in a 50 mL centrifuge tube. This configuration maintained approximately two-thirds liquid volume and one-third headspace to accommodate vapor expansion and pressure buildup. Prior to operation, all sealing surfaces were carefully cleaned and dried to ensure proper gasket performance. The reactor was then sealed using a torque-sequencing method to achieve a secure, leak-free closure, ensuring consistent and safe experimental conditions.

2.10 Post-Reaction Product Processing and Fraction Separation

2.10.1 Reactor Cooling and Initial Product Recovery

After completion of the hydrothermal liquefaction (HTL) experiment, the reactor vessel was allowed to cool naturally to near ambient temperature. To ensure operational safety and to prevent sudden depressurization, the reactor was left undisturbed overnight until both the internal temperature and pressure had fully equilibrated with laboratory

conditions. Once cooled, any residual pressure was released slowly through the manual valve, and the reactor lid was carefully opened.

The internal stainless-steel sample container was then removed from the reactor. The liquid reaction products were transferred into a clean collection vessel for processing and analysis. All reactor components, including the vessel interior and sample container, were thoroughly rinsed and cleaned after product removal.

2.10.2 Solid–Liquid Separation by Vacuum Filtration

The first step in post-reaction processing involved the separation of suspended solid residues from the liquid product mixture. The recovered liquid contained visible particulate matter, originating from unconverted biomass fragments, char, or inorganic residues formed during HTL.

Solid–liquid separation was carried out using vacuum filtration. A Büchner funnel fitted with appropriately sized qualitative filter paper was mounted onto a side-arm Erlenmeyer flask connected to a laboratory vacuum line. The liquid product was poured into the funnel, and reduced pressure was applied to accelerate filtration. This procedure allowed efficient removal of the solid fraction while minimizing liquid loss. The collected solids were retained on the filter paper, while the clarified filtrate was collected in the receiving flask for subsequent liquid–liquid extraction.

2.10.3 Liquid–Liquid Extraction and Phase Separation

Following solid removal, the clarified liquid consisted primarily of an aqueous phase containing dissolved organics and a bio-oil fraction. To isolate the oil phase, liquid–liquid extraction was performed using dichloromethane (DCM) as the organic solvent.

The filtered liquid was transferred into a clean glass separatory funnel equipped with a stopcock and stopper. Dichloromethane was added to the funnel, and the mixture was gently shaken to promote efficient partitioning of organic compounds into the solvent phase. During shaking, the separatory funnel was periodically vented by opening the stopcock to release accumulated pressure generated by solvent vapor or dissolved gases.

After agitation, the funnel was placed on a ring stand and allowed to rest until clear phase separation was observed. Two distinct layers formed within several minutes: a lower organic phase consisting of DCM containing the extracted bio-oil, and an upper aqueous phase. The extraction procedure was repeated three times to maximize oil recovery. After each extraction, the lower organic layer was carefully drained into a clean round-bottom flask, with the stopcock closed immediately before any aqueous phase entered the flask to prevent cross-contamination.

2.10.4 Solvent Removal by Rotary Evaporation

The combined organic extracts were concentrated by solvent removal using a rotary evaporator. The round-bottom flask containing the DCM–oil solution was attached to the rotary evaporator vapor duct and secured with a clip to ensure mechanical stability under vacuum conditions.

The evaporation bath was filled with reverse-osmosis (RO) water and maintained at a temperature between 25 and 30 °C, as elevated temperatures were not required due to the low boiling point of dichloromethane under reduced pressure. The flask was partially immersed in the water bath, and rotation was initiated at approximately 180 rpm to increase the surface area of the liquid film.

Vacuum was applied gradually, starting at approximately 10 mbar and subsequently reduced to 2–3 mbar to prevent bumping. Under these conditions, solvent evaporation occurred within several minutes, as evidenced by continuous bubble formation and solvent condensation in the receiving flask. After complete removal of dichloromethane, a viscous bio-oil residue remained coated along the inner surface of the round-bottom flask.

2.10.5 Bio-Oil Recovery and NMR Sample Preparation

Following solvent evaporation, the bio-oil residue was recovered by gently scraping the inner surface of the flask using a clean spatula. The collected oil was transferred into a labeled container and redissolved in deuterated chloroform (CDCl_3), depending on the subsequent NMR experiment.

In some cases, fine particulate matter remained suspended in the oil solution. The presence of such particles can interfere with magnetic field homogeneity and prevent proper shimming during NMR measurements. To address this issue, the solution was either further diluted or subjected to an additional fine filtration step. This secondary filtration was performed by passing the solution through a glass Pasteur micropipette packed with a small plug of cotton, which effectively removed remaining particulates without significant

sample loss. The clarified solution was then transferred into a clean NMR tube and sealed for spectroscopic analysis.

2.10.6 NMR Analysis

Prepared NMR samples were analyzed using appropriate one- and multidimensional experiments, with acquisition parameters optimized based on solvent, concentration, and experimental objectives to ensure reliable spectral quality and reproducibility.

Nonlinear least squares fitting of complex spectral data requires robust optimization strategies due to the presence of noise, overlapping peaks, and correlated parameters. Steepest descent methods update parameters along the negative gradient direction and are generally stable, but they often exhibit slow convergence in narrow and highly correlated parameter spaces.^{17,18} In contrast, the Gauss–Newton (GN) method accelerates convergence by linearizing the residuals and solving a sequence of least squares problems; however, its performance may degrade in ill-conditioned or highly nonlinear systems, particularly when peaks overlap or parameters are weakly identifiable. To address these limitations, modern GN approaches incorporate globalization techniques such as line search and trust-region strategies to improve stability and convergence behavior.¹⁹

The Levenberg–Marquardt (LM) algorithm provides a robust alternative by combining the strengths of gradient descent and Gauss–Newton through an adaptive damping parameter. This allows LM to behave like Gauss–Newton near the solution for rapid convergence, while reverting to a more stable gradient-based approach under noisy or poorly conditioned conditions.²⁰⁻²² Recent developments further interpret LM within

trust-region and majorization–minimization frameworks, offering both strong theoretical guarantees and practical efficiency.²³ Empirically, LM and related trust-region methods have demonstrated reliable performance in fitting noisy, constrained, and highly nonlinear systems, including applications in chemical kinetics and systems biology.¹⁹

Overall, given the challenges associated with nonlinear peak fitting of overlapping and noisy spectral data, LM provides the most effective balance between convergence speed, stability, and robustness, making it particularly well suited for the analysis performed in this study.^{19,21,22}

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CHAPTER 3: RESULTS AND DISCUSSION

3.0 Overview

This chapter presents and discusses the experimental results obtained from the hydrothermal liquefaction (HTL) experiments and the subsequent Nuclear Magnetic Resonance (NMR) analysis of both model systems and phytoplankton bio-oil samples. The primary objective of this chapter is to evaluate how different HTL operating conditions influence bio-oil yield and composition, and to examine whether molecular-level information obtained through NMR techniques can be used to interpret the structural and dynamic properties of the resulting products. By integrating HTL performance metrics with NMR analysis and spectroscopic characterization, this chapter provides a comprehensive assessment of the conversion behavior of phytoplankton biomass.

The chapter begins with a detailed discussion of HTL product yields obtained under different reaction conditions. The distribution of products amongst the bio-oil, aqueous, solid (char), and gas phases is analyzed to determine how operating parameters such as temperature, pressure, and reaction holding time affect the overall efficiency of the HTL process. These yield results provide direct insight into the phase distribution of the produced biocrude oil. Differences in yields with variations in experimental conditions are examined to identify trends related to oil phase distribution and the chemical reactions during liquefaction.

Following the HTL yield analysis, this chapter presents the NMR characterization of the two model systems: crab oil and petroleum asphaltenes. These materials were selected as reference systems representing low-molecular weight and high-molecular

weight components, respectively. First, a qualitative NMR analysis is used to identify the main compound classes present in each model system through one-dimensional NMR experiments. Relaxation measurements are then applied to examine differences in molecular mobility and phase behavior, including features associated with aggregation in asphaltene samples and micelle-like structures.⁸ Results are presented across multiple concentrations and in to evaluate solvent effects, concentration-dependent peak broadening, and molecular mobility. The analysis of these model systems plays a critical role in validating the NMR acquisition parameters, relaxation protocols, and data-processing strategies developed in Chapters 1 and 2.

The chapter then focused on the NMR analysis of bio-oils produced from phytoplankton biomass through HTL. Bio-oil samples obtained from different phytoplankton species, specifically *Chlorella* and *Spirulina*, are analyzed using ¹H NMR, ¹³C NMR and relaxation experiments. These techniques enable detailed examination of the aliphatic, aromatic, oxygenated, and heteroatom-containing fractions of the bio-oils. Comparisons are made between bio-oils produced from different species and under various HTL conditions to identify systematic changes in functional group distribution and molecular complexity. These comparisons provide insight into how feedstock composition and processing conditions influence the chemical nature and quality of the resulting bio-oils.

In addition to direct spectral interpretation, this chapter also applies nonlinear modeling to analyze overlapping NMR peaks in both the model systems and the HTL bio-oils. Because bio-oils and asphaltenes are inherently complex mixtures that produce broad and congested NMR spectra, parameter estimation is treated as a nonlinear least-squares

problem. Gauss–Newton methods are used as a baseline for local optimization, while damped Levenberg–Marquardt strategies are employed to improve stability and robustness in the presence of parameter coupling and experimental noise. These modeling approaches enable more reliable peak separation, quantitative comparison of spectral components, and consistent chemical-shift assignment across solvents and concentrations.

NMR relaxation measurements (T_1 and T_2) are further used as qualitative indicators of molecular mobility and intermolecular interactions within the model systems and bio-oils. The ratio of T_1 to T_2 is interpreted as a proxy for aggregation behavior, where larger ratios are associated with larger or more compact molecular aggregates and smaller ratios indicate greater molecular mobility and reduced aggregation. By comparing relaxation behavior across crab oil, asphaltenes, and HTL bio-oils, this chapter establishes important structure–mobility relationships that support the interpretation of compositional trends identified through conventional NMR analysis.

Overall, this chapter discusses phytoplankton bio-oil yield results, 1-D and 2-D NMR analysis, and relaxation experiments to provide a detailed and systematic evaluation of bio-oil formation and structure. Together, these results provide the experimental and analytical foundation for the broader mechanistic interpretation and optimization strategies discussed in the subsequent chapter.

3.1 HTL Product Distribution and Mass Yields of Phytoplankton Bio-Oil

Following hydrothermal liquefaction, the reaction products were separated into solid, bio-oil, gas, and aqueous phases in order to assess phase distribution and overall mass balance. Each phase was physically separated, independently collected, and quantified. While mass balances were performed for all experiments, some material losses were unavoidable during reaction, phase separation, and sample handling. As a result, the gravimetric yield analysis is presented primarily to provide context for product distribution, whereas the main focus of chemical interpretation in this study is based on Nuclear Magnetic Resonance (NMR) analysis of the recovered bio-oil fractions.

The dry mass of biomass was determined prior to HTL for each batch and used as the normalization basis for yield calculations. All phase yields are therefore reported as a percentage of the initial dry mass.

The solid phase (biochar) was separated from the reaction mixture by vacuum filtration. After filtration, the filter paper containing the retained solid material was dried and weighed. The mass of the solid phase was determined by subtracting the mass of the empty filter paper from the combined mass of the filter paper and retained solid. The bio-oil phase was recovered through liquid–liquid extraction and concentrated using rotary evaporation. After solvent removal, the bio-oil formed a thin film on the inner surface of a round-bottom flask. The mass of the dry bio-oil was calculated by subtracting the mass of the empty flask from the mass of the flask containing the dried bio-oil film.

The aqueous phase remaining after solid removal and organic extraction contained dissolved organic compounds and was therefore freeze dried prior to mass determination. The aqueous phase was dried to constant mass, and its dry mass was obtained by subtracting

the mass of the empty container from the mass of the container containing the dried aqueous residue.

The yield of each condensed phase was calculated by normalizing the measured dry mass of that phase to the initial dry mass of phytoplankton used in the HTL experiment, according to Equation 3.1:

$$\varpi_i (\%) = \frac{m_i}{m_{\text{phytoplankton, dry}}} \times 100 \quad (3.1)$$

Where ϖ_i represents the dry mass of the solid, bio-oil, or aqueous phase, and $\varpi_{\text{algae, dry}}$ is the initial dry biomass mass.

The gaseous phase yield was not collected directly and was therefore calculated by difference. The gas yield was determined by subtracting the combined yields of the measured condensed phases from the initial dry mass, as shown in Equation (3.2):

$$\varpi_{\text{gas}}(\%) = 100 - (\varpi_{\text{aq}} + \varpi_{\text{solid}} + \varpi_{\text{bio-oil}}) \quad (3.2)$$

A summary of the phase distributions expressed as percentages of the dry mass is provided in Table 3.1. In addition to the tabulated values, the relative product distributions are also illustrated using bar charts to facilitate visual comparison of the phase yields obtained under different experimental conditions. Presenting the data graphically allows trends in product formation to be more easily identified and compared across the different HTL experiments. Due to gas release and material losses associated with phase separation, transfer, and solvent removal, full mass recovery was not always achieved. Such minor discrepancies in the mass balance are common in hydrothermal liquefaction experiments because volatile compounds can escape during depressurization and small amounts of material may remain adhered to reactor surfaces, filtration equipment, or glassware during

product recovery. Consequently, the gravimetric yield data are used primarily to describe the overall distribution of products among the condensed phases rather than to establish an exact mass conversion for each experiment.

While these gravimetric results provide useful insight into the overall conversion efficiency and phase distribution of the HTL process, the detailed chemical interpretation of the bio-oil composition is primarily based on the Nuclear Magnetic Resonance (NMR) analyses presented in the subsequent sections of this chapter. The HTL yield data, therefore, serve as an important contextual framework that supports the molecular-level characterization of the bio-oil fractions discussed later in this work.

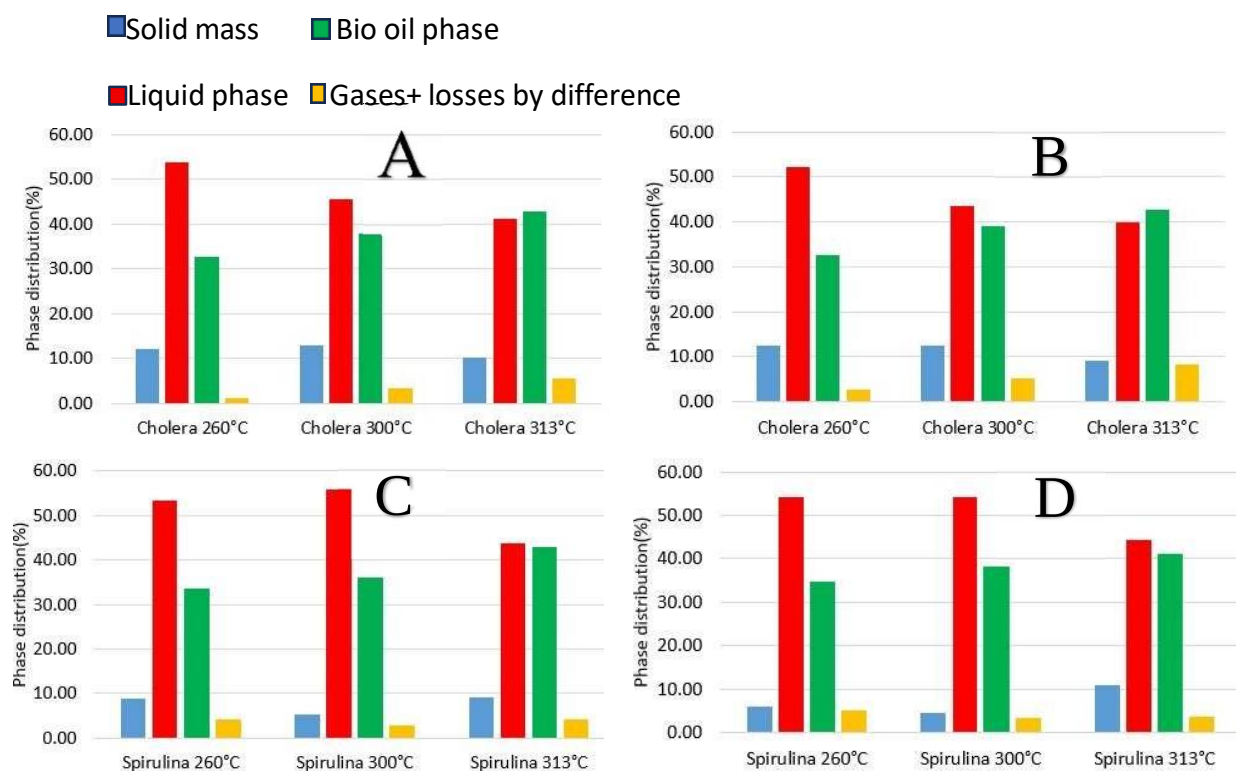


Figure 3.1. Phase distribution of hydrothermal liquefaction (HTL) products from **Chlorella** and **Spirulina** biomass at 260, 300, and 313 °C with holding times of 0.5 and 1 h, reported as percentages of the initial dry mass. Subfigures show: (A) *Chlorella*, 0.5 h; (B) *Chlorella*, 1 h; (C) *Spirulina*, 0.5 h; and (D) *Spirulina*, 1 h.

Table 3.1 Phase distribution and mass yields of *Chlorella* and *Spirulina* HTL products expressed as percentages of the initial dry biomass.

Batch Name	Temperature	Holding Time	Dry weight(g)	(%) Bio-oil	(%) Gas	(%) Aqueous	(%) Solid
<i>Chlorella</i>	260 °C	0.5h	10.0713±0.001	32.82±0.003	1.27±0.007	53.78±0.005	12.13±0.002
		1h	10.0663±0.001	32.74±0.003	2.67±0.006	52.1±0.005	12.49±0.002
	300 °C	0.5h	10.0153±0.001	37.95±0.004	3.36±0.006	45.6±0.005	13.08±0.002
		1h	10.0167±0.001	38.93±0.004	5.28±0.006	43.4±0.004	12.39±0.001
	313 °C	0.5h	10.0108±0.001	42.95±0.004	5.65±0.006	41.2±0.004	10.19±0.001
		1h	10.0773±0.001	42.65±0.004	8.25±0.006	39.9±0.004	9.20±0.001
<i>Spirulina</i>	260 °C	0.5h	10.106±0.001	33.57±0.003	4.14±0.006	53.4±0.005	8.89±0.001
		1h	10.118±0.001	34.51±0.004	5.07±0.007	54.3±0.005	6.12±0.001
	300 °C	0.5h	10.0164±0.001	35.94±0.004	2.87±0.007	55.8±0.006	5.39±0.001
		1h	10.0042±0.001	37.98±0.004	3.39±0.007	54.1±0.005	4.53±0.001
	313 °C	0.5h	10.0113±0.001	42.96±0.004	4.21±0.006	43.6±0.004	9.24±0.001
		1h	10.0143±0.001	40.93±0.004	3.73±0.006	44.3±0.005	11.03±0.001

These results suggest that the HTL experiments produced bio-oil yields ranging from approximately 32% to 43% of the initial dry mass. For the *Chlorella* experiments, the lowest bio-oil yields were observed at 260 °C, where the oil fraction remained close to 32–33 % regardless of reaction time. As the reaction temperature increased to 300 °C, the bio-oil yield increased noticeably to approximately 38 %, indicating more effective conversion of biomass into hydrophobic oil-phase products. This trend is consistent with previous *Chlorella*-based HTL studies, where *Chlorella sp.* produced a maximum bio-crude yield of 32.5% at 300 °C after 30 min, while *Chlorella OZ* showed a strong temperature-dependent increase in bio-oil yield from only 3.5–5.3% at 200–225 °C to 41.5% at 350 °C.^{1,16} The higher *Chlorella* yields observed in the present study at 313 °C therefore fall within the upper range reported for *Chlorella*-type microalgae and support the interpretation that increasing HTL temperature improves the conversion of both lipid and non-lipid

biomolecules into oil-soluble products. The highest bio-oil yields were obtained at 313 °C, reaching approximately 43 % for the 0.5-hour experiment and remaining slightly above 42 % for the 1-hour reaction. These results indicate that higher temperatures promote more extensive breakdown of phytoplankton macromolecules and facilitate the formation of oil-soluble compounds. The small difference between the 0.5-hour and 1-hour *Chlorella* reactions at 313 °C should not be overinterpreted, because gravimetric HTL yields can be affected by experimental uncertainty associated with phase separation, solvent removal, volatile loss during depressurization, and small amounts of material remaining on reactor or glassware surfaces. Therefore, the two 313 °C *Chlorella* yields are best interpreted as broadly comparable rather than as evidence of a strong residence-time effect.

A similar trend was observed for the *Spirulina* experiments. At 260 °C, the bio-oil yield was approximately 33–35 %, while increasing the temperature to 300 °C increased the oil yield to approximately 36–38 %. At 313 °C, the bio-oil yield increased further, reaching approximately 43 % for the 0.5-hour experiment. The *Spirulina* results are also comparable to previous HTL work on *Spirulina platensis*, where bio-crude yields up to $44.8 \pm 1.9\%$ were reported over the temperature range of 280–350 °C and reaction times of 15–45 min. The maximum *Spirulina* yield obtained in the present study is therefore very close to the highest value reported for *Spirulina platensis* in the literature, suggesting that the selected HTL conditions were effective for converting this protein- and lipid-containing biomass into bio-oil.^{23,14} Although the oil yield slightly decreased to approximately 41 % for the 1-hour reaction at the same temperature, the overall trend still demonstrates improved oil formation at elevated temperatures. As with the *Chlorella* samples, this slight

decrease at longer residence time may reflect a combination of real secondary reactions and experimental uncertainty.

The aqueous phase represented the largest fraction of the HTL products for most experimental conditions, with yields generally ranging from 40 % to 56 %. Higher aqueous yields were particularly evident at lower temperatures (260 °C), where incomplete conversion of biomass likely results in the formation of water-soluble compounds such as organic acids, small oxygenates, and nitrogen-containing species derived from proteins. This is consistent with previous HTL studies showing that proteins and carbohydrates can form water-soluble intermediates such as amino acids, carboxylic acids, aldehydes, amides, and other polar oxygenated compounds.^{22,27} As the temperature increased to 300–313 °C, the aqueous fraction gradually decreased, reflecting increased conversion of these intermediates into more hydrophobic oil-phase products. Similar temperature-dependent shifts toward higher bio-oil formation have been reported in HTL studies, although the extent depends on feedstock composition and reaction conditions.²²

The solid phase (biochar) accounted for approximately 4 % to 13 % of the initial biomass, depending on reaction conditions. For *Chlorella*, the char yield generally decreased slightly with increasing temperature, suggesting enhanced thermal decomposition and conversion of solid residues into liquid or gaseous products. Similarly, Biller and Ross reported that solid residue, aqueous products, bio-crude, and gas are all competing HTL product fractions, and changes in reaction conditions can shift material between these phases.²³ In contrast, the *Spirulina* experiments showed somewhat lower char formation overall, with solid yields falling below 10 % for several conditions, particularly at intermediate temperatures. This range is comparable to reported solid-

residue yields for HTL; for example, Christensen et al. reported solid-residue yields of approximately 5.5–8.4% for *Phaeodactylum tricornutum* over 275–420 °C, while Biller and Ross showed that *Chlorella* and *Spirulina* produced low solid-residue fractions under non-catalytic hydrothermal conditions.^{28,23} The gaseous fraction, calculated by difference, remained relatively small in comparison with the other phases, typically ranging from 1 % to 8 % of the initial biomass. Gas formation tended to increase slightly with increasing temperature and reaction time, consistent with enhanced decarboxylation, deamination, and cracking reactions occurring during HTL.

The trends observed in Table 3.1 therefore indicate that reaction temperature plays a dominant role in determining HTL product distribution. Increasing the temperature from 260 °C to 313 °C consistently improved bio-oil yields while reducing the aqueous and solid fractions. This behavior is consistent with previous HTL studies, where increasing reaction temperature in the subcritical range promoted bio-oil formation by enhancing hydrolysis, depolymerization, and conversion of biomass-derived intermediates into hydrophobic oil-phase products.^{23,28} Reaction time showed a smaller influence compared with temperature.

When comparing the two phytoplankton species, the overall product distributions are broadly similar, although minor differences are observed in char formation and aqueous yields. These variations likely arise from differences in biochemical composition between *Chlorella* and *Spirulina*, particularly their relative lipid, carbohydrate, and protein contents. Such compositional differences influence the dominant reaction pathways during hydrothermal liquefaction and ultimately affect the phase distribution of the resulting products.¹ Similar species-dependent behavior has been reported by Biller and Ross, who showed that *Chlorella* and *Spirulina* differ in biochemical composition, with *Chlorella*

containing higher lipid content and *Spirulina* containing higher protein and carbohydrate contents. These differences were linked to changes in bio-crude, aqueous, gas, and solid product fractions during HTL.¹⁰ Vardon *et al.* also noted that feedstock composition strongly affects HTL biocrude yield and chemistry, as well as conversion efficiency.³

The phase distribution results presented in Table 3.1, therefore, provide a general assessment of HTL conversion performance and establish the experimental context for the subsequent molecular-level characterization of the bio-oil fractions using NMR spectroscopy.

3.2 Visual Appearance & Physical Properties of HTL Oils

Immediately after hydrothermal liquefaction, the recovered HTL product appeared mainly as a brick-orange liquid with low apparent viscosity. The liquid phase appeared mostly clear, but brown particles were visible in the liquid.

Filtration of the crude HTL product resulted in the separation of a black solid fraction identified as biochar. This solid phase appeared as both large black clumps and finer suspended particles. Because many of these particles remained dispersed in the liquid, vacuum filtration was required to effectively separate the solid phase. After filtration, the remaining liquid phase retained its brick-orange color.

Liquid–liquid extraction was then carried out using dichloromethane (CH_2Cl_2). When the filtered HTL liquid was transferred to a separatory funnel and mixed with CH_2Cl_2 , two distinct liquid layers formed after settling. The upper layer became dark brown in color, while the lower organic layer showed a light-yellow appearance. This behavior

suggests that heavier and more polar compounds remained in the upper phase, whereas less polar oil components were preferentially extracted into the dichloromethane phase.^{2,3}

Light brown particles were visible at the interface between the layers, indicating the presence of partially separated organic material. These interfacial particles are likely composed of partially soluble compounds, such as oxygen-containing or nitrogen-containing species, which show limited solubility in both the aqueous and organic phases. Their accumulation at the interface indicates a mixture of compounds with intermediate polarity.^{4,5} In this work, these materials were not collected separately and were not analyzed by NMR, because the study focused specifically on the recovered oil phase. As a result, the interfacial material remained between the aqueous and oil layers during separation. Alternative solvent extraction was not attempted for this fraction, in order to keep the recovery method consistent across all HTL conditions.

After separation, the organic oil phase was concentrated using rotary evaporation. During solvent removal, the oil formed a thin coating on the inner surface of the round-bottom flask. The concentrated oil displayed a cloudy yellow color and viscous texture. When mechanically scraped from the flask surface, the oil showed a thick and sticky consistency, indicating the presence of higher-molecular-weight components. Despite this increased viscosity, the oil remained readily soluble in organic solvents, allowing preparation of samples for NMR analysis.

These visual and physical observations highlight the complex nature of HTL products and demonstrate the need for filtration and solvent extraction to obtain a clean bio-oil fraction suitable for analysis.⁶ The observed changes in color, viscosity, and phase

behavior provide important context for the molecular-level NMR results discussed in the following sections of this chapter.

3.3 Model System 1 – Crab Oil NMR Results

In this section, the Nuclear Magnetic Resonance (NMR) results obtained for crab oil are presented and discussed as a low-molecular weight reference system for comparison with HTL bio-oils. Its triglyceride- and fatty-acid-rich nature makes it a useful benchmark for assigning lipid-derived NMR signals.

The ^1H NMR spectra were first examined to identify characteristic aliphatic, olefinic, and carbonyl resonances associated with lipid structures. In addition, T_1 and T_2 relaxation experiments are analyzed to evaluate the molecular mobility and phase behavior, including possible differences between more mobile and less mobile components of crab oil under the selected sample concentrations.^{7,8} Together, these results establish a baseline for interpreting both the structural features and relaxation behavior of lipid components in the more chemically complex HTL bio-oils discussed in later sections of this chapter.

The relaxation NMR spectra of crab oil samples were recorded at 3 different concentrations of 5, 15, 50 mg/ml. Instead of analyzing each peak individually, three main groups of peaks were investigated. The three crab oil concentrations were selected to evaluate concentration-dependent changes in relaxation behavior, including possible effects of restricted motion and peak broadening in a low-molecular weight oil model. This approach follows the rationale used in complex petroleum-like systems, where NMR relaxation is sensitive to concentration-dependent aggregation.⁸

3.3.1 NMR Analysis of Crab Oil

The ^1H NMR spectrum of crab oil at 70 mg/mL in CDCl_3 shows mostly sharp and well-defined signals across the aliphatic, oxygenated/olefinic, and aromatic regions. As expected for a low-molecular-weight model system, the highest intensity is observed in the aliphatic region between ~ 0.8 and 2.5 ppm, mainly corresponding to methyl and methylene protons from fatty acid chains. Some broader features are also present in this region, which may be due to overlapping signals from fatty acid chains with different chain lengths, degrees of unsaturation, or similar methylene environments.

In the oxygenated/olefinic region, sharp signals are observed around ~ 5.0 – 5.4 ppm. These peaks may be assigned to olefinic protons from unsaturated fatty acid chains or protons near oxygen-containing glycerol/ester environments.

The aromatic region shows several sharp but low-intensity peaks, indicating that aromatic compounds are only minor components in the crab oil sample. The absence of strong broad aromatic signals suggests that crab oil does not contain a significant amount of condensed or aggregated aromatic structures.⁸ Broad aromatic peaks are more typical of aromatic-rich and aggregated systems such as asphaltenes, while crab oil is mainly composed of more mobile lipid-like molecules, giving sharper signals.⁹

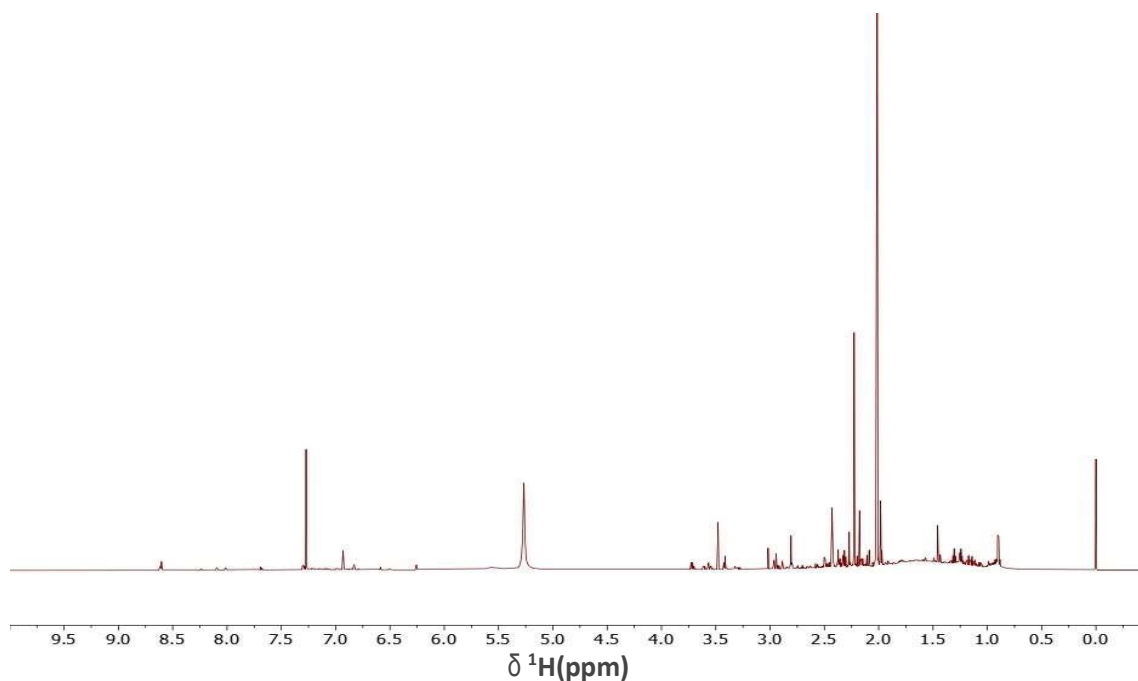


Figure 3.2 ^1H NMR spectrum of crab oil at 70 mg/mL in CDCl_3 .

3.3.2 T_1 and T_2 Relaxation Behavior of Crab Oil

Longitudinal (T_1) and transverse (T_2) relaxation measurements were performed on the crab oil samples to investigate proton mobility in different chemical environments. The oil samples were prepared at three different concentrations in CDCl_3 and all measurements were carried out at ambient temperature.

T_1 relaxation times were measured using the inversion recovery (IR) experiment, while T_2 relaxation times were obtained using the Carr–Purcell–Meiboom–Gill (CPMG) experiment. Representative inversion recovery and CPMG decay curves for the bio-oil samples are shown in this section.

The raw data obtained from both IR and CPMG experiments were normalized and plotted as a function of delay time. The relaxation curves were analyzed by fitting the experimental data using the Levenberg–Marquardt non-linear least-squares algorithm to

obtain the best agreement between the experimental data and the fitted curves. In cases where more than one relaxation component was present, bi-exponential fitting was applied to account for multiple proton environments with different relaxation behaviors.

T_1 and T_2 relaxation measurements were performed for each concentration in $CDCl_3$ to examine the effect of concentration on proton dynamics in the oil samples.

Table 3.2 ^1H NMR relaxation times of a crab oil sample at a concentration of 5 mg/ml in $CDCl_3$.

^1H ppm	Assignment	T_1 (s)	STD	SS	T_2 (s)	STD	SS	T_1/T_2
0.88- 1.02	Aliphatic	0.339(36.5%)	1.18	0.004	0.134(32.1%)	2.00	0.013	2.52
		1.97(63.5%)			1.46(67.9%)			0.231
1.25- 1.4	Aliphatic	0.302(38.0%)	1.42	0.006	0.205(30.3%)	1.65	0.009	1.47
		2.02(62.0%)			1.83(69.6%)			1.10
1.95- 2.1	Alicyclic	0.197(32.6%)	2.78	0.246	0.400(37.5%)	1.80	0.010	0.492
		3.37(68.4%)			4.48(62.5%)			0.752
7.25- 7.35	Aromatic	2.16(36.8%)	2.07	0.013	0.721(41.1%)	2.80	0.025	3.01
		5.33(63.2%)			8.95(58.8%)			0.596

Table 3.3 ^1H NMR relaxation times of a crab oil sample at a concentration of 15 mg/ml in $CDCl_3$.

^1H ppm	Assignment	T_1 (s)	STD	SS	T_2 (s)	STD	SS	T_1/T_2
0.88- 1.02	Aliphatic	0.403(16.1%)	7.35	0.173	0.109(27.6%)	1.52	0.007	3.69
		1.77(83.9%)			1.56(72.4%)			1.13
1.25- 1.4	Aliphatic	0.278(26.7%)	0.613	0.001	0.208(28.0%)	1.07	0.004	1.34
		1.78(73.3%)			1.62(71.9%)			1.09
1.95- 2.1	Alicyclic	0.253(37.1%)	2.35	0.173	0.173(35.3%)	1.49	0.008	1.46
		2.61(62.9%)			2.60(64.7%)			1.00
7.25- 7.35	Aromatic	0.429(79.9%)	0.637	0.001	0.044(22.5%)	0.996	0.003	9.75
		4.48(20.1%)			3.68(77.5%)			1.21

Table 3.4 ^1H NMR relaxation times of a crab oil sample at a concentration of 50 mg/ml in CDCl_3 .

^1H ppm	Assignment	T_1 (s)	STD	SS	T_2 (s)	STD	SS	T_1/T_2
0.88- 1.02	Aliphatic	0.41(64.4%)	0.448	0.000	0.036(37.6%)	1.58	0.008	11.5
		1.66(35.4%)			1.33(62.4%)			1.24
1.25- 1.4	Aliphatic	0.402(65.8%)	0.639	0.001	0.151(33.2%)	1.11	0.003	2.66
		1.76(34.2%)			1.38(66.8%)			1.27
1.95- 2.1	Alicyclic	0.348(44.4%)	0.351	0.000	0.124(40.5%)	2.38	0.018	2.80
		2.53(54.6%)			1.88(59.5%)			1.34
7.25- 7.35	Aromatic	1.19(36.7%)	0.843	0.002	0.030(35.5%)	0.956	0.003	39.8
		4.78(63.3%)			4.18(65.5%)			1.14

The relaxation values obtained for crab oil show that this model system is dominated by relatively mobile aliphatic proton environments. In the aliphatic methyl region at 0.88–1.02 ppm and the methylene region at 1.25–1.40 ppm, the longer T_2 components remain relatively high, with values of 1.46 and 1.83 s at 5 mg/mL, 1.56 and 1.62 s at 15 mg/mL, and 1.33 and 1.38 s at 50 mg/mL. The corresponding lower T_1/T_2 ratios for these longer components are 0.231–1.10 at 5 mg/mL, 1.13–1.09 at 15 mg/mL, and 1.24–1.27 at 50 mg/mL. These low ratios are consistent with relatively mobile fatty-acid-chain protons in crab oil.⁸

The same aliphatic regions also show short- T_2 components with higher T_1/T_2 ratios than the longer- T_2 components. This indicates that a smaller fraction of the aliphatic signal behaves as a more restricted component, likely due to overlapping lipid environments or less mobile molecular associations. The trend is most visible in the methyl region, where the short-component T_1/T_2 ratio is highest at 50 mg/mL, supporting increased restriction at higher concentration.

