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Biomedical Sciences

DETECTION OF CHEMOMETRIC MARKERS IN LIVER TISSUE USING INFRARED SPECTROSCOPY

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Master's Thesis

Supervisor

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Istanbul, 2023

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The thesis titled DETECTION OF CHEMOMETRIC MARKERS IN LIVER TISSUE USING INFRARED SPECTROSCOPY prepared by ARWA DAHHAM ABDULHADI QADAWEE and submitted on 21/03/2023 has been **accepted unanimously** for the degree of Master of Science in Biomedical Sciences.

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I hereby declare this thesis meets all format and submission requirements for Master's Thesis.
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I hereby declare that all information/data presented in this graduation project has been obtained in full accordance with academic rules and ethical conduct. I also declare all unoriginal materials and conclusions have been cited in the text and all references mentioned in the Reference List have been cited in the text, and vice versa as required by the abovementioned rules and conduct.

Arwa QADAWEE

Sign

DEDICATION

First, I want to thank Allah for his blessings and his gifts to me, he is my first and last support all the time. I want to say to my supervisor Assoc. Prof. Dr. Hakan Kaygusuz, and my co-advisor Asst. Prof. Dr. Şebnem Garip, thank you very much for every single thing and for your cooperation in writing this thesis and for their patience and valuable advice.

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ABSTRACT

DETECTION OF CHEMOMETRIC MARKERS IN LIVER TISSUE USING INFRARED SPECTROSCOPY

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Among the leading contributors of fetal alcohol spectrum disorder (FASD) is perinatal alcohol usage, which is characterized by neurodevelopmental dysfunction and growth retardation. Studies have mostly examined how alcohol affects the tissues of the brain. The effects of alcohol usage during pregnancy on the fetal liver are yet unknown at the molecular level. The aim of the study is to determine spectroscopic biomarkers referring to the results of prenatal exposure to alcohol in the liver of newborn mice. There were three main groups of samples: Negative control group, gavage control group (positive control), and group with gavage + alcohol. which is treated between postnatal days (PD) 3 and 20 with 3.0 g/kg body weight of ethanol in a liter of artificially enhanced milk (.02 ml/g). In accordance with that, there were three different groups of samples, for these groups, these three different groups of samples were measured using FTIR spectra, and in statistical analysis, these spectral data were evaluated. In the classification of spectral data, three main regions were significant: Fingerprint region (800 to 1800 cm^{-1}), lipid region (3000 to 2800 cm^{-1}) and water region and O-H stretch region (3000 to 3600 cm^{-1}). For normalization, a standard normal variate (SNV) normalization technique was evaluated after the baseline correction. For statistical analysis, principal component analysis (PCA) was evaluated using R statistical language with FactoMineR package. According to the

results of this study, it can be proposed that FTIR is an effective tool for development of chemometric markers in liver tissue, especially for the observation of the consequences of consuming alcohol on female rats. Possible chemometric biomarkers detected in this study are related to the lipids and found as follows: 2960 cm^{-1}, 2873 cm^{-1}, 1454 cm^{-1} and 1398 cm^{-1}. Further studies can reveal the levels of alcohol consumption and detection of other markers.

Keywords: FTIR Spectroscopy, PCA, Liver Tissue, SNV, Chemometric Markers.

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ABBREVIATIONS

ADH	:	Alcohol Dehydrogenase
ANN	:	Neural Network Analysis
ATR	:	Attenuated Total Reflectance
CART	:	Classification and Regression Trees
ECM	:	Extracellular Matrix
FAS	:	Fetal Alcohol Syndrome
FASD	:	Fetal Alcohol Spectrum Disorders
FTIR	:	Fourier Transform Infrared
HCA	:	Hierarchical Cluster Analysis
LDA	:	Linear Discriminant Analysis
PCA	:	Principal Component Analysis
SNV	:	Standard Normal Variate
ATR	:	Attenuated Total Reflectance
IR	:	Infrared
AUD	:	Alcohol Use Disorders
ALD	:	Alcoholic Liver Disease

1. INTRODUCTION AND PURPOSE

1.1 ALCOHOL-INDUCED FETAL DISORDERS

Alcoholism during pregnancy (FAS) Fetal alcohol syndrome is a syndrome in which the fetus is affected by a mother who drinks alcohol before or during pregnancy, which leads to several defects in terms of growth, cognitive functions, facial abnormalities and central nervous system weakening and damage to the internal organs (Dejong, Olyaei, & Lo, 2019)

FAS is frequently misdiagnosed or ignored, which delays the start of therapy for affected children. During prenatal care and preconception counseling, all women should be checked since there is no safe level of alcohol consumption during pregnancy. According to estimates, between 0.3% and 0.8% of children in the United States and 2.9% of children globally have FASD. It is the most prevalent cause of non-intellectual impairment that is not heritable (Leeanne Denny, Sarah Coles, & Robin Blitz, 2017) .Women who have already had fetal alcohol spectrum disorder (FASD) are more likely to experience another pregnancy with a child who is equally or more seriously impacted by alcohol (Jones, 2011)

Alcohol is considered a strong teratogenic substance. consumption of alcohol in prenatal period causes several disabilities in the newborn in terms of growth and brain(Krulewitch, 2005) Fetal alcohol spectrum disorders (FASD) can be diagnosed by focusing on the defects caused by drinking alcohol, rather than the physical or psychological effects it has on the body(Kaminen-Ahola, 2020) Diagnosing the disease is often very difficult because of the mothers' lack of credibility for drinking alcohol or taking drugs. The most reliable way is to diagnose the fetus with FASD. It shows the pathological features through which it is possible to know the Fetal alcohol syndrome prevalence and to infer that the mother was consuming alcohol during pregnancy (Caprara, Nash, Greenbaum, Rovet, & Koren, 2007)

The inability to interact with the society that surrounds the kid and discriminating between relatives and strangers is one of the psychological signs of this syndrome. These are all emotional symptoms, such as mood swings, emotional regulation, and adaptability issues,

they are one of the causes for the spread of borderline personality (Sappok, Tergeist, Kruse, & Wagner, 2022)

A range of neurobehavioral effects that can result from alcohol exposure while a baby is still growing are known as fetal alcohol spectrum disorders (FASD), Research on (FASD) has focused on establishing the exact neurobehavioral problems connected to prenatal alcohol exposure in the absence of physical dysmorphology in order to better identify those who are affected. The quantity and timing of prenatal alcohol use have an effect on development. Constant drinking may have fewer detrimental behavioral and cognitive effects than binge drinking, which is defined as four or more drinks consumed in less than two hours for females. To clarify precise dose and timing concerns associated to prenatal alcohol intake, animal models have been employed. Binge drinking during pregnancy has been linked to behavioral issues, higher mental health issues, and worse academic performance in children, according to observational studies in humans.

The fetus's central nervous system can be damaged by drinking at any time throughout pregnancy. Nevertheless, the developmental stage at which exposure occurs affects the degree of structural damage. The fetus is especially vulnerable to the negative effects of prenatal alcohol use because it is going through critical phases of brain development, many of which start very early in pregnancy. For instance, it seems that drinking too much alcohol during gastrulation, which occurs between weeks three and four of human pregnancy, causes facial dysmorphology. There might be a range of consequences on the child, ranging from mild to severe behavioral and cognitive impairments, depending on the pattern of alcohol consumption and the timing of exposure in relation to developmental stages.

Maternal risk factors for FASD include being older, having a history of alcohol abuse in the family and/or maternal partner, and going to fewer prenatal checks. A higher risk is also associated with lower maternal body weight, height, and BMI, as well as with poor nutrition, demographic traits such low maternal education, rural area, and lower socioeconomic status. Further research is needed to identify other maternal risk factors and possible targets for preventing FASD-related disabilities.

The fetal brain's development may be significantly impacted by maternal alcohol use during pregnancy. Several impacts have been discovered in studies, including a loss of gray matter, a reduction in overall brain volume, and a disruption of the central nervous system. The consequences of prenatal alcohol consumption appear to be particularly sensitive to several tissues, including the frontal and parietal lobes. Children with FASD also have aberrant network connection, with unusual activity seen in the insula, basal ganglia, cerebellum, and amygdala. Correlations between brain structural measurements and other significant factors, such as behavior and cognition, have been added to volumetric declines.

Cognitive deficiencies are correlated with reduced volume in various brain regions. Smaller left hippocampi, which are essential for memory consolidation and retrieval, are linked to lower verbal learning and spatial memory in children with FASD. For children exposed to alcohol during pregnancy, caudate volume is the greatest indicator of neuropsychological function since it corresponds with cognitive control, language learning, and memory abilities. In people exposed to alcohol, correlations between the volumes of the brain's structural components and facial dysmorphology have been discovered. Studies using functional neuroimaging have revealed that activities involving language learning, reaction inhibition, visual attention, and working memory result in different patterns of brain activation. These studies demonstrate the potential effects of prenatal alcohol exposure on the fetal brain's development and how it can influence adult cognitive ability (Mattson, Bernes, & Doyle, 2019)

Prenatal alcohol exposure causes a wide range of birth defects collectively known as "Fetal Alcohol Spectrum Disorders" (FASD) (EtOH). It can have a modest to severe negative impact on a developing fetus, resulting in physical, behavioral, and cognitive problems that last a lifetime. Estimates of FASD prevalence range from 0 to 176.77 per 1000 live infants; EtOH intake during pregnancy is completely avoidable. One of the most significant and avoidable non-genetic birth abnormalities linked to intellectual impairment is this one (Ehrhart et al., 2019)

Fetal alcohol spectrum disorder, which is brought on by prenatal alcohol exposure, includes a number of symptoms, such as minor craniofacial anomalies, growth retardation, neurological abnormalities, cognitive and behavioral impairment, and birth defects. It is as

common as autism spectrum disorder globally, with a frequency of 0–6%, yet underdiagnosis still occurs due to social stigma, diagnostic challenges, reliance on facial characteristics, and qualities that are similar to those of other diagnoses. Initiatives to improve diagnosis include studies of previously undiagnosed individuals, international research focusing on high-risk populations, improved three-dimensional face imaging for screening, and neurobehavioral screening tools for school-aged children. Especially in terms of epidemiology and clinical expression, this Review outlines advancements in the research of fetal alcohol spectrum disorders. Moreover, it discusses classifications, diagnostic techniques, specific brain anomalies, pathophysiology, and treatment options for the condition (Wozniak, Riley, & Charness, 2019)

Fetal alcohol spectrum disorder can be avoided with the support of public health programs such timely alcohol abstinence, alcohol abuse prevention, addiction treatment, and birth control. Its most distinguishing characteristics are short palpebral fissures, a smooth philtrum, and a thin upper lip vermilion. In patients who have been exposed to alcohol during pregnancy, minor morphological anomalies include railroad-track ears, ptosis, epicanthal folds, anteverted nares, midface hypoplasia, joint contractures, camptodactyly, and altered palmar creases might manifest. Despite the need of dysmorphology assessment, the number of persons with fetal alcohol spectrum disorder exceeds the capacity of specialty fetal alcohol disorder clinics. The Collaboration on Fetal Alcohol Spectrum Disorder Prevalence research consortium's recommendations, which are based on Institute of Medicine criteria, define the amount of alcohol exposure in addition to less stringent criteria for cognitive impairment. The four subtypes of the fetal alcohol spectrum disorder for which diagnostic criteria are necessary are fetal alcohol syndrome, partial fetal alcohol syndrome, alcohol-related neurodevelopmental disorder, and alcohol-related birth defects (Wozniak et al., 2019)

1.2 EFFECTS OF PRENATAL ALCOHOL CONSUMPTION ON DIFFERENT FETAL TISSUE

The most vulnerable organ to the consequences of prenatal alcohol use throughout pregnancy is the fetal brain. Alcohol use during pregnancy mostly causes central nervous system dysfunction. Excessive alcohol consumption increases the amount of habit stimuli in the body of the fetus and the weakening of the brain. The myelination rate of neurons is reduced, and memory is therefore impaired. Alcohol significantly affects the heart, as pre-born alcohol consumption is firmly linked to coronary artery disease and congenital heart defects. Alcohol has dangerous effects also on the lung tissues which causes losing control over viruses and germs entering the body. Thus, tuberculosis and pneumonia are easily exposed in this situation (Caputo, Wood, & Jabbour, 2016)

Even drinking alcohol before the pregnancy impairs the metabolism and metabolic balance of the pregnant mother, which leads to impaired fetal growth (Lee et al., 2020) Ethanol also affects the placenta of the fetus .The placenta, fetus, and newborn all undergo ethanol metabolism. Ethanol is broken down into two types of metabolism: oxidative and non-oxidative. According to a previous study, certain children's development of FAS may be influenced by polymorphisms in the enzymes involved in ethanol metabolism. Pure ethanol can be expelled through the skin, lungs, and kidneys. At two months of pregnancy, alcohol dehydrogenase (ADH), the main enzyme responsible for ethanol oxidation, is found in the newborn (L Burd, J Blair, & K Dropps, 2012) In several studies, maternal alcohol consumption reduce or raise placental weight and fetal weight suitable for gestational age. Alcohol exposure in the womb can cause a variety of biochemical and physiological changes in the developing embryo (Koçkaya & Akay, 2006)

1.3 ANIMAL MODEL OF HUMAN FAS

It was observed that mice develop fetal alcohol syndrome when exposed to alcohol before and during pregnancy (Koçkaya & Akay, 2006).Two weeks before conception and fertilization, mice were exposed to alcohol and the result was reduced pregnancy, the number of fetuses and the low weight of birth (Lee et al., 2020). When mice were sucked

and vaccinated with alcohol, an increase in activity was observed (Breit, Zamudio, & Thomas, 2019)

Rodents are the most prevalent models especially rat and mice. Small mice and rats make for simpler handling and less threatening models than adults (Kehrberg, Parrish, & Eby, 2017)

That the human third trimester corresponds to the two weeks following birth in rats (Dobbing & Sands, 1979) Studies on prenatal alcohol exposure, its neurobehavioral impacts, and other effects on bodily systems are being done in order to better understand the molecular basis of alcohol-induced disorders. For the development of more effective human therapies for alcoholism, animal research on alcohol is crucial. Animal models are also used to offer information on the possible consequences of alcohol poisoning on nutrition, environment, and genes. Rodents have a quicker metabolic rate than humans, therefore they digest alcohol more quickly even if the procedures for doing so in both species are the same (Ali Forat Abdulsattar AL-GBURI, 2022).