Although crab oil is dominated by mobile aliphatic fatty-acid-chain protons, the weak aromatic region shows a much more restricted relaxation response at higher concentration. At 50 mg/mL, the aromatic short component has a very short T_2 value of 0.030 s and a high T_1/T_2 ratio of 39.8, indicating restricted motion or strong spectral overlap in this low-intensity region. This does not suggest that crab oil is aromatic-rich; rather, it shows that the small aromatic contribution becomes more difficult to resolve and behaves as a more constrained component at higher concentration.

3.4 Model System 2 – Asphaltene NMR Results

In this section, the Nuclear Magnetic Resonance (NMR) results obtained for petroleum asphaltenes are presented and discussed as an aromatic-rich reference system for comparison with HTL bio-oils. Asphaltenes represent a geologically and thermally altered material with relatively low oxygen content and limited labile functional groups. Their sulfur and nitrogen contents are mainly associated with heterocyclic aromatic structures rather than side-chain functionalities, and they contain little to no olefinic components.¹⁰ As a result, asphaltenes provide a suitable benchmark for representing heavy, highly aromatic components commonly found in HTL bio-oils. The ^1H NMR spectra are first examined to identify characteristic aromatic and aliphatic resonances associated with asphaltene structures. In addition, T_1 and T_2 relaxation experiments are analyzed to assess molecular mobility, aggregation behavior, and dynamic restrictions within the asphaltene system.¹⁰ Together, these results establish an upper-bound reference for aromatic content and restricted molecular motion when interpreting the NMR behavior of HTL bio-oils discussed in later sections of this chapter.

3.4.1 NMR Analysis of Asphaltene

The ^1H NMR spectra of asphaltene samples were recorded at three different concentrations. In contrast to crab oil, the spectra are dominated by broad and overlapping signals, particularly in the aromatic region, reflecting the heterogeneous and aggregated nature of the asphaltene molecules. In asphaltene samples, individual peaks cannot be discerned.⁸ The spectra are composed of bands of signals corresponding to general structural features. The spectra were analyzed by grouping signals into three main regions: aromatic, alicyclic, and aliphatic.⁸ These regions were selected because they are representative of the mentioned groups' proton environments.

The ^1H NMR spectrum of asphaltene shows predominantly broad and overlapping signals across the aliphatic and aromatic regions. This behavior is consistent with the complex chemical nature of asphaltenes, which are composed mainly of polyaromatic structures with aliphatic substitution and a strong tendency toward molecular aggregation.⁸ Compared with crab oil, which shows sharper lipid-derived signals, the asphaltene spectrum contains much broader features due to the presence of multiple similar aromatic and aliphatic environments, extensive π - π interactions, and restricted molecular motion. These effects cause many resonances to overlap and reduce spectral resolution. The aromatic region around ~ 6.5 – 8.0 ppm shows noticeably broad signals, which is expected because asphaltenes are aromatic-rich materials.⁷ Therefore, compared with crab oil, a higher aromatic contribution is expected in the asphaltene spectrum. The broadness in this region suggests a distribution of substituted aromatic structures rather than a few isolated aromatic compounds. The strong broad envelope in the aliphatic region also indicates the presence of alkyl side chains attached to aromatic cores and other saturated hydrocarbon

environments. Although alicyclic protons are often overlooked because they overlap strongly with other aliphatic signals, they are important structural components of asphaltenes. Their relaxation behavior provides key evidence for the aggregation model, since restricted alicyclic and alkyl environments can show shorter relaxation times associated with reduced molecular mobility near aromatic cores or within aggregated structures. Overall, the spectrum confirms that asphaltene behaves as a heavier, more aromatic, and more aggregated model system compared with crab oil.⁸

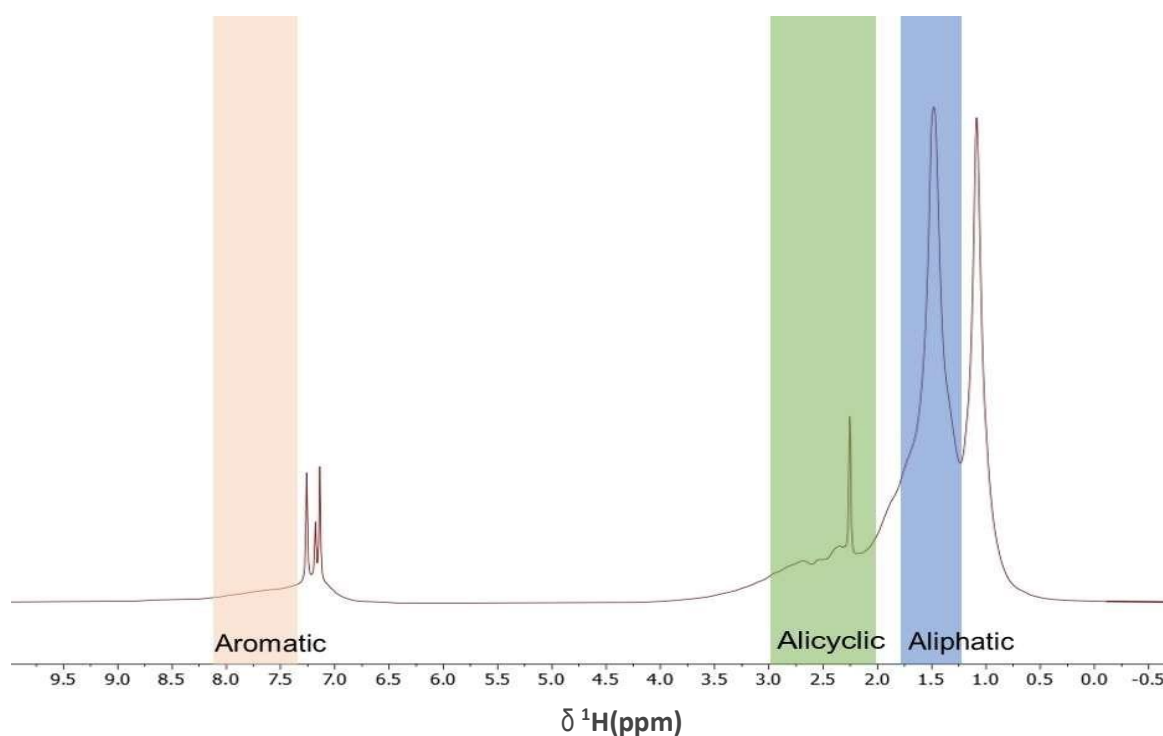


Figure 3.3 ¹H NMR spectrum of Asphaltene at 70 mg/mL in CDCl₃.

3.4.2 T₁ and T₂ Relaxation Behavior of Asphaltene

Longitudinal (T₁) and transverse (T₂) relaxation measurements were performed on the Asphaltene samples to investigate proton mobility in different chemical environments. The oil samples were prepared at three different concentrations in CDCl₃ and all measurements were carried out at ambient temperature.

T₁ relaxation times were measured using the inversion recovery (IR) experiment, while T₂ relaxation times were obtained using the Carr–Purcell–Meiboom–Gill (CPMG) experiment. Representative inversion recovery and CPMG decay curves for the bio-oil samples are shown in this section.

The raw data obtained from both IR and CPMG experiments were normalized and plotted as a function of delay time. The relaxation curves were analyzed by fitting the experimental data using the Levenberg–Marquardt non-linear least-squares algorithm to obtain the best agreement between the experimental data and the fitted curves. In cases where more than one relaxation component was present, bi-exponential fitting was applied to account for multiple proton environments with different relaxation behaviors.

T₁ and T₂ relaxation measurements were performed for each concentration in CDCl₃ to examine the effect of concentration on proton dynamics in the samples.

Table 3.5 ¹H NMR relaxation times of an Asphaltene sample at a concentration of 5 mg/ml in CDCl₃.

¹ H ppm	Assignment	T ₁ (s)	STD	SS	T ₂ (s)	STD	SS	T ₁ /T ₂
0.99-1.45	Aliphatic	0.45(45.4%) 1.62(54.5%)	1.17	0.000	0.065(40.2%) 0.602(59.8%)	0.461	0.007	6.92 2.69
2.00-2.80	Alicyclic	1.96(36.8%) 0.69(63.7%)	0.310	0.000	0.067(39.1%) 0.806(60.8%)	2.05	0.013	29.2 0.86
7.35-7.88	Aromatic	6.62(19.8%) 0.85(80.2%)	0.356	0.001	0.184(22.1%) 1.18(78.9%)	0.764	0.052	35.97 0.52

Table 3.6 ^1H NMR relaxation times of an Asphaltene sample at a concentration of 15 mg/ml in CDCl_3 .

^1H ppm	Assignment	T_1 (s)	STD	SS	T_2 (s)	STD	SS	T_1/T_2
0.99-1.45	Aliphatic	0.55(46.6%)	0.143	0.001	0.048(45.5%)	0.931	0.003	11.46
		1.37(53.4%)			0.211(54.5%)			6.49
2.00-2.80	Alicyclic	2.05(42.6%)	0.361	0.076	0.139(43.2%)	0.735	0.002	36.33
		0.69(57.4%)			0.230(56.8%)			2.78
7.35-7.88	Aromatic	3.06(46.4%)	0.637	0.001	0.073(44.3%)	1.29	0.005	41.92
		1.42(53.6%)			0.324(55.7%)			4.38

Table 3.7 ^1H NMR relaxation times of an Asphaltene sample at a concentration of 50 mg/ml in CDCl_3 .

^1H ppm	Assignment	T_1 (s)	STD	SS	T_2 (s)	STD	SS	T_1/T_2
0.99-1.45	Aliphatic	1.51(34.4%)	0.143	0.001	0.048(37.3%)	1.37	0.006	31.46
		4.66(65.6%)			0.524(62.7%)			8.89
2.00-2.80	Alicyclic	3.95(43.2%)	0.224	0.001	0.060(41.8%)	1.54	0.007	65.83
		0.99(56.8%)			0.734(58.2%)			1.35
7.35-7.88	Aromatic	5.87(20.1%)	1.31	0.144	0.036(28.5%)	1.14	0.004	163
		1.97(79.8%)			1.055(71.5%)			1.87

The relaxation values obtained for asphaltene show more restricted and heterogeneous dynamic behavior than crab oil. In all three concentrations, the aliphatic, alicyclic, and aromatic regions required two T_2 components, indicating the presence of both relatively mobile and more restricted proton environments. At 5 mg/mL, the aliphatic region shows T_2 components of 0.065 and 0.602 s, while the alicyclic region shows 0.067 and 0.806 s. The aromatic region also contains two T_2 components, 0.184 and 1.18 s. These values indicate that asphaltene contains multiple mobility environments across all chemical-shift regions, rather than behaving as a purely mobile hydrocarbon system.

The concentration dependence becomes clearer as the sample concentration increases. In the aliphatic region, the short-component T_1/T_2 ratio increases from 6.92 at 5 mg/mL to 11.46 at 15 mg/mL and 31.46 at 50 mg/mL. A similar increase is observed in the aromatic region, where the short-component T_1/T_2 ratio increases from 35.97 at 5 mg/mL to 41.92 at 15 mg/mL and 163 at 50 mg/mL. These increasing ratios are mainly associated with short T_2 components, showing that restricted relaxation behavior becomes more pronounced at higher concentration.

The aromatic region is particularly important because aromatic protons are associated with the more rigid asphaltene core. At 50 mg/mL, the aromatic short component has a T_2 value of 0.036 s and a T_1/T_2 ratio of 163, while the longer aromatic component has a T_2 value of 1.055 s and a lower ratio of 1.87. The increase in aromatic T_1 at higher concentration also supports slower molecular motion, which is consistent with stronger intermolecular association and the presence of larger aggregated asphaltene structures. Together, the longer T_1 response and the short- T_2 restricted component support the presence of two aromatic mobility regimes: a restricted aggregated component and a relatively more mobile component. This behavior is consistent with Ratna's interpretation of asphaltene relaxation, where biexponential T_1 and T_2 behavior was assigned to two mobility populations within aggregated asphaltene structures.⁸ Therefore, the asphaltene sample provides an aromatic-rich and aggregation-prone reference system for comparison with HTL bio-oils. If HTL oils show shorter T_2 values and higher T_1/T_2 ratios, especially in the aromatic region, it is reasonable to believe the formation of heavier, more condensed, or more strongly interacting products during HTL.³

3.5 Algal HTL Bio-Oil NMR Results

3.5.1 NMR Analysis of *Chlorella pyrenoidosa* Derived HTL Bio-Oil

In this section, the structural changes occurring during hydrothermal liquefaction, HTL, are discussed based on the evolution of different functional groups in *Chlorella* bio-oil. A combination of ^1H NMR, DEPT, COSY, HSQC, HMBC, and DOSY experiments was used to evaluate the transformation of aliphatic, oxygenated, and aromatic compounds during HTL.¹¹

3.5.1.1 Aliphatic Region (0.5–2.5 ppm): Hydrocarbon Structures in *Chlorella* HTL Oil

The aliphatic region shows a high density of signals in the ^1H NMR spectra, with multiple overlapping multiplets and singlets. These signals are consistent with fatty acid-derived compounds showing a distribution of chain lengths and aliphatic environments. Under moderate HTL conditions at 300 °C, several aliphatic peaks decrease in intensity, suggesting the onset of chain scission and the breakdown of long-chain lipid structures. Under higher temperature HTL conditions (313 °C), the spectra show increased intensity and sharper, more resolved features. This indicates the formation of smaller and more uniform hydrocarbon-like molecules as complex lipid structures are converted into simpler aliphatic products.¹² At the same time, clear evidence for branching cannot be confirmed from the ^1H spectra alone because of strong signal overlap in the methyl and methylene regions.

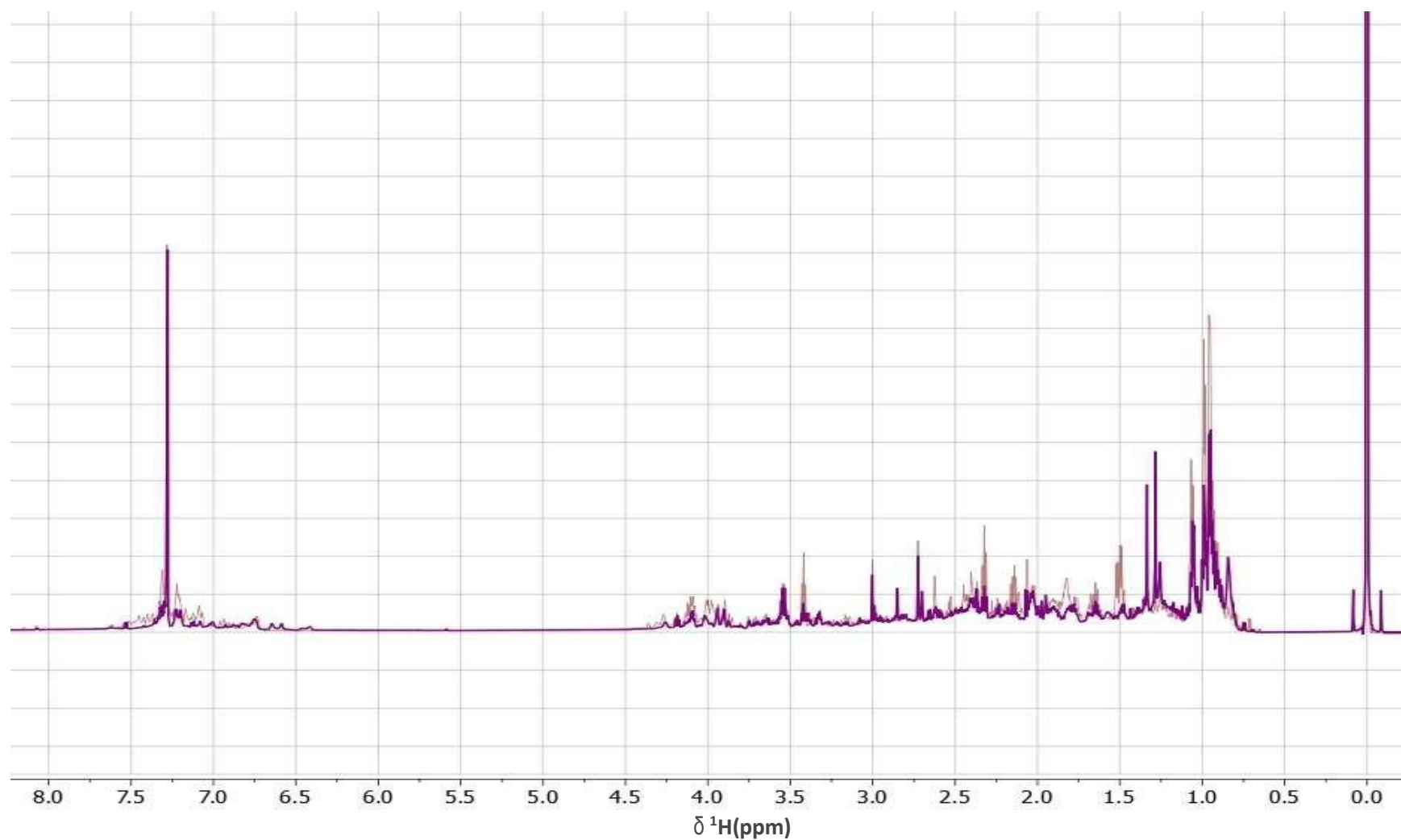


Figure 3.4 Superimposed full ^1H NMR spectra of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h (red) and *Chlorella* 313 °C, 1 h (purple).

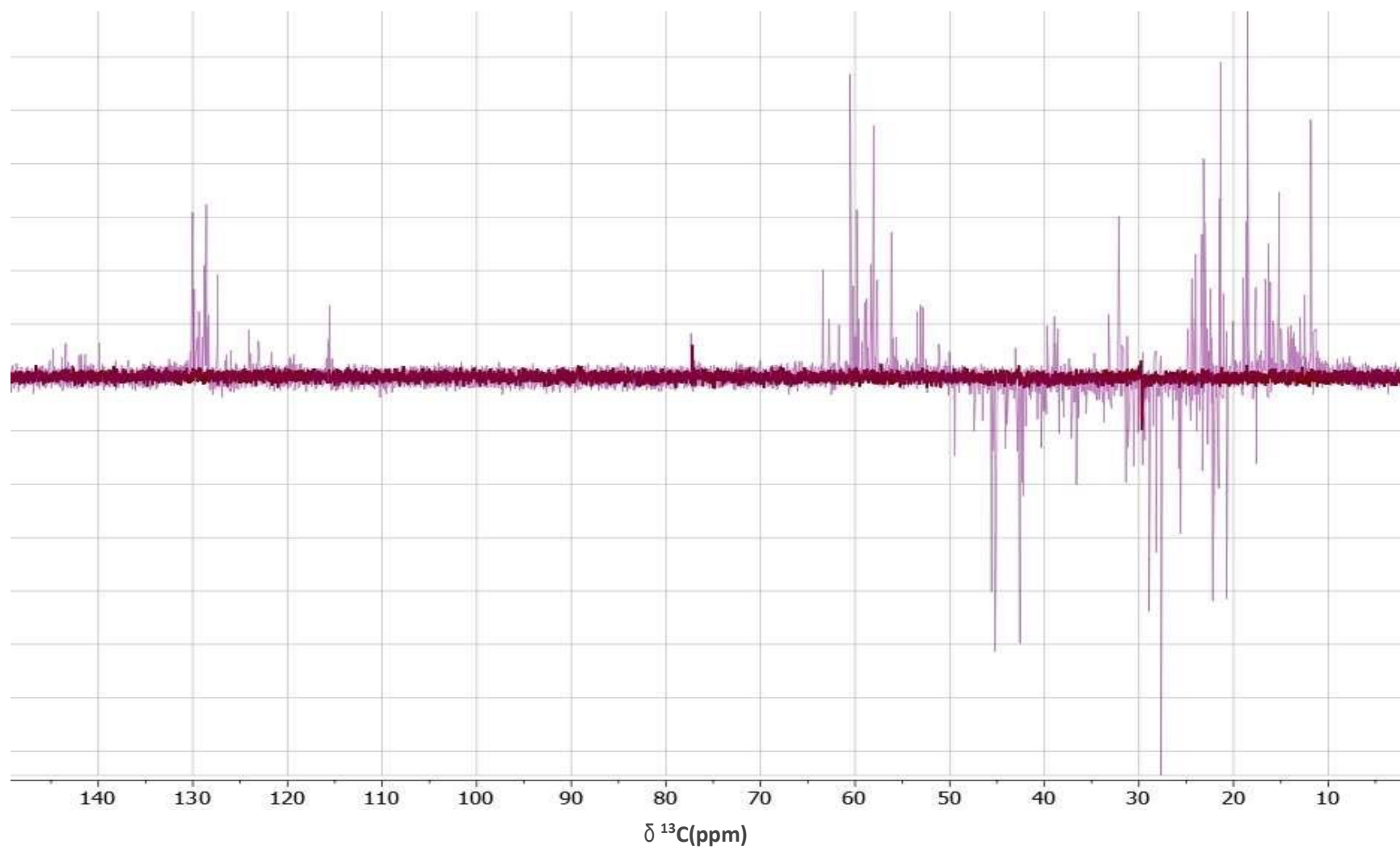


Figure 3.5 Superimposed full DEPT-135 NMR spectra of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h holding time (red) and *Chlorella* 313 °C, 1 h holding time (purple).

COSY spectra confirm proton–proton connectivity along alkyl chains, with correlations such as 1.33/1.77 ppm to 1.34/3.24 and 1.35/2.86, 0.66/2.05 to 0.84/2.05 ppm. These cross-peaks support the presence of long-chain fatty acid structures. In contrast, the disappearance of signals such as 0.77/1.38, 1.24/2.55, 1.33/1.77, and 1.76/2.82 ppm at higher temperature HTL conditions suggests cleavage of these chains into smaller fragments. (Figure 3.6) HSQC correlations also support this interpretation, with CH or CH₃ signals such as 1.05/15.23, 0.95/16.60, and 0.93/12.00 ppm, and CH₂ environments such as 1.77/21.46, 1.91/22.56, and 0.99/24.39 ppm. The disappearance of some CH₂ correlations, such as 2.89/3.31 and 3.02/2.23 ppm, is consistent with the breakdown of extended methylene sequences.

At the same time, new HSQC correlations, including 1.28/37.49, 0.88/45.49, and 1.66/30.04 ppm, as shown in Figure 3.7, suggest the formation of new aliphatic environments. These may correspond to branched hydrocarbons, short-chain alkanes, or methylene groups adjacent to substituted or rearranged carbon centers. DEPT-135 further supports this trend. At lower temperature, strong negative-phase CH₂ signals in the 20–25 ppm range are consistent with long, relatively linear fatty-acid-derived chains. As temperature increases, these CH₂ signals become more dispersed and less apparent across 20–40 ppm, while CH and CH₃ signals become more apparent. This agrees with Vardon *et al.*, who reported that HTL biocrude oils can contain straight and branched aliphatic compounds and that long branched aliphatic contents vary depending on feedstock composition.²² In the present study, the appearance of new HSQC correlations, together with increased CH₃ and CH contributions in DEPT-135, suggests the formation of shorter and more substituted aliphatic structures.

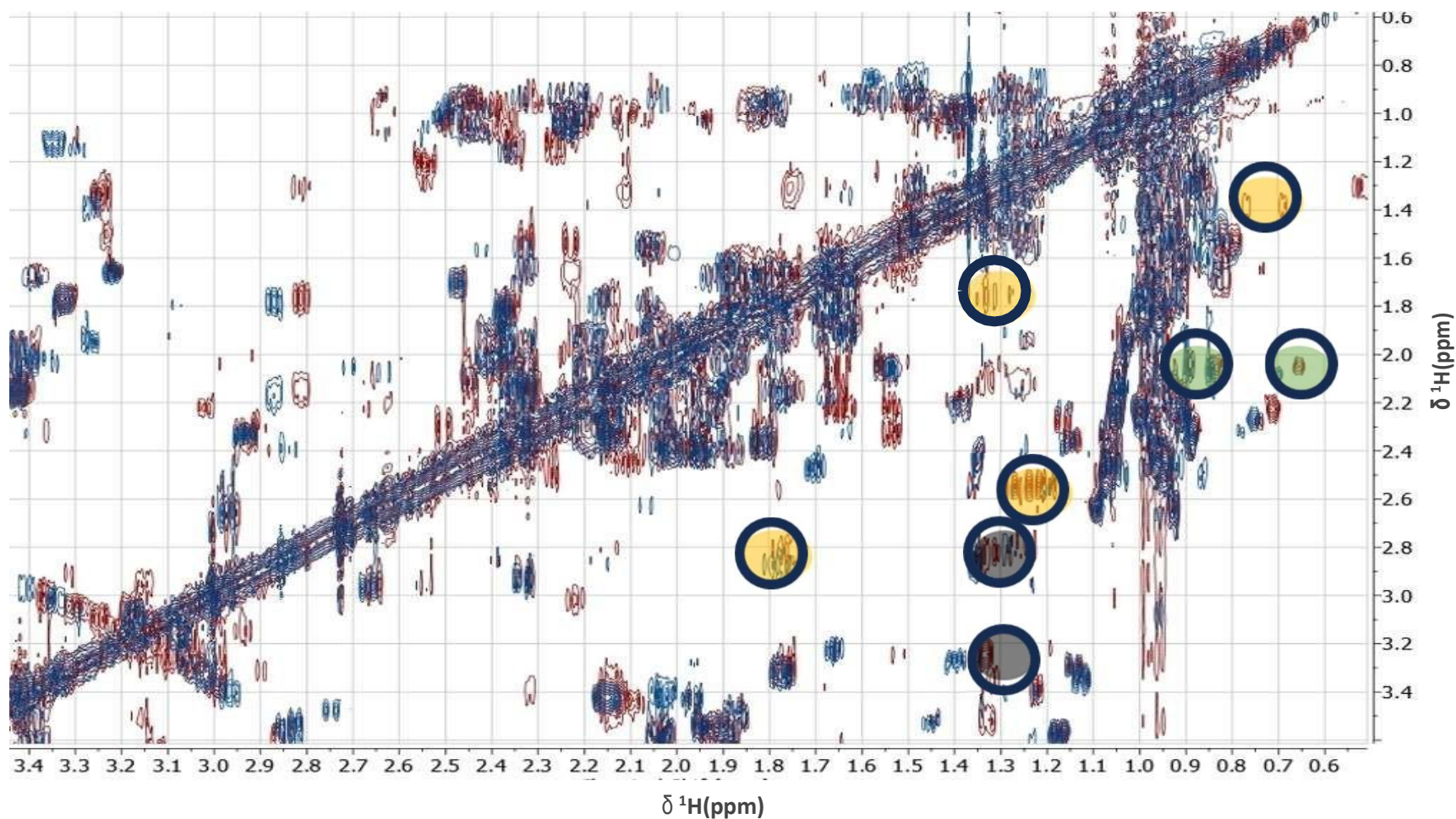


Figure 3.6 Superimposed COSY spectrum of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h (red) and *Chlorella* 313 °C, 1 h (blue). Yellow-marked disappearing correlations, including 0.77/1.38, 1.24/2.55, 1.33/1.77, and 1.76/2.82 ppm, suggest cleavage of alkyl chains under higher temperature HTL conditions. Green and dark-grey marked correlations, including 1.33/1.77 to 1.34/3.24 ppm and 1.35/2.86, 0.66/2.05 to 0.84/2.05 ppm, confirm proton–proton connectivity along alkyl chains.

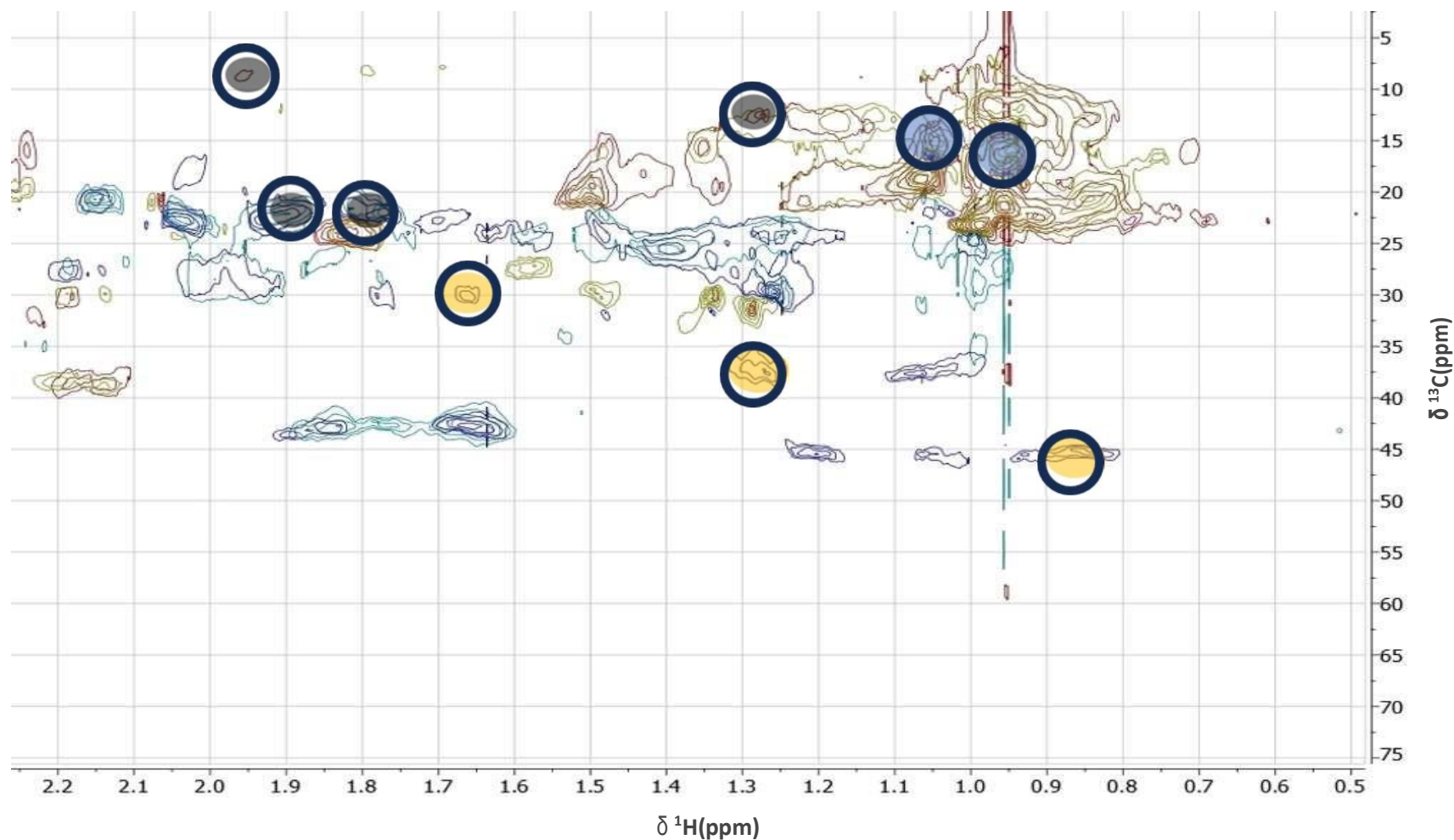


Figure 3.7 Superimposed HSQC spectrum of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h, with CH₂ signals in red and CH/CH₃ signals in light blue, and *Chlorella* 313 °C, 1 h, with CH₂ signals in mustard and CH/CH₃ signals in dark blue. Blue and dark-grey correlations support aliphatic CH/CH₃ and CH₂ environments, while yellow-marked new signals at 1.28/37.49, 0.88/45.49, and 1.66/30.04 ppm suggest the formation of new aliphatic environment.

HMBC analysis also supports the transformation of aliphatic structures. In the lower proton chemical-shift range, especially around 0.85 and 0.99–1.00 ppm, long-range correlations are mainly observed with carbons between 10 and 40 ppm. These are consistent with terminal CH₃ groups and saturated aliphatic CH/CH₂ environments. These correlations become more prominent at higher temperature, suggesting an increase in methyl-containing saturated aliphatic fragments. This may result from chain scission of longer lipid structures and the formation of shorter fatty acid-derived chains. Some of the new methyl signals could also indicate branched aliphatic structures.¹⁶ At higher proton chemical shifts, such as 1.50, 2.30, and 2.50 ppm, as shown in Figure 3.8, correlations are observed not only with aliphatic carbons but also with higher carbon shifts near 170 ppm. Since carbons in this region are commonly associated with carbonyl groups, these HMBC correlations suggest that some aliphatic protons are located close to carboxyl, ester, amide, or other substituted carbonyl-containing environments. This indicates that part of the aliphatic fraction is not composed only of simple hydrocarbon chains, but also includes lipid- or protein-derived fragments that still contain oxygenated or nitrogen-containing functional groups. Similar carbonyl-containing compounds, including fatty acids, amides, and esters, have been reported in HTL bio-oils by Leonardis et al., supporting this interpretation.¹¹

In the DOSY spectra, most cross-peaks occur in the aliphatic region, indicating that alkyl-rich and hydrocarbon-like compounds dominate the detectable bio-crude fraction. Their persistence is partly due to their longer T₁ and T₂ relaxation times, which make these mobile aliphatic components more visible in DOSY than broader, less mobile fractions.⁸

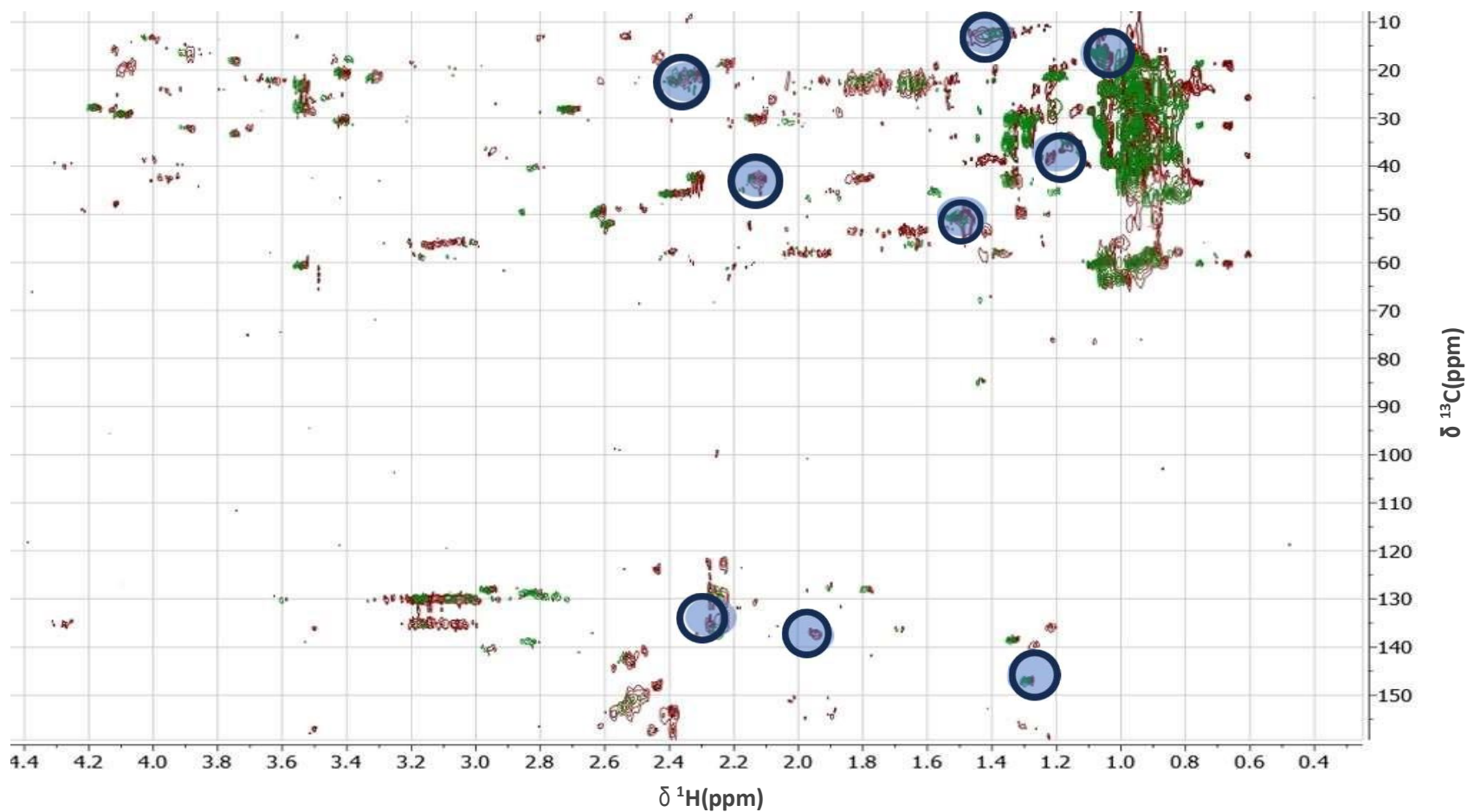


Figure 3.8 Superimposed HMBC spectrum of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h (red) and *Chlorella* 313 °C, 1 h (green). The blue-marked correlations highlight methyl-rich aliphatic fragments and carbonyl-connected aliphatic environments, supporting chain scission and formation of shorter or substituted lipid/protein-derived fragments under higher temperature HTL conditions.

In the aliphatic DOSY region, most cross-peaks occur between approximately 0.5 and 4.0 ppm, with diffusion coefficients mainly around 5.0×10^{-10} to 8.0×10^{-10} m²/s. at higher temperatures, some signals extend to smaller diffusion coefficients, approximately 3.0×10^{-10} m²/s, while others remain closer to $8.0\text{--}9.0 \times 10^{-10}$ m²/s. Because smaller diffusion coefficients indicate larger apparent hydrodynamic size, this broader diffusion range suggests that part of the aliphatic fraction becomes associated with larger structures. For example, the slower-diffusing aliphatic features may reflect aggregation or polymerization/condensation of hydrocarbon-rich fragments as HTL condition temperature increases. Overall, the aliphatic DOSY region indicates that high-temperature HTL conditions produce a more heterogeneous hydrocarbon-rich fraction with both relatively mobile smaller components and less mobile larger/aggregated species.⁸

3.5.1.2 Oxygenated Region, 2.5–5.5 ppm: Oxygen-Containing Functional Groups in *Chlorella* HTL Oil

The oxygenated region shows clear changes in signal intensity and distribution across HTL conditions. In the ¹H NMR spectra, a general decrease in intensity is observed at intermediate temperature, followed by partial intensity recovery at higher temperature. The disappearance of signals around 2.62, 3.00, 3.95, and 4.26 ppm suggests that oxygenated aliphatic structures are decomposed as HTL temperature increases. The HSQC correlations at 2.62/38.14 ppm and 3.00/60.13 ppm support the presence of substituted and oxygen-adjacent CH/CH₂ environments, respectively. Their loss in the ¹H spectra therefore reflects the breakdown of oxygen-containing structures through hydrolysis, followed by deoxygenation. These reactions convert oxygenated compounds into hydrocarbons, CO₂, and water. This interpretation is consistent with Biller et al., who reported the conversion

of algal lipid-derived compounds during hydrothermal processing, including the formation of fatty acid intermediates and further conversion toward hydrocarbon-like products under deoxygenating conditions.¹⁴ Shifts near 3.85 and 2.06 ppm also indicate changes in the local chemical environment, since chemical shifts depend on the magnetic environment around each proton. These peak movements support the modification of oxygenated aliphatic structures during HTL, consistent with deoxygenation and structural rearrangement reactions.

COSY spectra support these transformations. The disappearance of COSY cross-peaks such as 3.37/6.96ppm, 3.39/6.72 ppm, and 2.78/5.66 ppm suggests the loss of proton–proton connectivity (Figure 3.9) associated with oxygenated aliphatic groups. Since Sarpal *et al.* assigned the 3–5 ppm region to OCH, OCH₂, CHOH, and CH₂OH environments in glycerol and lipid-derived structures, the loss of these cross-peaks indicates decomposition or rearrangement of oxygenated lipid-derived fragments.¹⁶ This supports the interpretation that oxygenated structures are modified during HTL, with deoxygenation being one possible reaction pathway. The appearance of new COSY correlations at higher temperature further suggests that some oxygenated fragments are reorganized into new alcohol-, ether or ester-like environments rather than being completely removed.^{13,16}

HSQC spectra provide additional evidence for these changes. Correlations such as 2.24/15.68, 2.40/19.81, and 2.15/38.60 ppm are consistent with methylene groups adjacent to carbonyl. CH and CH₃ correlations at 2.13/20.72, 3.55/45.47, and 3.42/43.64 ppm suggest the formation of short-chain alcohols, ethers, or partially reduced oxygenated hydrocarbons (Figure 3.10).

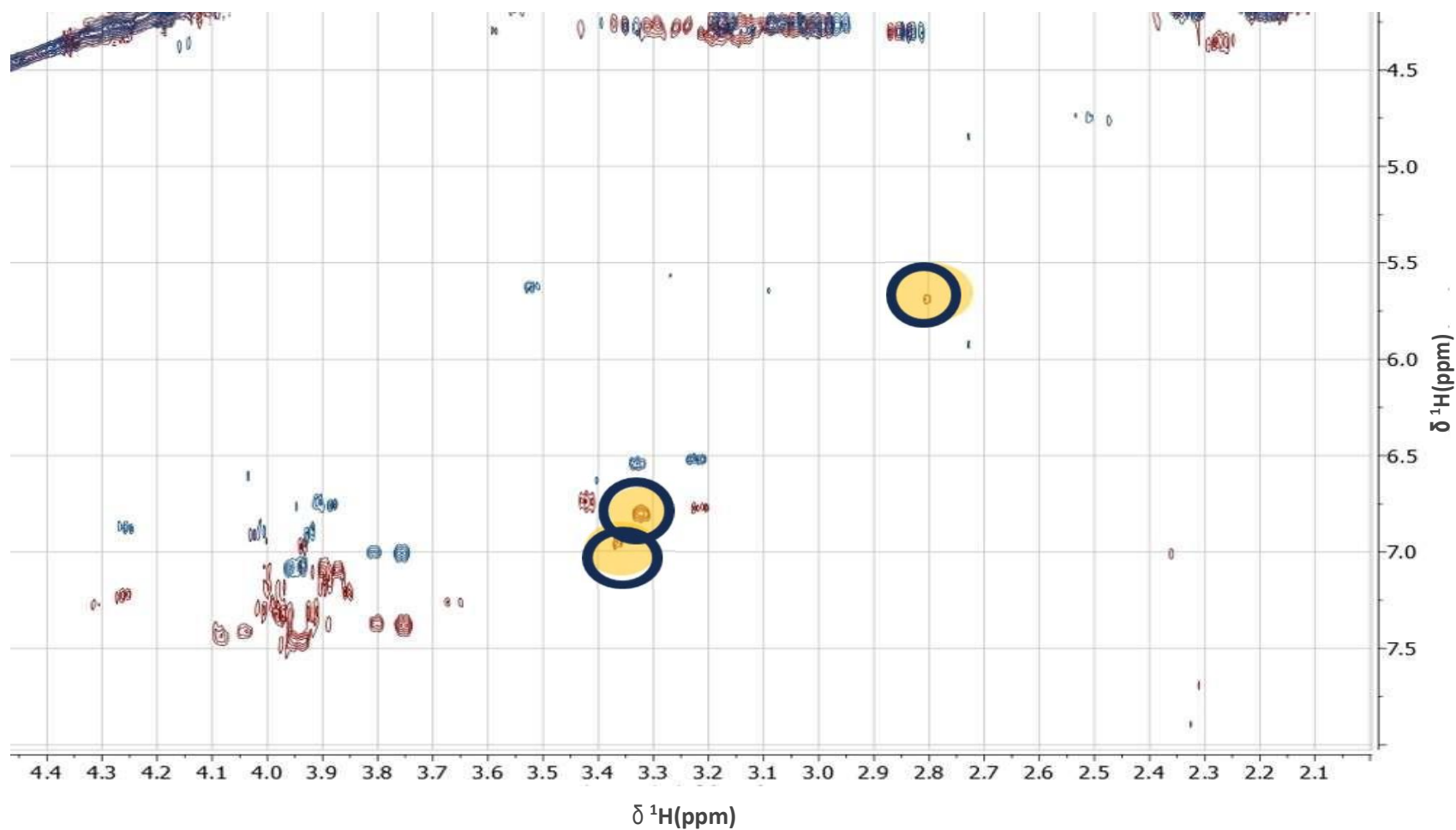


Figure 3.9 Superimposed COSY spectrum of the oxygenated region of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h (red) and *Chlorella* 313 °C, 1 h (blue cross-peaks). Yellow-highlighted disappearing cross-peaks, including 3.37/6.96 ppm, 3.39/6.72 ppm, and 2.78/5.66 ppm, suggest the loss of proton–proton connectivity associated with oxygenated aliphatic groups.

New correlations, including 4.64/43.26 and 3.56/34.01 ppm, indicate new oxygen-adjacent carbon environments formed during advanced HTL reactions. This is consistent with Wang et al., who described algal HTL bio-oil as a complex mixture containing oxygen-containing compounds such as fatty acids, ketones, esters, furans, and phenols, and Villadsen et al., who reported fatty acids, phenols, indanones, cyclopentenones/cyclic ketones, and aromatic oxygen-containing compounds in HTL bio-oils.^{3,26}

The disappearance of HSQC signals such as 3.35/50.05 and 2.45/22.56 ppm supports the removal or transformation of specific oxygenated or substituted aliphatic environments. This interpretation is consistent with Wang et al., who reported that macromolecular components decompose and recombine during HTL to form hydrocarbons, oxygen-containing compounds such as fatty acids, ketones, and esters, and nitrogen-containing compounds. They also noted that higher-temperature HTL can promote further decomposition and secondary reactions of bio-oil products. Therefore, the loss of these HSQC correlations in the present spectra suggests that these oxygenated environments are not stable throughout high HTL temperatures, but are consumed, rearranged, or converted into other aliphatic, carbonyl-containing, or less oxygenated products.³ Phase changes in signals, such as the signal near 3.32/50.05 ppm, reflect changes between CH₂ and CH/CH₃ phases, which are caused by different components overlapping each other or partial structural transformations.

DEPT analysis also shows that oxygen-adjacent carbon environments are limited and transient. Weak CH₂ contributions in the 50–90 ppm range are observed mainly at intermediate HTL temperatures, whereas these signals decrease at the higher temperature. This suggests that oxygenated structures are progressively transformed through

deoxygenation, dehydration, and decarboxylation into simpler, less oxygenated compounds.¹⁶

HMBC correlations further confirm this trend. Several protons in the 2.5–4.1 ppm range show long-range correlations with substituted aliphatic and carbonyl-region carbons. Correlations such as 2.34/179.46 and 2.73/177.45 ppm remain visible, suggesting that some oxygenated or heteroatom-substituted fragments persist even under severe HTL conditions. The persistence of these signals is reasonable because Zimmermann et al. showed that HTL biocrude still contains highly functionalized oxygen- and nitrogen-containing compounds, including aromatic N-heterocycles, aliphatic amides, and oxygen-functionalized N-compounds.⁴ Correlations such as 3.95/167.70 and 4.05/170.18 ppm are important because they suggest connectivity between oxygenated protons and carbonyl-region carbons, consistent with ester-like, carboxyl-derived, amide-like, or related structures (Figure 3.11).

In the oxygenated DOSY region, only a small number of cross-peaks are observed, mainly at 3–4 ppm, with little extension toward 6 ppm. These signals have diffusion coefficients around 6.0×10^{-10} to 1.0×10^{-9} m²/s, indicating intermediate to relatively slow mobility compared with the more mobile aliphatic fraction. At higher HTL temperature, some oxygenated features shift by about 0.2 log units toward lower diffusion, corresponding to an approximately 1.6-fold decrease in D and an apparent hydrodynamic radius about 60% larger, assuming a similar molecular shape. The broader or thicker features, therefore, suggest oxygenated groups associated with less mobile bio-crude fractions. Thus, the oxygenated DOSY region supports both removal and rearrangement of oxygen-containing structures rather than simple one-step deoxygenation.

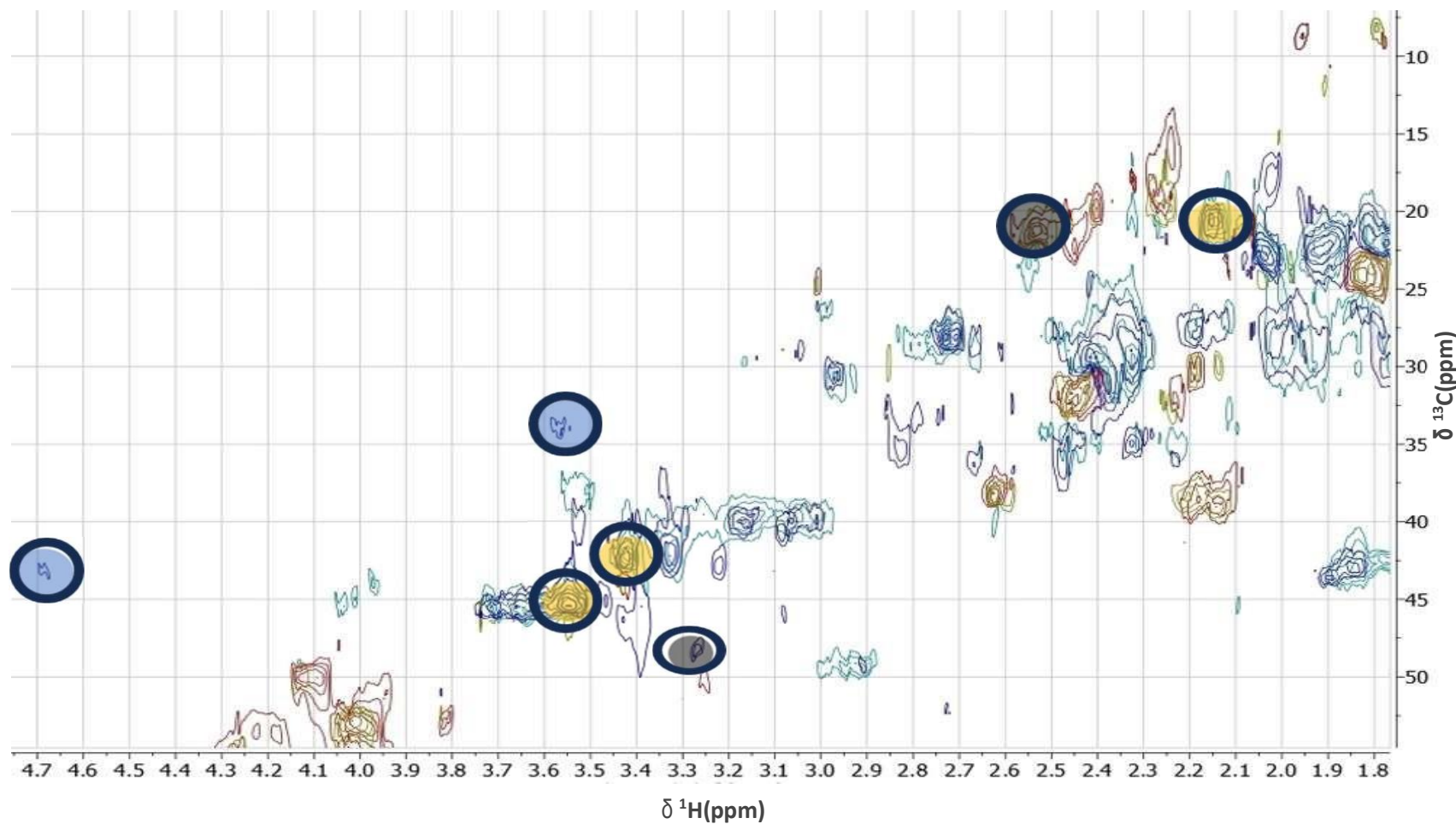


Figure 3.10 Superimposed HSQC spectrum of the oxygenated region of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h, with CH₂ signals in red and CH/CH₃ signals in light blue, and *Chlorella* 313 °C, 1 h, with CH₂ signals in mustard and CH/CH₃ signals in dark blue. Blue-highlighted new correlations indicate newly formed oxygen-adjacent environments, dark-grey disappearing signals support removal or transformation of oxygenated/substituted aliphatic groups, and yellow correlations indicate methylene groups adjacent to carbonyl or substituted environments.

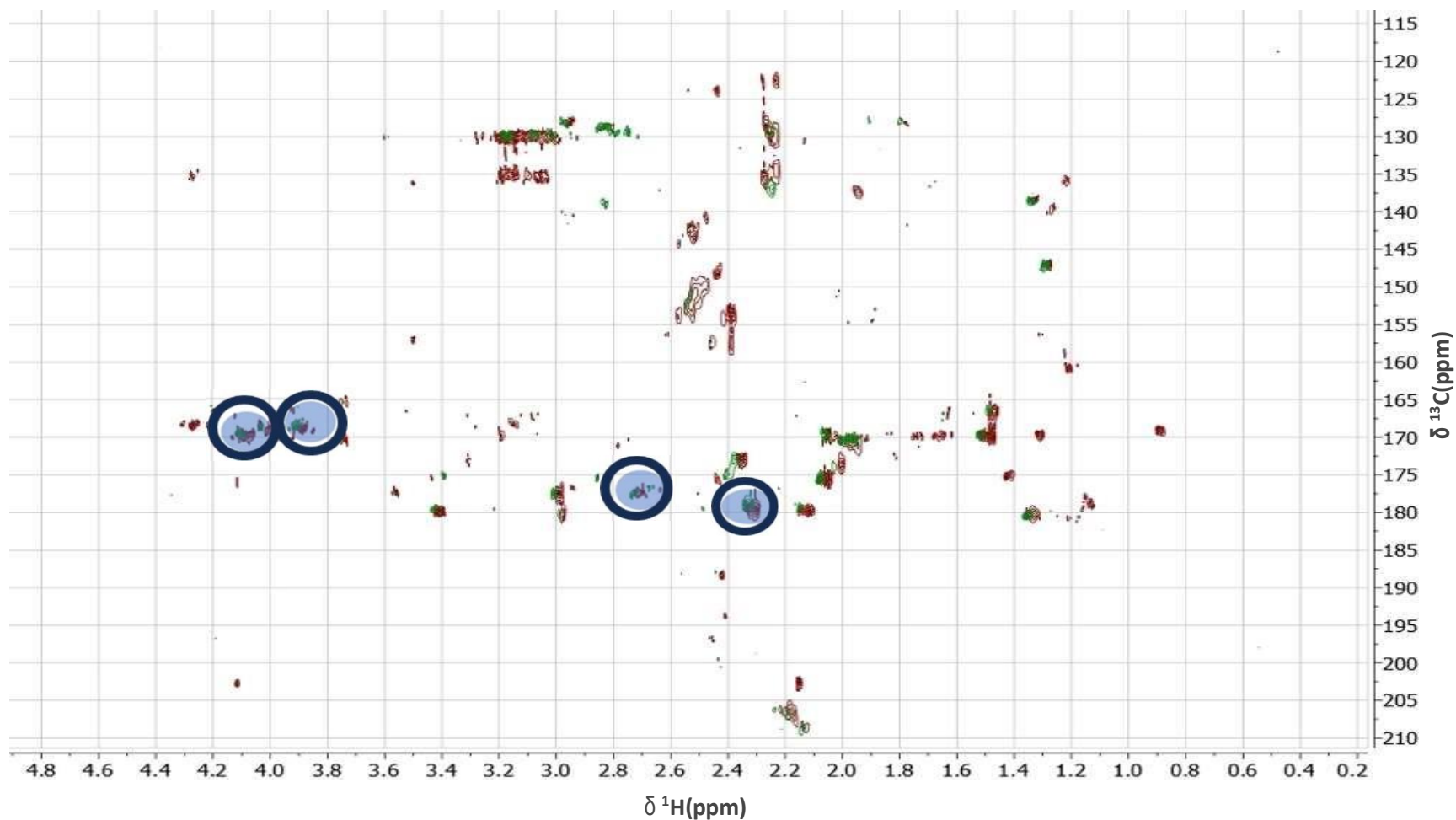


Figure 3.11 Superimposed HMBC spectrum of the oxygenated region of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h (red) and *Chlorella* 313 °C, 1 h (green). Blue correlations such as 2.34/179.46 and 2.73/177.45 ppm remain visible, suggesting persistence of some oxygenated or heteroatom-substituted fragments, while 3.95/167.70 and 4.05/170.18 ppm indicate connectivity between oxygenated protons and carbonyl-region carbons.

3.5.1.3 Aromatic and Olefinic Region, 5.5–8 ppm: Secondary Reaction Products in *Chlorella* HTL Oil

The aromatic and olefinic region show clear changes with increasing HTL temperature, indicating progressive formation and transformation of aromatic compounds. In the ^1H NMR spectra, several signals increase in intensity or newly appear at higher temperature, especially around 6.55–6.65, 6.73, and 7.12 ppm. These changes suggest the formation of aromatic proton environments through cyclization and condensation of aliphatic and oxygenated precursors.

At higher HTL temperatures, many peaks become broader and more overlapped, which is consistent with the formation of more complex mixtures containing substituted aromatics and possibly polycyclic aromatic structures. This interpretation is consistent with Xu *et al.*, who reported ^1H NMR peaks at 7.2–8.0 ppm from aromatic protons or conjugated dienes in HTL biocrude, and with Bjelic *et al.*, who found that HTL-derived asphaltene fractions were more aromatic and contained condensed aromatic/polyaromatic species.^{14,27}

COSY spectra show new aromatic cross-peaks at higher temperature, including 7.15/8.08, 7.72/6.48, and 6.29/6.94 ppm as instructed in Figure 3.12. These correlations indicate the formation of new coupled aromatic proton systems and support the development of substituted aromatic rings or extended conjugated structures.

HSQC spectra confirm the development of aromatic C–H environments, with correlations such as 7.54/118.11, 7.36/124.29, and 6.88/115.12 ppm. These signals support the presence of substituted benzene-like and conjugated structures. The disappearance of some correlations, including 7.31/134.83, 6.90/131.65, and 6.99/124.29 ppm, indicates loss or transformation of specific carbon environments during deoxygenation, decarboxylation, and condensation. In addition, Bjelic *et al.* reported that HTL-derived asphaltene fractions

were less saturated and more aromatic, with increased aromatic and condensed aromatic/polyaromatic species, supporting the possibility that some disappearing aromatic correlations are incorporated into heavier condensed structures.³⁰

DEPT spectra show that the aromatic and olefinic carbon region, 100–160 ppm, is mainly composed of CH and quaternary carbon environments, with minimal CH₂ contribution. This confirms that methylene groups are not strongly incorporated into the aromatic framework. The limited CH₂ contribution in the 100–160 ppm region suggests that the aromatic fraction is mainly composed of cyclic aromatic structures with protonated and substituted/quaternary aromatic carbons. This is consistent with HTL reactions in which methylene-rich fragments undergo cracking, dehydrogenation, cyclization, and condensation rather than being directly incorporated as CH₂ groups into the aromatic framework.¹⁶ At higher temperature, increased intensity and clustering of aromatic CH signals suggest enhanced formation of substituted and condensed aromatic systems.

HMBC analysis also supports increasing aromatic complexity with increasing HTL temperature. At lower temperatures, aromatic-region correlations are limited, but with increasing temperature, additional correlations appear, supporting secondary cyclization, aromatization, and condensation reactions. The signal near 7.22 ppm is particularly informative because it shows long-range correlations to carbons near 40, 127, and 168 ppm. The 7.22/168 ppm, 7.47/169ppm and 7.62/138ppm correlation indicate association with a carbonyl-region carbon, suggesting oxygenated, ester-like, carboxyl-derived, amide-like, or heteroatom-substituted aromatic structures (Figure 3.15).

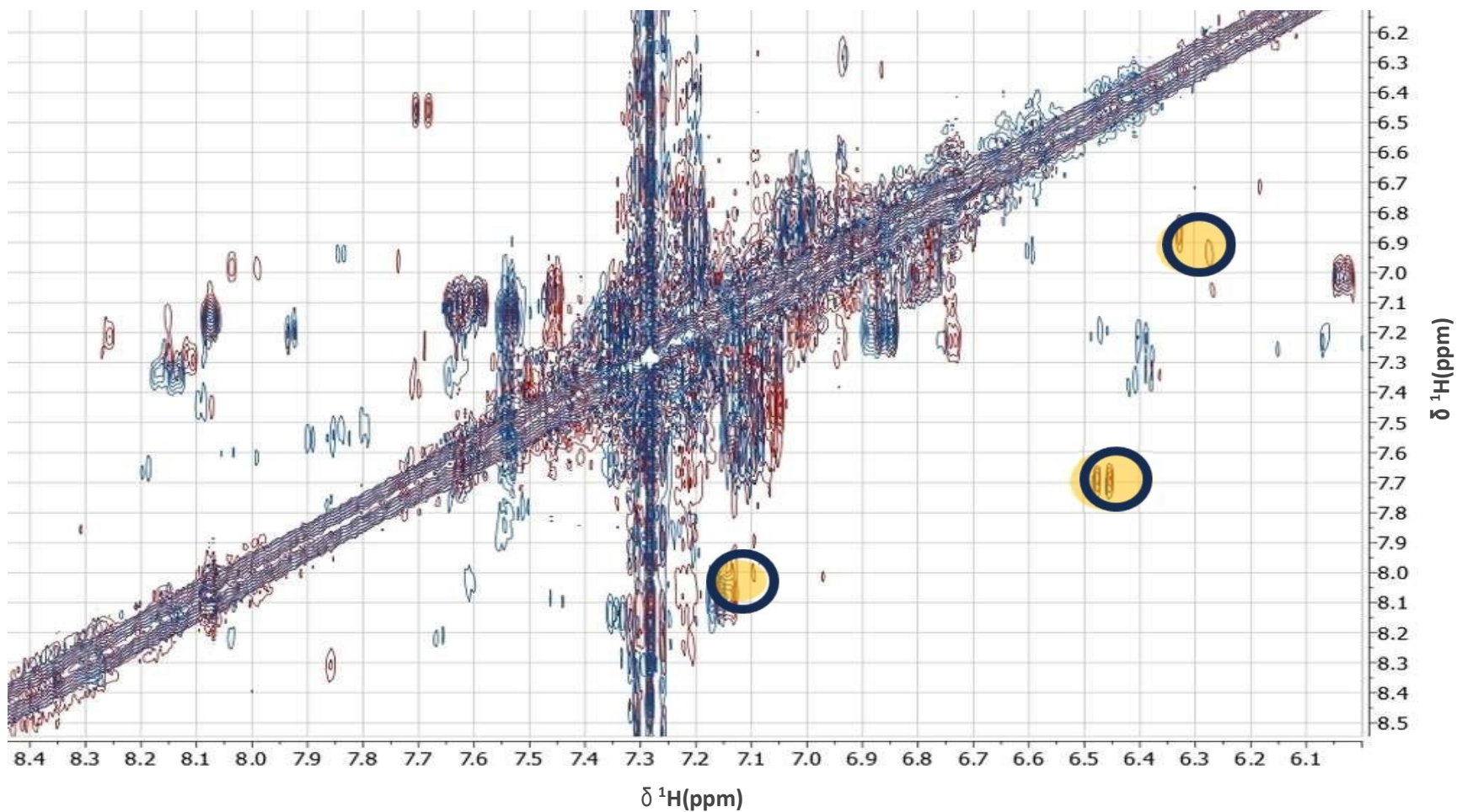


Figure 3.12 Superimposed COSY spectrum of the aromatic region of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h (red) and *Chlorella* 313 °C, 1 h (blue). Yellow-highlighted new cross-peaks at 7.15/8.08, 7.72/6.48, and 6.29/6.94 ppm indicate new coupled aromatic proton systems, supporting the formation of substituted aromatic rings or extended conjugated structures.

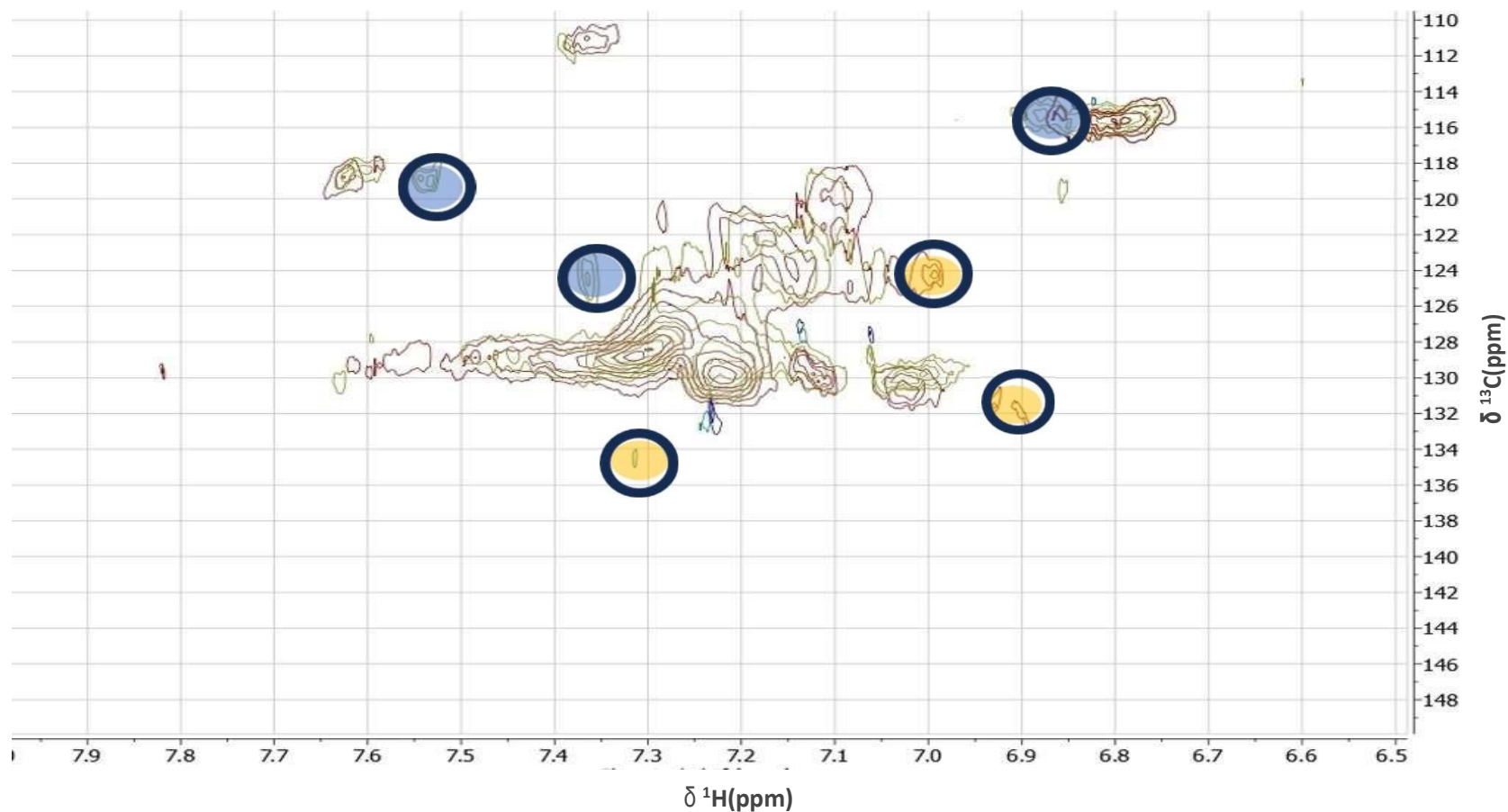


Figure 3.13 Superimposed HSQC spectrum of the aromatic region of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h, with CH₂ signals in red and CH/CH₃ signals in light blue, and *Chlorella* 313 °C, 1 h, with CH₂ signals in mustard and CH/CH₃ signals in dark blue. Blue correlations such as 7.54/118.11, 7.36/124.29, and 6.88/115.12 ppm confirm aromatic C–H environments, while yellow disappearing correlations, including 7.31/134.83, 6.90/131.65, and 6.99/124.29 ppm, indicate loss or transformation of specific carbon environments during deoxygenation and decarboxylation.

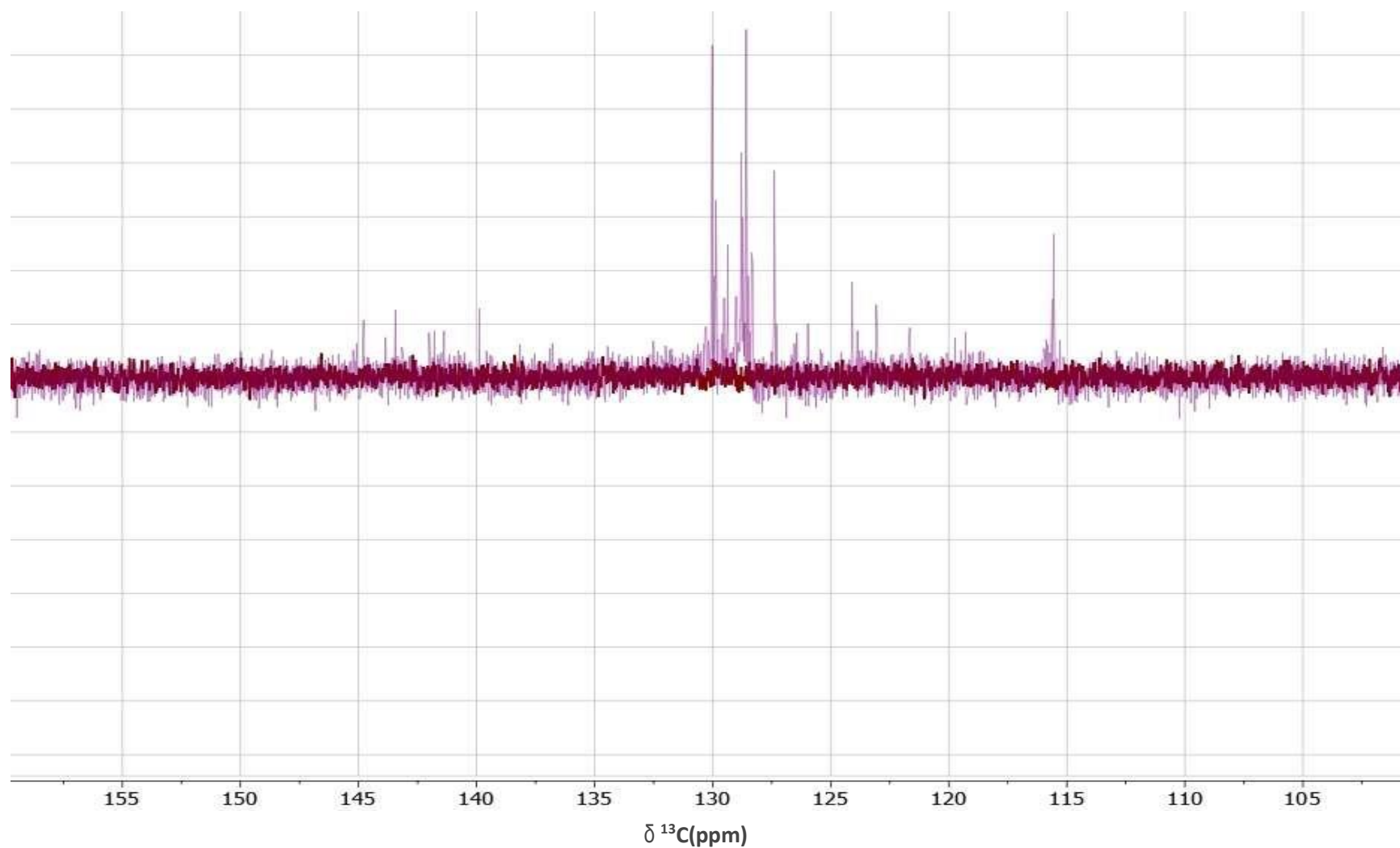


Figure 3.14 Superimposed DEPT-135 spectrum of the aromatic region of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h (red) and *Chlorella* 313 °C, 1 h (purple). The limited CH₂ contribution and stronger aromatic CH signals suggest formation of substituted or condensed aromatic structures at higher HTL temperature.

This interpretation agrees with Vardon *et al.*, who reported the presence of aromatics, phenolic derivatives, carboxylic acids, esters, and nitrogenous ring structures in HTL biocrude oils, and with Wang *et al.*, who described the conversion of furan compounds to aromatic compounds into aromatic compounds during high-temperature hydrothermal processing.^{22,3} In the 7.3–7.6 ppm range, correlations such as 7.18/168, 7.47/169, and 7.62/168 ppm weaken or disappear at the highest temperature, suggesting transformation of early oxygenated or carbonyl-substituted aromatic intermediates rather than complete loss of aromatic material due to decomposition.

In the aromatic DOSY region, as shown in Figure 3.16, fewer cross-peaks are observed than in the aliphatic region, showing that aromatic species are less prominent in the soluble bio-oil fraction. At lower HTL temperature, aromatic signals occur mainly at 6.6–7.8 ppm with diffusion coefficients of approximately 5.6×10^{-10} to 8.0×10^{-10} m²/s. At higher HTL temperature, the aromatic region extends to about 6.6–8.5 ppm, but most diffusion coefficients remain in the same range, indicating no large change in overall mobility.

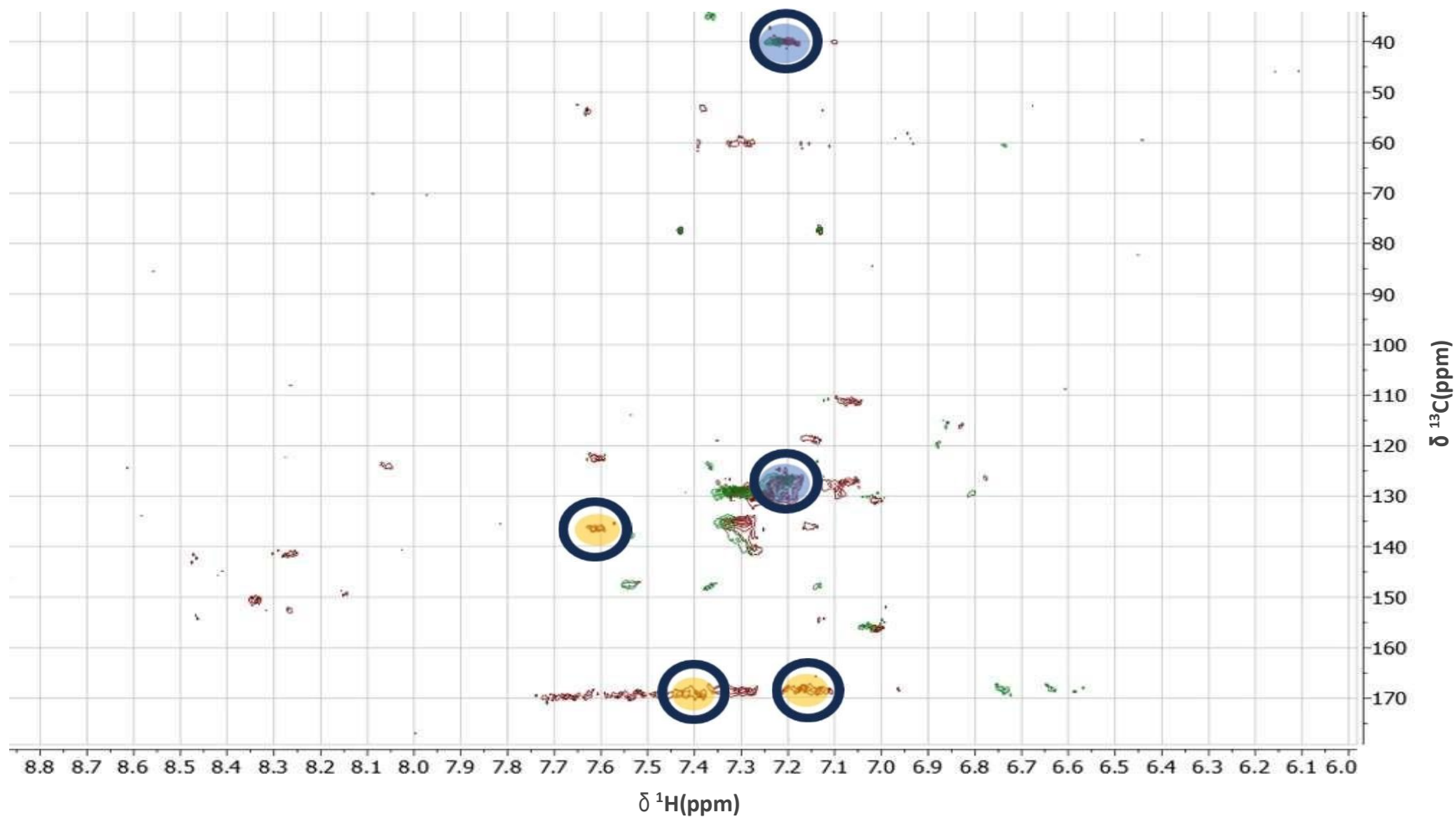


Figure 3.15 Superimposed HMBC spectrum of the aromatic region of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h (red) and *Chlorella* 313 °C, 1 h (green). Blue correlations at 7.22/127 and 7.22/40 ppm support aromatic and alkyl-substituted aromatic environments, while yellow correlations at 7.22/168, 7.47/169, and 7.62/138 ppm indicate oxygenated or heteroatom-substituted aromatic structures.