1.4 LIVER TISSUE

1.4.1 What Is Liver

The right ribcage covers and protects the upper right corner of the belly, where the biggest internal organ in the human body, the liver, is located. It has reddish brown color. The size of the liver is approximately 12-15 cm, while its weight is about 1500 g, equivalent to 2.5% of the body weight, it aids in metabolism, digestion, immunity, and strong essential nutrients within the body shown in Figure 1.1 (The World Health Organization, n.d.)

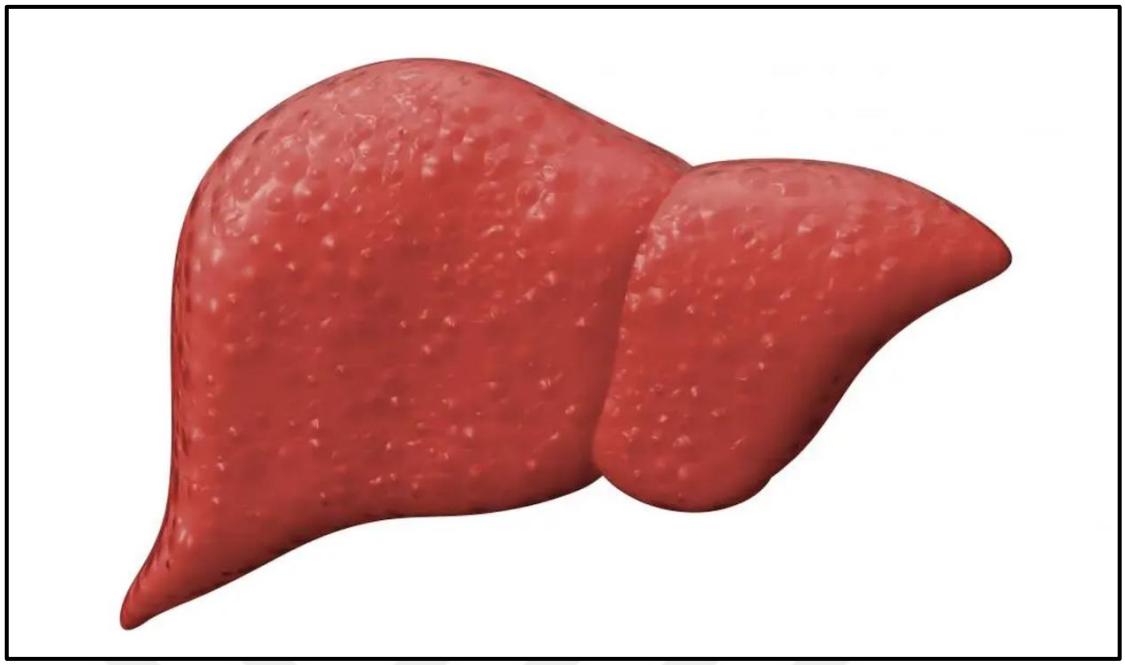

Figure 1.1: Liver organ external image (Tim Newman, 2018)

The liver has a trapezoidal form and is separated into two lobes, the larger of which is on the right and the smaller of which is on the left. The falciform ligament, a band of connective tissue that holds the diaphragm together, divides the lobes. The outer layer of the liver is protected by Glisson's capsule, a fibrous tissue layer. The peritoneum, a membrane that forms the lining of the abdominal cavity, also protects this capsule. This prevents the liver from being physically harmed and retains it in place. The liver is the only regenerable organ and the biggest solid organ in the human body. It belongs to the class of glands (Tim Newman, 2018)

Hepatocytes, sinusoid endothelium, Kupffer, stellate, biliary epithelial, and pit cells are among the cell types found in the liver shown in Figure 1.2 (Hoganson, Pryor, & Vacanti, 2008)

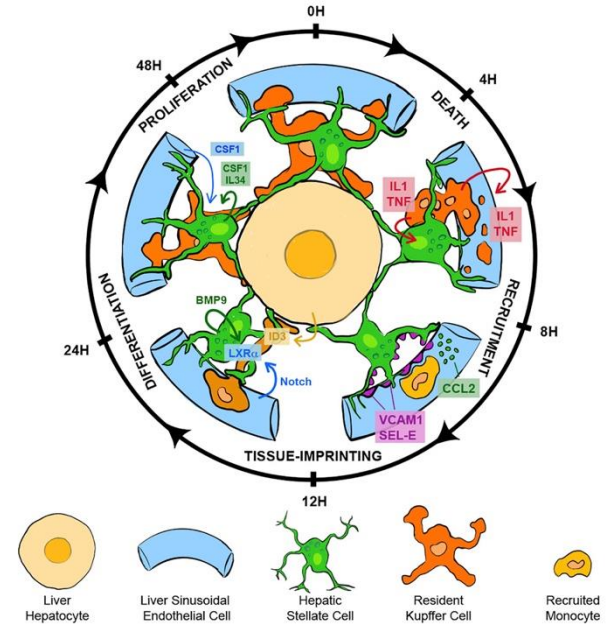

Figure 1.2: Human liver cell types (Bonnardel et al., 2019)

1.4.2 Functions and Importance of Liver Tissue

Liver is a gland that has several functions (Tim Newman, 2018).

The major functions of the liver include:

a. An active member of the metabolism

i. Glucose metabolism: During the period of feeding

ii. During fasting, move glucose into glycogen storage.

iii. Move sugar from stores to blood.

iv. Excretory function: Bile pigments (bilirubin)- Bile salts (vital for fat absorption)

b. Storage for vitamins and minerals: stores vitamins A, D, E, K, and B12.

c. Immunological function:

Because the liver contains Kupffer cells which participates in maintaining the body's immunity as it works to get rid of any factors that may cause diseases to the liver entering through the gut

d. d. Supporting blood clots: Coagulants, which help blood clot, are produced when vitamin K is present. The liver produces bile, which is important for absorbing vitamin K. Insufficient bile production by the liver prevents the production of clotting factors.

e. Bile production: Bile aids in the breakdown and absorption of lipids, cholesterol, and certain vitamins in the small intestine. Cholesterol, bilirubin, bile salts, electrolytes, and water make up bile (The World Health Organization, n.d.).

1.4.3 Morphogenesis of the Fetal Liver

Human and mouse fetal development goes through similar phases; However, the relative timings are different. The last trimester of fetal gestation is when males experience the brain's most rapid development, which is linked to the creation of a complex multicellular organization known as the brain growth phase. The rodents, on the other hand, only have a growth spike during the postnatal period, which starts around two weeks after birth. It is referred to as the animal's third trimester or its equivalent (Dobbing & Sands, 1979)

The epithelial cells that border the intrahepatic bile ducts are hepatocytes and cholangiocytes, are the main constituents of the liver. The bipotential progenitors of the liver eventually develop to hepatocytes and cholangiocytes in three nearby areas of the endodermal. The first evidence for liver bud formation is the thickening of the endoderm of the ventral foregut.

The creation of different lobes in the liver may include hepatoblast growth or directed migration. The visceral ligaments partition the liver into many lobes of a certain form and size in the mature organ. The margin of the expanding organ and the end of the liver lobes was indicated by increased cell division. The liver size is strictly controlled by the adult and ensures a strong homeostatic and renewable response. A comparable potential seems to develop liver to modulate organ size by altering the number of progenitor cells (Ober & Lemaigre, 2018)

1.5 THE EFFECTS OF ALCOHOL INTAKE ON FETAL LIVER TISSUE DURING THE PRENATAL STAGE

The liver is one of the most important and first organs to be affected by alcohol abuse, and the first to be affected by the body’s organs, because it is primarily responsible for the interior environment, or homeostasis, of complex living things. Alcoholic liver disease develops in humans through several phases, from fatty liver to alcoholic hepatitis and

cirrhosis, depending on the level of alcohol consumption. Only 15–25% of heavy drinkers develop cirrhosis, indicating that there are other risk factors that contribute to the development of the disease. For the liver to heal from many damages, including those caused by alcohol, its capacity to repair itself is crucial. Both acute and chronic alcohol use reduce the liver's ability to produce new DNA, which is genetic material. However, the surviving liver cells cannot divide as quickly to rebuild the liver if there is insufficient DNA production. On the other hand, it may play an important function in persons with liver illness (Ponnappa & Rubin, 2000)

The fetal liver is a vital organ that experiences major morphological and functional adjustments after birth, with the quantity and size of liver cells increasing while the number of hematopoietic cells decreases. Alcohol's poisonous effects are especially dangerous to the liver, and major changes in liver histology and extracellular matrix (ECM) may occur because of alcohol consuming. During pregnancy, the maternal tissue primarily performs the function of alcohol intake in the fetus. The decreased activity of the alcohol-oxidizing isoenzymes suggests that maternal alcohol intake causes alcohol metabolism problems in all kids. As a result, alcohol consumption may harm fetuses' organs more than adult organs. And studies still on going to know if drinking alcohol during pregnancy affects the prenatal and postnatal growth and metabolism of gastrointestinal organs like the liver (Koçkaya & Akay, 2006) .One of the enzymes that influence the fetal liver is alcohol dehydrogenase (ADH) enzyme. Alcohol dehydrogenase is the enzyme that largely oxidizes ethanol in the liver. In other tissues, enzyme activity is minimal or non-existent. Because ethanol quickly travels through biological membranes, it is possible for the fetus to come into touch with it during pregnancy. If ADH is present in the embryonic liver, ethanol is oxidized, which may affect the fetus' metabolism (Pikkarainen & Räihä, 1967)

As a teratogen, alcohol can cause birth abnormalities in the fetus. Alcohol is swiftly absorbed by the digestive system despite being lipid soluble. Pregnant women experience the same levels of maternal and fetal blood alcohol because the placental barrier is permeable to alcohol molecules (Ali Forat Abdulsattar AL-GBURI, 2022)

The peak periods of organ system development or maturation are correlated with the most critical times for alcohol intake. For instance, drinking alcohol during the first trimester is

associated with musculoskeletal and organ abnormalities, including distinct facial abnormalities, while drinking alcohol during the second and third trimesters (the brain growth spurt period) is associated with growth retardation, as well as intellectual and behavioral deficits, and drinking alcohol during the final trimester can be associated with abnormal liver function, including fibrosis and fatty hepatic degeneration in children with Down syndrome (FAS)(Aronson & Olegård, 1987)

Infrared Spectroscopy Principle

Infrared spectroscopy is the study of the absorption, emission, and reflection of infrared light by various materials. This approach is used to study and identify chemical compounds or functional groups in solid, liquid, or gaseous forms. The most prevalent wavenumber units used in IR spectra are reciprocal centimeters, indicated by the sign cm^{1} (Zeitler et al., 2010), It is possible to swiftly record (IR) spectra in the solid, liquid, solution, and steam phases at a range of temperatures. It is now possible to record molecules in samples using modern (IR) technologies. Due to its versatility, (IR spectroscopy) has become the "workhorse" of analytical research. No other technique allows materials to be investigated and recognized in such a variety of physical situations (Ali Forat Abdulsattar AL-GBURI, 2022).

Because molecules absorb frequencies that are specific to their structure, infrared spectroscopy takes use of this phenomenon.

There are three regions of IR (Near - Mid - Far)

1. Overtone or combination modes of molecular vibration can be induced by higher-energy near-IR, which has a wavelength of 0.7–2.5 m and a cm^{1} range of 14,000–4,000.