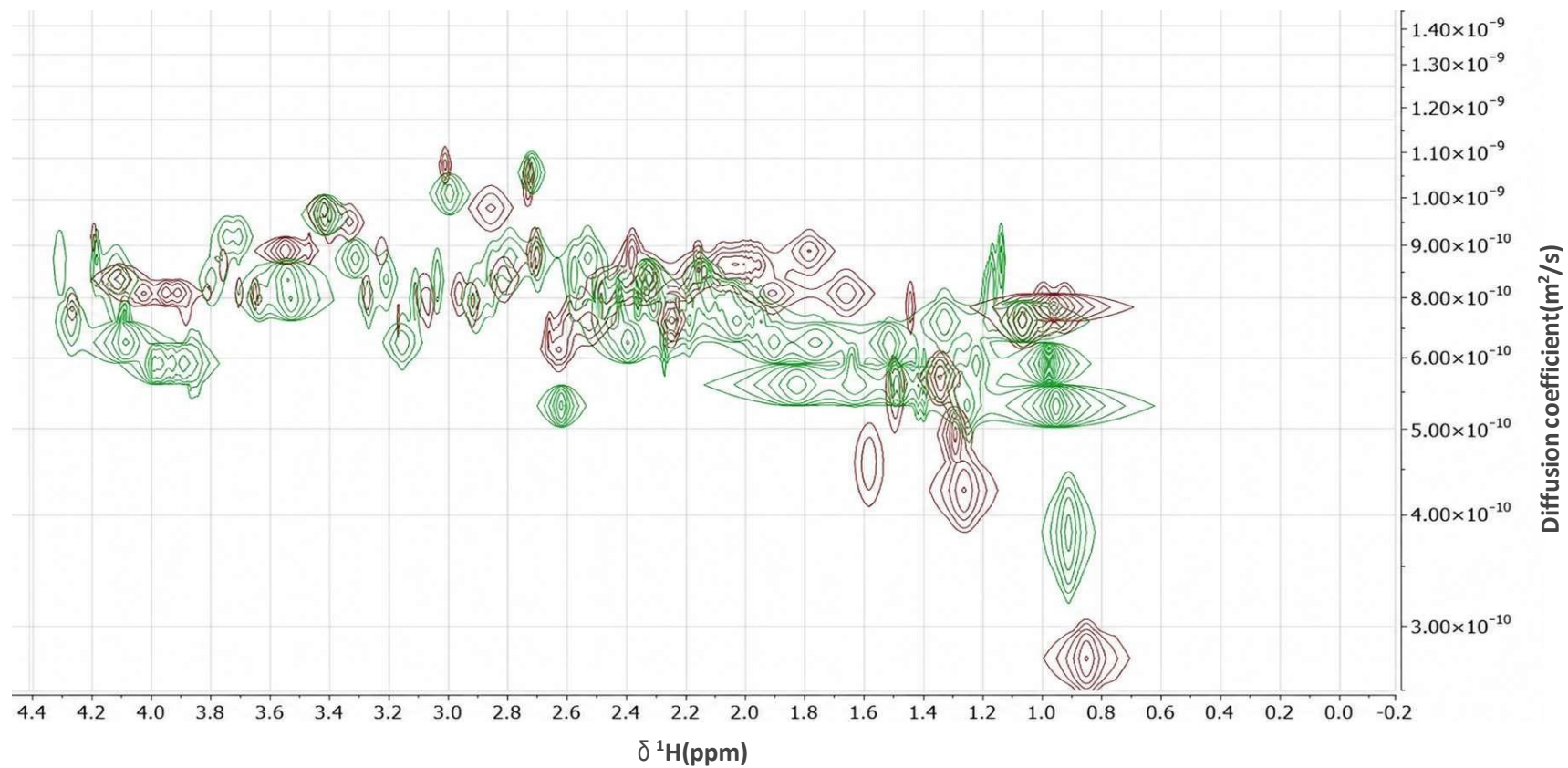


Figure 3.16 Superimposed DOSY spectrum of *Chlorella*-derived HTL bio-oil comparing *Chlorella* 260 °C, 0.5 h holding time (red) and *Chlorella* 313 °C, 1 h holding time (green).

3.5.2 NMR Analysis of *Spirulina platensis* Derived HTL Bio-Oil

3.5.2.1 Aliphatic Region (0.5–2.5 ppm): Hydrocarbon Evolution in *Spirulina* HTL Oil

The aliphatic region of the ^1H NMR spectra shows systematic changes with increasing HTL temperature, reflecting the transformation of lipid-derived structures. At lower temperatures, this region is dominated by broad and overlapping signals, consistent with long-chain fatty acids and complex aliphatic environments. As temperature increases, sharper and redistributed peaks appear, especially around 0.99–1.06 ppm. This sharpening suggests the formation of more uniform aliphatic environments, likely from shorter hydrocarbon chains produced through chain scission reactions.¹²

At higher chemical shifts, especially around 1.2–1.7 ppm, the spectra become broader at 313 °C and show a more uniform signal distribution. This broadening may reflect greater structural heterogeneity and overlapping signals from newly formed aliphatic species, including branched hydrocarbons and substituted chains. Whereas, sharp peaks around 1.28 and 1.32 ppm persist, suggesting that some aliphatic chain environments remain stable during HTL. The appearance of short multiplets around 1.50–1.60 ppm at higher temperature suggests the formation or rearrangement of new aliphatic environments, likely associated with alkyl-chain, branched, or cyclic aliphatic structures. This assignment is consistent with Xu *et al.*, who reported that the 0.5–1.8 ppm region of HTL biocrude is dominated by methyl and methylene protons and that corresponding aliphatic carbon signals reflect different chain lengths and branching degrees.²⁵ Near 2.0 ppm, the increase in sub-peaks indicates more complex aliphatic environments, such as protons near substituted or functionalized carbon centers.

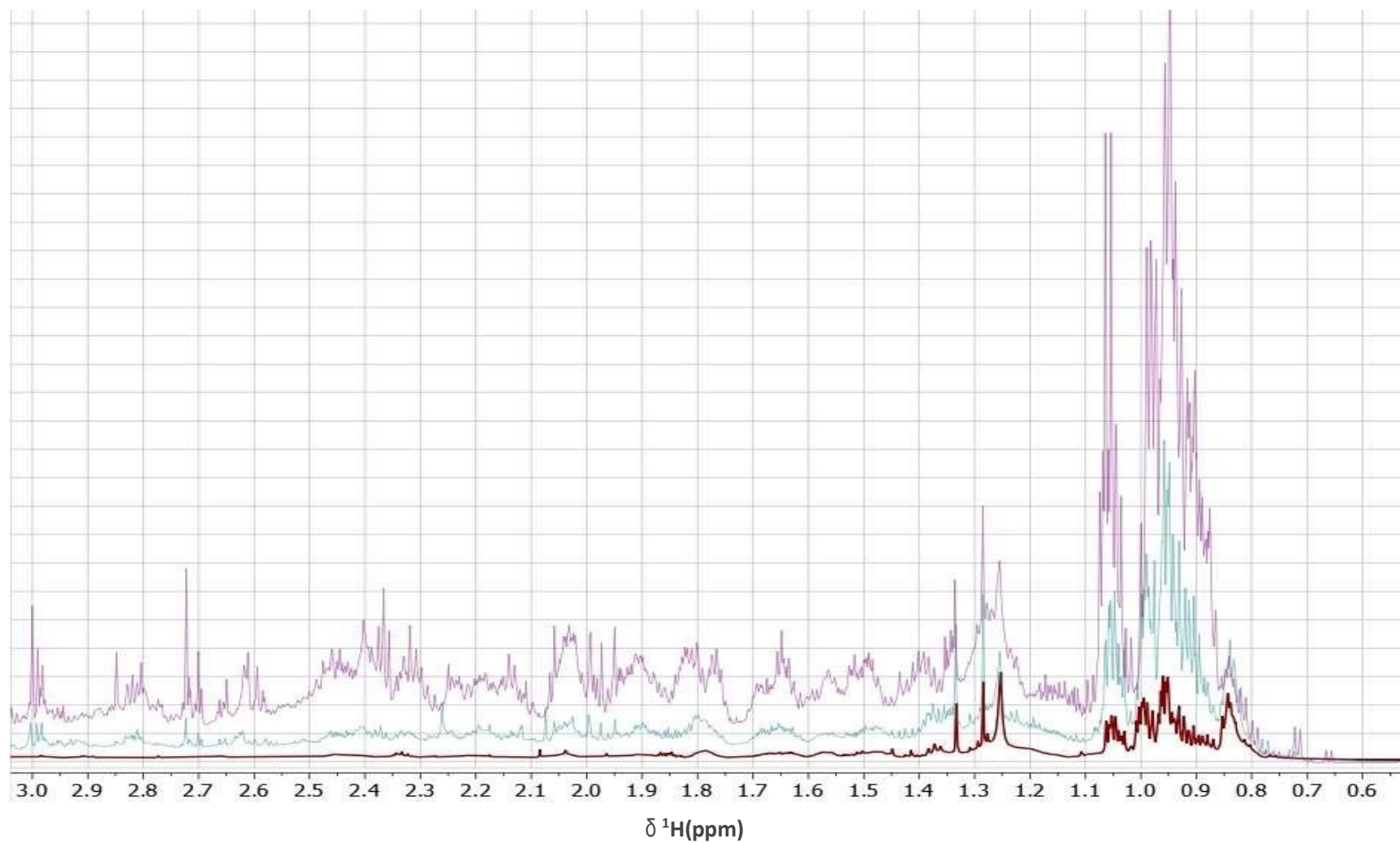


Figure 3.17 Superimposed ^1H NMR spectra of the aliphatic region of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C with 0.5 h holding time (red), *Spirulina* 300 °C with 1 h holding time (blue), and *Spirulina* 313 °C with 1 h holding time (purple).

COSY spectra support these trends. Several proton–proton correlations persist across the reaction conditions, including 1.35/2.87, 1.66/1.91, 1.41/2.20, and 1.02/1.43 ppm (Figure 3.18). The persistence of these cross-peaks indicates that part of the aliphatic chain connectivity remains intact even at higher HTL temperatures. The disappearance of some correlations, such as those involving 1.55/3.28 ppm, suggests localized structural breakdown. Minor chemical-shift changes across the aliphatic region also indicate changes in the local chemical environment, likely caused by branching, substitution, or proximity to newly formed functional groups. Yang et al. further showed that some larger fatty-acid-derived or high-carbon compounds disappear under catalytic HTL because of decarboxylation and cracking to smaller molecules, supporting the interpretation that loss of specific correlations can indicate localized structural breakdown.²⁸

HSQC spectra provide further evidence for aliphatic transformation. The disappearance of correlations near 1.12 and 1.38 ppm indicates the loss or transformation of original aliphatic structures. At the same time, new CH and CH₃ correlations, such as 1.80/8.30, 1.91/11.30, and 1.57/42.81 ppm, as shown in Figure 3.19, suggest the formation of new hydrocarbon species. Similar aliphatic diversity has been reported for HTL biocrudes: Vardon et al. found that *Spirulina* HTL biocrude contained alkene and branched alcohol products such as tridecene and trimethyl-dodecanol, while ¹³C NMR showed short aliphatic and long-branched aliphatic carbon regions. Xu *et al.* also reported that methyl and methylene carbon signals in HTL biocrude reflect different chain lengths and branching degrees. Therefore, the observed CH₂ transitions, including 0.96/13.85 to 0.96/17.98 ppm and 1.29/27.14 to 1.29/31.26 ppm, suggest rearrangement of aliphatic structures through fragmentation, branching, and secondary recombination during HTL.^{22,25}

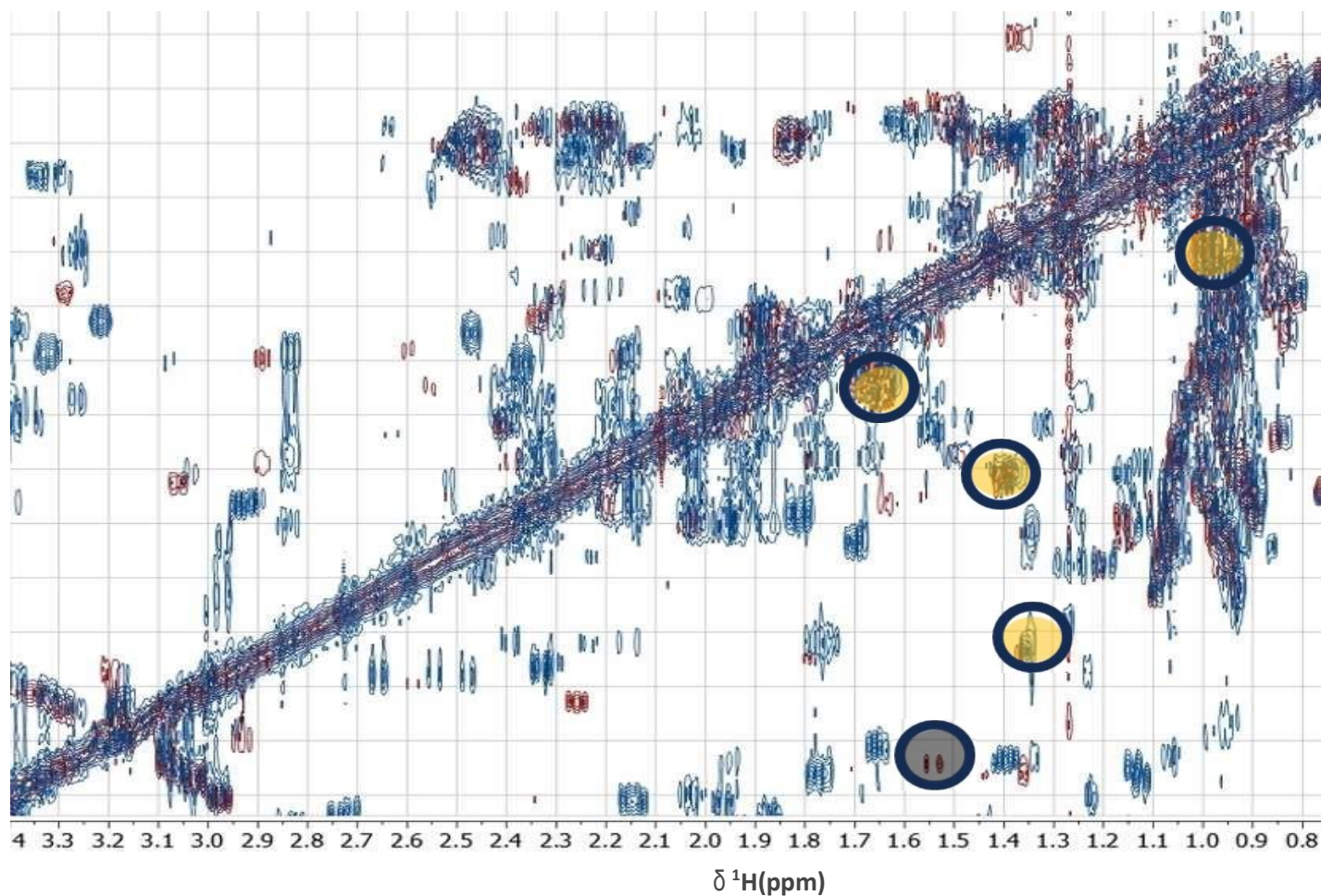


Figure 3.18 Superimposed COSY spectrum of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h and *Spirulina* 313 °C, 1 h holding time (blue). Yellow correlations, including 1.35/2.87, 1.66/1.91, 1.41/2.20, and 1.55/3.28 ppm, are present across reaction conditions, while the dark-grey correlation at 1.55/3.28 ppm disappears at higher HTL temperature.

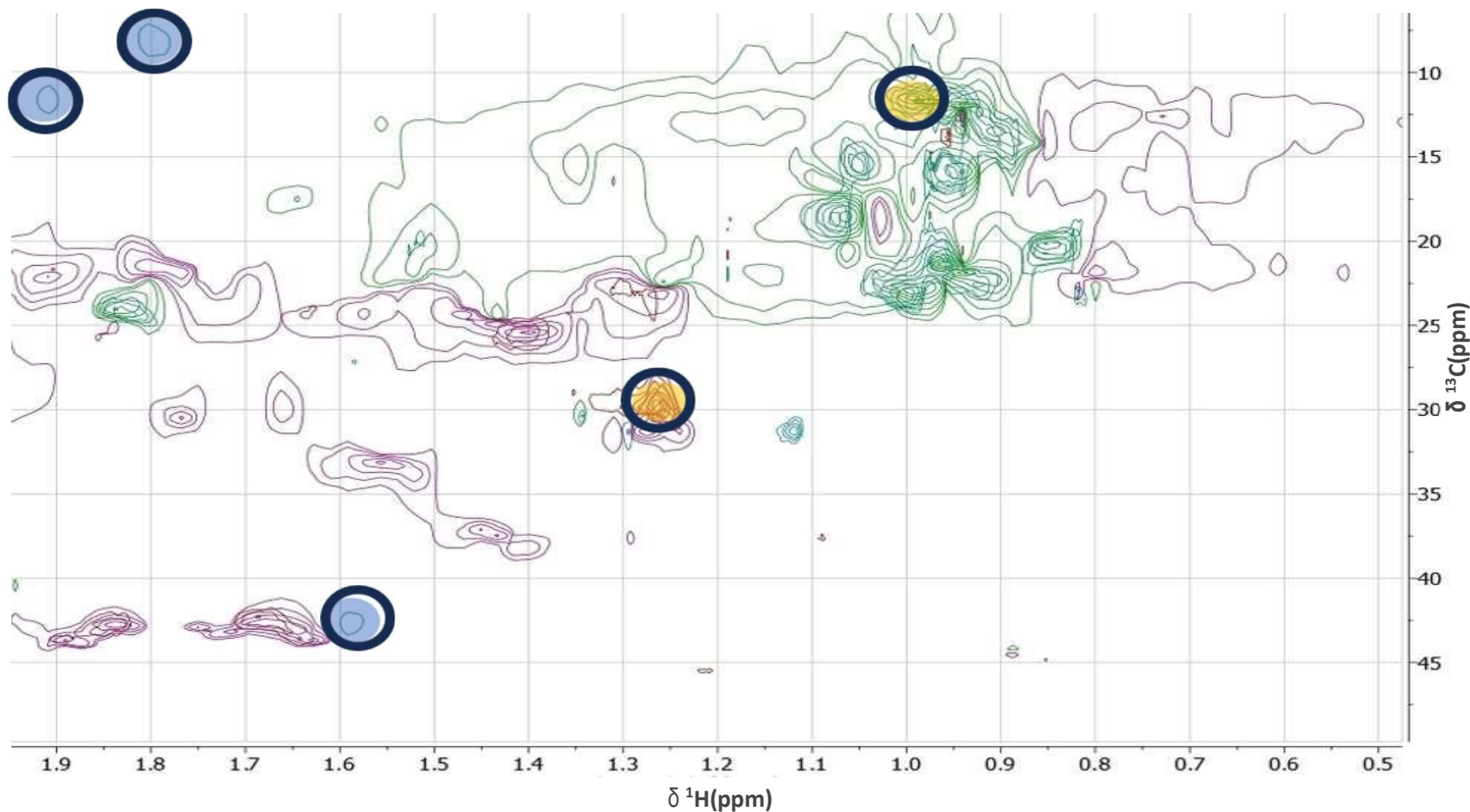


Figure 3.19 Superimposed HSQC spectrum of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time, with CH₂ signals in red and CH/CH₃ signals in light blue, and *Spirulina* 313 °C, 1 h holding time, with CH₂ signals in purple and CH/CH₃ signals in green. Blue-marked correlations such as 1.80/8.30, 1.91/11.30, and 1.57/42.81 ppm suggest formation of new hydrocarbon species, while the yellow correlations at 0.96/13.85 and 1.29/27.14 ppm indicate rearrangement of aliphatic structures based on chemical-shift changes.

DEPT analysis supports this interpretation. Under milder HTL conditions, negative-phase CH₂ signals appear mainly around 18, 23, and 25 ppm, consistent with methylene groups from lipid-derived chains. As HTL temperature increases, CH₂ signals become more visible at higher carbon shifts, *c.a.* 28, 40, and 45 ppm at 313 °C. CH and CH₃ signals also become more noticeable around 300 °C and at higher temperatures, suggesting the formation of branched or rearranged hydrocarbon structures. This is reasonable because *Spirulina* HTL has been reported to produce fatty-acid ester-rich and nitrogen-containing biocrudes, while HTL pathways involve hydrolysis, cracking, decarboxylation, isomerization, and recombination reactions.²²

HMBC analysis further confirms this trend. At lower proton shifts, such as 0.94/18 ppm and 0.95/45 ppm, correlations are mainly associated with aliphatic carbon environments, consistent with terminal CH₃ groups and saturated hydrocarbon fragments. Only a few clear correlations are observed near 1.3 ppm with oxygenated, aromatic, or carbonyl-region carbons, suggesting that this region is mainly associated with simple alkyl-chain environments, consistent with previous reports of methyl- and methylene-rich straight or branched aliphatic compounds in HTL biocrudes.^{22,25} Above 1.3 ppm, additional correlations appear to higher carbon shifts, including 1.34/138.50, 1.35/180, 1.55/51, and 1.56/169 ppm (Figure 3.19). These correlations indicate that some aliphatic protons are near substituted, unsaturated, or carbonyl-containing environments. From this region to approximately 2.55 ppm, many cross-peaks are mainly observed in the sample at 313 degrees with a 1-hour holding time, supporting the formation of shorter and more substituted aliphatic fragments.²⁵

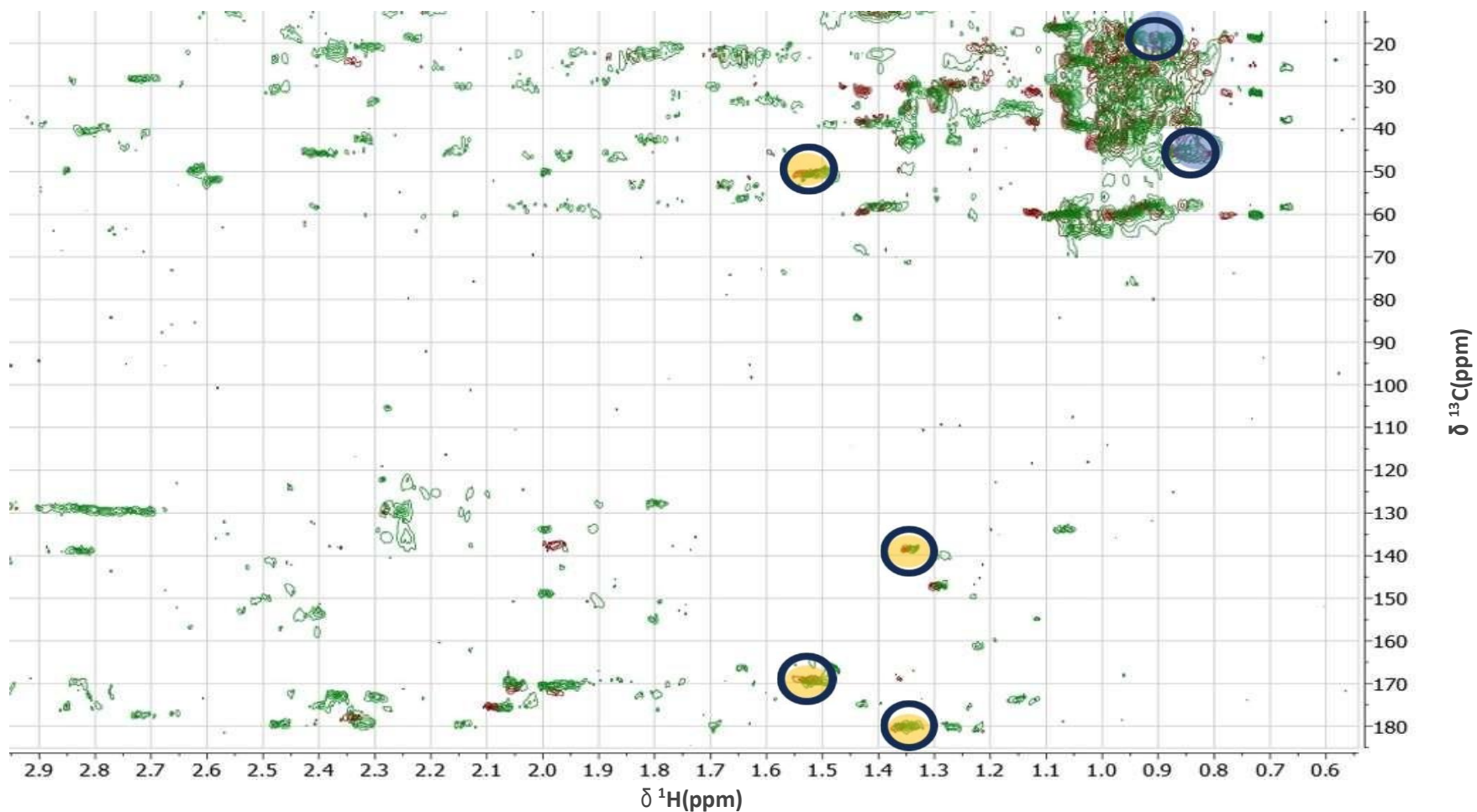


Figure 3.20 Superimposed HMBC spectrum of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time (red) and *Spirulina* 313 °C, 1 h holding time (green). Blue correlations such as 0.94/18 and 0.95/45 ppm are consistent with terminal CH₃ groups and saturated aliphatic fragments, while yellow correlations including 1.34/138.50, 1.35/180, 1.55/51, and 1.56/169 ppm indicate additional connectivity to higher-shift carbon environments.

In the DOSY spectra, the aliphatic region contains the highest number of signals, mainly between 0.5 and 3.0 ppm. Most cross-peaks are distributed around 7.0×10^{-10} to 1.0×10^{-9} m²/s, indicating a relatively mobile alkyl-rich fraction. At high-temperature HTL conditions, many aliphatic signals remain in this range, but some features extend to smaller diffusion coefficients, approximately 3.0×10^{-10} to 5.0×10^{-10} m²/s. Because smaller diffusion coefficients indicate larger apparent hydrodynamic size, these slower-diffusing features may reflect aggregation, condensation, or polymerization of some aliphatic fragments. For example, the slower features near 1.1–1.5 ppm suggest that part of the aliphatic fraction is associated with larger, less mobile hydrocarbon-rich structures rather than only small degradation products. This interpretation is consistent with Dutta Majumdar et al., who showed that asphaltene-like systems contain aggregated structures with restricted molecular mobility.²⁴

3.5.2.2 Oxygenated Region, 2.5–5.5 ppm: Deoxygenation and Intermediate Formation

The oxygenated region of the ¹H NMR spectra shows pronounced changes with increasing HTL temperature, reflecting formation and transformation of oxygen-containing species. At lower temperatures, several peaks become broadened, suggesting the formation of oxygenated intermediates such as alcohols, esters, or related functional groups. As temperature increases, some peaks disappear, including those around 2.26 and 2.08 ppm, indicating breakdown or transformation of initial oxygen-containing structures. At the same time, new sub-peaks appear with greater intensity and uniformity, especially around 2.38–2.40 ppm, where a distinct triplet forms. This suggests the formation of more stable aliphatic environments adjacent to functional groups.¹³

COSY provide further evidence of these changes. Correlations such as 1.78/3.32 and 1.52/4.12 ppm indicate connectivity between oxygen-adjacent protons and hydrocarbon chains. Meanwhile, the disappearance of cross-peaks at 4.26/6.81, 3.43/6.65, and 3.28/6.66 ppm supports the transformation of oxygen-containing functionalities into less polar structures. Transient correlations at 300 °C, such as 3.76/7.21 and 3.80/7.20 ppm, represent short-lived oxygenated intermediates that are formed during partial hydrolysis, dehydration, or rearrangement reactions and later consumed at higher HTL temperatures. A similar reaction pattern was described by Changi *et al.*, who reviewed hydrothermal reactions of microalgae-relevant biomolecules and reported that fatty acids produced from acylglyceride hydrolysis can reach maximum concentrations near 300 °C before further decomposing or converting to other compounds above 300 °C. Edifor *et al.* also observed that triglyceride-derived ester signals, aldehydes, and other oxygenated products formed from model lipid feedstocks disappear with increasing HTL temperature.^{33,34} Additional correlations including 2.80/3.64 and 3.93/6.94 ppm, which support the formation of new oxygen-containing or rearranged molecular environments (Figure 3.21).

HSQC analysis confirms the transformation of oxygenated carbon–hydrogen environments. The disappearance of correlations such as 4.03/55.09 and 3.89/60.13 ppm indicates the removal or transformation of specific oxygenated functional groups. New CH and CH₃ correlations, including 2.81/57.21, 2.07/40.76, and 3.57/63.13 ppm, suggest the formation of new oxygen-adjacent or partially deoxygenated environments. New CH₂ correlations, such as 3.26/47.97, 2.91/49.29, 2.78/51.97, 3.79/50.04, 2.82/35.49, 3.32/42.25, and 2.97/30.85 ppm, further indicate the generation of secondary alcohols, ethers, or rearranged oxygenated intermediates.^{29,30}

DEPT spectra show that CH₂ environments dominate the 50–60 ppm region up to 313 °C, consistent with methylene groups adjacent to oxygen-containing functionalities. With higher temperatures during the HTL, CH and CH₃ signals also become more visible in this region, suggesting that oxygenated structures are not only decomposed but also reorganized into new carbon environments. The oxygenated region appears most structurally diverse around 300 °C and then becomes partly simplified at 313 °C as some oxygen-adjacent CH environments are further converted through dehydration, decarboxylation, deoxygenation, and gas-forming reactions. Xu *et al.* described HTL as a sequence of hydrolysis, cracking, dehydration, and cross-reactions in which hydrophilic groups such as hydroxyl and carboxyl groups are removed to form more lipophilic biocrude products, mainly through dehydration and decarboxylation.²⁵

HMBC spectra show that many oxygenated-region cross-peaks become more visible at the highest HTL temperature. Correlations in the 2–3 ppm range, including 2.71/29.13, 2.72/129.33, 2.62/49.65, 3.00/130.23, and 3.22/180.41 ppm, indicate a mixture of substituted aliphatic, unsaturated/aromatic, and carbonyl-containing environments. In the 3–4 ppm range, correlations around 3.40, 3.76, and 4.04 ppm extend toward approximately 170 ppm, suggesting long-range connectivity to carbonyl-containing structures such as ester-like, carboxyl-derived, amide-like, or heteroatom-substituted groups (Figure 3.24). This behavior is reasonable for *Spirulina*-derived oil, as its high protein content can promote nitrogen-containing and amide-like products during HTL. Vardon *et al.* found that *Spirulina* HTL biocrude contained a high proportion of nitrogenous compounds, with FTIR evidence of N–H functionality and carbonyl-region bands associated with carboxylic acids, esters, amides, and ketones.²²

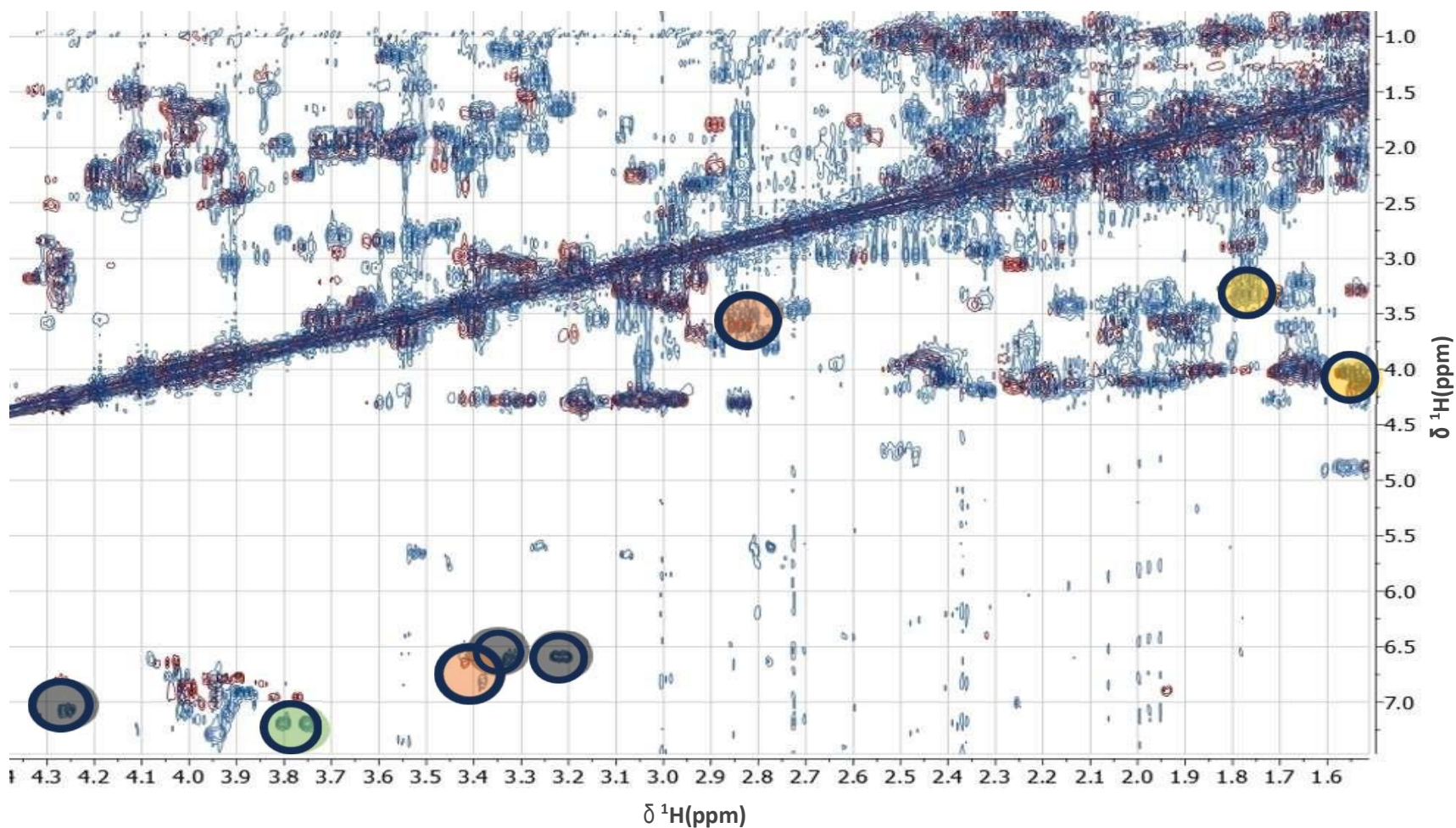


Figure 3.21 Superimposed COSY spectrum of the oxygenated region of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time (red) and *Spirulina* 313 °C, 1 h holding time (blue). Yellow correlations at 1.78/3.32 and 1.52/4.12 ppm indicate connectivity between oxygen-adjacent protons and hydrocarbon chains; dark-grey cross-peaks at 4.26/6.81, 3.43/6.65, and 3.28/6.66 ppm disappear; and green correlations at 2.80/3.64 and 3.93/6.94 ppm indicate additional oxygenated-region connectivity.