2- The mid-infrared, which has a wavelength between 4,000 and 400 cm^{-1}, is frequently used to study the fundamental vibrations and associated rotational-vibrational structure (2.5 to 25 m).

3- The far-infrared, which has a wavelength between 400 and 10 cm^{-1}, is low energy and ideal for rotational spectroscopy and low frequency vibrations (25 to 1,000 m)

For analyzing materials with covalent bonding, this approach is widely used. Simple spectra are produced by samples with high degrees of purity and few IR active bonds. Increasingly sophisticated molecular architectures result in more absorption bands and

intricate spectra. This method is commonly used in laboratory equipment such as Fourier transform infrared (FTIR) spectrometers (Harwood, 1989)

1.6 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

Fourier transform infrared (FTIR) spectroscopy is one of the most effective techniques for analyzing and comprehending the molecular structure of various biological membranes and tissues. It may be used to understand how modifications to functional groups impact the structure of tissues and membranes and have a profound impact on how well these systems operate. Each data point contains information on each infrared frequency from the source, resulting in an incredibly rich signal (Ali Forat Abdulsattar AL-GBURI, 2022) .

Vibrational spectroscopy is among the most popular and common techniques used in several fields such as studying protein solutions and visual testing of molecular tissue changes, especially abnormal tissues. Spectral bands in the vibrational spectrum give direct biological composition information (Movasaghi, Rehman, & ur Rehman, 2008). FTIR spectroscopy and micro spectroscopy has been utilized to assess the molecular changes in liver tissues due to a pathologic condition, exposure to toxic agents or drugs (Algburi, Dursun, & Ustaoglu, 2022; Drozdz et al., 2020; Garip et al., 2016; Perez-Guaita, Richardson, Heraud, & Wood, 2020)

The key advantage of these techniques is that even from extremely small sample quantities, they may provide a non-invasive, label-free molecular fingerprint of tissue and cells. Both FTIR and Raman methods, which produce comparable and complimentary chemical composition and structural data, may be used to observe the same tissue (Garip et al., 2016)

Infrared light may be directed at samples in several ways, allowing for the study of a wide range of samples. Thus, the technique can be studied both in transmission and reflection Attenuated Total Reflectance (ATR) mode. FTIR spectroscopy examines samples from the surface of the internal reflection element (IRE) at a penetration depth of 0.5-5 μm as in reflection mode. One of the advantages of FTIR is that it measures many technical applications on samples with high efficiency, accuracy in performance and low cost (Dunuwila & Berglund, 1997; Movasaghi et al., 2008) Moreover, the sample amount required for recording the spectra is very small for both liquid samples as blood, serum and urine and tissue samples. High signal-to-noise ratio, high resolution (0.1 0.005 cm^{-1}), quick

scan times, independence from the user, and non-invasiveness are further benefits (Garip Ustaoğlu, Kaygusuz, Bilgin, & Severcan, 2021)

The one disadvantage of the technique is for studying the aqueous samples due to the significant water bands of absorption. Those water bands overlap all the other bands of the functional groups in the spectra of the sample. The solution for that problem is to use nitrogen gas for removing the intermolecular water molecules in the sample. The exposure to nitrogen gas in a sample-specific optimized time (few minutes) results in monitoring the functional group bands clearly in the spectra of the sample (Dogan, Gurbanov, Severcan, & Severcan, 2021)

Peaks are quite narrow, and the vibrators of a given chemical bond (or functional group) may in many cases be correlated with a particular molecule. And this dipolar moment shifts during molecular vibration are the result of FTIR Spectroscopy. It is also used to detect the secondary structure of a protein by combining with the Neural network analysis (ANN) and chemometrics (Movasaghi et al., 2008; Tiernan, Byrne, & Kazarian, 2020)

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy is a modern and promising method for tissue analysis that has a great range of biological uses such as imaging and identification of living cells and pathological tissues. This technique can be used to examine the penetration depth of drugs using an appropriate spectral signature (Tiernan et al., 2020) .ATR is suitable for highly absorbent materials and for measuring surfaces and thin film ‹by using ATR-FTIR. We can reduce the measurement time and increase the effectiveness of the measurements by improving the noise signal ratio (Binder et al., 2018; Tiernan et al., 2020)

ATR-FTIR Spectroscopy perhaps utilized effectively to assess in situ, with adequate accuracy and the metastable limit, solubility, and precise super saturation. Because the ATR measurement's infrared light penetration depth to the sample is independent of sample thickness, low or no sample preparation is required for recording spectra from the samples (Dunuwila & Berglund, 1997)

1.6.1 Chemometrics Analysis

Chemometrics is a technique for exploratory data analysis that clusters samples according to how closely they resemble patterns discovered by multivariate pattern recognition algorithms. The two most popular chemometric investigations are principal component analysis (PCA) and hierarchical cluster analysis (HCA). HCA groups samples based on cluster distance, a similarity metric that highlights connections between samples. An HCA also reveals samples that are responsible for significant variation. So, to improve the clustering results, these samples might be ignored. A PCA reduces a complex data matrix into a limited number of components or factors, with the other components being noise or useless information. A PCA also reveals samples that could be outliers. An essential component of chemometric research is data visualization. Tribes and families identified by an HCA are simply shown in a dendrogram that draws comparisons between data based on cluster distance and naturally occurring groups. A PCA employs 2D or 3D scatterplots to illustrate visually significant differences across data clusters and evaluate the HCA groupings. So, without making any assumptions about how the data would be distributed into patterns that may be comprehended, chemometric analysis simplifies complex data (Meyer, Adhikari, Olson, Overton, & Miles, 2018) Chemometrics is the study of data-driven methods for information extraction from chemical systems. Chemometrics is naturally multidisciplinary and frequently draws on methods from multivariate statistics, applied mathematics, and computer science to address problems in chemistry, biochemistry, medicine, biology, and chemical engineering. It is comparable to other interdisciplinary fields like econometrics and psychometrics (Wold, 1995)

1.6.2 Chemometric Methods Combined with Spectroscopic Data

Chemometric techniques are strong instruments utilized in the analytical sciences and other fields. The way they apply is steadily rising, in part because new concerns may be explored because to technological advancements. The most often used chemometric instruments in the most current studies, which appear at the intersection of many scientific fields and instrumental methods, incorporating hyphenated mass spectrometry techniques, electrochemistry, and vibrational spectroscopy, Their use is always expanding, in part

because of technical developments that make it possible to investigate new problems (Peris-Díaz & Krężel, 2021)

Before using chemometric methods, all spectra were subjected to pre-treatment techniques such baseline correction and normalization (SNV method). We collected the average spectra for further chemometric analysis (Sharma, Verma, Kumar, Chauhan, & Sharma, 2020)

1.6.3 Supervised and Unsupervised Methods

To maximize the effectiveness of the classification approach, supervised algorithms strive for the best group separation possible, Without the possible bias of knowing in advance which samples belong to which category, unsupervised approaches seek to expose the underlying data structure (Rácz, Bajusz, & Héberger, 2018)

Some examples about supervised and unsupervised methods:

a. In the field of chemometrics, linear discriminant analysis is one of the supervised classification techniques that is most frequently used linear discriminant analysis (LDA), Recursive partitioning, also referred to as classification and regression trees, is the foundation for a different family of supervised learning techniques called classification and regression trees (CART).

b. In unsupervised pattern one of the most used chemometric techniques is principal component analysis (PCA). It is based on dimension reduction and does this using matrix decomposition. Data is divided up into a specific set of coefficients termed loading vectors P′ by principal component scores in PCA (abstract factors or latent variables), Cluster analysis, often known as clustering, is another frequently used technique in which data are organized into groups (or "clusters") according to how far off they are from one another. The similarity between two objects increases with their proximity (Rácz et al., 2018)

1.6.4 PCA Method

The most exploratory technique utilized in the chemical and physical sciences is undoubtedly PCA Principal Components Analysis. Finding patterns in two-dimensional

space is made possible by defining new variables called Principal Components (PC) from linear combinations of the original ones.

When there are more variables than samples, PCA may be used without producing false positives. PCA also aims to reduce the number of dimensions by eliminating unnecessary data while keeping all nonzero PCs. A method for decrease in size aims to offer a discriminating behavior based on overall variability. This method scales back and compresses the original data (Peris-Díaz & Krężel, 2021)

PCA is a projection technique that seeks for routes in the multivariate space that, over time, best suit the data distribution, or that, in the least squares sense, most closely resemble the data. This explains why, when performing exploratory data analysis, PCA is typically the method of choice. PCA allowed to clearly identify clusters in the scores plot (Biancolillo & Marini, 2018)

(PCA) transforms multidimensional data in such a manner that the first few changed dimensions retain the bulk of the changes made to the original multidimensional space. It may be used to examine complicated spectra that could contain hundreds of absorbance values by condensing each spectrum to a single point in a lower-dimensional space (Elibol, Severcan, Jakubowska- Dogru, Dursun, & Severcan, 2022)

(PCA) makes it possible to see the general "form" of the data and aids in classifying comparable and dissimilar sample groups. Principal Component Analysis (PCA) breaks down the initial variables into fewer Principal Components (PC), or the directions with the most variance. This simplifies the dataset. A dataset can be reformulated in terms of eigenvalues and eigenvectors without changing the underlying data. Kaiser criteria are used in this situation to choose the number of PCs, with PCs with Eigen values greater than 1 being prioritized above those with eigen values below 1. Thus, eigenvalue denotes the mechanisms involved in data compression. Only for the purpose of spectral peak clarity are reformulating spectra given (Sharma et al., 2020)

Using exploratory multivariate analytic techniques like (PCA) or FTIR spectroscopy in conjunction with sample identification is a rapid method (HCA). Both PCA and HCA are unsupervised techniques that may be used in tandem to examine the data structure and identify patterns across several samples (Matwijczuk et al., 2022)

1.6.5 SNV Method

Weighted normalization is used in the Standard Normal Variate (SNV) normalization approach. SNV scales the rows of the matrix rather than the columns to get the standard deviation of all the variables that have been collect for a certain sample. SNV correction reduces undesired spectral contributions and makes it easier to evaluate the analytical signal, sometimes even more so than derivative spectra. Monitoring of blending operations can be made easier by the SNV method because it reduces noise in the signal. In this study, we applied the SNV correction to lower undesired noise in the infrared Fourier spectra (Blanco, 2002)

The primary goal of the data pre-treatment is to eliminate any potential noise or disruption in each spectrum. This makes it possible for only variance caused by chemical ingredients to predominate and accurately reflect the features of the samples. Pre-treatments come in a variety of forms, and the type that is chosen depends on the sample's makeup. SNV (Standard Normal Variate) pretreatment for scattering correction is quite successful. The slope variation in the spectrum can be eliminated with the use of SNV transformation. The employment of a derivative approach improves resolution and eliminates constant and linear drift across samples by estimating the existence of overlapping and hidden peaks. The most common order derivatives are first and second order (Sharma et al., 2020)

PCA can provide light on the underlying biochemical differences or molecular composition of the analyzed data sets, and the findings are equivalent to the difference spectra that can be produced using straightforward subtraction. The location of the spectra on the scores plot and the magnitude of the loadings are directly correlated. Despite corresponding to two distinct directions in the scatter plot, the negative scores are just as instructive as the positive ones. The negative and positive peaks may be assigned to the various plot groups using the scale that is available for each PC, and utilizing various reference spectra, these peaks can then be matched with certain characteristics for the molecular characterisation of the materials (Bonnier & Byrne, 2012)

1.6.6 HCA Method

HCA is a traditional classification technique that use graphical techniques to display the clustering of the data in two dimensions. Heterogeneity is a metric for similarity. The

Ward's approach was used in this study to apply HCA. Utilizing the squared Euclidean distance between the samples, Ward's approach creates clusters with the lowest within-class variation (Elibol et al., 2022)

An exploratory approach it is used to locate unnoticed natural groups (or clusters) within a data collection. The best time to utilize it is when you only need to group a few hundred thingsCluster distance, a measure of similarity that illustrates the links between samples, is used by HCA to group samples. HCA may also identify samples that are significantly increasing variance. These samples can thus be disregarded to enhance the cluster findings (Szekely & Rizzo, 2005)

The foundation of HCA is the measurement of the dissimilarity between sets of data and the smallest distances between objects (such as spectroscopic spectra). Using a distance matrix and the degree of similarity between the items, it is one of the exploratory techniques used to categorize things into clusters (groups) based on distance. The correct technique for gauging the distance between observational pairs should be employed, together with linking criteria, in hierarchical clustering. As opposed to previous clustering methods, this one does not need predetermination of the number of clusters to be produced (Matwijczuk et al., 2022)

1.7 PURPOSE OF THE STUDY

The aim of the study is to determine spectroscopic biomarkers related to the effects of early postnatal exposure of alcohol in the liver of the newborn mice.