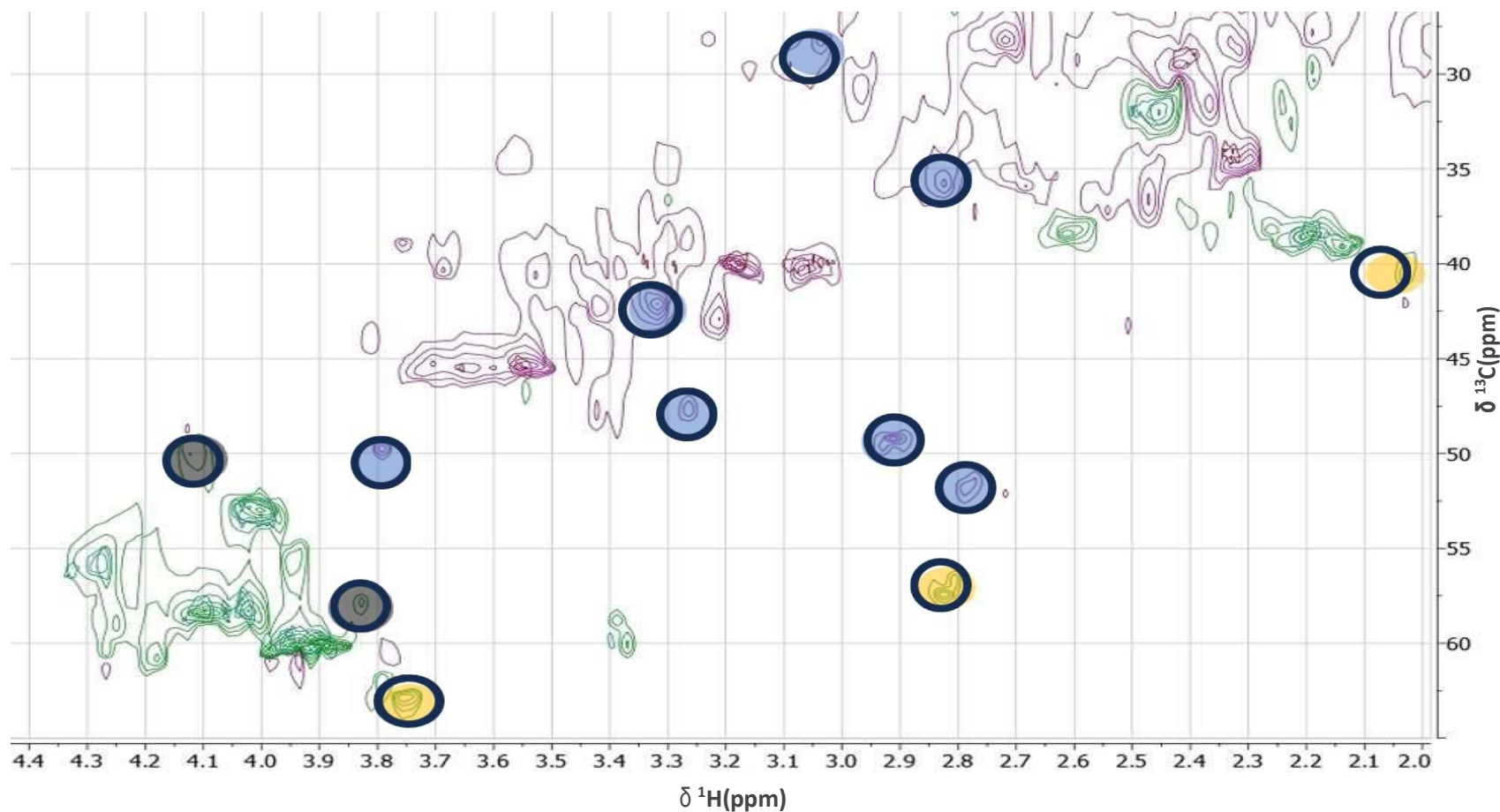


Figure 3.22 Superimposed HSQC spectrum of the oxygenated region of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time, with CH₂ signals in red and CH/CH₃ signals in light blue, and *Spirulina* 313 °C, 1 h holding time, with CH₂ signals in purple and CH/CH₃ signals in green. Dark-grey signals at 4.03/55.09 and 3.89/60.13 ppm disappear, while yellow signals at 2.81/57.21, 2.07/40.76, and 3.57/63.13 ppm suggest new oxygen-adjacent or partially deoxygenated environments. Blue correlations are newly formed.

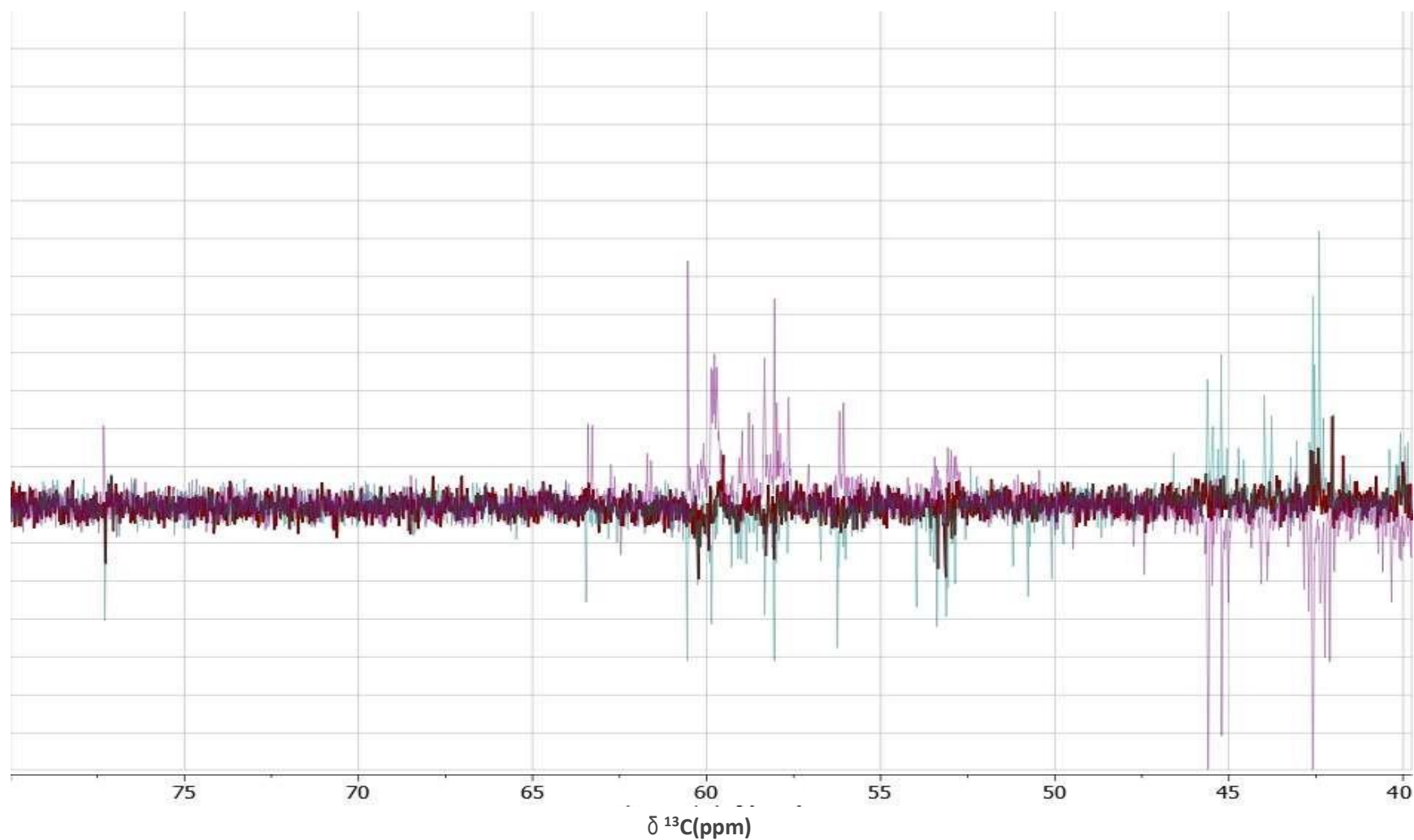


Figure 3.23 Superimposed DEPT-135 spectra of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time (red), *Spirulina* 300 °C, 1 h holding time (blue), and *Spirulina* 313 °C, 1 h holding time (purple).

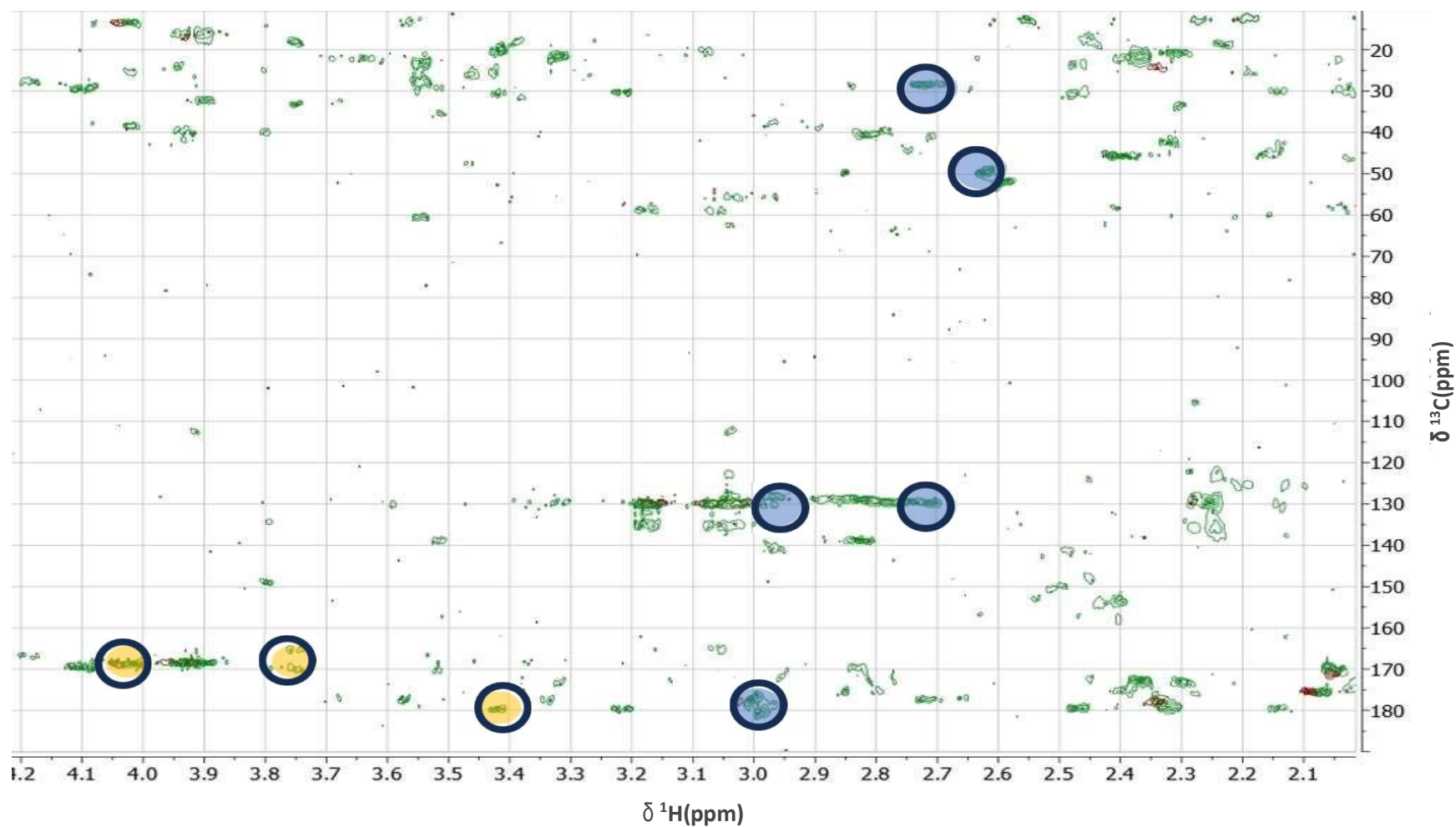


Figure 3.24 Superimposed HMBC spectrum of the oxygenated region of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time (red) and *Spirulina* 313 °C, 1 h holding time (green). Blue correlations indicate substituted aliphatic, unsaturated/aromatic, and carbonyl-containing environments, while yellow correlations near 3.40–4.04 ppm extending toward ~170 ppm suggest carbonyl connectivity.

In the oxygenated DOSY region, fewer cross-peaks are observed than in the aliphatic region, mainly between 3.0 and 5.5 ppm. Most oxygenated signals have diffusion coefficients of approximately 8.0×10^{-10} to 1.0×10^{-9} m²/s, indicating intermediate mobility. The spread from 8.0×10^{-10} to 1.0×10^{-9} m²/s corresponds to about a 25% increase in diffusion coefficient, suggesting that these oxygenated components have slightly different apparent mobilities but remain within the same general size range. At higher HTL temperature, some oxygenated signals remain visible rather than disappearing completely, indicating that oxygenated or heteroatom-containing fragments are partly rearranged into new substituted structures.

3.5.2.3 Aromatic Region, 5.5–8 ppm: Formation of Aromatic Structures

The aromatic and olefinic region of the ¹H NMR spectra is mainly characterized by broad and overlapping signals, reflecting the complex aromatic products formed during HTL. At lower temperature, fewer features are observed, while increasing lead to a higher number of peaks and sub-peaks. These signals become broader, especially around 6.8 and 7.0 ppm, indicating a wider distribution of aromatic environments and increasing molecular complexity.³ At intermediate conditions, around 300 °C, semi-resolved peaks begin to appear near 7.22 ppm, suggesting the formation of more defined aromatic structures. At 313 °C, the intensity increases and several sub-peaks become visible. Instead of one broad overlapping envelope, the spectra develop into a mixture of broad and sharper peaks, indicating multiple aromatic species with different substitution patterns. Subtle shifts near the residual solvent region around 7.26 ppm also suggest changes in the electronic environment of aromatic protons caused by substitution, deoxygenation, and condensation reactions.

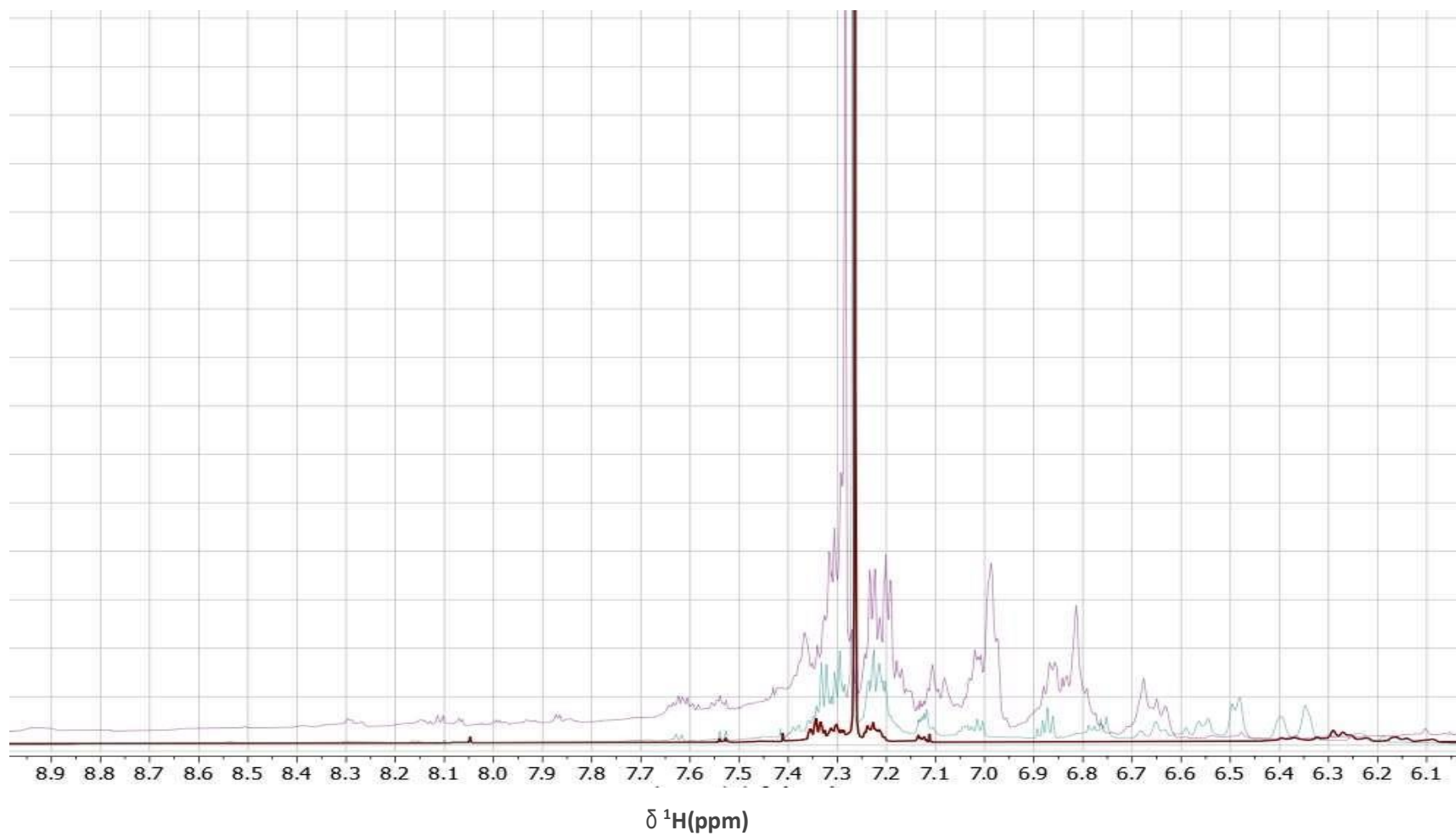


Figure 3.25 Superimposed ^1H NMR spectra of the aromatic region of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time (red), *Spirulina* 300 °C, 1 h holding time (blue), and *Spirulina* 313 °C, 1 h holding time (purple).

COSY spectra show both disappearance and formation of aromatic connectivities. Cross-peaks such as 7.47/8.12 and 7.03/7.78 ppm, as shown in Figure 3.26, disappear with increasing temperature, indicating loss or transformation of specific coupled proton environments. At the same time, new correlations involving signals around 6.90 and 7.22 ppm appear and show connectivity to oxygenated-region protons. This suggests that some aromatic structures are initially connected to oxygen-containing functionalities and later transform as deoxygenation progresses. These changes indicate rearrangement of aromatic systems, including changes in substitution patterns and development of new conjugated structures. This suggests that some aromatic structures are initially associated with oxygen-containing functionalities and later transform as deoxygenation progresses. Similar behavior has been reported by Villadsen et al., who identified phenols as benzene structures substituted with alcohol or ester groups and aliphatic substituents in HTL bio-oils, along with indoles, cyclic ketones, indanones, and other aromatic compounds.²⁶

HSQC spectra confirm the formation of new aromatic carbon–hydrogen environments. New HSQC correlations at 6.37/16.55, 8.11/121.83, 7.00/129.33, and 7.31/111.52 ppm indicate the formation of protonated aromatic carbons in substituted aromatic and heteroaromatic environments (Figure 3.27). Although the exact HSQC correlations cannot be assigned to individual compounds from these data alone, their aromatic/heteroaromatic chemical-shift positions are compatible with N-containing aromatic structures. Zimmermann et al. reported several N-heteroaromatic compound classes in HTL biocrude fractions, including indole, carbazole, tetrahydroquinoline, phenyl-pyridine, furopyridine, pyrrole-carboxylic acid, and O-substituted pyridine-type structures.⁴

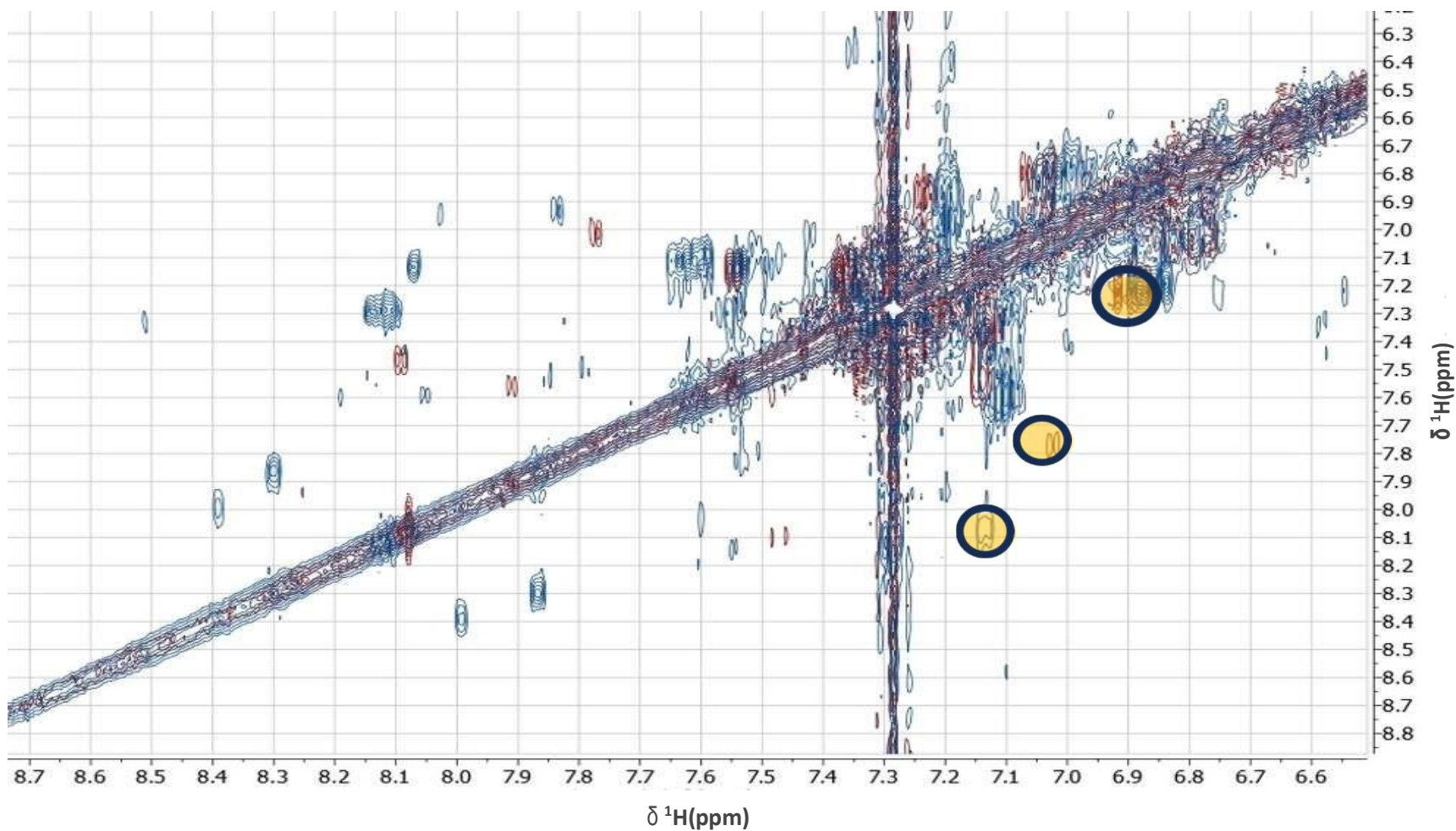


Figure 3.26 Superimposed COSY spectrum of the aromatic region of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time (red) and *Spirulina* 313 °C, 1 h holding time (blue). Yellow-highlighted disappearing cross-peaks, such as 7.47/8.12 and 7.03/7.78 ppm, indicate loss or transformation of specific coupled proton environments, while new correlations involving signals around 6.90 and 7.22 ppm suggest connectivity to oxygenated-region protons.

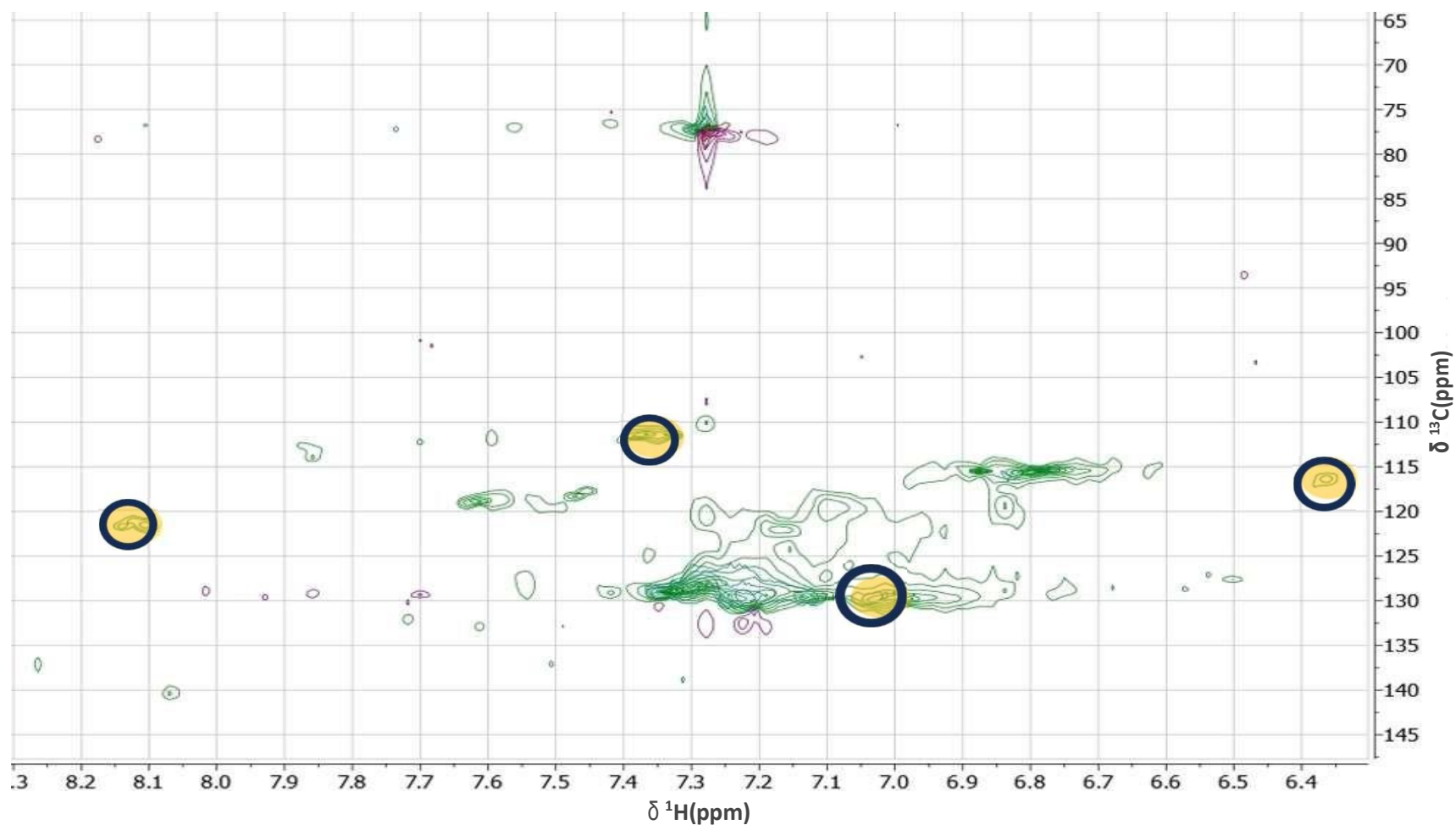


Figure 3.27 Superimposed HSQC spectrum of the aromatic region of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time, with CH₂ signals in red and CH/CH₃ signals in light blue, and *Spirulina* 313 °C, 1 h holding time, with CH₂ signals in purple and CH/CH₃ signals in green. Yellow-highlighted new correlations at 6.37/116.55, 8.11/121.83, 7.00/129.33, and 7.31/111.52 ppm indicate formation of protonated aromatic carbons in substituted aromatic and heteroaromatic environments.

DEPT analysis shows early formation of aromatic carbon environments around 130 ppm. At lower temperature and shorter holding time, some CH₂-type contributions are observed, suggesting early aromatic or olefinic intermediates with aliphatic substituents. These CH₂ contributions become less evident at the highest temperature, indicating that methylene-containing aromatic intermediates are consumed or transformed during advanced HTL reactions. At higher temperature, the aromatic region becomes mainly dominated by CH and CH₃-type signals, supporting the formation of more substituted and structurally complex aromatic compounds. Xu *et al.* identified aromatic compounds in HTL biocrude, including methyl-substituted phenolic structures, while Bjelic *et al.* reported increased aromatic and condensed aromatic/polyaromatic character in heavier HTL-derived fractions.^{23,27}

HMBC analysis also supports an increase in aromatic complexity. At lower temperature, many aromatic cross-peaks are weak or less developed, while at higher HTL temperature, they become more visible and more dispersed. Some correlations persist throughout the HTL series, including 7.32/128.12, 7.34/136.22, and 7.22/40.39 ppm (Figure 3.29). The correlations near 128–136 ppm are consistent with aromatic or olefinic carbons, while 7.22/40.39 ppm suggests alkyl-substituted aromatic structures. The correlations at 6.99/155.11, 6.88/120, 7.21/158, and 6.99/129.23 ppm suggest substituted aromatic environments, likely including phenolic, oxygen-substituted, or heteroaromatic structures, while the 6.82/60.40 ppm feature indicates an associated methoxyl/hydroxyl or oxygenated aliphatic environment. These assignments are supported by reports of substituted phenols, indoles, pyrroles, indanones, and condensed aromatic/polyaromatic species in HTL bio-oils and heavier HTL-derived fractions.²⁹

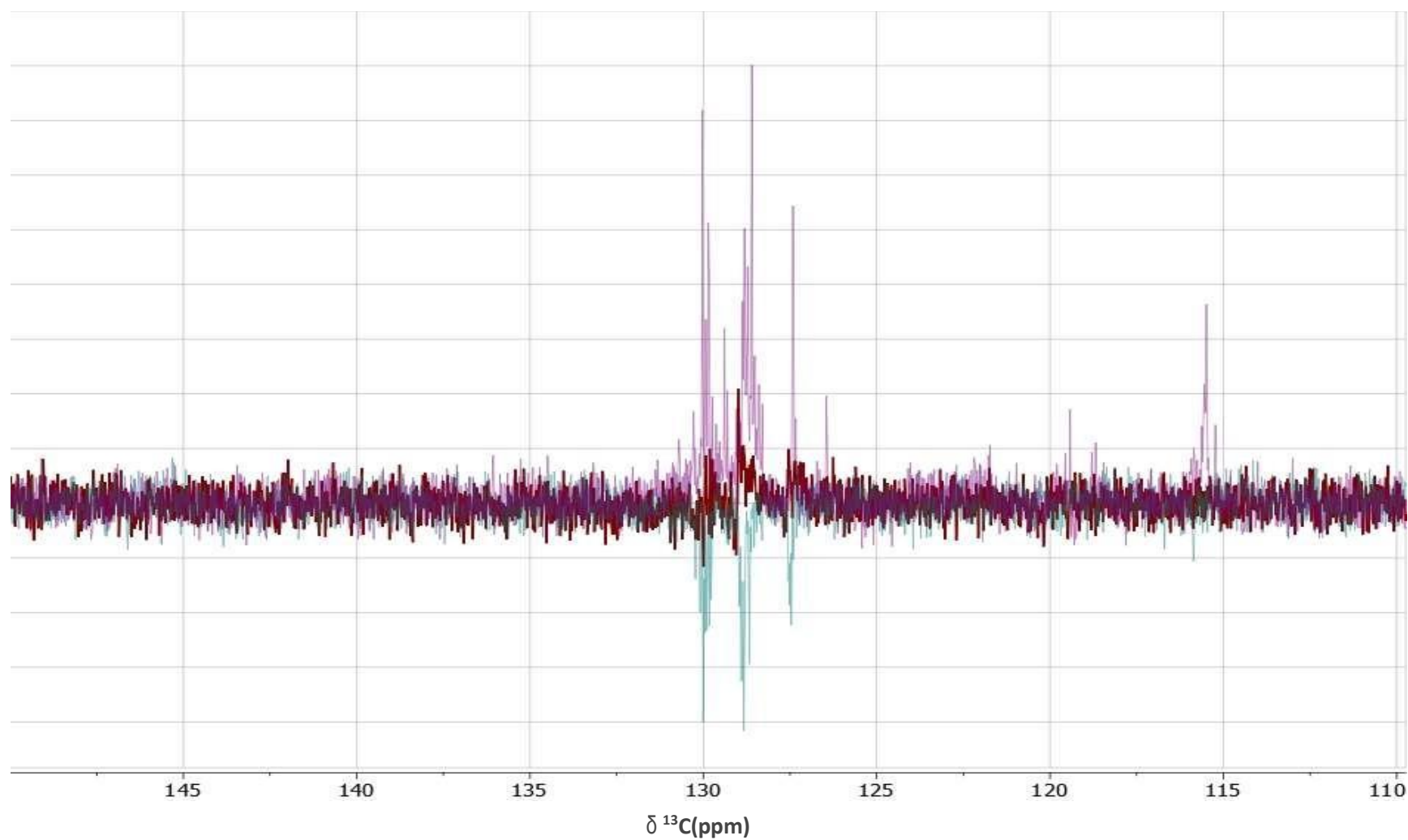


Figure 3.28 Superimposed DEPT-135 spectra of the aromatic region of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time (red), *Spirulina* 300 °C, 1 h holding time (blue), and *Spirulina* 313 °C, 1 h holding time (purple).

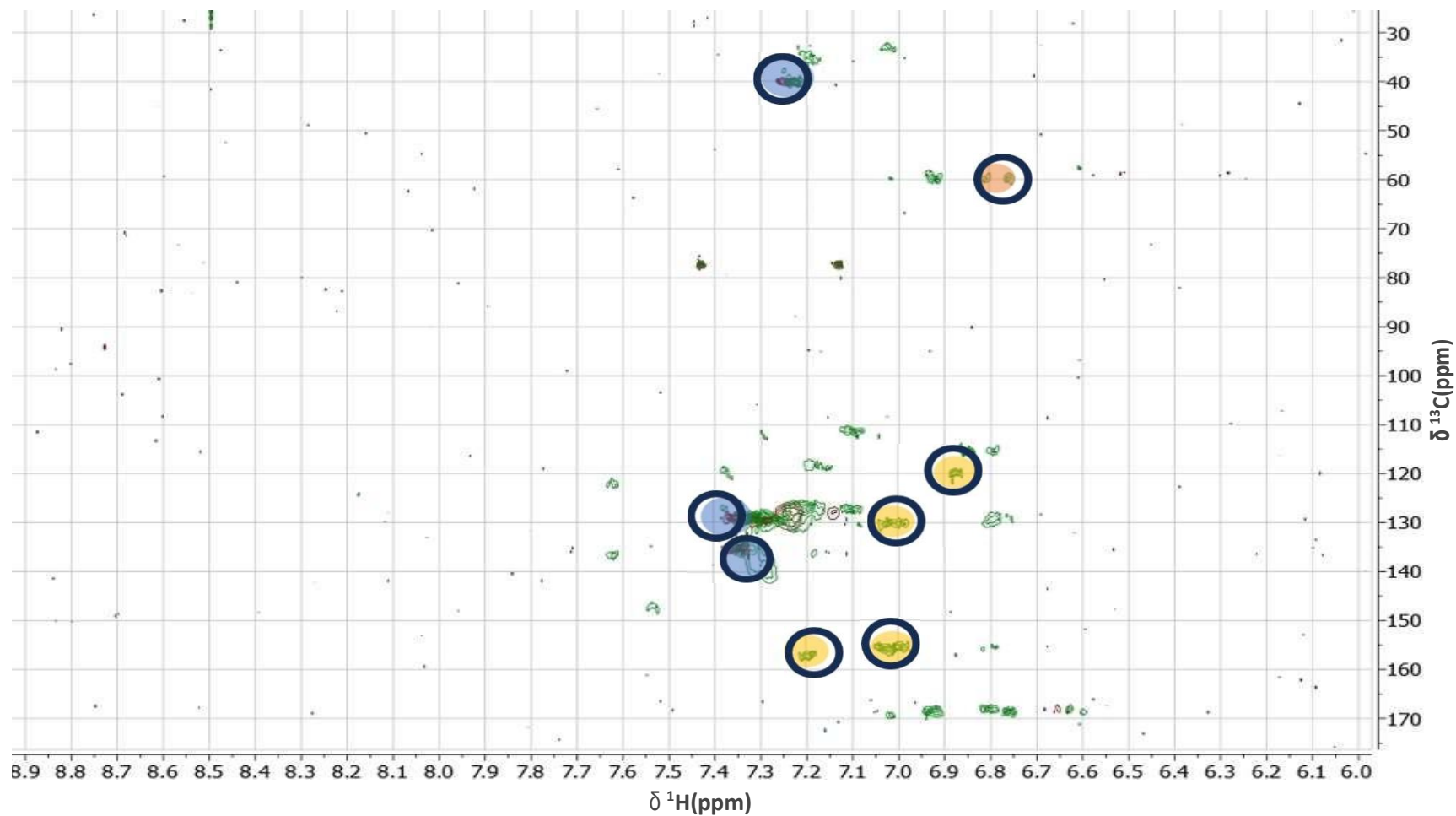


Figure 3.29 Superimposed HMBC spectrum of the aromatic region of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time (red) and *Spirulina* 313 °C, 1 h holding time (green). Yellow correlations, including 7.32/128.12, 7.34/136.22, and 7.22/40.39 ppm, persist across the HTL series. Blue correlations at 6.99/155.11, 6.88/120, 7.21/158, and 6.99/129.23 ppm suggest substituted aromatic environments, while the red feature at 6.82/60.40 ppm indicates an associated oxygenated aliphatic environment.

In the aromatic DOSY region, as illustrated in Figure 3.30, mainly 6.0–8.5 ppm, more cross-peaks are observed at higher HTL temperatures, consistent with the stronger aromatic overlap seen in the 1-D, COSY, HSQC, and HMBC spectra. Most aromatic features fall around 6.0×10^{-10} to 1.0×10^{-9} m²/s, while some broader features extend toward lower diffusion coefficients, indicating less mobile and possibly larger substituted or heteroatom-containing aromatic structures. Therefore, the DOSY data support increased aromatic complexity in *Spirulina*, but the diffusion range suggests these species are still not as large or immobile as strongly aggregated asphaltene-like material.^{14,8}

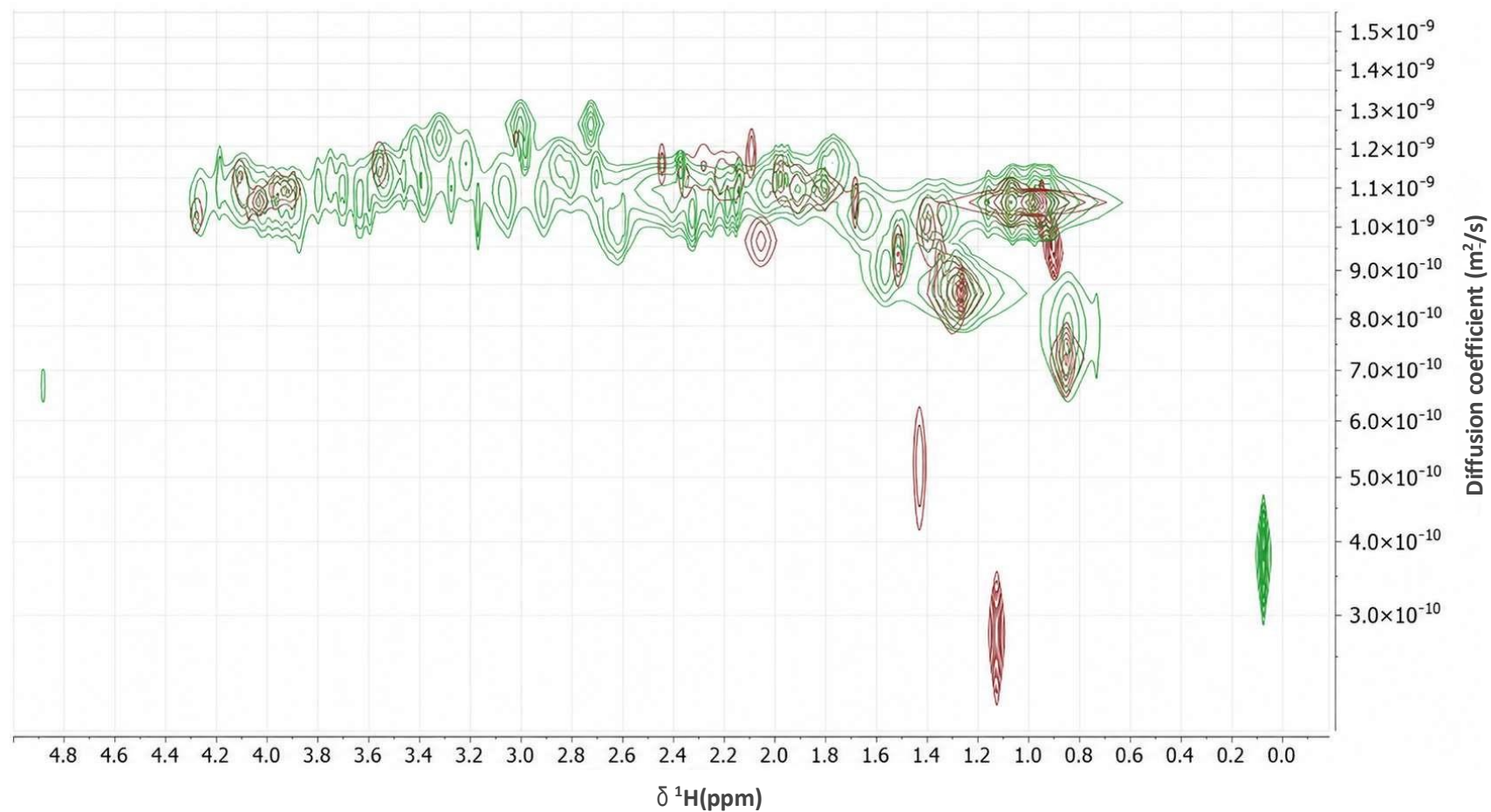


Figure 3.30 Superimposed DOSY spectrum of *Spirulina*-derived HTL bio-oil comparing *Spirulina* 260 °C, 0.5 h holding time (red) and *Spirulina* 313 °C, 1 h holding time (green).

3.6 NMR Relaxation Analysis of *Chlorella* and *Spirulina* HTL Bio-Oils

NMR relaxation experiments were conducted to examine the molecular mobility and intermolecular interactions present in the HTL bio-oil samples. Longitudinal relaxation times (T_1) were determined using inversion recovery pulse sequences, while transverse relaxation times (T_2) were measured using Carr–Purcell–Meiboom–Gill (CPMG) experiments. These measurements provide information on molecular motion, structural heterogeneity, and aggregation behavior within the complex bio-oil matrices.

The inversion recovery experiments were performed using 8 scans with a recycle delay of 60 s to ensure sufficient recovery of longitudinal magnetization between successive acquisitions. A total of 32 variable delay times, extending up to 20 s, were used to fully capture the recovery of the longitudinal magnetization and allow accurate determination of T_1 values for the different molecular environments present in the samples.

Transverse relaxation measurements were obtained using CPMG pulse sequences under similar acquisition conditions. Experiments were conducted using 8 scans and a recycle delay of 60 s, with an echo spacing (d_{20}) of 1 μ s. A total of 32 variable delay points, extending up to 15 s, were used to monitor the decay of transverse magnetization over time. This wide delay range enabled detection of both rapidly relaxing and more mobile components within the bio-oil mixtures.

The raw datasets for T_1 and T_2 determination were obtained directly from the inversion recovery and CPMG experiments, respectively. Signal intensities were normalized to account for variations in initial magnetization and acquisition conditions, and the normalized intensities were plotted as a function of delay time for inversion recovery and echo time for the CPMG experiments. The resulting recovery and decay

curves were subsequently used to extract relaxation parameters, providing insight into molecular mobility, intermolecular interactions, and the degree of structural complexity within the HTL bio-oil systems.

Table 3.8 ^1H relaxation times of the *Chlorella* sample made 260 °C with a 0.5-hour holding time.

^1H ppm	Assignment	T ₁ (s)	T ₂ (s)	T ₁ /T ₂
0.90-0.95	Aliphatic	1.234	0.028	43.402
		0.821	1.049	0.783
1.00-1.05		1.153	0.649	1.778
		0.761	1.212	0.628
1.25-1.30		1.194	0.618	1.931
		0.840	1.340	0.627
1.45-1.50		1.253	0.003	412.388
		0.872	0.885	0.985
1.95-2.00		1.380	0.025	55.356
		1.053	1.297	0.812
2.20-2.30		1.127	0.344	3.276
		0.935	1.674	0.558
2.60-2.65		0.961	0.363	2.649
		0.747	1.517	0.492
2.95-3.00	Oxygenated	0.990	0.715	1.386
		0.859	3.172	0.271
3.40-3.50		1.280	0.075	17.005
		0.925	1.142	0.810
3.85-3.90		1.329	0.054	24.486
		1.064	0.912	1.167
4.05-4.10		1.142	0.060	18.968
		1.077	1.100	0.979
7.15-7.20	Aromatic	1.040	0.463	2.247
		1.040	3.219	0.323
7.40-7.50		0.148	0.040	1.336
		0.815	1.336	0.610

The relaxation behavior of the HTL bio-oils reflects the chemical heterogeneity observed in the one- and two-dimensional NMR spectra. Across the selected peaks, both mono-exponential and bi-exponential relaxation behavior were observed. Mono-exponential behavior indicates that the fitted signal is dominated by one main proton environment with a relatively uniform relaxation response. In contrast, bi-exponential behavior suggests that the same chemical-shift region contains at least two overlapping proton populations with different molecular mobilities.¹⁵ This is expected for HTL oils because each selected peak region may contain several compounds with similar ¹H chemical shifts, including lipid-derived hydrocarbons, oxygenated intermediates, aromatic products, and heteroatom-containing species. Hence, the presence of two fitted components does not necessarily indicate two isolated compounds, but rather two dynamic populations within an overlapping spectral region.^{11,16}

The long T_2 component in the aliphatic region, together with low T_1/T_2 ratios, supports the presence of relatively mobile lipid-derived hydrocarbon chains. This assignment is supported by reports of abundant methyl and methylene protons from alkyl-chain and fatty-acid-derived structures in HTL biocrudes, and by relaxation studies showing that aliphatic groups have greater internal mobility and lower T_1/T_2 ratios than more restricted aromatic or alicyclic environments.^{8,25} This agrees with the ¹H NMR spectra and COSY/HSQC correlations, where CH₃ and CH₂ environments from alkyl-chain structures were observed. In contrast, some aliphatic and aliphatic/alicyclic peaks also contain very short T_2 components with high T_1/T_2 ratios.

The oxygenated and olefinic/oxygenated regions show a less consistent relaxation response. In the table, the oxygenated aliphatic peaks around 2.95–3.00 ppm and 3.40–3.50

ppm often show short T_2 components with high T_1/T_2 ratios, while the second components show longer T_2 values and are therefore more mobile. This agrees with the ^1H , HSQC, and HMBC results, where oxygenated structures were not simply removed, but transformed and reorganized into alcohol-like, ether-like, ester-like, carbonyl-connected, or heteroatom-containing environments.⁴ The aromatic region also shows evidence of restricted motion, especially where short T_2 components appear around 7.15–7.20 ppm and 7.40–7.50 ppm. This supports the interpretation that aromatic and substituted aromatic structures form during HTL and become part of a more complex, less mobile fraction.

Thus, the shorter T_2 components and higher T_1/T_2 ratios indicate more restricted local motion, stronger intermolecular interactions, or a higher degree of structural complexity. The longer T_2 components, with lower T_1/T_2 ratios, represent more mobile fractions of the oil. The relaxation results are also consistent with the functional-group changes observed with increasing HTL temperature. The table therefore provides a representative summary of how relaxation behavior changes across selected functional-group regions, while the complete relaxation datasets for the remaining eleven HTL samples are provided in Appendix B.

3.7 Comparison of *Chlorella* and *Spirulina* HTL Bio-Oils

Based on the NMR data acquired, both *Chlorella* and *Spirulina* show clear structural changes during HTL, but their spectral evolution is slightly different. In the aliphatic region, *Chlorella* appears to have sharper and more intense peaks around 1.0 ppm at lower temperature, suggesting a stronger contribution from well-resolved alkyl-chain compounds. *Chlorella* shows a spikier aliphatic pattern with sharper, better-resolved

multiplets, suggesting a stronger contribution from relatively uniform lipid-derived alkyl-chain environments. *Spirulina* also shows sharp peaks near 0.8 and 1.3 ppm, but the remaining aliphatic region is broader and more overlapped, indicating a more heterogeneous mixture of aliphatic environments. This interpretation is supported by Sarpal *et al.*, who identified clear lipid-chain and triglyceride signals in phytoplankton oils, including terminal CH₃, long-chain methylene, CH₂C=O, CH=CH, and OCH/OCH₂ groups also reported that *Spirulina* showed more prominent polar lipid-related signals such as CH₂O, CH₂OH, CHO, and CHOH groups. Together with Vardon *et al.*'s finding that *Spirulina* HTL biocrude contains higher α -to-heteroatom/unsaturated functionality, this suggests that the broader *Spirulina* aliphatic pattern may arise from overlapping nitrogenous, oxygenated, unsaturated, and polar lipid-derived environments rather than a uniform alkyl-chain population.^{14,16,22}

With increasing HTL temperature, the aliphatic regions of both microalgae and cyanobacteria become more similar, suggesting that the breakdown and rearrangement of lipid-derived structures produce more comparable hydrocarbon-like compounds. However, *Chlorella* shows clearer evidence of new aliphatic signals forming, such as peaks around 0.84, 1.28, and 1.33 ppm, along with the disappearance of some original peaks. In *Spirulina*, this region mostly shows intensity loss and shifting rather than strong formation of new intense peaks. The COSY spectra support this pattern: in *Chlorella*, many aliphatic correlations disappear, while newly formed peaks may be hidden by overlap in the crowded aliphatic region. In *Spirulina*, some new COSY cross-peaks appear, which may be due to chemical-shift changes caused by structural rearrangement during HTL.

In the oxygenated region, both phytoplankton species show evidence of newly formed oxygen-containing environments in the 1-D spectra, while COSY and HSQC more clearly show loss or transformation of specific oxygenated correlations. *Spirulina* displays a more active oxygenated region, with new COSY cross-peaks appearing at greater HTL temperatures, whereas *Chlorella* shows a stronger disappearance of oxygenated correlations. This difference may reflect different reaction pathways in the two feedstocks. Villadsen *et al.* reported that HTL bio-oils contain complex mixtures of oxygen- and nitrogen-containing products, including fatty acids, phenols, cyclopentenones, indoles, pyrroles, and amides.²⁹ Therefore, the more active *Spirulina* oxygenated region reflects continued formation of polar, oxygenated, and heteroatom-containing intermediates, while the stronger disappearance of *Chlorella* oxygenated correlations suggests greater conversion of oxygen-containing structures into less oxygenated products.^{26,29}

In the aromatic region, both species develop more complex aromatic features with increasing HTL temperature. The 1-D spectra show broader, more overlapping, and more uniform aromatic patterns at higher temperature, consistent with the formation of complex aromatic or unsaturated compounds. The 2-D spectra show some differences: *Chlorella* appears to develop newer aromatic COSY and HSQC correlations, while *Spirulina* shows fewer newly resolved aromatic cross-peaks. Overall, both species follow similar HTL pathways involving lipid breakdown, deoxygenation, and aromatic formation, but *Chlorella* shows stronger evidence of new aliphatic and aromatic product formation, while *Spirulina* shows more noticeable changes in the oxygenated region.^{17,18}

3.7.1 Comparison of *Chlorella* and *Spirulina* Relaxation Behavior

The relaxation behavior of *spirulina* and *Spirulina* HTL bio-oils reflects the differences already observed in the NMR spectral analysis of the two phytoplankton species. Both species show evidence of mobile aliphatic components and more restricted oxygenated or aromatic components, but the balance between these regions is different. In *Chlorella*, the relaxation values are generally consistent with a strong lipid-derived hydrocarbon contribution.¹⁹ The aliphatic regions contain mobile components with relatively long T_2 values and lower T_1/T_2 ratios, supporting the interpretation from the ^1H NMR, COSY, and HSQC spectra that *Chlorella* produces abundant fatty acid-derived and hydrocarbon-like structures. At the same time, some aliphatic/alicyclic and oxygenated regions show short T_2 components and higher T_1/T_2 ratios, indicating that part of the *Chlorella* oil also contains restricted or structurally modified environments formed through chain breakage, deoxygenation, and rearrangement.²⁰

Spirulina shows a less consistent relaxation pattern, especially in oxygenated, olefinic, and aromatic regions. Several *Spirulina* regions show elevated T_1 values and short T_2 components, suggesting a larger contribution from restricted molecular environments. DOSY supports this interpretation because *Spirulina* shows fewer but chemically broader oxygenated/aromatic features, while the aliphatic region remains the dominant mobile fraction. The *Spirulina* DOSY spectra show many aliphatic signals between 0.5–3.0 ppm, but oxygenated and aromatic regions contain fewer cross-peaks, with some signals extending to lower diffusion coefficients, indicating less mobile or larger apparent structures. This agrees with the elevated T_1/T_2 behavior, suggesting that part of the *Spirulina* bio-oil contains restricted, heteroatom-substituted, or aggregated environments

rather than only freely mobile lipid-derived fragments. This is consistent with the NMR analysis, where *Spirulina* showed broad and overlapping aromatic features, new oxygenated-region correlations, and evidence for substituted or heteroatom-containing structures.^{18,21} The HSQC and HMBC results support this interpretation because *Spirulina* contains several correlations involving oxygenated, carbonyl-connected, aromatic, or heteroatom-substituted environments. These features are reasonable because *Spirulina* is protein-rich compared with many lipid-dominant systems, and protein-derived nitrogen-containing products may contribute to more complex aromatic or heteroatom-containing HTL products.

In summary, the relaxation comparison supports the structural trends observed in the main NMR analysis. *Chlorella* behaves more strongly as a hydrocarbon-rich, lipid-derived bio-oil, especially in the aliphatic region, while *Spirulina* shows a greater degree of structural heterogeneity and restricted motion in oxygenated and aromatic regions. With increasing temperature, both species show evidence of transformation from mobile lipid-derived structures toward more complex substituted and aromatic products. Although *Spirulina* appears to produce a more chemically diverse relaxation response, *Chlorella* shows clearer lipid-to-hydrocarbon conversion behavior. This supports the conclusion that feedstock composition influences not only the functional-group distribution of the HTL oils, but also their molecular mobility and aggregation-related behavior.^{22,23}

3.8 Comparison of HTL Bio-Oils and model Systems

The ¹H NMR spectra of the HTL bio-oils were compared with crab oil and asphaltene as model systems representing aromatic-rich materials, respectively. Crab oil

showed mostly sharp, well-resolved peaks, especially in the aliphatic region, which is consistent with a more mobile system. In contrast, asphaltene showed broad and overlapping resonances, particularly in the aliphatic and aromatic regions, indicating a much more complex and aggregated aromatic-rich system. The aromatic region of asphaltene appeared as a mixture of broad and sharper features over a relatively limited chemical-shift range, suggesting overlapping aromatic environments and restricted molecular mobility.

The *Chlorella* and *Spirulina* HTL bio-oils initially showed spectral behavior that was closer to crab oil, especially through the presence of relatively sharp aliphatic and oxygenated-region peaks. This suggests that lipid-like and mobile hydrocarbon-rich compounds are important components of the phytoplankton oils.²² Nevertheless, the phytoplankton spectra were more complex than crab oil, with a larger number of overlapping signals across the aliphatic, oxygenated, and aromatic regions. As HTL temperature increased, both samples showed stronger broadening and increased overlap, especially in the lower half of the spectrum, indicating the formation of a more heterogeneous mixture of hydrocarbon-rich and substituted compounds.

At high-temperature HTL conditions, the bio-oils appeared to shift away from the simple lipid-like behavior of crab oil and toward more complex behavior resembling some features of asphaltene. This was reflected by the development of broader peaks, increased aromatic-region signals, and greater overlap, suggesting the formation of more substituted, condensed, or aggregated structures during HTL. This trend is expected because higher temperatures can convert biomass-derived oxygenates and lipid-like structures into more complex aromatic and oligomeric products. Pedersen *et al.* reported that HTL biocrude

contains both volatile ketones/oxygenated aromatics and a non-volatile residue with high-molecular-weight aromatic structures, supporting the development of broader and more overlapped aromatic-region signals. Nevertheless, the phytoplankton oils remained different from true asphaltenes because they retained sharp, mobile aliphatic signals, whereas Dutta Majumdar *et al.* showed that petroleum asphaltenes are dominated by aggregated large polyaromatic cores with only smaller mobile alkyl side chains.^{17,24} Therefore, the HTL oils can be interpreted as intermediate systems: they retain strong lipid-like characteristics similar to crab oil, while increasing temperature promotes the development of more complex and partially aromatic structures that move them closer to asphaltene-like behavior.

3.8.1 Comparison of HTL Bio-Oils Relaxation with Crab Oil and Asphaltene

The relaxation behavior of the HTL bio-oils falls between the two model systems, crab oil and asphaltene. Crab oil represents a relatively mobile reference system, while asphaltene represents a heavier, aromatic-rich, and more aggregation-prone reference.¹⁰ The phytoplankton oils do not behave exactly like either model system because they contain a mixture of lipid-derived hydrocarbons, oxygenated fragments, aromatic products, and heteroatom-containing compounds. Thus, comparison with the model systems helps clarify which regions of the HTL bio-oil spectra are more mobile and lipid-like, and which regions are more restricted and aromatic-like.

The relatively mobile behavior of the crab oil aliphatic region is expected because crab oil is dominated by triglyceride and fatty-acid-chain structures. Similar oils are characterized by methyl and methylene signals from long hydrocarbon chains, and Sarpal

et al. reported that algal oils contain neutral lipids, triglycerides, free fatty acids and unsaturated fatty-acid chains with clear NMR aliphatic-chain features. Therefore, the longer T_2 components and lower T_1/T_2 ratios observed for crab oil are consistent with flexible methyl and methylene environments from lipid-derived hydrocarbon chains, rather than restricted or aggregated aromatic structures.¹⁶ Although a weak association between lipid molecules may occur at higher concentrations, the persistence of sharp aliphatic peaks and long T_2 components indicates that crab oil is not dominated by strong aggregation. Stronger aggregation would be expected to shorten T_2 and broaden the signals, as observed more commonly in aromatic-rich asphaltene systems.⁸ This agrees with the ^1H NMR spectra, where the aliphatic region contains many intense peaks, and with the COSY and HSQC results, which confirm alkyl-chain connectivity and CH_3/CH_2 environments. These similarities suggest that part of the HTL oil retains lipid-derived character, especially in the hydrocarbon-rich fraction.^{22,25}

In contrast, the aromatic and some oxygenated/olefinic regions of the HTL-made oils show partial similarity to asphaltene. These regions often contain shorter T_2 components and higher T_1/T_2 ratios, which indicate more restricted motion and stronger intermolecular interactions. This behavior is consistent with the broader aromatic signals observed in the ^1H NMR spectra and with the HMBC and HSQC evidence for substituted aromatic and carbonyl-connected structures. However, the phytoplankton oils do not reach the same degree of aromatic dominance as asphaltene. The aliphatic contribution remains strong, and the presence of mobile long- T_2 components shows that the phytoplankton oils still contain a significant fraction of smaller or more flexible molecules.²⁶ Therefore, the relaxation data suggest that HTL-made oils behave as intermediate systems: their

hydrocarbon-rich regions resemble crab oil, while their aromatic and structurally complex regions move toward asphaltene-like behavior as HTL temperature increases.

This chapter presented the HTL conversion of *Chlorella* and *Spirulina* biomass and evaluated the resulting products using phase distribution, NMR spectroscopy, relaxation analysis, and DOSY. The phase-distribution results showed that temperature was the main factor controlling product formation, with higher HTL temperatures generally promoting bio-oil formation and reducing the relative contribution of aqueous and solid phases. The highest bio-oil yields were observed at 313 °C, with *Chlorella* reaching approximately 42.95% at 0.5 h and *Spirulina* reaching approximately 42.96% at 0.5 h, showing that both species produced very similar maximum oil yields under the high-temperature and short-holding-time conditions.

Crab oil and asphaltene were used as reference systems to support interpretation of the phytoplankton oils. Crab oil represented a low-molecular weight, aliphatic reference with sharp signals, while asphaltene represented a more aromatic, broad, overlapping, and less mobile reference. The phytoplankton bio-oils fell between these two systems, retaining strong mobile aliphatic character while also developing oxygenated, aromatic, and heteroatom-substituted environments.

Overall, *Chlorella* showed clearer lipid-to-hydrocarbon conversion, with strong aliphatic features and relatively mobile hydrocarbon-rich fractions. *Spirulina* showed a more heterogeneous response, with greater contribution from oxygenated, aromatic, carbonyl-connected, and heteroatom-substituted structures. Relaxation and DOSY results supported this interpretation by showing that both oils retained mobile aliphatic components, but *Spirulina* contained more restricted and chemically diverse environments.

Together, these results show that HTL transforms biomass through chain scission, deoxygenation, rearrangement, and aromatic formation, with feedstock composition strongly influencing the final bio-oil structure and mobility.

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CHAPTER 4: CONCLUSION AND FUTURE DIRECTIONS

4.1 Conclusion

This study was conducted to investigate how different hydrothermal liquefaction (HTL) conditions influence the production and molecular composition of bio-oils derived from two phytoplankton feedstocks, *Chlorella pyrenoidosa* and *Spirulina platensis*. The main objective was not only to evaluate product formation at different HTL temperatures, but also to determine how NMR spectroscopy can provide molecular-level information about how functional groups in the aliphatic, oxygenated, and aromatic regions of the spectra behave under different levels of HTL temperatures. This work focused on how aliphatic, oxygenated, olefinic, and aromatic regions evolve as a function of reaction temperature, residence time, and phytoplankton species. This approach addressed the broader research gap that, although HTL has been widely studied in terms of yield and bulk composition, fewer studies have used detailed NMR analysis and relaxation behavior to interpret the structural and dynamic properties of these complex bio-oil mixtures.^{1,2}

A reliable workflow was developed for recovering and analyzing HTL bio-oils. Following the reaction, the products were separated into solid, aqueous, gas, and bio-oil phases. The bio-oil fraction was recovered through filtration, liquid–liquid extraction, solvent separation, and rotary evaporation. This procedure made it possible to isolate a concentrated oil fraction suitable for NMR analysis. The phase-distribution results showed that bio-oil yields generally increased with increasing HTL temperature. For both species, the lowest oil yields were observed at 260 °C, while the highest yields were obtained at 313 °C. The aqueous phase represented the largest fraction for most conditions, particularly

at lower temperature, suggesting that incomplete conversion or partial degradation of biomass initially produced water-soluble intermediates. As temperature increased, a greater portion of the biomass was converted into hydrophobic oil-phase products.

The NMR results showed that HTL bio-oils are highly complex mixtures containing multiple overlapping chemical environments. The ^1H NMR, COSY, HSQC, HMBC, DOSY, and relaxation measurements indicated that HTL does not simply produce a limited series of specific individual compounds, but instead generates a complex mixture of lipid-derived aliphatic structures, oxygenated intermediates, olefinic structures, aromatic products, and heteroatom-containing components. These observations are consistent with previous reports that HTL biocrudes contain straight and branched aliphatic compounds, aromatics and phenolic derivatives, carboxylic acids, esters, and nitrogen-containing structures.³⁻⁵

Model systems were used to support the interpretation of the HTL bio-oil spectra and to help optimize NMR acquisition and relaxation parameters. Crab oil served as a low-molecular reference system, dominated mainly by sharp aliphatic signals associated with mobile fatty acid chain environments. In contrast, asphaltene served as an aromatic-rich and aggregation-prone reference system, characterized by broader, more overlapping signals and more restricted relaxation behavior. These model systems provided two useful end-member references: crab oil represented a more mobile, lipid-like system, while asphaltene represented a heavier, more aromatic, and more aggregated system.

Compared with these model systems, the HTL bio-oils exhibited intermediate behavior. The aliphatic regions of the HTL oils showed similarities to crab oil, especially through the presence of strong aliphatic signals and mobile long- T_2 relaxation components.

This indicates that a significant fraction of the algal and cyanobacteria-made bio-oils remains hydrocarbon-rich and relatively flexible. In contrast, the oxygenated, olefinic, and aromatic regions showed more complex behavior, including broader peaks, increased spectral overlap, and shorter T_2 components. These features suggest the presence of more restricted molecular environments, likely associated with substituted structures, heteroatom-containing components, aromatic products, and possible aggregation-related behavior. The phytoplankton oils did not show the same behavior as the asphaltene reference, because the aliphatic region remained comparatively mobile and showed limited evidence of aggregation. The strongest signs of increasing complexity were instead observed in the oxygenated and aromatic regions, where broader peaks and shorter T_2 components suggest the formation of larger, more substituted, or less mobile components at high-temperature HTL conditions.^{6,7}

The relaxation data for the *Chlorella* and *Spirulina* HTL bio-oils, provided in Appendix B, further support the Chapter 3 interpretation of how the aliphatic, oxygenated, and aromatic regions behave under different levels of HTL temperature. Across the relaxation dataset, most selected peak regions showed two-component T_1 and T_2 relaxation behavior, indicating that the same spectral region may contain both relatively mobile and more restricted molecular populations. The relationship between T_1 and T_2 is useful because both relaxation times are influenced by molecular motion, which is commonly described by the rotational correlation time. Correlation time reflects how quickly a molecule or molecular segment reorients in solution. Smaller, more mobile molecules generally have shorter correlation times, while larger molecules, aggregates, or more restricted structures have longer correlation times and larger apparent hydrodynamic radii. Therefore, higher

T_1/T_2 ratios, especially for short- T_2 components, suggest more restricted motion, stronger local interactions, or overlapping heavier fractions with reduced mobility. In contrast, lower T_1/T_2 ratios for long- T_2 components reflect more mobile molecular populations with smaller apparent size or less restricted motion.^{8,9} This behavior suggests that the same chemical-shift regions contain more than one dynamic environment, as expected for complex HTL bio-oils, where several compounds can overlap within the same spectral window. In addition, the T_1 , T_2 , and T_1/T_2 results show that relaxation analysis added information beyond chemical-shift assignment by identifying differences in molecular mobility between *Chlorella* and *Spirulina*, across the three HTL temperature levels, and within the aliphatic, oxygenated, and aromatic regions.

Comparison of the NMR spectral data and relaxation results between the two samples showed that *Chlorella pyrenoidosa* and *Spirulina platensis* produced broadly similar HTL bio-oils, but with important differences in signal distribution, spectral complexity, and molecular mobility. *Chlorella* generally showed stronger lipid-derived and hydrocarbon-like characteristics, especially in the aliphatic region. Its spectra suggested progressive conversion of long-chain lipid structures into shorter and more mobile hydrocarbon-rich fragments as HTL temperature increased. While oxygenated and aromatic features were also present, the overall behavior of *Chlorella* remained more closely associated with lipid-to-hydrocarbon transformation.¹⁰

The *Spirulina platensis* bio-oils showed a more heterogeneous response compared to *Chlorella* samples, reflected by greater NMR peak overlap, broader signals, and more variable relaxation behavior. The spectra contained broader and more overlapping signals in these regions, and the relaxation data support evidence of restricted motion. This

behavior is likely related to the higher protein content and greater contribution of nitrogen- and heteroatom-containing compounds in *Spirulina*-derived bio-oils.^{3,11} The oxygenated region in *Spirulina* appeared especially active, showing formation, disappearance, and rearrangement of several oxygen-adjacent and carbonyl-connected environments. This difference suggests that, while *Chlorella* appeared to follow a more hydrocarbon-rich conversion pathway, *Spirulina* produced a more chemically diverse and dynamically heterogeneous bio-oil.

The phase-distribution results indicate that elevated HTL temperature improved bio-oil production for both feedstocks. The highest bio-oil yield for *Chlorella* was obtained at 313 °C for 0.5-hour, reaching 42.95%, while the 313 °C, 1-hour condition gave a very similar yield of 42.65%. *Spirulina* showed the same maximum yield of 42.95% at 313 °C for 0.5-hour, followed by a slightly lower yield of 40.94% at 313 °C for 1-hour. Therefore, the 313 °C, 0.5-hour condition gave the highest bio-oil yield for both species, but the difference between the highest-yielding *Chlorella* and *Spirulina* samples was not significant based on the reported gravimetric yields.

The distinction between the two feedstocks is therefore more clearly reflected in the NMR-based chemical interpretation than in the maximum oil yield alone. Literature discussed in Chapter 1 shows that *Chlorella vulgaris* has been widely examined in HTL studies because of its balanced lipid, protein, and carbohydrate composition, while proteins are known to contribute to nitrogen-containing heterocycles in HTL bio-oils.^{1,7} In Chapter 3 results, *Chlorella pyrenoidosa* showed stronger lipid-derived and hydrocarbon-like behavior, especially in the aliphatic region, with evidence of conversion of long-chain lipid structures into shorter, mobile hydrocarbon-rich fragments. In contrast, *Spirulina platensis*

showed broader and more overlapping features in the oxygenated, olefinic, and aromatic regions, together with relaxation evidence for more restricted motion. These observations support a careful conclusion that *Chlorella* produced the more aliphatic-rich and mobile bio-oil in this dataset, whereas *Spirulina* produced a more chemically heterogeneous bio-oil with stronger oxygenated, aromatic, and heteroatom-associated contributions. This interpretation is supported by Biller and Ross, who reported higher protein and carbohydrate contents for *Spirulina* than for *Chlorella* species.¹

4.2 Limitations of the study

Although this study provides useful insight into the molecular composition, product distribution, and relaxation behavior of HTL bio-oils, several limitations should be recognized when interpreting the results. These limitations do not reduce the value of the findings, but they define the experimental boundaries of the work and indicate where future studies could improve reproducibility, scalability, and mechanistic understanding. In particular, the main limitations are related to the chemical composition differences of the feedstock species, the preparation and handling of the biomass before HTL, the operating range of the laboratory-scale HTL apparatus, and the limited number of comparable studies that apply detailed NMR relaxation analysis to phytoplankton-derived HTL products.

These factors are important because HTL bio-oil composition is influenced not only by reaction temperature and holding time, but also by the original biochemical composition of the phytoplankton and the conditions under which the biomass was cultivated, harvested, dried, and stored. Differences in lipid, protein, carbohydrate, moisture, and ash content can affect oil yield, aqueous-phase formation, oxygenated and nitrogen-containing products,

and the development of aromatic or condensed structures during HTL.^{3,10} Similarly, the available HTL apparatus limited the range of temperatures and pressures that could be safely and consistently tested, meaning that the results should be interpreted as a laboratory-scale comparison rather than a complete optimization of all possible HTL conditions. Finally, because relatively few studies have combined HTL with detailed NMR relaxation measurements, some interpretations in this work are necessarily comparative and should be further tested using broader datasets, additional feedstocks, and expanded reaction conditions.

One limitation is the scarcity of previous studies that combine phytoplankton-derived HTL bio-oils with detailed NMR relaxation analysis. Although HTL has been widely studied in terms of yield, elemental composition, GC–MS analysis, and general bio-oil quality, fewer studies have used T_1 and T_2 relaxation to examine molecular mobility, aggregation-related behavior, and phase heterogeneity. This information is important for processing and refining because less mobile or aggregation-prone fractions may affect viscosity, phase stability, separation, and upgrading. This is similar to crude oil systems, where asphaltenes strongly influence flow behavior, precipitation, and refining performance.⁸ Thus, in this study, asphaltene was used as a heavier aromatic-rich reference, while crab oil represented a more mobile reference. Due to the limited number of comparable studies, the relaxation-based interpretations remain mainly qualitative and comparative.

Another limitation is that the influence of feedstock composition and cultivation conditions was not directly investigated. Factors such as nutrient availability, lipid content, and the relative proportions of lipids, proteins, and carbohydrates can affect the

biochemical composition of phytoplankton species and may influence their behavior during HTL. The *Chlorella pyrenoidosa* and *Spirulina platensis* samples used in this study were commercially obtained as dried powdered biomass, which improved reproducibility and simplified sample preparation. Since these species were not cultivated under local southern Alberta environmental conditions, the results may not fully represent the behavior of phytoplankton grown under regional ambient conditions, where light intensity, seasonal temperature variation, water chemistry, and nutrient availability could affect biomass composition and HTL performance. In practice, these species may require controlled or closed cultivation systems to maintain consistent growth and biomass quality under Alberta conditions.¹²⁻¹⁴

A further methodological constraint was the operating range of the laboratory-scale HTL apparatus used in this study. The experiments were carried out under controlled high-temperature and elevated-pressure conditions suitable for safe laboratory-scale operation; however, the system was not designed to reproduce the full pressure range available in more advanced pilot-scale or industrial hydrothermal processing systems. Near-critical water refers to water operated close to its critical point, while supercritical water refers to water above its critical temperature and pressure, approximately 374 °C and 22.1 MPa. Under these conditions, water has altered solvent properties that can influence biomass conversion, product distribution, and reaction pathways.⁷ Access to a more industrialized or higher-pressure reactor system would allow future studies to examine more severe HTL conditions and determine whether increased pressure, in combination with temperature and residence time, could further influence bio-oil yield, deoxygenation, aromatic formation, and relaxation behavior. The present findings provide a reliable laboratory-scale

comparison of HTL bio-oils, but they should be interpreted within the operating limits of the available apparatus.¹⁵

4.3 Future Directions

In this study, biofuels were successfully produced from biomass through HTL. The process parameters were selected to evaluate the chemical conversion behavior of the feedstock and to investigate the products using NMR techniques. Model systems such as crab oil and asphaltene were chosen as comparison samples because they represent simpler reference systems with structural features that overlap with different regions of the HTL bio-oils. These samples provided a clearer basis for understanding how complex mixtures such as phytoplankton-derived bio-oils behave, while also highlighting differences in molecular structure and mobility between low-molecular weight samples and high-molecular weight organic materials.

The powdered *Chlorella* and *Spirulina* samples used in this study were selected for convenience and reproducibility. Using commercially available dried feedstock simplified the experiments and minimized cultivation-related variability. Nevertheless, it is acknowledged that this choice may make the results more specific to the samples used in this study, because algae and cyanobacteria in natural or freshly cultivated conditions may respond differently during HTL due to variations in moisture, nutrient content, and biochemical composition.

In summary, this work demonstrated that hydrothermal liquefaction combined with NMR spectroscopy provides an effective approach for studying how phytoplankton biomass transforms during HTL and how different molecular features develop in the

resulting bio-oils. The inclusion of model systems supported the validation of spectral interpretations and provided a comparative framework for understanding complex bio-oil formation.

Although this study provided a foundation for understanding HTL bio-oil chemistry, several areas need further investigation to improve fuel yield, quality, and while reducing energy consumption during the HTL process.

4.3.1 Exploring Solvent and Catalyst Effects

Future studies should examine how different solvent systems and catalysts affect the yield and composition of phytoplankton bio-oils. Previous studies have shown that both solvent selection and catalyst use can strongly influence HTL product distribution, bio-oil yield, and fuel quality. For example, Sharma *et al.* reported that catalysts can increase bio-oil yield, improve high heating value, and influence the chemical composition of HTL products, while solvent systems such as water, methanol, ethanol, and water–ethanol mixtures also affect bio-oil productivity.¹⁶ Wang *et al.* also showed that ethanol-containing solvent systems and two-step HTL processing can improve bio-oil yield and reduce solid residue formation.²⁰ Hence, exploring heterogeneous metal or metal-supported catalysts could be beneficial for improving the stability and energy density of the final bio-oil. Optimizing these parameters would help improve conversion efficiency, increase fuel quality, and provide better control over the chemical methods involved in the process of HTL.