2. GENERAL INFORMATION

The most common teratogen in humans is ethanol, and severe drinking during pregnancy can have major consequences for the fetus. The effect of alcohol on mice has significant nutritional consequences as ethanol causes malnutrition when it replaces nutrients due to its high calorie content and body weight loss in ethanol-saturated mice depends on the amount of dietary fat consumed (Koçkaya & Akay, 2006).

For example, the placenta of mice exposed to alcohol is much heavier than that of the controls, due to weight gain، to stop maternal blood cells in the labyrinthine blood channels, in addition to increasing the size and number of giant cells (Eguchi et al., 1989)

The effects of mother breastfeeding in the presence of ethanol and offspring development were studied using a foscross-fostering approach. At birth, rats in the ethanol group were exposed to higher doses of ethanol (5 % in the first week to 20% in the fourth week). Offspring exposed to ethanol while breastfeeding or during pregnancy consume less milk than usual. The weights of the liver, kidneys, and spleen, as well as milk intake at the conclusion of lactation, were also investigated. Although there were no negative effects of ethanol on body weight at delivery, alcohol during nursing caused a growth deficit despite a normal birth weight (Murillo-Fuentes, Artillo, Carreras, & Murillo, 2001)

In adult rats, high-dose ethanol exposure has been linked to reduced nephron number, hypertension, insulin resistance, and glucose intolerance (Probyn, Zanini, Ward, Bertram, & Moritz, 2012) .During pregnancy, postprandial blood glucose levels in ethanol-fed mice were significantly higher. Pre-exposed mice displayed to ethanol various regulatory influences during early pregnancy affect the capacity to absorb glucose and the responsiveness of the intracellular glucose disposal system [(Lee et al., 2020). Humans and animals with fetal alcohol syndrome are similar in terms of facial shape and brain structure tricks exposed to alcohol in the prenatal period, as alcohol was given to mice (C57B1 / 6J) which resulted in a change in the shape of the face in terms of small eyes, head size and long upper lips. Microscopic examination revealed after giving alcohol after 24 hours and for a week indicates a change in the composition of the brain and eyes because of alcohol consumption (Jones, 2011)

We don't know the chemical mechanisms through which ethanol harms unborn children, although one possible mechanism is the production of oxidative stress. In whole animal and tissue culture studies, antioxidants have been demonstrated to prevent ethanol-induced oxidative damage in fetuses. It is crucial to carefully assess the safety of vitamins C and E during pregnancy as well as the experimental use of antioxidants in alcohol-consuming women in order to prevent fetal alcohol harm and based on proven experimental findings (Cohen-Kerem & Koren, 2003)

Pregnancy exposed to alcohol also affects liver functions in fetal liver protein synthesis and enzyme activity (Liu et al., 2016) .With almost every part of the brain being adversely altered by prenatal alcohol consumption, the brain is the organ most badly affected. The gastrointestinal tract, endocrine, renal, liver, and cardiac systems are also problematic. FAS can be significantly influenced by prenatal alcohol exposure, although more study is required to pinpoint which infants are affected and when. It will be ground-breaking to discover strategies to lessen or stop the physical and neurological abnormalities that occur within the fetus, for pregnant women at risk of alcohol consumption, nutritional supplementation may be a viable prophylactic strategy (Caputo et al., 2016)

According to a research of rat offspring exposed to alcohol *in utero*, total protein expression was downregulated in 7-day-old and 3-month-old rats. Alcohol intake during pregnancy increased fructose-1, 6-bisphosphatase's activity, which is a gluconeogenic enzyme. Cell signaling, cellular stress, protein synthesis, cytoskeleton, aminoacidic, adenosine, and energy metabolism are among the pathways impacted by these protein changes (Fofana et al., 2010) With an estimated $3.6 billion yearly cost of care and a $2.9 million lifetime cost per case, FASD has significant societal repercussions (Hofer & Burd, 2009).Alcohol intake by pregnant women is a major contributor to birth abnormalities nowadays. Congenital heart disease and development deficiencies are only two examples of the negative impact prenatal alcohol consumption may have on the developing fetus (Liu et al., 2016)

Intrauterine growth retardation, central nervous system malformation and malfunction, cardiac and skeletal abnormalities, etc. are some of the key clinical characteristics of FAS. Several embryonic organs, including the pituitary, neurological system, brain, liver, and

placenta, have been linked to ethanol consumption (Perez, Velasco, Monte, Gonzalez-Buitrago, & Marin, 2006)

Men and women are affected by alcohol usage in various ways. Although more men than women experience alcohol use disorders (AUD), women usually progress faster toward dependence and experience the negative effects of alcoholism earlier than men. In addition, males tend to be less reactive and demonstrate less alcohol appetite than women do. Significantly more ethanol was consumed by female rats than male rats. At two hours after the ethanol drinking session began, the blood alcohol levels in the female rats were considerably greater than those in the male rats. Researchers collected vaginal smears to track the estrous cycle for two weeks to ascertain if hormonal changes affected alcohol use. The amount of ethanol consumed by the women at different estrous cycle phases did not change noticeably. However, ethanol consumption by females was higher than that of males during the whole estrous cycle (Li et al., 2019)

Preclinical alcohol model data indicate that while women are quicker than men to self-administer alcohol and consume more of it during maintenance phases, they also have less severe physical and negative affective withdrawal symptoms. Although it cannot be completely ruled out that organizational steroid effects play a role in regulating sex differences in alcohol responses, the main focus of this review is on the effects of adulthood levels of estrogen, progesterone, and neuroactive metabolites on alcohol consumption and pertinent addiction-related phenotypes in females.

This sex difference appears to be influenced by the facilitative effects of estrogen in females and the inhibitory effects of testosterone in males.

It was probably necessary to adopt Dozier and colleagues' method of in-depth menstrual cycle description during periods of active drinking to show the significant menstrual cycle-related shift in alcohol consumption.

Alcohol consumption may be influenced by hormonal changes that occur throughout the menstrual cycle but not the estrous cycle. Rodents lack a comparable luteal phase, although humans do, which explains this. Greater alcohol consumption during the late stage of the female menstrual cycle was strongly connected with premenstrual discomfort and depressive moods in women.

Progesterone has been used as a "prodrug" to boost allopregnanolone levels in small cohorts of male and female patients with co-occurring AUD and cocaine use disorder. This approach has shown promise in lowering cue-induced desire and cortisol responses. Males and females with the highest allopregnanolone levels had the greatest fall in desire following progesterone injection. As a result, methods to maintain or increase levels of GABAA receptor-active neurosteroids like allopregnanolone, or to utilize allopregnanolone analogs with longer half-lives, may offer novel effective therapies for both men and women who have AUD (Finn, 2020)

PCA can be represented utilizing its information on the most important factors and provides data reduction with little information loss (Mustapha & Aris, 2012)

To identify the biochemical markers separating alcohol groups and control and intubations groups in liver tissue samples, the unsupervised exploratory PCA discovered substantially unique morphological regions that may be individually examined.

To monitor liver tissue disease, biochemical data from tissue imaging using FTIR spectroscopy would be very helpful (Kaznowska, Depciuch, Szmuc, & Cebulski, 2017)

Baseline correction is a step in the preprocessing process, which researchers refer to as eliminating as many undesired elements as possible from the raw data. In other words, preprocessing is carried out differently depending on the circumstance.

In investigations that look at the impact of an experimental modification, baseline correction is helpful. By including random fluctuations, baseline correction increases statistical power in these trials (Mathôt, Fabius, Van Heusden, & Van der Stigchel, 2018)

Male mice fed alcohol continuously cause a greater death rate in female mice. While ethanol consumption by male and female mice was comparable when corrected to body weight, female mice showed considerable inflammation (increases in TNF- and IL-6) as well as death. Male control and ethanol-fed mice died at similar rates, on the other hand. It's interesting to note that for the first 4 weeks of ethanol administration, there was no difference in mortality between ethanol-fed male and female mice.

These findings suggest that female mice are more susceptible to the long-term consequences of ethanol use. We have identified the sex-specific, alcohol-induced reduction in CTRP3 as a potential mechanism for the increased susceptibility of females to alcoholic cirrhosis. It is significant to note that ethanol consumption led to increased levels of PAI-1, indicating in both sexes that alcohol consumption causes tissue damage and disruptions to overall tissue homeostasis (DeGroat et al., 2018)

Preclinical research suggest that females learn to self-administer alcohol more quickly and consume more alcohol during maintenance periods than men do. After progesterone injection, male and female individuals with the highest allopregnanolone levels showed the largest reduction in desire. Hence, novel effective therapies for both males and females with AUD may involve using allopregnanolone analogs with longer half-lives or stabilizing or enhancing levels of GABAA receptor-active neurosteroids like allopregnanolone (Finn, 2020)

Females are more prone to alcoholic liver damage than men in both humans and animals, At several levels, the immune systems of men and women differ from one another. For instance, women are thought to be more prone to autoimmune disorders—an estimated 80% of autoimmune diseases affect women. Alcohol and its metabolites affect immunological cells as well as local tissue cells, which results in alcoholic liver disease. Alcohol-related liver damage is more likely to affect women than men. Alcohol consumption disorder is the sixth most prevalent cause of death in both Europe and the United States. Alcohol use has negative effects on human tissues and organs in a number of different ways. In both humans and animals, women are more likely than men to have their livers damaged by alcohol (Alharshawi et al., 2021)

Alcohol consumption disorder is the sixth leading cause of death in both the US and Europe. Moreover, 3.3 million individuals worldwide pass away each year from alcohol-related causes, making up 5.9% of all fatalities. Alcohol use has a deleterious effect on a number of human organs and tissues. Alcohol induces tissue damage as a result of its immune-system-damaging effects as well as those of its metabolites, including acetaldehyde, reactive oxygen species, and nitrogen species. The clinical and histological elements of alcoholic liver disease include steatosis, steatohepatitis, alcoholic hepatitis, and alcoholic liver cirrhosis (ALD). both in mice and in humans (Alharshawi et al., 2021)

Using FTIR, we obtain information and data with high reliability and accuracy. The one disadvantage of the technique is for studying the aqueous samples due to the significant water bands of absorption. Those water bands overlap all the other bands of the functional groups in the spectra of the sample. The solution for that problem is to use nitrogen gas for removing the intermolecular water molecules in the sample. The exposure to nitrogen gas in a sample-specific optimized time (few minutes) results in monitoring the functional group bands clearly in the spectra of the sample (DOGAN et al., 2021)

Plasma alcohol levels were still significantly elevated in both male and female ethanol-treated mice 12 hours after the alcohol challenge, which was accompanied by a significant buildup of lipids in the liver and an increase in plasma transaminases; however, lipid accumulation was about three times greater in female mice than in male ones.

According to the findings of several research conducted on animals, rats who are female are more likely than male rodents to develop chronic alcohol-induced liver damage (ALD).

According to the findings of several human research, women are more susceptible than males to alcoholic liver disorders. In fact, research has revealed that just half of the quantity of alcohol drunk on average is needed to harm women's health in a comparable way. In addition, despite a reported shorter history of alcohol misuse and a lower consumption of alcohol than men, female alcohol abusers had more advanced liver damage at the time of diagnosis.

It has been demonstrated that female rats are more susceptible to ALD than male animals in an intragastric model of chronic alcohol feeding, and that the gonadal hormone estrogen plays a role in the early development of ALD. Animal studies have shown that estrogen may have a significant role in the greater sensitivity of female mice to alcohol. For instance, persistent alcohol use in female rats was found to increase intestinal permeability and plasma endotoxin levels in the portal vein(Wagnerberger et al., 2013)

The impact of sex hormones on oxidative and metabolic pathways may contribute to gender disparities in the prevalence, course, and consequences of prevalent liver disorders. Gender inequalities in liver illness may also be influenced by variations in access to care and treatment as well as diagnostic factors.

Although the fact that males misuse or depend on alcohol more than women do, women are more vulnerable to the harmful effects of alcohol on the liver at any given amount. Women are more likely to acquire alcohol-related liver disease, according to a 12-year prospective study of alcohol consumption in more than 13,000 individuals in Denmark. (Jennifer Guy, 2013)

The development of an alcohol consumption problem may be influenced by gender variations in how the brain is affected by excessive or chronic alcohol use (AUD). The alterations in important brain systems that control executive function, memory, and stress are what cause AUD, a multidimensional and complicated condition.

Progesterone and its metabolites have also been connected to the modulation of mesocorticolimbic dopamine neurons in response to alcohol. In research on male rats, progesterone increases the levels of dopamine extracellularly by 55% in the medial prefrontal cortex after the experimenter administers alcohol.

A nonhuman monkey study found that women drink more when their blood levels of estradiol, progesterone, and their metabolites are higher (i.e., during the luteal phase compared to the follicular phase of the menstrual cycle). The maximum drinking occurred during the progesterone peak's decreasing phase during the luteal phase, and there was a tendency for serum allopregnanolone levels and alcohol consumption to be positively correlated. Progesterone and neuroactive hormones may affect mesocorticolimbic dopaminergic neurons involved in reward processing, potentially altering drinking behavior. However, further study is required to determine whether these effects change depending on gender (Flores-Bonilla, 2020)

Compared to their body weight, mature female rats drink more than adult male rodents. It has been demonstrated that predator odor stress increases drinking in male rats to the degree seen in females. Only when the baseline drinking was stratified into low vs high amounts was this stress impact observed in female mice (Flores-Bonilla, 2020)

Reduced alcohol consumption among expectant mothers. The likelihood of drinking while pregnant increases with depression and being alone. Although while drinking declines during the first trimester, 1 in 10 pregnant women still indulge in alcohol each month.

Children who drink had lower IQ scores at age 8, especially if they have one of four hereditary abnormalities in the genes responsible for alcohol metabolism (White, 2020)

Alcohol and gender

Alcohol consumption has always been a male-dominated activity. Worldwide, males use more alcohol than women, and they are also more accountable for the harm that alcohol causes to oneself and others. In 2016, alcohol usage by people worldwide aged 15 and older was 32% among women (0.88 billion) and 54% among men (1.46 billion).

The ratio of male to female drinkers is 1:1, yet there may appear to be a gender difference in alcohol intake. The size of the gap varies by culture and nation.

In addition, 7% more men than women (4%) are diagnosed with alcohol use disorder (AUD) each year. Males and females with AUD receive treatment at about the same rates (9% and 9%) respectively. 6 Women are more likely than males to suffer damages because of another person's drinking, according to research (White, 2020)

3. METHODOLOGY

3.1 SAMPLE PREPARATION-ATR METURMENT

Three groups of newborn mice were created: the control group, the intubation group, and the alcohol group. From postnatal days 3 to 20, we took mice pups and weighed it daily prior to intubation. In the alcohol-treated group, 3.0 g/kg body weight of amount of alcohol was intubated in a quantity of 0.02 ml/g of phony milk. The control group had two daily intubations using the same technique as the alcohol group.

After the euthanasia of the mice on PD 90, their liver tissues were taken away and saved at -80 oC for spectroscopic research. After removing any remaining blood from the tissues by rinsing them in phosphate buffer saline, liver samples were separated into three pieces for each animal in the control, intubation control, and alcohol-treated groups (PBS). The Bruker Alpha II 100 FTIR spectrometer (Bruker, Berlin, Germany) was used to read the liver tissue samples on the Diamond/ZnSe (Di/ZnSe) Zinc Selenide crystal plate of the Universal ATR to create IR spectra.

The spectra are collected between 4000-850 cm^{-1} at ambient temperature. Each interferogram needed 128 images and had a resolution of 4 cm^{-1}. Each sample's three tissue slices were used to capture the spectra in duplicates. The average of these three spectra was then used to analyze the molecular characteristics of the liver tissues from diverse animal groups.

Band area/intensity data give information on the concentration of the target group, whereas band location and bandwidth values reveal structural and functional characteristics.

The band positions (wavenumber values) were determined by using the centers of the peaks that comprised 80% of the peaks.

Three tissue pieces from each sample were used to record the spectra in duplicates. The molecular features of the liver tissues of the various animal groups were then examined using the average of these three spectra (Ali Forat Abdulsattar AL-GBURI, 2022)

Experimental protocol of this study was approved by the local scientific ethical committee of Bezmialem Vakif University, Istanbul, Turkey (2017 / 99)

3.2 SAMPLE CLASSIFICATION

There were three main groups: Negative control group, gavage control group (positive control) and group with gavage + alcohol, which is handled between postnatal days (PD) 3 and 20 with 3.0 g/kg body weight of ethanol in a liter of artificially enhanced milk (0.02 ml/g). In accordance with that, there were three different groups of samples, with the following codes attached to them:

a. Female negative control (FCN) 5 samples
b. Female positive control (FCG) 7 samples
c. Female alcohol (FA) 5 samples

For these groups, at least five different samples were measured using FTIR spectra, and in statistical analysis these 17 spectral data was evaluated.
In classification of spectral data, three main regions were significant:

a. Fingerprint region 1800 to 800 cm^{-1}
b. lipid region 3000 to 2800 cm^{-1}
c. and water region 3600 to 3000 cm^{-1}

3.3 STATISTICAL ANALYSIS

Spectral data of all 17 samples were evaluated after a baseline correction by using SpectraGryph program (Elibol et al., 2022)

For normalization, a standard normal variate (SNV) normalization technique, which was described earlier, was evaluated.

After SNV, processed spectral data was saved and used for further statistical analysis.

After SNV was applied for the 17 data by depending on this study (Elibol et al., 2022) we divided the spectrum to three regions to find a suitable peaks:

a. (1800-900 cm^{-1})
b. (3030-2800 cm^{-1})
c. (3800-3030 cm^{-1})

We choses the peaks from every region and we get 14 peaks.

a. 1022, 1080, 1150, 1207, 1238, 1398, 1454, 1547, 1638 $cm^{-1}$from the first region
b. 2853, 2873, 2925, 2960 cm^{-1} from the second region
c. 3282 cm^{-1} from the third region

For statistical analysis, principal component analysis (PCA) was evaluated using R statistical language with FactoMineR package (Giuseppe Marano, 2013), It is used for factor analysis and data mining with R (Lê, Josse, & Husson, 2008)

In PCA, a correlation matrix method is used for the following wavenumbers: 1022, 1080, 1150, 1207, 1238, 1398, 1454, 1547, 1638, 2853, 2873, 2925, 2960, 3282 cm^{-1}.

We applied PCA first for all spectrum for all peaks and then we applied them separately (fingerprint, lipid) to be more specific.

i. Fingerprint region (1022, 1080, 1150, 1207, 1238, 1398, 1454, 1547, 1638cm^{-1}).
ii. Lipid region (2853, 2873, 2925, 2960cm^{-1}).

Each region is corresponding to a specific bond and/or presence of a biochemical group in FTIR. With 17 total number of samples and 14 different wavenumbers (14 variables & 17 samples). A 14x17 matrix was calculated.

Similar studies were conducted for different data sets by (Kalaycıoğlu, Kaygusuz, Döker, Kolaylı, & Erim, 2017; Kaygusuz, 2021; Kaygusuz et al., 2016)

For clustering analysis, a hierarchical cluster analysis (HCA) was employed for the predominancy principal component projections of the whole data.

4. RESULTS

In this study we determined spectroscopic features related to the effects of early postnatal exposure of alcohol in the liver tissue of the newborn mice.

There were three different groups of samples: Negative control group, gavage control group (positive control) and group with gavage + alcohol. Obtained FTIR spectra of 17 female mice samples was evaluated using SpectraGryph program as shown in figures, which indicates the baseline corrected and SNV-normalized data for all samples. Figure 4.1 present Baseline figure for female samples of three different groups.

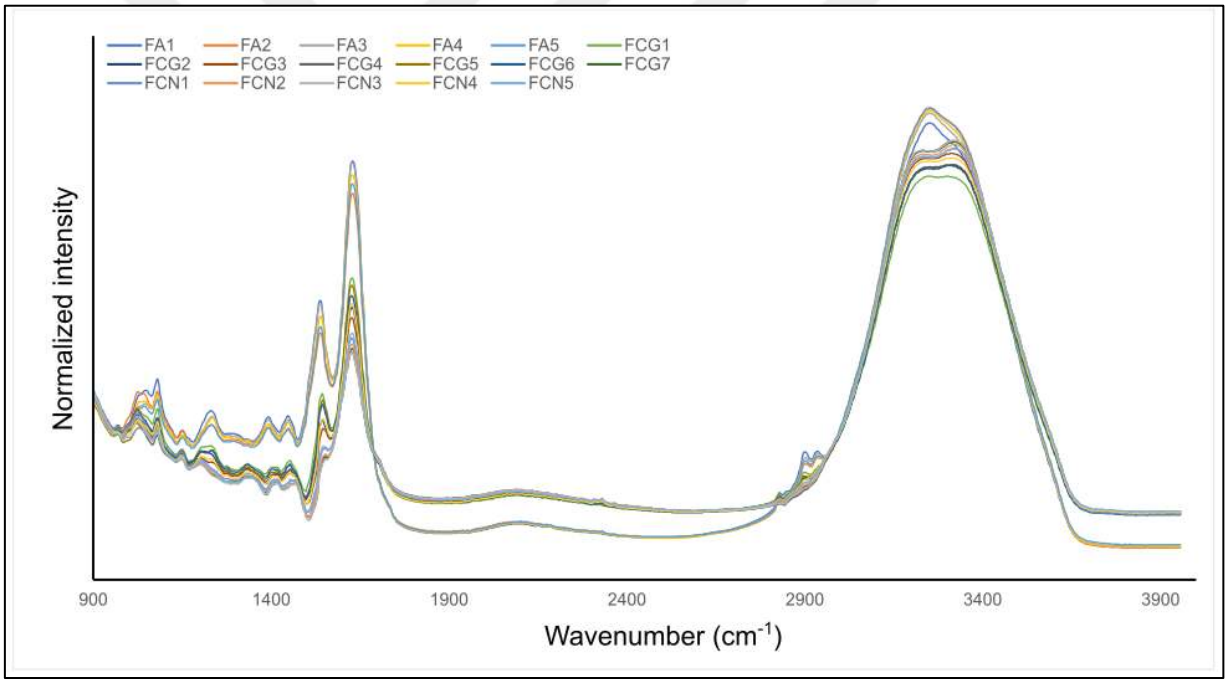

Figure 4.1: Baseline figure for female samples of three different groups

After baseline correction, SNV normalization was applied for those groups for female samples as shown in the Figure 4.2.

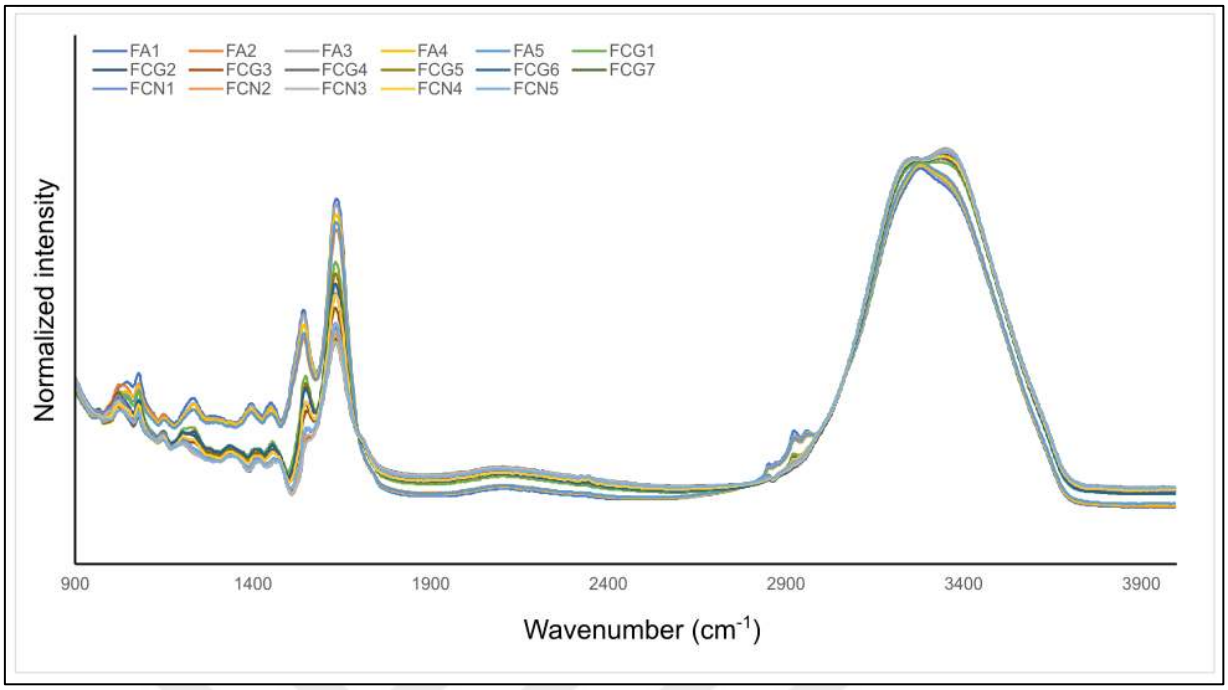

Figure 4.2: (SNV)Normalized FTIR spectra of female samples.

After the normalization step, spectrum was analyzed according to the literature (Elibol et al., 2022) and divided into three regions:

a. 1800-900 cm^{-1}

b. 3030-2800 cm^{-1}

c. 3800-3030 cm^{-1}

As it shown in the Figures 4.3, each region was separately analyzed to find the corresponding peaks.