4.3.2 Investigating Locally Adapted Species

Future studies should investigate locally adaptable phytoplankton strains using cultivation systems that are suitable for regional environmental conditions. Gaurav *et al.* reviewed microalgae-based biodiesel production and discussed cultivation using open pond systems and several types of photobioreactors, including tubular, column, stirred-tank, and flat-panel photobioreactors. Open ponds are generally lower-cost and easier to scale, but they are more exposed to contamination, evaporation, temperature variation, and limited environmental control. In contrast, photobioreactors provide better control over light, temperature, pH, gas exchange, and contamination, although they require higher capital and operational costs. Based on these considerations, future work in southern Alberta could compare locally isolated phytoplanktons with established biofuel strains under both open-pond and controlled photobioreactor conditions to determine which approach provides the best balance between biomass productivity, lipid accumulation, energy input, and fuel potential.¹⁷

4.3.3 Enhancing Lipid Accumulation

A further direction for future work would be to investigate how controlled cultivation stress can be used to adjust the biochemical composition of phytoplanktons before HTL. Nitrogen limitation is one commonly reported strategy because reduced nitrogen availability can slow protein synthesis and cell division while encouraging the accumulation of storage lipids, especially triglycerides. Kröger and Müller-Langer described nitrogen deprivation as a well-known approach for increasing triglyceride accumulation in phytoplanktons, while also noting that excessive nutrient stress can reduce

overall biomass productivity.²¹ Similarly, Mat Aron *et al.* noted that third-generation biofuel feedstocks such as microalgae can accumulate high lipid content, particularly under optimized or stress-based cultivation conditions, but further improvements are needed to increase energy efficiency and reduce production costs.²² A follow-up study on this matter would be beneficial because low-molecular weight biomass has been linked to higher bio-crude yield during hydrothermal liquefaction, while reduced protein content may limit the formation of nitrogen-containing bio-oil components that lower fuel quality and increase the need for downstream upgrading.¹

4.3.4 Improving Post-Harvest Processing

Post-harvest handling should also be examined more carefully because drying and storage conditions can change the quality of biomass before HTL and affect the overall energy efficiency of the process. Although HTL can process wet biomass, drying may still be needed when biomass must be stored, transported, weighed accurately, or compared between experiments. Freeze-drying is useful at the laboratory scale because it can produce a stable dry mass with limited thermal alteration; for example, Bauer *et al.* harvested *Chlorella zofingiensis* by centrifugation and then freeze-dried the biomass before further processing.¹⁸ Mat Aron *et al.* also noted that biomass drying is one of the energy-demanding steps in microalgae and cyanobacteria biofuel production, which can reduce overall sustainability.²² More scalable methods, such as low-temperature oven drying, air drying, or solar drying, could reduce cost and energy demand, but they may also increase the risk of lipid oxidation and moisture reabsorption if storage is not well controlled. Future studies should compare wet paste, partially dried biomass, and freeze-dried biomass under the

same HTL conditions to determine how each method affects bio-crude yield, phase distribution, nitrogen and oxygen content, and NMR-detectable structural features. If wet or partially dried biomass produces similar bio-crude yield and quality to fully dried biomass, reducing the drying step could improve the overall energy efficiency of HTL biofuel production.

4.3.5 Advanced Product Upgrading

Advanced product upgrading should also be considered in future work because HTL bio-oils often contain oxygenated compounds, unsaturated structures, and heteroatom-containing components that can reduce fuel stability and performance. Hydro-treatment is one important upgrading step, in which phytoplankton oil reacts with hydrogen at elevated temperature and pressure in the presence of catalysts. This process removes oxygen-containing functional groups through hydrodeoxygenation and can also hydrogenate unsaturated olefinic compounds, producing more stable hydrocarbon-rich fuels. Hsieh et al. reported that hydrotreated oil mainly contained C₁₅–C₁₈ linear alkanes, especially pentadecane, hexadecane, heptadecane, and octadecane, which are desirable diesel-range hydrocarbons. These hydrocarbons are useful because they have good ignition quality and high fuel value. A second step, hydroisomerization, could then be used to slightly branch some of these straight-chain hydrocarbons. This is useful because straight-chain hydrocarbons can have poor cold-flow properties, meaning they may crystallize more easily at low temperatures. Forming some branched hydrocarbons can improve low-temperature flow while keeping the fuel suitable for diesel use. Together, hydrotreatment and hydroisomerization could help convert phytoplankton bio-oils into fuels with lower

oxygen content, better stability, improved cold-flow properties, and composition closer to petroleum diesel.¹⁹

Chapter 4 synthesizes the main findings, limitations, and future directions of this thesis. The results show that hydrothermal liquefaction can convert *Chlorella pyrenoidosa* and *Spirulina platensis* into measurable bio-oil fractions, with higher temperatures generally improving oil production. More importantly, the NMR results demonstrate that bio-oil yield alone does not fully describe product quality. Instead, the molecular composition, spectral complexity, and relaxation behavior provide important information about how each feedstock responds to HTL. In this study, *Chlorella*-derived bio-oils showed stronger lipid-derived and aliphatic-rich characteristics, while *Spirulina*-derived bio-oils showed greater chemical heterogeneity, with more pronounced oxygenated, aromatic, and heteroatom-associated features.

Collectively, these findings demonstrate the value of integrating HTL product recovery with advanced NMR analysis to better characterize phytoplankton-derived bio-oils. Although the study was limited by the use of commercially dried biomass, laboratory-scale reaction conditions, and a limited number of comparable relaxation studies, it provides a useful foundation for future work. Further investigation of catalysts, solvents, locally adapted strains, lipid-enhancement strategies, wet-biomass processing, and upgrading methods could improve both bio-oil yield and fuel quality. In this way, the findings of this thesis contribute to the broader development of algal and cyanobacterial HTL as a sustainable pathway for producing renewable liquid fuels.

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Appendix A: Operational Profiles of Hydrothermal Liquefaction Experiments

This appendix presents representative operational data recorded during hydrothermal liquefaction (HTL) experiments, including reactor temperature and pressure as functions of reaction time. These profiles are provided to document reactor performance, stability of operating conditions, and reproducibility of experimental control across different feedstocks, target temperatures, and residence times. The operational data illustrate the heating, holding, and stabilization behavior of the reactor during HTL processing and confirm that the desired reaction conditions were consistently achieved. In particular, the temperature and pressure profiles demonstrate controlled ramping to the target setpoints, maintenance of stable isothermal conditions during the designated residence periods (30 or 60 min), and predictable pressure evolution characteristic of subcritical water under confined conditions. Together, these data support the reliability and repeatability of the HTL procedures described in Chapter 2 and provide experimental context for the interpretation of product yields and compositional analyses discussed in subsequent chapters

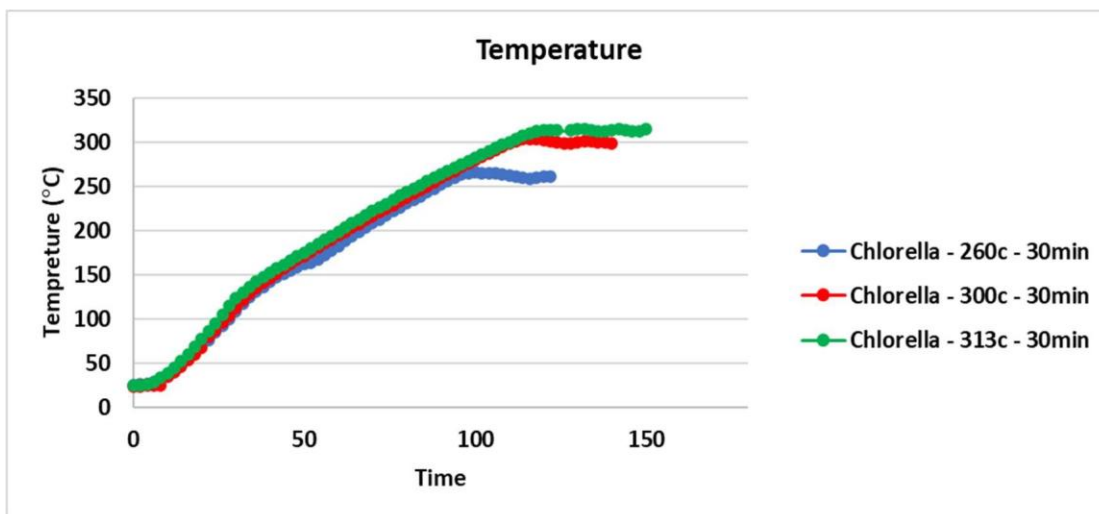


Figure A1. Temperature profile as a function of time during hydrothermal liquefaction of *Chlorella pyrenoidosa* at target temperatures of 260, 300, and 313 °C (30 min residence time). Line colors correspond to reaction temperature: blue (260 °C), red (300 °C), and green (313 °C).

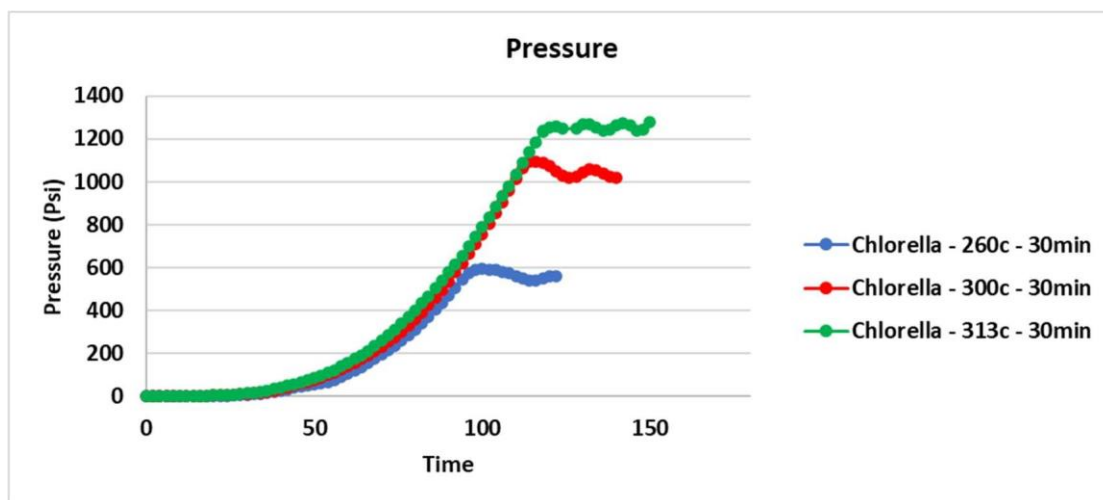


Figure A2. Pressure profile as a function of time during hydrothermal liquefaction of *Chlorella pyrenoidosa* at target temperatures of 260, 300, and 313 °C (30 min residence time). Line colors correspond to reaction temperature: blue (260 °C), red (300 °C), and green (313 °C).

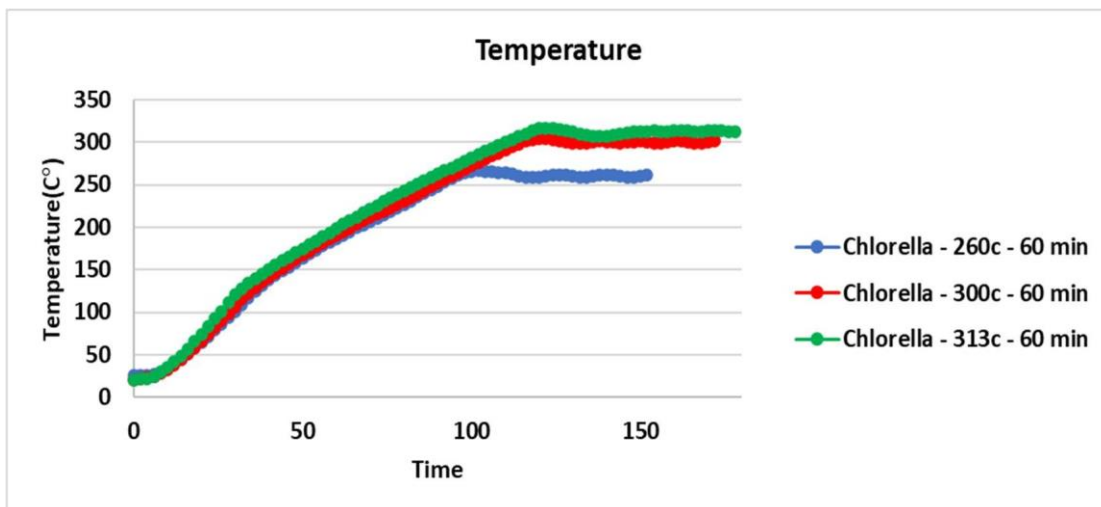


Figure A3. Temperature profile as a function of time during hydrothermal liquefaction of *Chlorella pyrenoidosa* at target temperatures of 260, 300, and 313 °C (60 min residence time). Line colors correspond to reaction temperature: blue (260 °C), red (300 °C), and green (313 °C).

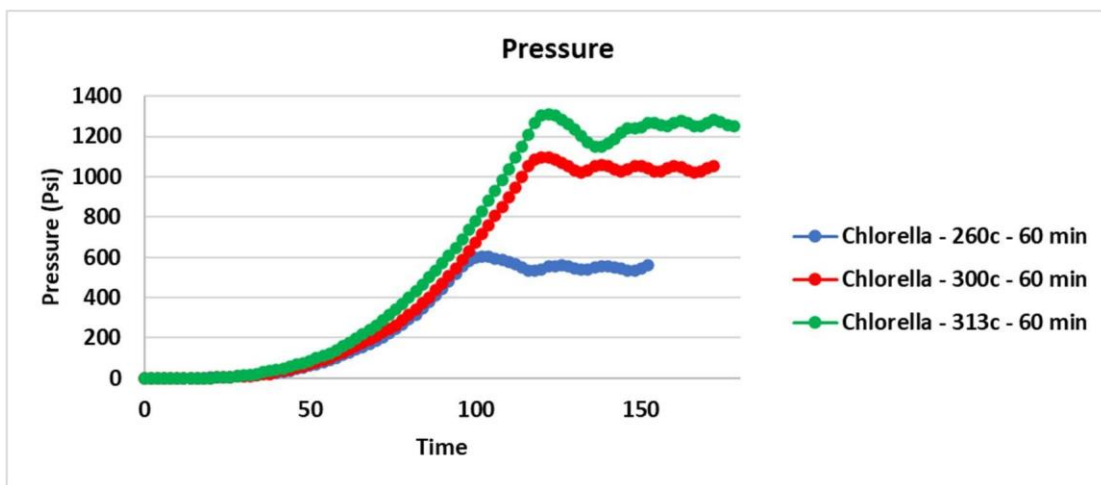


Figure A4. Pressure profile as a function of time during hydrothermal liquefaction of *Chlorella pyrenoidosa* at target temperatures of 260, 300, and 313 °C (60 min residence time). Line colors correspond to reaction temperature: blue (260 °C), red (300 °C), and green (313 °C).

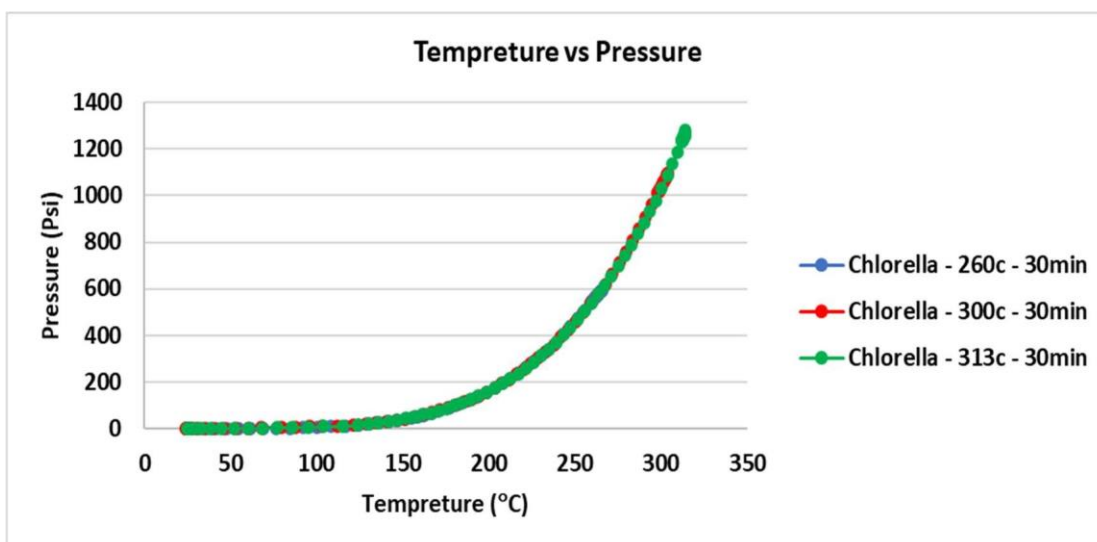


Figure A5. Pressure as a function of temperature during hydrothermal liquefaction of *Chlorella pyrenoidosa* at nominal reaction temperatures of 260, 300, and 313 °C and residence times of 30 min. Line colors indicate target reaction temperature: blue (260 °C), red (300 °C), and green (313 °C). The overlapping pressure–temperature trends observed across experiments indicate consistent reactor behavior, with pressure primarily governed by the thermophysical properties of water under confined HTL conditions rather than feedstock type or residence time.

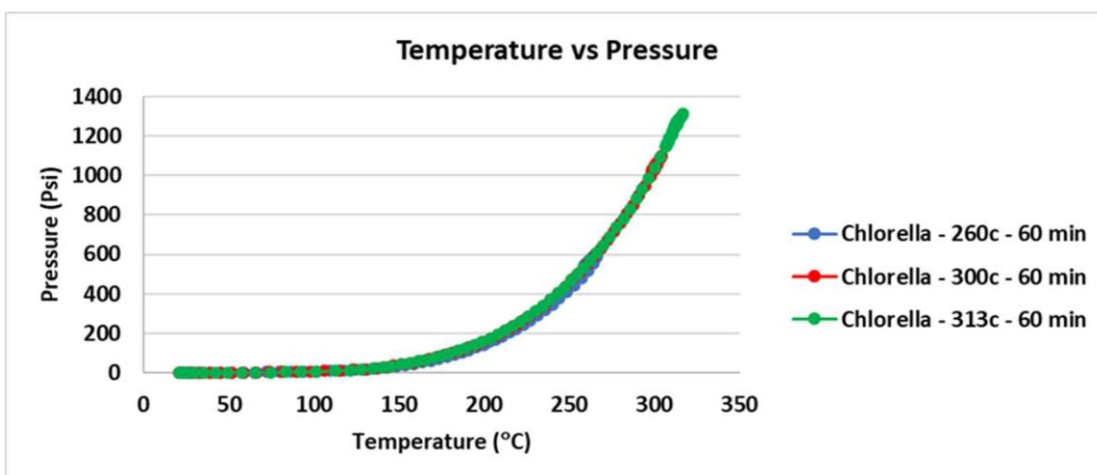


Figure A6. Pressure as a function of temperature during hydrothermal liquefaction of *Chlorella pyrenoidosa* at nominal reaction temperatures of 260, 300, and 313 °C and residence times of 60 min. Line colors indicate target reaction temperature: blue (260 °C), red (300 °C), and green (313 °C). The overlapping pressure–temperature trends observed across experiments indicate consistent reactor behavior, with pressure primarily governed by the thermophysical properties of water under confined HTL conditions rather than feedstock type or residence time.

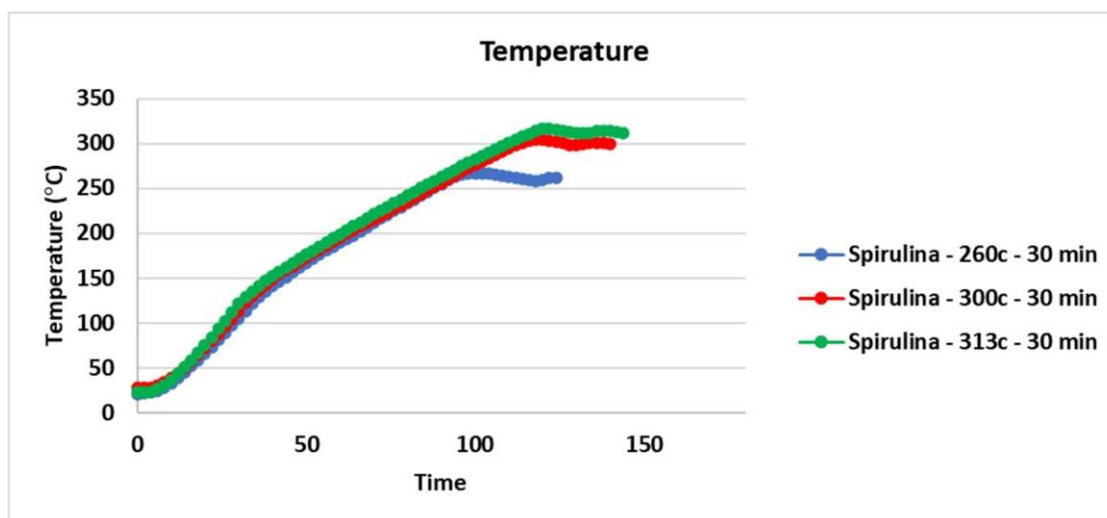


Figure A7. Temperature profile as a function of time during hydrothermal liquefaction of *Spirulina platensis* at target temperatures of 260, 300, and 313 °C (30 min residence time). Line colors correspond to reaction temperature: blue (260 °C), red (300 °C), and green (313 °C).

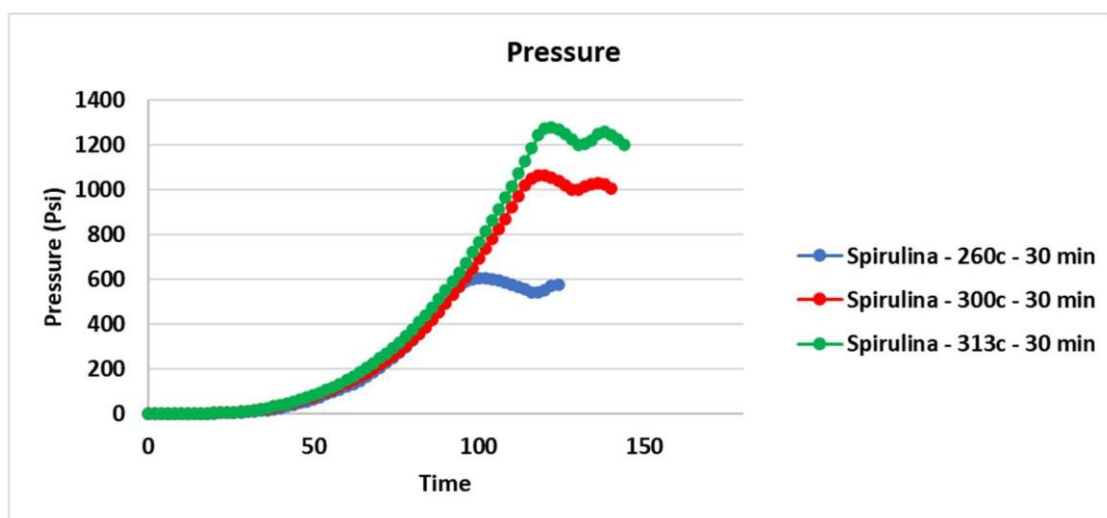


Figure A8. Pressure profile as a function of time during hydrothermal liquefaction of *Spirulina platensis* at target temperatures of 260, 300, and 313 °C (30 min residence time). Line colors correspond to reaction temperature: blue (260 °C), red (300 °C), and green (313 °C).

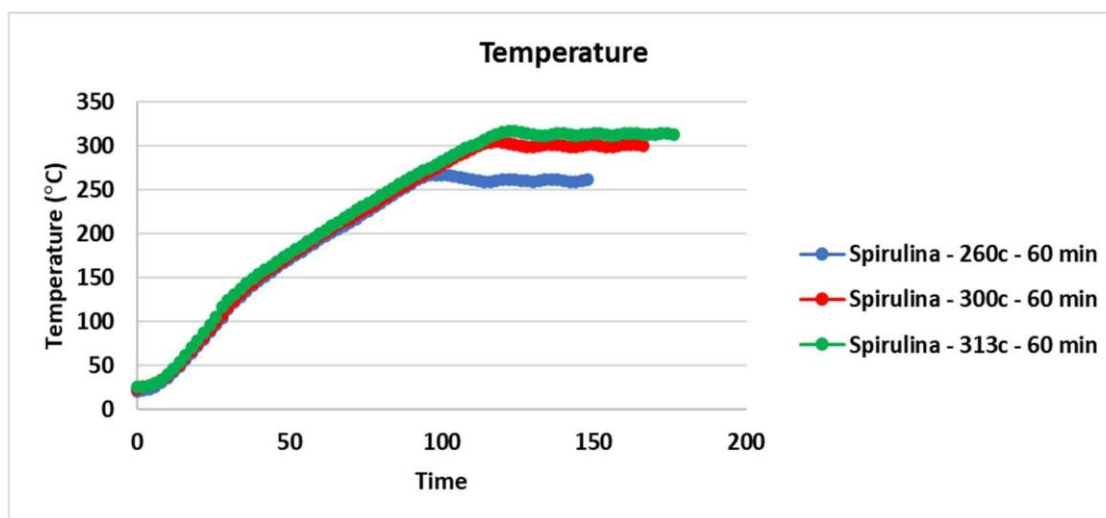


Figure A9. Temperature profile as a function of time during hydrothermal liquefaction of *Spirulina platensis* at target temperatures of 260, 300, and 313 °C (60 min residence time). Line colors correspond to reaction temperature: blue (260 °C), red (300 °C), and green (313 °C).

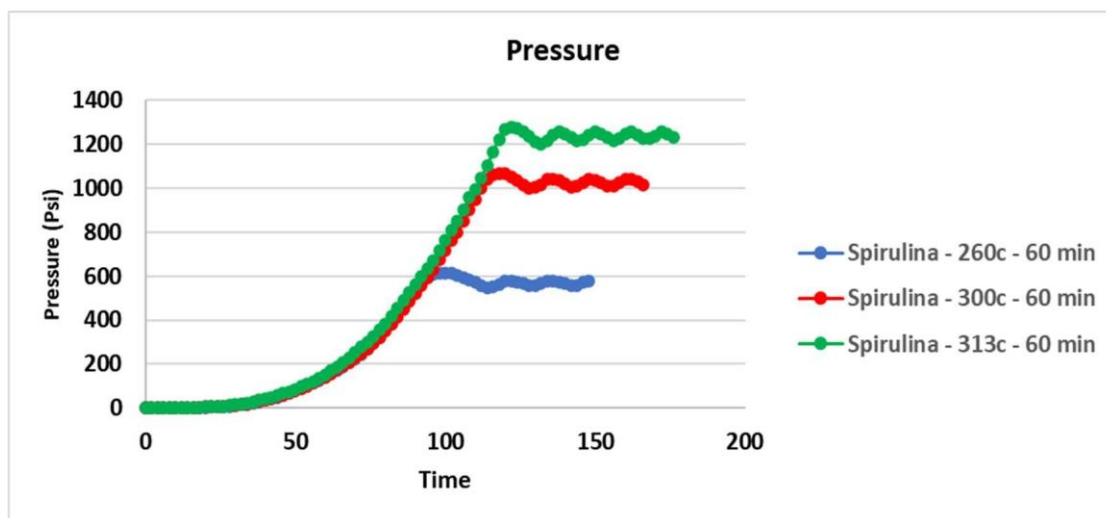


Figure A10. Pressure profile as a function of time during hydrothermal liquefaction of *Spirulina platensis* at target temperatures of 260, 300, and 313 °C (60 min residence time). Line colors correspond to reaction temperature: blue (260 °C), red (300 °C), and green (313 °C).

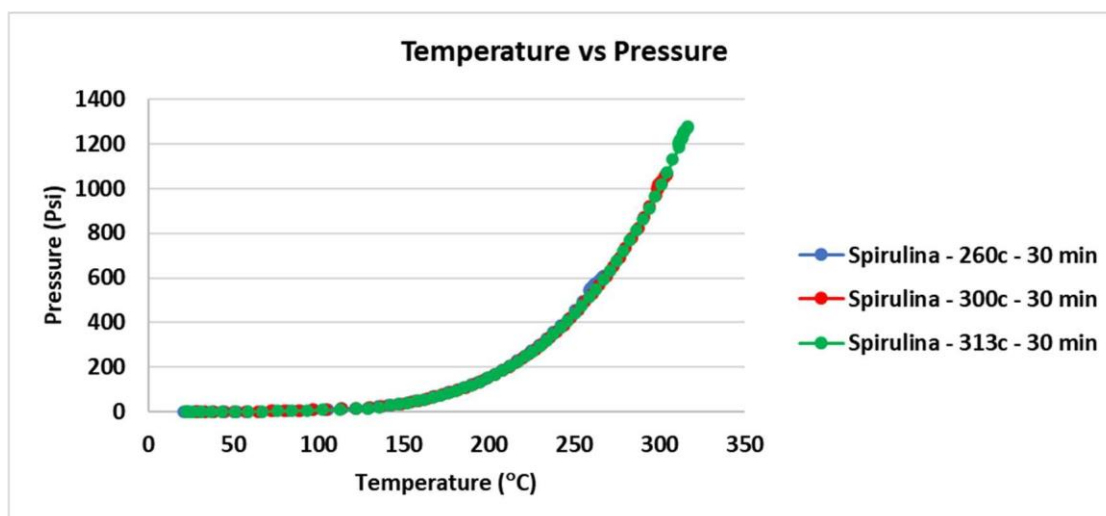


Figure A11. Pressure as a function of temperature during hydrothermal liquefaction of *Spirulina platensis* at nominal reaction temperatures of 260, 300, and 313 °C and residence times of 30 min. Line colors indicate target reaction temperature: blue (260 °C), red (300 °C), and green (313 °C). The overlapping pressure–temperature trends observed across experiments indicate consistent reactor behavior, with pressure primarily governed by the thermophysical properties of water under confined HTL conditions rather than feedstock type or residence time.

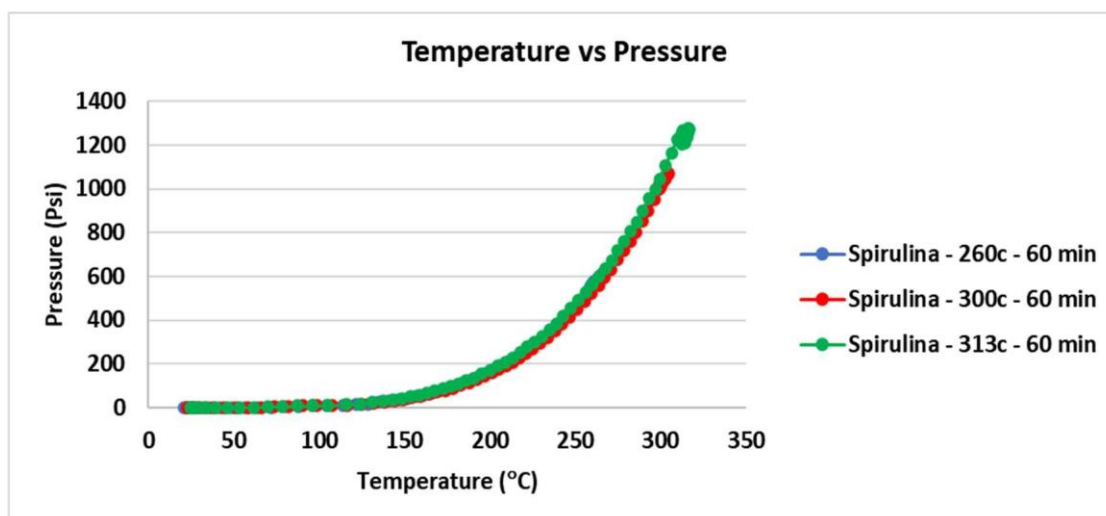


Figure A12. Pressure as a function of temperature during hydrothermal liquefaction of *Spirulina platensis* at nominal reaction temperatures of 260, 300, and 313 °C and residence times of 60 min. Line colors indicate reaction temperature: blue (260 °C), red (300 °C), and green (313 °C). The overlapping pressure–temperature trends observed across experiments indicate pressure primarily governed by the thermophysical properties of water under confined HTL conditions rather than feedstock type or residence time.

Appendix B: Complete Relaxation Dataset for Algal HTL Bio-Oils

This appendix presents the complete relaxation dataset obtained from the selected ^1H NMR peak regions of *Chlorella pyrenoidosa* and *Spirulina platensis* hydrothermal liquefaction (HTL) bio-oils. The dataset includes the chemical-shift regions, functional-group assignments, fitted T_1 and T_2 relaxation values, and calculated T_1/T_2 ratios for the full set of HTL conditions, including different reaction temperatures and residence times. Where applicable, both mono-exponential and bi-exponential fitting results are reported to account for proton environments with different relaxation behavior within overlapping spectral regions. These data are provided as supporting information for the relaxation analysis discussed in Chapter 3 and allow direct comparison of molecular mobility, restricted motion, and spectral heterogeneity across the algal bio-oil samples. Only representative relaxation values and trends are discussed in the main text to maintain clarity, while the full dataset is included here for completeness and transparency.

Table B.1. Complete T₁ and T₂ relaxation values for selected ¹H NMR peak regions of *Chlorella pyrenoidosa* HTL bio-oils.