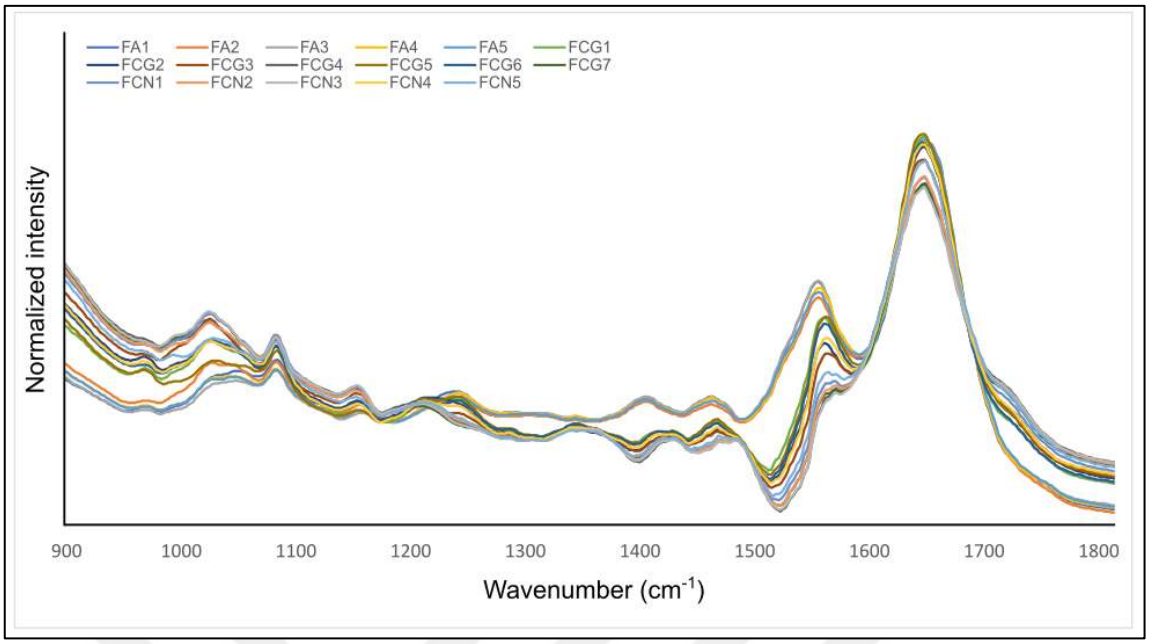

Figure 4.3: 1800-900 cm^{-1} first region

In Figure 4.4 it was more than five peaks with a functional group related to fingerprint region and protein region which every peak is related to a specific band.

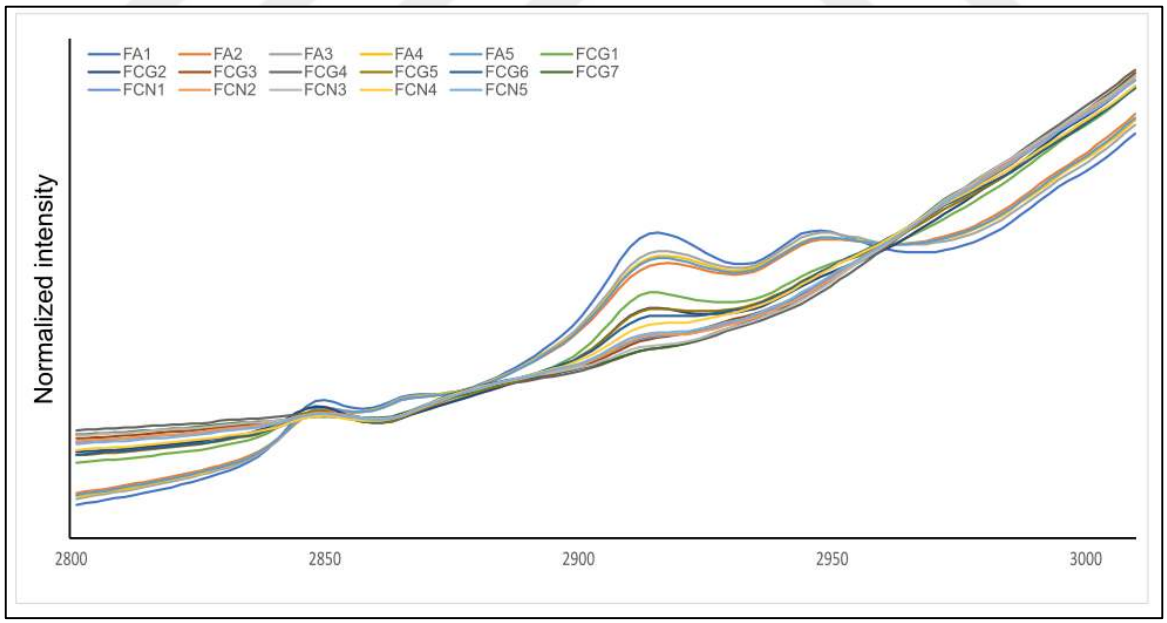

Figure 4.4: 3030-2800 cm^{-1} second region

In second region four peaks are visible, which are related to the bands in lipid region and protein interactions shown in Figure 4.5.

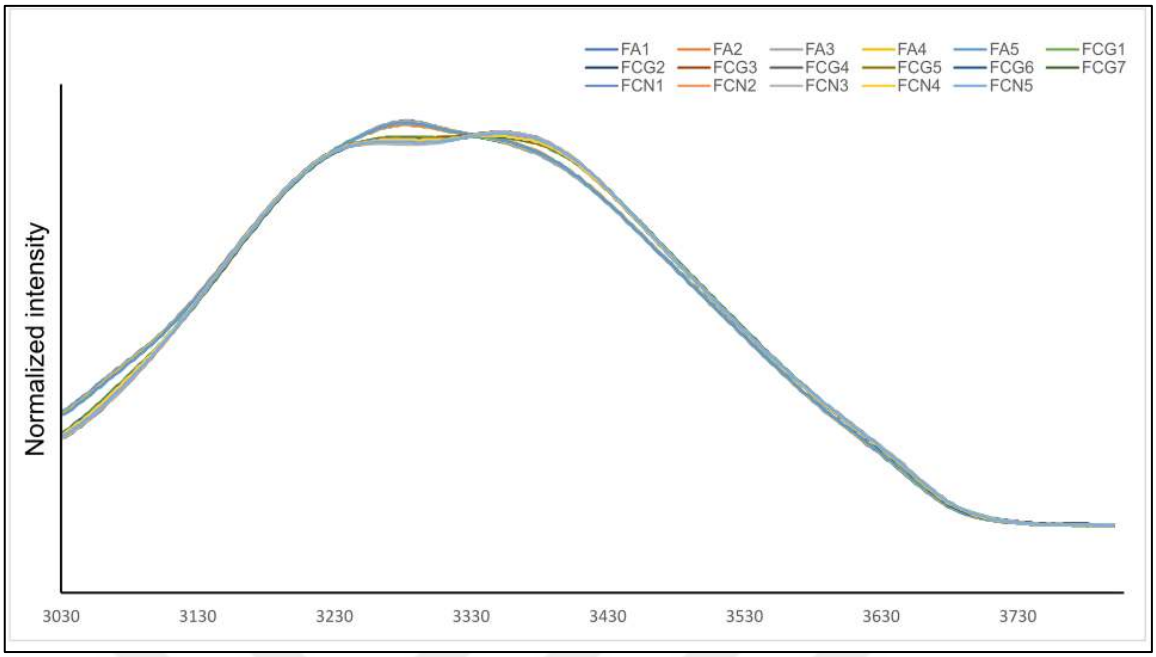

Figure 4.5: 3800-3030 cm^{-1} third region

In the third region the strongest bands are water and O-H stretch band, as seen in the figure.

14 different peaks were selected from the analysis of the three different regions:

a. (1022, 1080, 1150, 1207, 1238, 1398, 1454, 1547, 1638) cm^{-1} from the first region.
b. (2853, 2873, 2925, 2960) cm^{-1} from the second region.
c. (3282) cm^{-1} from the third region.

In the second part of this study, using principal component analysis, statistical analysis was performed on 17 samples (PCA).

PCA was applied for the selected 14 normalized peak values. For this aim, first we applied the 14 peaks in full spectrum and in the second step by separating spectrum into two regions (fingerprint, lipid) to be more specific.

a. Full spectrum PCA: (1022, 1080, 1150, 1207, 1238, 1398, 1454, 1547, 1638, 2853, 2873, 2925, 2960, 3282 cm^{-1}).
b. PCA for fingerprint region (1022, 1080, 1150, 1207, 1238, 1398, 1454,1547, 1638 cm^{-1})
c. PCA for lipid region (2853, 2873, 2925, 2960 cm^{-1})

Tables 4.1, 4.2 and 4.3 display the preliminary findings of (PCA) for full spectrum, fingerprint region and lipid region. These findings suggest that the first two components account for almost all the remaining variation. It displays loadings and score graphs to help make sense of the principal component analysis.

Table 4.1: Eigenvalues and variance results of the PCA for full spectrum.

PC	**Eigenvalues**	**Variance %**	**Cumulative % variance**
PC-1	1.154277e+01	8.244839e+01	82.44839
PC-2	1.656071e+00	1.182908e+01	94.27747
PC-3	5.408792e-01	3.863423e+00	98.14089
PC-4	1.552642e-01	1.109030e+00	99.24992
PC-5	5.990260e-02	4.278757e-01	99.67780
PC-6	2.930924e-02	2.093517e-01	99.88715
PC-7	7.155191e-03	5.110851e-02	99.93826
PC-8	5.574272e-03	3.981623e-02	99.97807
PC-9	1.365018e-03	9.750127e-03	99.98782
PC-10	1.063594e-03	7.597098e-03	99.99542
PC-11	3.923454e-04	2.802467e-03	99.99822
PC-12	1.601486e-04	1.143919e-03	99.99937
PC-13	6.429119e-05	4.592228e-04	99.99983
PC-14	2.432579e-05	1.737557e-04	100.00000

Table 4.2: Eigenvalues and variance results of the PCA for fingerprint region.

PC	**Eigenvalues**	**Variance %**	**Cumulative % variance**
PC-1	7.4930643214	83.256270238	83.25627
PC-2	1.2491221907	13.879135452	97.13541
PC-3	0.1497087129	1.663430143	98.79884
PC-4	0.0577147070	0.641274522	99.44011
PC-5	0.0417889722	0.464321913	99.90443
PC-6	0.0051839483	0.057599426	99.96203
PC-7	0.0017743503	0.019715003	99.98175
PC-8	0.0011887680	0.013208534	99.99496
PC-9	0.0004540292	0.005044769	100.00000

Table 4.3: Eigenvalues and variance results of the PCA for lipid region.

PC	**Eigenvalues**	**Variance %**	**Cumulative % variance**
PC-1	3.4095034567	85.237586416	85.23759
PC-2	0.5234305922	13.085764806	98.32335
PC-3	0.0668667152	1.671667880	99.99502
PC-4	0.0001992359	0.004980898	100.00000

The loadings and score plots of the PCA analysis are shown in the Figures 4.6:

a. PCA result for full spectrum:

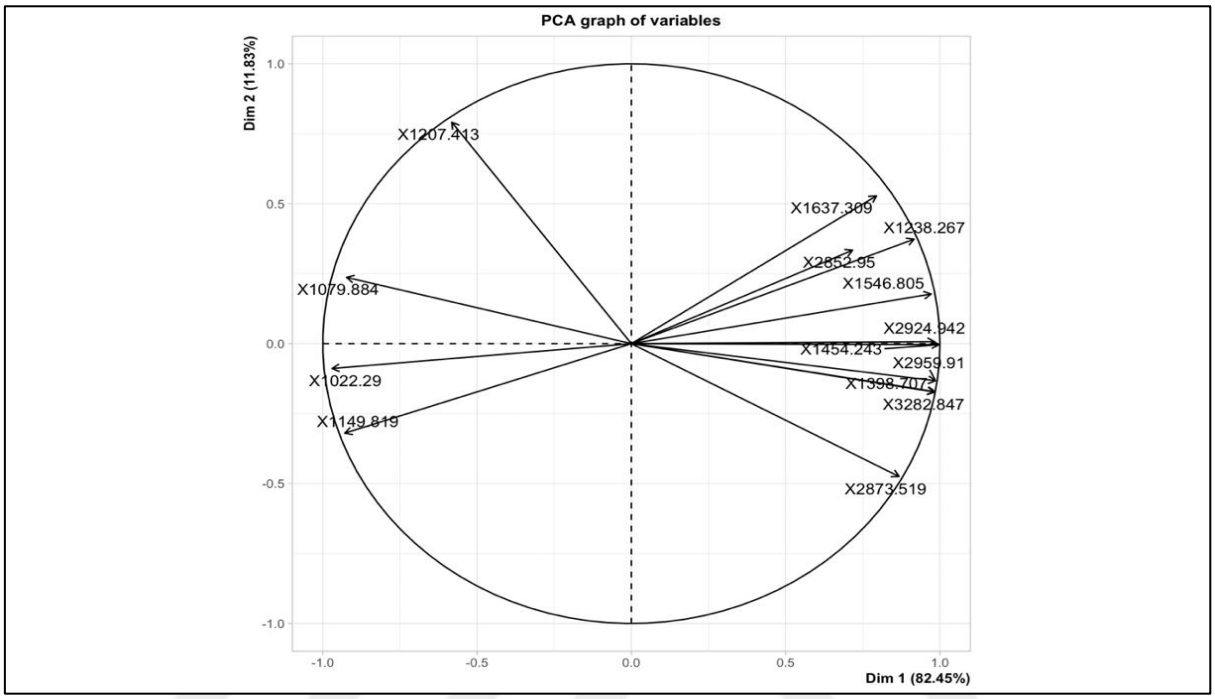

Figure 4.6: PCA loadings for Full spectrum

For the full spectrum PCA, first principal component (PC1) is corresponding to 82.45% of the total variance and (PC2) is corresponding to 11.83% of the total variance, which means the first two principal components explain 94.28% of the total variance, as shown Figure 4.6.

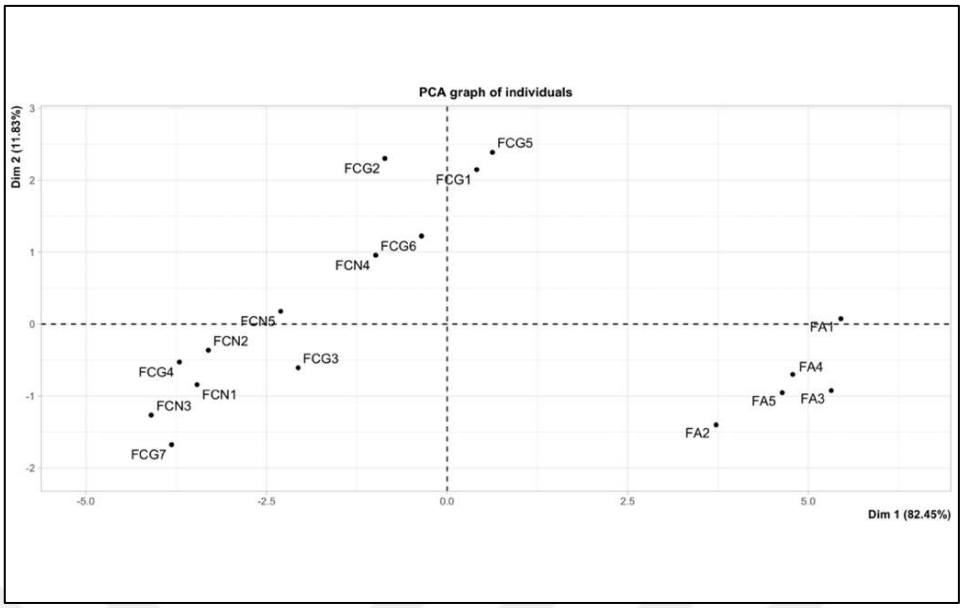

Figure 4.7: PCA score plot for full spectrum

As seen from this Figure 4.7 of score plot of PCA for full spectrum, alcohol groups are totally separated from the control and gavage group. All alcohol group samples are located in the right side of the score plot, where control and gavage group are located on the opposite side.

b. PCA result of fingerprint region:

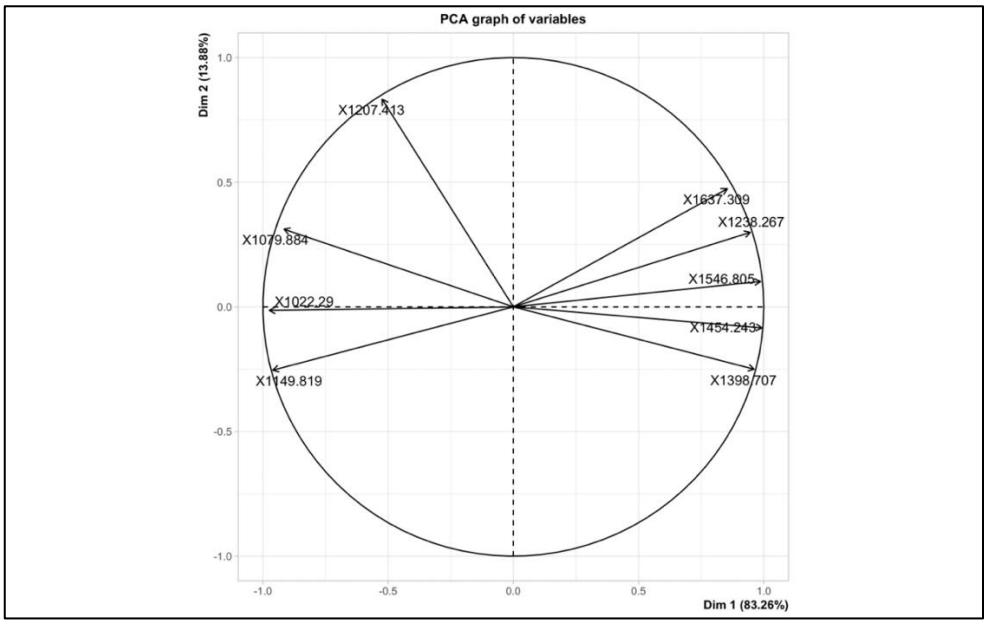

Figure 4.8: PCA loadings for fingerprint region

For the fingerprint region PCA, first principal component (PC1) is corresponding to 83.26% of the total variance and PC2 is corresponding to 13.88% of the total variance, which means the first two principal components explain 97.14% of the total variance presented in Figure 4.8.

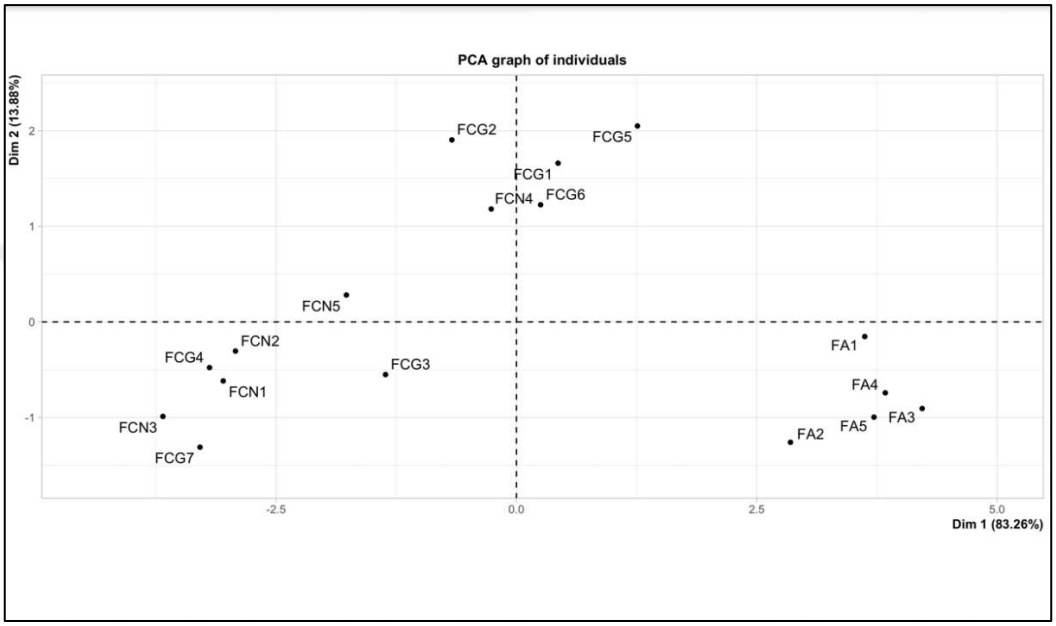

Figure 4.9: PCA score plot for Fingerprint region

In the PCA score plot for the fingerprint region, it is clearly visible that alcohol group is separated from control and gavage groups by being placed on the positive region of the PC1, where in the control and gavage groups two different subgroups are visible. First of these subgroups is located along the positive part of PC2 and second subgroup is on the left side of the score plot. However, these subgroups contain both control and gavage samples, therefore no separation for control and gavage group is obtained.

Since the alcohol group is separated by being placed on the fourth quadrant, there is a clear contribution of two peaks: 1454 cm^{-1} which is related to (C-H) vibration of lipid, and the band at 1398 cm^{-1} which represent fatty acids contribution with amino acid presented in Figure 4.9.

c. Lipid region spectrum PCA result:

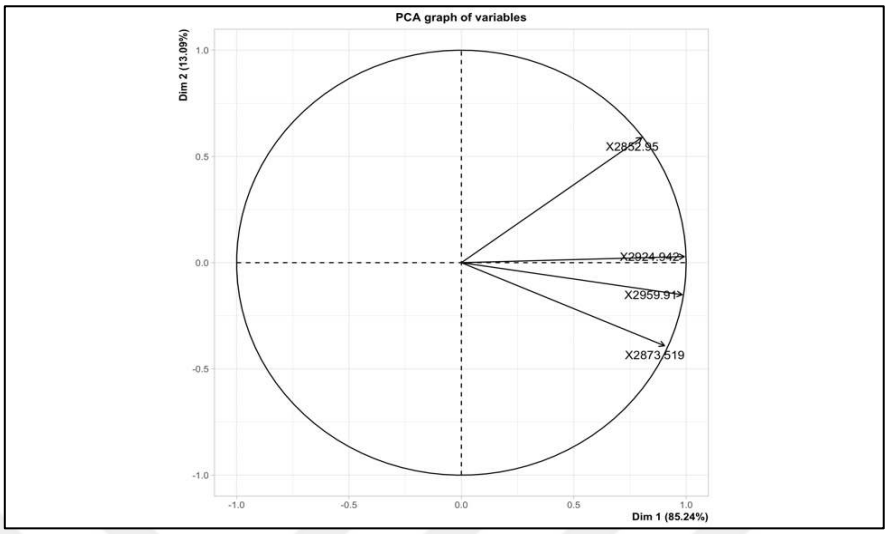

Figure 4.10: PCA loadings for Lipid region

For the lipid region PCA, first principal component (PC1) is corresponding to 85.24% of the total variance and PC2 is corresponding to 13.09% of the total variance, which means the first two principal components explain 98.33% of the total variance shown in Figure 4.10.

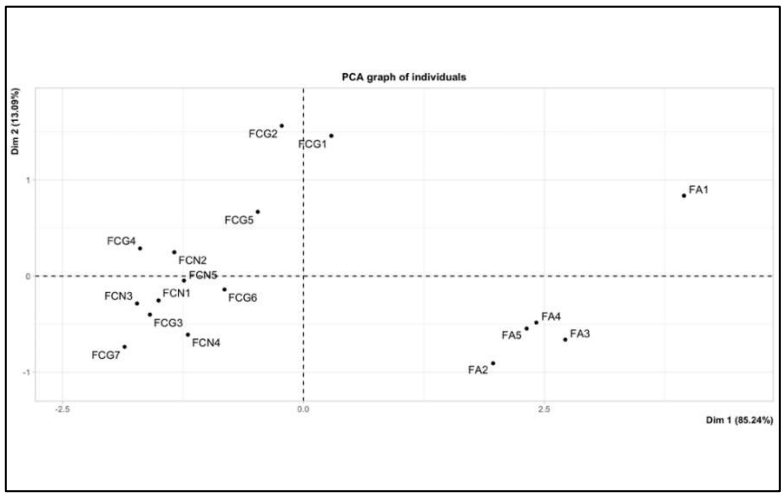

Figure 4.11: PCA score plot for Lipid region

According to the PCA results, there is a very clear and significant clustering of female mice by the means of the results, i.e., all female positive and negative control groups are on

the negative part of score plot, this indicating a clear difference in liver tissues of alcohol consumed rats. The loadings plot indicates the contribution of the different wavelengths in this separation. For alcohol group, one sample (FA1) is located on the first quadrant as an outlier, however other alcohol samples are clearly grouped on the fourth quadrant presented in Figure 4.11.

Excluding the outlier, the contribution to the separation on the fourth quadrant is related to 2873 cm^{-1} which represent CH_3 symmetric stretching (mainly protein) and 2960 cm^{-1} which represent CH_3 antisymmetric stretching (protein and lipid).

Result of the HCA are shown in the following figures: An exploratory approach called hierarchical cluster analysis (HCA) is used to locate unnoticed natural groups (or clusters) within a data collection. The best time to utilize it is when you only need to group a few hundred thinks (Szekely & Rizzo, 2005) shown in Figures 4.12, 4.13, and 4.14.