Algae species	HTL temperature (°C)	Holding time (h)	Condition ID	Peak #	Component	Peak position (ppm)	Region	T1 (s)	T2 (s)	T1/T2
Chlorella	260	0.5	Chlorella 260°C 0.5 h	1	Value 1	0.9-0.95	Aliphatic	1.234	0.028	43.402
Chlorella	260	0.5	Chlorella 260°C 0.5 h	1	Value 2	0.9-0.95	Aliphatic	0.821	1.049	0.783
Chlorella	260	0.5	Chlorella 260°C 0.5 h	2	Value 1	1.0-1.05	Aliphatic	1.153	0.649	1.778
Chlorella	260	0.5	Chlorella 260°C 0.5 h	2	Value 2	1.0-1.05	Aliphatic	0.761	1.212	0.628
Chlorella	260	0.5	Chlorella 260°C 0.5 h	3	Value 1	1.25-1.3	Aliphatic	1.194	0.618	1.931
Chlorella	260	0.5	Chlorella 260°C 0.5 h	3	Value 2	1.25-1.3	Aliphatic	0.840	1.340	0.627
Chlorella	260	0.5	Chlorella 260°C 0.5 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	1.253	0.003	412.388
Chlorella	260	0.5	Chlorella 260°C 0.5 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	0.872	0.885	0.985
Chlorella	260	0.5	Chlorella 260°C 0.5 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	1.380	0.025	55.356
Chlorella	260	0.5	Chlorella 260°C 0.5 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	1.053	1.297	0.812
Chlorella	260	0.5	Chlorella 260°C 0.5 h	6	Value 1	2.2-2.3	Aliphatic	1.127	0.344	3.276
Chlorella	260	0.5	Chlorella 260°C 0.5 h	6	Value 2	2.2-2.3	Aliphatic	0.935	1.674	0.558
Chlorella	260	0.5	Chlorella 260°C 0.5 h	7	Value 1	2.6-2.65	Aliphatic / oxygen-adjacent	0.961	0.363	2.649
Chlorella	260	0.5	Chlorella 260°C 0.5 h	7	Value 2	2.6-2.65	Aliphatic / oxygen-adjacent	0.747	1.517	0.492
Chlorella	260	0.5	Chlorella 260°C 0.5 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	0.990	0.715	1.386
Chlorella	260	0.5	Chlorella 260°C 0.5 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	0.859	3.172	0.271

Chlorella	260	0.5	Chlorella 260°C 0.5 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	1.280	0.075	17.005
Chlorella	260	0.5	Chlorella 260°C 0.5 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	0.925	1.142	0.810
Chlorella	260	0.5	Chlorella 260°C 0.5 h	10	Value 1	3.85-3.90	Olefinic / oxygenated	1.329	0.054	24.486
Chlorella	260	0.5	Chlorella 260°C 0.5 h	10	Value 2	3.85-3.90	Olefinic / oxygenated	1.064	0.912	1.167
Chlorella	260	0.5	Chlorella 260°C 0.5 h	11	Value 1	4.05-4.10	Olefinic / oxygenated	1.142	0.060	18.968
Chlorella	260	0.5	Chlorella 260°C 0.5 h	11	Value 2	4.05-4.10	Olefinic / oxygenated	1.077	1.100	0.979
Chlorella	260	0.5	Chlorella 260°C 0.5 h	12	Value 1	7.15-7.2	Aromatic	1.040	0.463	2.247
Chlorella	260	0.5	Chlorella 260°C 0.5 h	12	Value 2	7.15-7.2	Aromatic	1.040	3.219	0.323
Chlorella	260	0.5	Chlorella 260°C 0.5 h	13	Value 1	7.40-7.50	Aromatic	0.148	0.040	3.695
Chlorella	260	0.5	Chlorella 260°C 0.5 h	13	Value 2	7.40-7.50	Aromatic	0.815	1.336	0.610
Chlorella	260	1	Chlorella 260°C 1 h	1	Value 1	0.9-0.95	Aliphatic	0.878	0.050	17.597
Chlorella	260	1	Chlorella 260°C 1 h	1	Value 2	0.9-0.95	Aliphatic	0.878	0.603	1.455
Chlorella	260	1	Chlorella 260°C 1 h	2	Value 1	1.0-1.05	Aliphatic	1.060	0.348	3.045
Chlorella	260	1	Chlorella 260°C 1 h	2	Value 2	1.0-1.05	Aliphatic	0.514	0.465	1.106
Chlorella	260	1	Chlorella 260°C 1 h	3	Value 1	1.25-1.3	Aliphatic	1.367	0.026	51.704
Chlorella	260	1	Chlorella 260°C 1 h	3	Value 2	1.25-1.3	Aliphatic	0.596	0.545	1.094
Chlorella	260	1	Chlorella 260°C 1 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	1.112	0.038	29.660
Chlorella	260	1	Chlorella 260°C 1 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	0.399	0.426	0.936

Chlorella	260	1	Chlorella 260°C 1 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	1.759	0.111	15.779
Chlorella	260	1	Chlorella 260°C 1 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	0.609	1.058	0.575
Chlorella	260	1	Chlorella 260°C 1 h	6	Value 1	2.2-2.3	Aliphatic	1.986	0.086	23.150
Chlorella	260	1	Chlorella 260°C 1 h	6	Value 2	2.2-2.3	Aliphatic	0.478	0.874	0.546
Chlorella	260	1	Chlorella 260°C 1 h	7	Value 1	2.6-2.65	Aliphatic / oxygen- adjacent	2.205	0.150	14.706
Chlorella	260	1	Chlorella 260°C 1 h	7	Value 2	2.6-2.65	Aliphatic / oxygen- adjacent	0.289	1.123	0.258
Chlorella	260	1	Chlorella 260°C 1 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	2.590	0.209	12.404
Chlorella	260	1	Chlorella 260°C 1 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	0.416	1.827	0.228
Chlorella	260	1	Chlorella 260°C 1 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	1.707	0.035	48.654
Chlorella	260	1	Chlorella 260°C 1 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	0.498	0.711	0.701
Chlorella	260	1	Chlorella 260°C 1 h	10	Value 1	3.85-3.90	Olefinic / oxygenated	1.683	0.268	6.276
Chlorella	260	1	Chlorella 260°C 1 h	10	Value 2	3.85-3.90	Olefinic / oxygenated	0.424	0.732	0.579
Chlorella	260	1	Chlorella 260°C 1 h	11	Value 1	4.05-4.10	Olefinic / oxygenated	0.512	0.440	1.163
Chlorella	260	1	Chlorella 260°C 1 h	11	Value 2	4.05-4.10	Olefinic / oxygenated	2.002	0.116	17.263
Chlorella	260	1	Chlorella 260°C 1 h	12	Value 1	7.15-7.2	Aromatic	2.400	0.559	4.292
Chlorella	260	1	Chlorella 260°C 1 h	12	Value 2	7.15-7.2	Aromatic	0.328	0.071	4.587
Chlorella	260	1	Chlorella 260°C 1 h	13	Value 1	7.40-7.50	Aromatic	0.364	0.025	14.680
Chlorella	260	1	Chlorella 260°C 1 h	13	Value 2	7.40-7.50	Aromatic	0.037	0.313	0.119

Chlorella	300	0.5	Chlorella 300°C 0.5 h	1	Value 1	0.9-0.95	Aliphatic	0.941	0.021344122	44.105
Chlorella	300	0.5	Chlorella 300°C 0.5 h	1	Value 2	0.9-0.95	Aliphatic	0.941	0.815845228	1.154
Chlorella	300	0.5	Chlorella 300°C 0.5 h	2	Value 1	1.0-1.05	Aliphatic	0.823	0.610233061	1.348
Chlorella	300	0.5	Chlorella 300°C 0.5 h	2	Value 2	1.0-1.05	Aliphatic	0.823	1.878852906	0.438
Chlorella	300	0.5	Chlorella 300°C 0.5 h	3	Value 1	1.25-1.3	Aliphatic	1.528	0.036532726	41.836
Chlorella	300	0.5	Chlorella 300°C 0.5 h	3	Value 2	1.25-1.3	Aliphatic	0.893	1.100087706	0.812
Chlorella	300	0.5	Chlorella 300°C 0.5 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	0.827	0.063128592	13.106
Chlorella	300	0.5	Chlorella 300°C 0.5 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	0.827	0.666151374	1.242
Chlorella	300	0.5	Chlorella 300°C 0.5 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	1.376	0.054309096	25.338
Chlorella	300	0.5	Chlorella 300°C 0.5 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	1.056	1.138951757	0.927
Chlorella	300	0.5	Chlorella 300°C 0.5 h	6	Value 1	2.2-2.3	Aliphatic	1.538	0.082483901	18.651
Chlorella	300	0.5	Chlorella 300°C 0.5 h	6	Value 2	2.2-2.3	Aliphatic	0.838	1.218313407	0.688
Chlorella	300	0.5	Chlorella 300°C 0.5 h	7	Value 1	2.6-2.65	Aliphatic / oxygen- adjacent	1.924	0.206649649	9.310
Chlorella	300	0.5	Chlorella 300°C 0.5 h	7	Value 2	2.6-2.65	Aliphatic / oxygen- adjacent	0.486	1.775497844	0.274
Chlorella	300	0.5	Chlorella 300°C 0.5 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	2.093	0.131175616	15.958
Chlorella	300	0.5	Chlorella 300°C 0.5 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	0.325	2.238757815	0.145
Chlorella	300	0.5	Chlorella 300°C 0.5 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	1.205	0.060703535	19.850
Chlorella	300	0.5	Chlorella 300°C 0.5 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	0.610	0.977960586	0.623

Chlorella	300	0.5	Chlorella 300°C 0.5 h	10	Value 1	3.85-3.90	Olefinic / oxygenated	1.107	0.025836707	42.854
Chlorella	300	0.5	Chlorella 300°C 0.5 h	10	Value 2	3.85-3.90	Olefinic / oxygenated	1.107	0.705729692	1.569
Chlorella	300	0.5	Chlorella 300°C 0.5 h	11	Value 1	4.05-4.10	Olefinic / oxygenated	1.985	0.059739895	33.221
Chlorella	300	0.5	Chlorella 300°C 0.5 h	11	Value 2	4.05-4.10	Olefinic / oxygenated	0.857	0.871548744	0.983
Chlorella	300	0.5	Chlorella 300°C 0.5 h	12	Value 1	7.15-7.2	Aromatic	1.341	0.083640569	16.038
Chlorella	300	0.5	Chlorella 300°C 0.5 h	12	Value 2	7.15-7.2	Aromatic	0.476	1.231035496	0.387
Chlorella	300	0.5	Chlorella 300°C 0.5 h	13	Value 1	7.40-7.50	Aromatic	3.036	0.202835128	14.970
Chlorella	300	0.5	Chlorella 300°C 0.5 h	13	Value 2	7.40-7.50	Aromatic	1.089	3.928659761	0.277
Chlorella	300	1	Chlorella 300°C 1 h	1	Value 1	0.9-0.95	Aliphatic	0.904	0.024	37.565
Chlorella	300	1	Chlorella 300°C 1 h	1	Value 2	0.9-0.95	Aliphatic	0.904	0.752	1.202
Chlorella	300	1	Chlorella 300°C 1 h	2	Value 1	1.0-1.05	Aliphatic	0.818	0.551	1.483
Chlorella	300	1	Chlorella 300°C 1 h	2	Value 2	1.0-1.05	Aliphatic	0.818	2.149	0.380
Chlorella	300	1	Chlorella 300°C 1 h	3	Value 1	1.25-1.3	Aliphatic	0.834	0.321	2.597
Chlorella	300	1	Chlorella 300°C 1 h	3	Value 2	1.25-1.3	Aliphatic	1.778	3.118	0.570
Chlorella	300	1	Chlorella 300°C 1 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	1.468	0.071	20.747
Chlorella	300	1	Chlorella 300°C 1 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	0.635	0.632	1.006
Chlorella	300	1	Chlorella 300°C 1 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	2.006	0.058	34.718
Chlorella	300	1	Chlorella 300°C 1 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	0.910	1.360	0.669

Chlorella	300	1	Chlorella 300°C 1 h	6	Value 1	2.2-2.3	Aliphatic	1.829	0.092	19.909
Chlorella	300	1	Chlorella 300°C 1 h	6	Value 2	2.2-2.3	Aliphatic	0.698	1.259	0.555
Chlorella	300	1	Chlorella 300°C 1 h	7	Value 1	2.6-2.65	Aliphatic / oxygen- adjacent	1.903	0.003	702.602
Chlorella	300	1	Chlorella 300°C 1 h	7	Value 2	2.6-2.65	Aliphatic / oxygen- adjacent	0.362	1.740	0.208
Chlorella	300	1	Chlorella 300°C 1 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	2.326	0.001	2280.573
Chlorella	300	1	Chlorella 300°C 1 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	0.114	2.958	0.039
Chlorella	300	1	Chlorella 300°C 1 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	1.639	0.057	28.878
Chlorella	300	1	Chlorella 300°C 1 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	0.819	0.944	0.867
Chlorella	300	1	Chlorella 300°C 1 h	10	Value 1	3.85-3.90	Olefinic / oxygenated	0.991	0.060	16.586
Chlorella	300	1	Chlorella 300°C 1 h	10	Value 2	3.85-3.90	Olefinic / oxygenated	0.991	0.634	1.562
Chlorella	300	1	Chlorella 300°C 1 h	11	Value 1	4.05-4.10	Olefinic / oxygenated	1.723	0.061	28.415
Chlorella	300	1	Chlorella 300°C 1 h	11	Value 2	4.05-4.10	Olefinic / oxygenated	0.902	0.674	1.340
Chlorella	300	1	Chlorella 300°C 1 h	12	Value 1	7.15-7.2	Aromatic	1.770	1.610	1.099
Chlorella	300	1	Chlorella 300°C 1 h	12	Value 2	7.15-7.2	Aromatic	0.547	0.153	3.562
Chlorella	300	1	Chlorella 300°C 1 h	13	Value 1	7.40-7.50	Aromatic	2.771	0.149	18.553
Chlorella	300	1	Chlorella 300°C 1 h	13	Value 2	7.40-7.50	Aromatic	0.730	3.494	0.209
Chlorella	313	0.5	Chlorella 313°C 0.5 h	1	Value 1	0.9-0.95	Aliphatic	1.223	0.020	62.022
Chlorella	313	0.5	Chlorella 313°C 0.5 h	1	Value 2	0.9-0.95	Aliphatic	0.718	0.727	0.988

Chlorella	313	0.5	Chlorella 313°C 0.5 h	2	Value 1	1.0-1.05	Aliphatic	1.088	0.102	10.649
Chlorella	313	0.5	Chlorella 313°C 0.5 h	2	Value 2	1.0-1.05	Aliphatic	0.596	0.677	0.880
Chlorella	313	0.5	Chlorella 313°C 0.5 h	3	Value 1	1.25-1.3	Aliphatic	1.563	0.036	44.016
Chlorella	313	0.5	Chlorella 313°C 0.5 h	3	Value 2	1.25-1.3	Aliphatic	0.655	0.774	0.846
Chlorella	313	0.5	Chlorella 313°C 0.5 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	1.245	0.029	42.678
Chlorella	313	0.5	Chlorella 313°C 0.5 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	0.550	0.409	1.346
Chlorella	313	0.5	Chlorella 313°C 0.5 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	2.325	0.040	58.068
Chlorella	313	0.5	Chlorella 313°C 0.5 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	0.779	0.888	0.877
Chlorella	313	0.5	Chlorella 313°C 0.5 h	6	Value 1	2.2-2.3	Aliphatic	2.082	0.050	41.431
Chlorella	313	0.5	Chlorella 313°C 0.5 h	6	Value 2	2.2-2.3	Aliphatic	0.773	0.888	0.870
Chlorella	313	0.5	Chlorella 313°C 0.5 h	7	Value 1	2.6-2.65	Aliphatic / oxygen- adjacent	1.850	0.199	9.294
Chlorella	313	0.5	Chlorella 313°C 0.5 h	7	Value 2	2.6-2.65	Aliphatic / oxygen- adjacent	0.650	2.624	0.247
Chlorella	313	0.5	Chlorella 313°C 0.5 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	4.335	0.392	11.058
Chlorella	313	0.5	Chlorella 313°C 0.5 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	0.931	4.190	0.222
Chlorella	313	0.5	Chlorella 313°C 0.5 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	1.813	0.043	41.970
Chlorella	313	0.5	Chlorella 313°C 0.5 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	0.949	1.024	0.926
Chlorella	313	0.5	Chlorella 313°C 0.5 h	10	Value 1	3.85-3.90	Olefinic / oxygenated	2.046	0.285	7.175
Chlorella	313	0.5	Chlorella 313°C 0.5 h	10	Value 2	3.85-3.90	Olefinic / oxygenated	0.992	0.285	3.478

Chlorella	313	0.5	Chlorella 313°C 0.5 h	11	Value 1	4.05-4.10	Aromatic	3.564	0.659	5.408
Chlorella	313	0.5	Chlorella 313°C 0.5 h	11	Value 2	4.05-4.10	Olefinic / oxygenated	1.090	0.048	22.575
Chlorella	313	0.5	Chlorella 313°C 0.5 h	12	Value 1	7.15-7.2	Olefinic / oxygenated	3.301	1.616	2.043
Chlorella	313	0.5	Chlorella 313°C 0.5 h	12	Value 2	7.15-7.2	Aromatic	1.057	0.188	5.613
Chlorella	313	0.5	Chlorella 313°C 0.5 h	13	Value 1	7.40-7.50	Aromatic	3.225	1.334	2.417
Chlorella	313	0.5	Chlorella 313°C 0.5 h	13	Value 2	7.40-7.50	Aromatic	0.830	0.027	30.214
Chlorella	313	1	Chlorella 313°C 1 h	1	Value 1	0.9-0.95	Aliphatic	1.189	0.008	147.355
Chlorella	313	1	Chlorella 313°C 1 h	1	Value 2	0.9-0.95	Aliphatic	0.620	0.651	0.953
Chlorella	313	1	Chlorella 313°C 1 h	2	Value 1	1.0-1.05	Aliphatic	1.088	3.698	0.294
Chlorella	313	1	Chlorella 313°C 1 h	2	Value 2	1.0-1.05	Aliphatic	0.539	0.543	0.992
Chlorella	313	1	Chlorella 313°C 1 h	3	Value 1	1.25-1.3	Aliphatic	1.016	0.838	1.212
Chlorella	313	1	Chlorella 313°C 1 h	3	Value 2	1.25-1.3	Aliphatic	1.016	0.029	35.369
Chlorella	313	1	Chlorella 313°C 1 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	1.119	0.020	55.280
Chlorella	313	1	Chlorella 313°C 1 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	0.558	0.436	1.281
Chlorella	313	1	Chlorella 313°C 1 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	2.334	0.034	69.460
Chlorella	313	1	Chlorella 313°C 1 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	0.724	1.008	0.718
Chlorella	313	1	Chlorella 313°C 1 h	6	Value 1	2.2-2.3	Aliphatic	2.483	0.020	122.643
Chlorella	313	1	Chlorella 313°C 1 h	6	Value 2	2.2-2.3	Aliphatic	0.734	0.436	1.684

Chlorella	313	1	Chlorella 313°C 1 h	7	Value 1	2.6-2.65	Aliphatic / oxygen- adjacent	1.581	0.051	30.819
Chlorella	313	1	Chlorella 313°C 1 h	7	Value 2	2.6-2.65	Aliphatic / oxygen- adjacent	0.902	1.140	0.792
Chlorella	313	1	Chlorella 313°C 1 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	3.843	0.002	2313.589
Chlorella	313	1	Chlorella 313°C 1 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	0.748	2.380	0.314
Chlorella	313	1	Chlorella 313°C 1 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	1.275	0.043	29.706
Chlorella	313	1	Chlorella 313°C 1 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	1.275	0.836	1.525
Chlorella	313	1	Chlorella 313°C 1 h	10	Value 1	3.85-3.90	Olefinic / oxygenated	0.881	0.034	26.274
Chlorella	313	1	Chlorella 313°C 1 h	10	Value 2	3.85-3.90	Olefinic / oxygenated	0.882	0.443	1.989
Chlorella	313	1	Chlorella 313°C 1 h	11	Value 1	4.05-4.10	Olefinic / oxygenated	0.061	0.045	1.369
Chlorella	313	1	Chlorella 313°C 1 h	11	Value 2	4.05-4.10	Olefinic / oxygenated	1.663	0.410	4.060
Chlorella	313	1	Chlorella 313°C 1 h	12	Value 1	7.15-7.2	Aromatic	0.663	0.102	6.532
Chlorella	313	1	Chlorella 313°C 1 h	12	Value 2	7.15-7.2	Aromatic	2.957	1.109	2.666
Chlorella	313	1	Chlorella 313°C 1 h	13	Value 1	7.40-7.50	Aromatic	0.220	0.060	3.651
Chlorella	313	1	Chlorella 313°C 1 h	13	Value 2	7.40-7.50	Aromatic	0.576	0.909	0.633

Table B.2. Complete T₁ and T₂ relaxation values for selected ¹H NMR peak regions of *Spirulina platensis* HTL bio-oils.

Algae species	HTL temperature (°C)	Holding time (h)	Condition ID	Peak #	Component	Peak position (ppm)	Region	T1 (s)	T2 (s)	T1/T2
Spirulina	260	0.5	Spirulina 260°C 0.5 h	1	Value 1	0.9-0.95	Aliphatic	1.268	0.029	44.348
Spirulina	260	0.5	Spirulina 260°C 0.5 h	1	Value 2	0.9-0.95	Aliphatic	0.711	0.649	1.096
Spirulina	260	0.5	Spirulina 260°C 0.5 h	2	Value 1	1.0-1.05	Aliphatic	1.070	0.028	38.734
Spirulina	260	0.5	Spirulina 260°C 0.5 h	2	Value 2	1.0-1.05	Aliphatic	0.385	0.629	0.612
Spirulina	260	0.5	Spirulina 260°C 0.5 h	3	Value 1	1.25-1.3	Aliphatic	1.102	0.094	11.777
Spirulina	260	0.5	Spirulina 260°C 0.5 h	3	Value 2	1.25-1.3	Aliphatic	0.522	0.686	0.761
Spirulina	260	0.5	Spirulina 260°C 0.5 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	1.158	0.015	76.207
Spirulina	260	0.5	Spirulina 260°C 0.5 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	0.445	0.498	0.893
Spirulina	260	0.5	Spirulina 260°C 0.5 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	1.116	0.041	27.103
Spirulina	260	0.5	Spirulina 260°C 0.5 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	0.712	0.505	1.409

Spirulina	260	0.5	Spirulina 260°C 0.5 h	6	Value 1	2.2-2.3	Aliphatic	1.573	0.066	24.019
Spirulina	260	0.5	Spirulina 260°C 0.5 h	6	Value 2	2.2-2.3	Aliphatic	0.706	1.102	0.641
Spirulina	260	0.5	Spirulina 260°C 0.5 h	7	Value 1	2.6-2.65	Aliphatic / oxygen-adjacent	1.988	0.199	9.995
Spirulina	260	0.5	Spirulina 260°C 0.5 h	7	Value 2	2.6-2.65	Aliphatic / oxygen-adjacent	0.371	1.058	0.351
Spirulina	260	0.5	Spirulina 260°C 0.5 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	1.863	0.304	6.136
Spirulina	260	0.5	Spirulina 260°C 0.5 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	0.317	1.960	0.162
Spirulina	260	0.5	Spirulina 260°C 0.5 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	1.485	0.052	28.601
Spirulina	260	0.5	Spirulina 260°C 0.5 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	0.493	0.620	0.795
Spirulina	260	0.5	Spirulina 260°C 0.5 h	10	Value 1	3.85- 3.90	Olefinic / oxygenated	1.784	0.185	9.620
Spirulina	260	0.5	Spirulina 260°C 0.5 h	10	Value 2	3.85- 3.90	Olefinic / oxygenated	0.946	0.954	0.992
Spirulina	260	0.5	Spirulina 260°C 0.5 h	11	Value 1	4.05- 4.10	Olefinic / oxygenated	1.930	0.011	170.656
Spirulina	260	0.5	Spirulina 260°C 0.5 h	11	Value 2	4.05- 4.10	Olefinic / oxygenated	0.606	0.688	0.881

Spirulina	260	0.5	Spirulina 260°C 0.5 h	12	Value 1	7.15-7.2	Aromatic	3.367	0.460	7.315
Spirulina	260	0.5	Spirulina 260°C 0.5 h	12	Value 2	7.15-7.2	Aromatic	1.411	2.837	0.497
Spirulina	260	0.5	Spirulina 260°C 0.5 h	13	Value 1	7.40- 7.50	Aromatic	2.816	0.025	114.659
Spirulina	260	0.5	Spirulina 260°C 0.5 h	13	Value 2	7.40- 7.50	Aromatic	0.530	0.977	0.543
Spirulina	260	1	Spirulina 260°C 1 h	1	Value 1	0.9-0.95	Aliphatic	1.455	0.548	2.657
Spirulina	260	1	Spirulina 260°C 1 h	1	Value 2	0.9-0.95	Aliphatic	1.455	2.762	0.527
Spirulina	260	1	Spirulina 260°C 1 h	2	Value 1	1.0-1.05	Aliphatic	0.321	0.003	111.375
Spirulina	260	1	Spirulina 260°C 1 h	2	Value 2	1.0-1.05	Aliphatic	1.776	0.660	2.691
Spirulina	260	1	Spirulina 260°C 1 h	3	Value 1	1.25-1.3	Aliphatic	1.950	0.001	1949.822
Spirulina	260	1	Spirulina 260°C 1 h	3	Value 2	1.25-1.3	Aliphatic	0.286	0.442	0.646
Spirulina	260	1	Spirulina 260°C 1 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	2.371	0.002	1516.069
Spirulina	260	1	Spirulina 260°C 1 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	0.565	0.577	0.978
Spirulina	260	1	Spirulina 260°C 1 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	1.904	0.003	760.499
Spirulina	260	1	Spirulina 260°C 1 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	0.356	1.854	0.192
Spirulina	260	1	Spirulina 260°C 1 h	6	Value 1	2.2-2.3	Aliphatic	1.744	0.003	685.156
Spirulina	260	1	Spirulina 260°C 1 h	6	Value 2	2.2-2.3	Aliphatic	0.310	1.161	0.267

Spirulina	260	1	Spirulina 260°C 1 h	7	Value 1	2.6-2.65	Aliphatic / oxygen-adjacent	1.257	0.365	3.444
Spirulina	260	1	Spirulina 260°C 1 h	7	Value 2	2.6-2.65	Aliphatic / oxygen-adjacent	0.406	2.261	0.179
Spirulina	260	1	Spirulina 260°C 1 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	3.135	0.030	105.765
Spirulina	260	1	Spirulina 260°C 1 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	3.135	1.132	2.769
Spirulina	260	1	Spirulina 260°C 1 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	1.621	0.363	4.465
Spirulina	260	1	Spirulina 260°C 1 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	1.621	3.382	0.479
Spirulina	260	1	Spirulina 260°C 1 h	10	Value 1	3.85- 3.90	Olefinic / oxygenated	1.360	0.026	51.764
Spirulina	260	1	Spirulina 260°C 1 h	10	Value 2	3.85- 3.90	Olefinic / oxygenated	1.360	0.665	2.046
Spirulina	260	1	Spirulina 260°C 1 h	11	Value 1	4.05- 4.10	Olefinic / oxygenated	1.461	0.002	679.092
Spirulina	260	1	Spirulina 260°C 1 h	11	Value 2	4.05- 4.10	Olefinic / oxygenated	1.461	0.523	2.796
Spirulina	260	1	Spirulina 260°C 1 h	12	Value 1	7.15-7.2	Aromatic	0.921	0.101	9.133
Spirulina	260	1	Spirulina 260°C 1 h	12	Value 2	7.15-7.2	Aromatic	2.553	1.329	1.921
Spirulina	260	1	Spirulina 260°C 1 h	13	Value 1	7.40- 7.50	Aromatic	0.568	0.070	8.135
Spirulina	260	1	Spirulina 260°C 1 h	13	Value 2	7.40- 7.50	Aromatic	1.651	1.061	1.556
Spirulina	300	0.5	Spirulina 300°C 0.5 h	1	Value 1	0.9-0.95	Aliphatic	1.845	0.055	33.572

Spirulina	300	0.5	Spirulina 300°C 0.5 h	1	Value 2	0.9-0.95	Aliphatic	1.845	0.812	2.271
Spirulina	300	0.5	Spirulina 300°C 0.5 h	2	Value 1	1.0-1.05	Aliphatic	1.613	0.482	3.349
Spirulina	300	0.5	Spirulina 300°C 0.5 h	2	Value 2	1.0-1.05	Aliphatic	0.479	0.482	0.995
Spirulina	300	0.5	Spirulina 300°C 0.5 h	3	Value 1	1.25-1.3	Aliphatic	2.737	0.015	185.463
Spirulina	300	0.5	Spirulina 300°C 0.5 h	3	Value 2	1.25-1.3	Aliphatic	2.737	1.652	1.657
Spirulina	300	0.5	Spirulina 300°C 0.5 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	1.235	0.003	453.410
Spirulina	300	0.5	Spirulina 300°C 0.5 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	1.235	0.950	1.300
Spirulina	300	0.5	Spirulina 300°C 0.5 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	1.847	0.003	711.131
Spirulina	300	0.5	Spirulina 300°C 0.5 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	0.413	1.328	0.311
Spirulina	300	0.5	Spirulina 300°C 0.5 h	6	Value 1	2.2-2.3	Aliphatic	3.264	0.001	1358.794
Spirulina	300	0.5	Spirulina 300°C 0.5 h	6	Value 2	2.2-2.3	Aliphatic	0.618	1.598	0.258
Spirulina	300	0.5	Spirulina 300°C 0.5 h	7	Value 1	2.6-2.65	Aliphatic / oxygen-adjacent	3.506	0.001	3264.002

Spirulina	300	0.5	Spirulina 300°C 0.5 h	7	Value 2	2.6-2.65	Aliphatic / oxygen-adjacent	0.554	2.058	0.300
Spirulina	300	0.5	Spirulina 300°C 0.5 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	7.728	0.003	1307.184
Spirulina	300	0.5	Spirulina 300°C 0.5 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	2.981	0.642	0.862
Spirulina	300	0.5	Spirulina 300°C 0.5 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	4.651	0.016	471.251
Spirulina	300	0.5	Spirulina 300°C 0.5 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	0.759	1.449	2.057
Spirulina	300	0.5	Spirulina 300°C 0.5 h	10	Value 1	3.85- 3.90	Olefinic / oxygenated	2.903	0.002	1990.024
Spirulina	300	0.5	Spirulina 300°C 0.5 h	10	Value 2	3.85- 3.90	Olefinic / oxygenated	0.690	0.398	1.906
Spirulina	300	0.5	Spirulina 300°C 0.5 h	11	Value 1	4.05- 4.10	Olefinic / oxygenated	1.876	0.055	53.220
Spirulina	300	0.5	Spirulina 300°C 0.5 h	11	Value 2	4.05- 4.10	Olefinic / oxygenated	1.349	0.386	1.787
Spirulina	300	0.5	Spirulina 300°C 0.5 h	12	Value 1	7.15-7.2	Aromatic	5.894	1.360	4.335
Spirulina	300	0.5	Spirulina 300°C 0.5 h	12	Value 2	7.15-7.2	Aromatic	1.984	1.360	1.459
Spirulina	300	0.5	Spirulina 300°C 0.5 h	13	Value 1	7.40- 7.50	Aromatic	4.023	1.322	3.044

Spirulina	300	0.5	Spirulina 300°C 0.5 h	13	Value 2	7.40- 7.50	Aromatic	0.803	1.322	0.608
Spirulina	300	1	Spirulina 300°C 1 h	1	Value 1	0.9-0.95	Aliphatic	1.771	0.712	2.488
Spirulina	300	1	Spirulina 300°C 1 h	1	Value 2	0.9-0.95	Aliphatic	1.771	0.712	2.488
Spirulina	300	1	Spirulina 300°C 1 h	2	Value 1	1.0-1.05	Aliphatic	0.884	0.540	1.636
Spirulina	300	1	Spirulina 300°C 1 h	2	Value 2	1.0-1.05	Aliphatic	0.884	0.540	1.636
Spirulina	300	1	Spirulina 300°C 1 h	3	Value 1	1.25-1.3	Aliphatic	0.964	0.968	0.996
Spirulina	300	1	Spirulina 300°C 1 h	3	Value 2	1.25-1.3	Aliphatic	0.964	0.968	0.996
Spirulina	300	1	Spirulina 300°C 1 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	0.603	0.372	1.620
Spirulina	300	1	Spirulina 300°C 1 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	0.603	0.372	1.620
Spirulina	300	1	Spirulina 300°C 1 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	0.982	0.011	89.986
Spirulina	300	1	Spirulina 300°C 1 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	0.982	0.692	1.420
Spirulina	300	1	Spirulina 300°C 1 h	6	Value 1	2.2-2.3	Aliphatic	0.947	0.085	11.131
Spirulina	300	1	Spirulina 300°C 1 h	6	Value 2	2.2-2.3	Aliphatic	3.393	1.316	2.579
Spirulina	300	1	Spirulina 300°C 1 h	7	Value 1	2.6-2.65	Aliphatic / oxygen-adjacent	0.715	0.235	3.050
Spirulina	300	1	Spirulina 300°C 1 h	7	Value 2	2.6-2.65	Aliphatic / oxygen-adjacent	0.715	1.634	0.438
Spirulina	300	1	Spirulina 300°C 1 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	0.600	0.716	0.837
Spirulina	300	1	Spirulina 300°C 1 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	3.277	3.001	1.092

Spirulina	300	1	Spirulina 300°C 1 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	1.153	0.024	48.382
Spirulina	300	1	Spirulina 300°C 1 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	1.514	1.041	1.455
Spirulina	300	1	Spirulina 300°C 1 h	10	Value 1	3.85- 3.90	Olefinic / oxygenated	0.436	0.024	18.285
Spirulina	300	1	Spirulina 300°C 1 h	10	Value 2	3.85- 3.90	Olefinic / oxygenated	2.586	1.041	2.485
Spirulina	300	1	Spirulina 300°C 1 h	11	Value 1	4.05- 4.10	Olefinic / oxygenated	2.284	0.041	55.955
Spirulina	300	1	Spirulina 300°C 1 h	11	Value 2	4.05- 4.10	Olefinic / oxygenated	1.007	2.715	0.371
Spirulina	300	1	Spirulina 300°C 1 h	12	Value 1	7.15-7.2	Aromatic	2.113	0.001	2112.539
Spirulina	300	1	Spirulina 300°C 1 h	12	Value 2	7.15-7.2	Aromatic	0.649	0.689	0.942
Spirulina	300	1	Spirulina 300°C 1 h	13	Value 1	7.40- 7.50	Aromatic	2.122	0.049	43.527
Spirulina	300	1	Spirulina 300°C 1 h	13	Value 2	7.40- 7.50	Aromatic	0.628	0.267	2.353
Spirulina	313	0.5	Spirulina 313°C 0.5 h	1	Value 1	0.9-0.95	Aliphatic	1.483	0.102	14.558
Spirulina	313	0.5	Spirulina 313°C 0.5 h	1	Value 2	0.9-0.95	Aliphatic	1.483	0.677	2.189
Spirulina	313	0.5	Spirulina 313°C 0.5 h	2	Value 1	1.0-1.05	Aliphatic	0.589	0.336	1.753
Spirulina	313	0.5	Spirulina 313°C 0.5 h	2	Value 2	1.0-1.05	Aliphatic	0.589	0.605	0.974

Spirulina	313	0.5	Spirulina 313°C 0.5 h	3	Value 1	1.25-1.3	Aliphatic	2.096	0.018	113.888
Spirulina	313	0.5	Spirulina 313°C 0.5 h	3	Value 2	1.25-1.3	Aliphatic	2.096	0.913	2.296
Spirulina	313	0.5	Spirulina 313°C 0.5 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	0.284	0.104	2.744
Spirulina	313	0.5	Spirulina 313°C 0.5 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	0.284	0.933	0.305
Spirulina	313	0.5	Spirulina 313°C 0.5 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	0.788	0.026	30.861
Spirulina	313	0.5	Spirulina 313°C 0.5 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	0.788	0.848	0.930
Spirulina	313	0.5	Spirulina 313°C 0.5 h	6	Value 1	2.2-2.3	Aliphatic	0.726	0.026	28.408
Spirulina	313	0.5	Spirulina 313°C 0.5 h	6	Value 2	2.2-2.3	Aliphatic	2.034	0.848	2.399
Spirulina	313	0.5	Spirulina 313°C 0.5 h	7	Value 1	2.6-2.65	Aliphatic / oxygen-adjacent	1.688	0.038	44.366
Spirulina	313	0.5	Spirulina 313°C 0.5 h	7	Value 2	2.6-2.65	Aliphatic / oxygen-adjacent	0.510	0.858	0.594
Spirulina	313	0.5	Spirulina 313°C 0.5 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	0.692	4.063	0.170
Spirulina	313	0.5	Spirulina 313°C 0.5 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	6.655	1.371	4.854

Spirulina	313	0.5	Spirulina 313°C 0.5 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	0.575	3.965	0.145
Spirulina	313	0.5	Spirulina 313°C 0.5 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	1.699	1.340	1.268
Spirulina	313	0.5	Spirulina 313°C 0.5 h	10	Value 1	3.85- 3.90	Olefinic / oxygenated	0.476	0.001	475.540
Spirulina	313	0.5	Spirulina 313°C 0.5 h	10	Value 2	3.85- 3.90	Olefinic / oxygenated	1.665	0.625	2.664
Spirulina	313	0.5	Spirulina 313°C 0.5 h	11	Value 1	4.05- 4.10	Olefinic / oxygenated	0.683	0.092	7.410
Spirulina	313	0.5	Spirulina 313°C 0.5 h	11	Value 2	4.05- 4.10	Olefinic / oxygenated	3.166	0.092	34.318
Spirulina	313	0.5	Spirulina 313°C 0.5 h	12	Value 1	7.15-7.2	Aromatic	2.531	0.337	7.515
Spirulina	313	0.5	Spirulina 313°C 0.5 h	12	Value 2	7.15-7.2	Aromatic	0.689	2.563	0.269
Spirulina	313	0.5	Spirulina 313°C 0.5 h	13	Value 1	7.40- 7.50	Aromatic	2.420	0.028	87.073
Spirulina	313	0.5	Spirulina 313°C 0.5 h	13	Value 2	7.40- 7.50	Aromatic	0.312	1.230	0.253
Spirulina	313	1	Spirulina 313°C 1 h	1	Value 1	0.9-0.95	Aliphatic	1.623	0.030	54.594
Spirulina	313	1	Spirulina 313°C 1 h	1	Value 2	0.9-0.95	Aliphatic	1.623	0.635	2.555
Spirulina	313	1	Spirulina 313°C 1 h	2	Value 1	1.0-1.05	Aliphatic	1.191	0.003	469.742

Spirulina	313	1	Spirulina 313°C 1 h	2	Value 2	1.0-1.05	Aliphatic	0.468	0.527	0.889
Spirulina	313	1	Spirulina 313°C 1 h	3	Value 1	1.25-1.3	Aliphatic	1.691	0.003	614.263
Spirulina	313	1	Spirulina 313°C 1 h	3	Value 2	1.25-1.3	Aliphatic	0.647	1.899	0.341
Spirulina	313	1	Spirulina 313°C 1 h	4	Value 1	1.45-1.5	Aliphatic/Alicyclic	0.473	0.029	16.565
Spirulina	313	1	Spirulina 313°C 1 h	4	Value 2	1.45-1.5	Aliphatic/Alicyclic	0.473	0.395	1.197
Spirulina	313	1	Spirulina 313°C 1 h	5	Value 1	1.95-2.0	Aliphatic/Alicyclic	1.228	0.002	594.295
Spirulina	313	1	Spirulina 313°C 1 h	5	Value 2	1.95-2.0	Aliphatic/Alicyclic	1.228	0.824	1.492
Spirulina	313	1	Spirulina 313°C 1 h	6	Value 1	2.2-2.3	Aliphatic	1.111	0.012	93.806
Spirulina	313	1	Spirulina 313°C 1 h	6	Value 2	2.2-2.3	Aliphatic	2.199	0.802	2.743
Spirulina	313	1	Spirulina 313°C 1 h	7	Value 1	2.6-2.65	Aliphatic / oxygen-adjacent	0.680	0.011	61.064
Spirulina	313	1	Spirulina 313°C 1 h	7	Value 2	2.6-2.65	Aliphatic / oxygen-adjacent	2.524	1.096	2.303
Spirulina	313	1	Spirulina 313°C 1 h	8	Value 1	2.95-3.0	Oxygenated aliphatic	3.935	0.161	24.513
Spirulina	313	1	Spirulina 313°C 1 h	8	Value 2	2.95-3.0	Oxygenated aliphatic	0.709	3.082	0.230
Spirulina	313	1	Spirulina 313°C 1 h	9	Value 1	3.4-3.5	Oxygenated aliphatic	0.703	0.022	31.379
Spirulina	313	1	Spirulina 313°C 1 h	9	Value 2	3.4-3.5	Oxygenated aliphatic	1.809	1.163	1.556
Spirulina	313	1	Spirulina 313°C 1 h	10	Value 1	3.85- 3.90	Olefinic / oxygenated	1.317	0.027	48.134

Spirulina	313	1	Spirulina 313°C 1 h	10	Value 2	3.85- 3.90	Olefinic / oxygenated	1.317	0.656	2.008
Spirulina	313	1	Spirulina 313°C 1 h	11	Value 1	4.05- 4.10	Olefinic / oxygenated	2.890	0.029	99.865
Spirulina	313	1	Spirulina 313°C 1 h	11	Value 2	4.05- 4.10	Olefinic / oxygenated	2.890	0.541	5.340
Spirulina	313	1	Spirulina 313°C 1 h	12	Value 1	7.15-7.2	Aromatic	3.453	0.204	16.934
Spirulina	313	1	Spirulina 313°C 1 h	12	Value 2	7.15-7.2	Aromatic	0.964	1.617	0.596
Spirulina	313	1	Spirulina 313°C 1 h	13	Value 1	7.40- 7.50	Aromatic	2.281	0.204	11.186
Spirulina	313	1	Spirulina 313°C 1 h	13	Value 2	7.40- 7.50	Aromatic	0.470	1.617	0.290