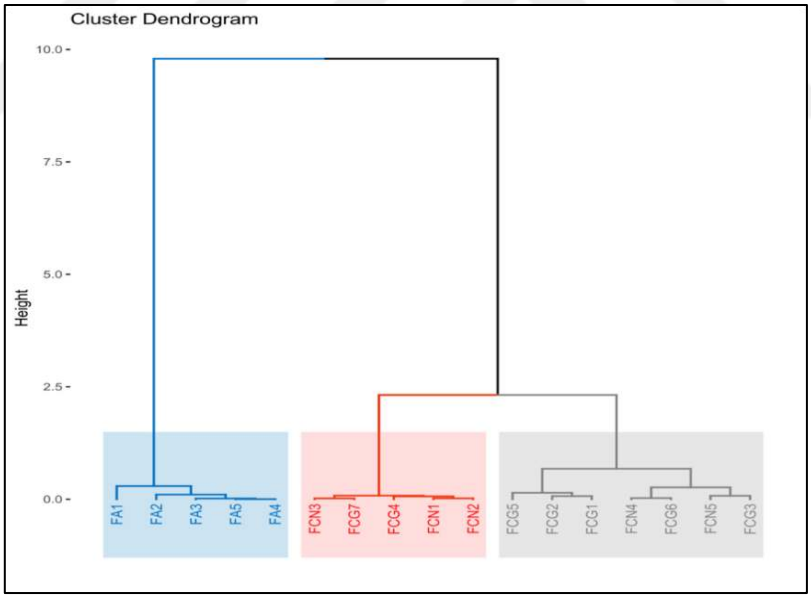

Figure 4.12: HCA results of the samples for full spectrum, based on the first two PC's.

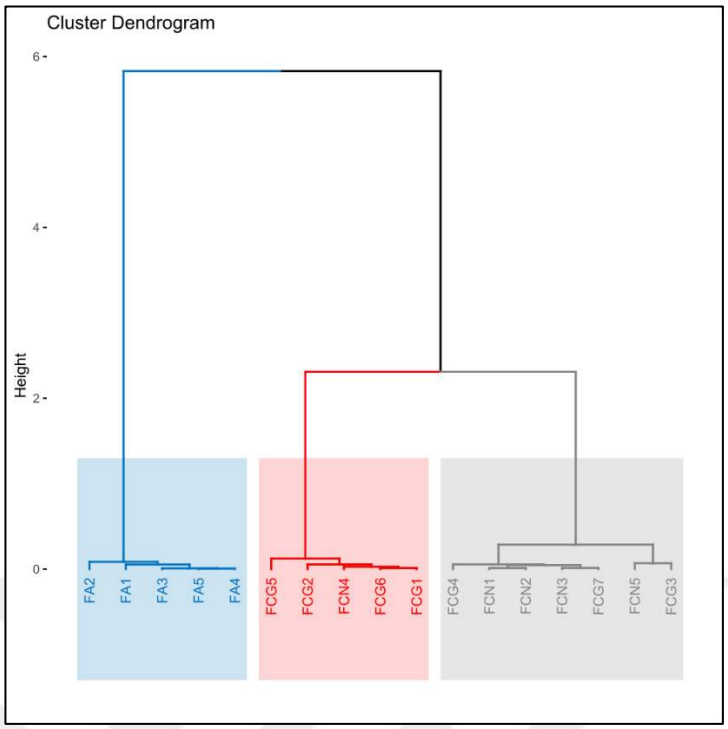

Figure 4.13: HCA results of the samples for fingerprint region, based on the first two PC's.

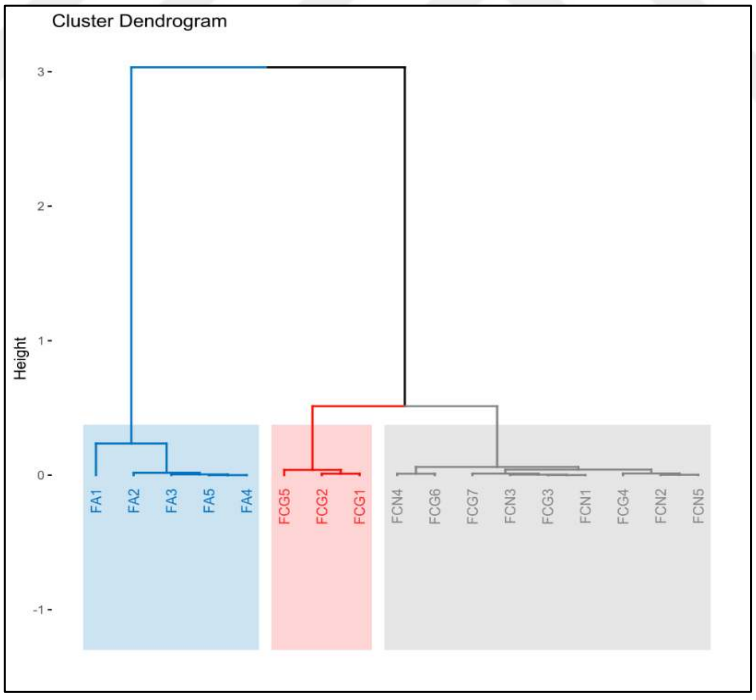

Figure 4.14: HCA results of the samples for lipid region, based on the first two PC's.

According to the HCA results, alcohol groups are clearly clustered, and even in lipid region the FA1 sample (which was seen as an outlier on the PCA score plot) is on the same cluster with other alcohol groups.

5. DISCUSSION AND CONCLUSION

The study's objective is to identify spectroscopic biomarkers referring to the results of prenatal exposure to alcohol in the liver of newborn mice. According to the results of this study, it can be proposed that FTIR is an effective tool for development of chemometric markers in liver tissue, especially for the observation of the consequences of consuming alcohol on female mice. Further studies can reveal the levels of alcohol consumption and detection of other markers.

The liver is a vital organ that affects digestion, immunity, and every aspect of metabolism. It affects practically all the organ systems in the body. It affects the endocrine and digestive systems, promoting metabolism and assisting with digesting. It serves as a barrier between the circulatory and digestive systems. It carries out a wide range of metabolic processes, including the breakdown of toxic substances before they reach the general circulation, the storage and conversion of nutrients for later use, the synthesis of most plasma proteins, the secretion of bile into the small intestine to break down fats, the synthesis of coagulation factor and protein, and involvement in hematology.

The original contraption of this study its by using FTIR for trying to find a suitable differences between alcohol consumption and these groups (gavage, control) and also aimed to find a suitable biomarker by using chemometric methods (analytical, statistical).

Without the need for significant sample numbers, FTIR spectroscopy is a special method that allows accurate simultaneously tracking the functional groups of macromolecules in biological systems. FTIR spectroscopy allows for the rapid and accurate detection of these changes without the need for outside assistance. A complete internal reflection underlies the ATR mode's operation. An infrared transparent material with a high refractive index transmits the electromagnetic wave in the infrared region to the sample on the crystal surface. Total internal reflection happens when the infrared ray's angle of incidence exceeds the critical angle. The measurement of biofluids, dry films, liquid and solid samples, and homogenous soft and hard tissues are all applications of ATR-FTIR spectroscopy that are widely used. The ATR unit's crystal may be analyzed immediately in solid or liquid form by dabbing a tiny quantity of material onto it; no extra preparation is necessary (Özer, 2022)

The liver is involved in several bodily functions, and when it is harmed, it may significantly affect the body's metabolism, immunological system, detoxification, and antimicrobial defenses. There are two artificial categories that may be used to categorize the mechanisms that induce liver damage: immunological and chemical injuries for instance, alcohol intake (as a chemical damage) affects the liver's cellular and molecular makeup and causes the release of enzymes, putting the liver at risk for harm. As a result, it alters the liver tissue's biomolecular composition and structure. In addition to hepatocytes, the liver tissue also consists of fibroelastic connective tissue, veins, arteries, ducts, and nerves. Hepatocytes are the name for the liver's main active cells. Many bands that correspond to different functional groups in proteins, polysaccharides, and nucleic acids may be present in the spectra obtained from liver tissue. The "Results" section of the thesis goes into great detail into how drinking alcohol during pregnancy affects the liver's lipids, proteins, and nucleic acids. Alcohol use also significantly changed the composition and structure of lipids and proteins in addition to nucleic acids (Ali Forat Abdulsattar AL-GBURI, 2022)

Due to the many functional groups, such as the symmetric and antisymmetric CH3 and CH2 fatty acids and amino acids, glycogen, amide I, amide II, and amide III, CH2 bending lipid, nucleic acids, and phospholipids, there are several bands. The values of the spectral band areas reflect the concentration of the functional groups connected to the relevant molecules; for example, a rise in band area values correlates to an increase in the concentration of the molecules allocated to the spectral bands (Ali Forat Abdulsattar AL-GBURI, 2022; Elibol et al., 2022; Özer, 2022) Spectral assignments for these bands are listed in Table 5.1.

Table 5.1: General band assignment of a liver tissue between 3800-900 cm^{-1} wavenumber region (Ali Forat Abdulsattar AL-GBURI, 2022; Elibol et al., 2022; Özer, 2022)

Band Number	Wavenumber cm^{-1}	Band assignment
1	3282	N-H O-H protein – polysaccharide
2	2960	"CH_3 antisymmetric stretching (protein and lipid)" equal contribution from lipids and proteins with minor contribution from carbohydrates and nucleic acids
3	2925	"CH_2 antisymmetric stretching (mainly lipid)" with minor contribution from proteins, carbohydrates, nucleic acids
4	2873	"CH_3 symmetric stretching (mainly protein)" with minor contribution from lipids, carbohydrates, nucleic acids
5	2853	"CH_2 symmetric stretching (mainly lipid)" with minor contribution from proteins, carbohydrates, nucleic acids
6	1638	"Amide I band mainly due to C=O stretching vibration of amide groups: protein"
7	1547	"Amide II band due to N-H bending strongly coupled to C-N stretching vibration of amide groups: protein"
8	1454	"CH_2 bending (lipid)"
9	1398	COO^- symmetric stretch: fatty acids, amino acid side groups
10	1238	"PO_2^- antisymmetric stretching: mainly nucleic acid with a little contribution of phospholipids"
11	1207	Amid III "Collagen"
12	1150	"Glycogen"
13	1080	"PO_2^- symmetric stretching: phospholipids"
14	1022	"Glycogen"

Bands between 3800 and 3030 cm^{-1} : The largest peaks in this region are caused by the O-H length of alcohols (including phenols) and carboxylic acids, the N-H stretches of amines and amides, and the C-H stretches of terminal alkynes. (Edu Spec, n.d.)

The effect of water is too much on the bands in 3000-3800 cm^{-1} band range, although drying of samples while using FTIR but it still has water, so it's not trusted bands.

The vibrations of CH3 antisymmetric stretching, CH2 antisymmetric stretching, CH3 symmetric stretching, and CH2 symmetric stretching are what create the bands in the 3030-2800 cm^{-1} range (Özer, 2022)

In first region which is fingerprint region between 1800-900 cm^{-1} there are many spectral bands.

Amide I band at 1638 cm^{-1} is mainly due to C=O stretching vibration of amide groups: protein" It is made up of 80% vibrations of the C=O type, 10% vibrations of the C-N type, and 10% vibrations of the N-H type. Amide II band at 1547 cm^{-1} caused by protein's N-H bending strongly related to C-N stretching vibration. Amid I and Amid II band wavenumber values fluctuation reveals changes in protein secondary structures. The CH2 bending band, which is positioned at a wavelength of 1454 cm^{-1} and contains mostly lipids with a negligible contribution from proteins, provides information regarding lipid structure and lipid concentration (Ali Forat Abdulsattar AL-GBURI, 2022; Özer, 2022)

Fatty acids and amino acids undergo COO-symmetric stretching, which causes the peak at 1398 cm^{-1} wavenumber. (El-Naggar, Hamouda, Rabei, Mousa, & Abdel-Hamid, 2019)

The absorbance of P=O bonds of phosphate (PO^{-2}) groups in nucleic acids and phospholipids makes up the majority of the bands at 1238 cm^{-1} and 1080 cm^{-1} in the spectra of tissues. These bands offer crucial details on the head groups of membrane phospholipids. The bands at 1150 and 1022 cm^{-1} are generated by the tissues' glycogen being stretched during C-O vibrations. Information on the amount of glycogen in the tissues may be gleaned from this band's area value. The bands at 1207 cm^{-1} gives information about collagen and amide III (Özer, 2022).

PCA results indicate that there is a very clear and significant clustering of female mice by the means of results, i.e., all female positive and negative control groups are on the negative part of score plot, this indicating a clear difference in liver tissues of alcohol consumed rats. The loadings plot indicates the contribution of the different wavelengths in this separation.

According to the results of PCA analysis, four significant bands are found to be contributing to the clustering of alcohol consumed mice. These are: 1398 cm^{-1} (COO^- symmetric stretch: fatty acids, amino acid side groups) 1454 cm^{-1} (CH_2 bending-lipid), 2873 cm^{-1} (CH_3 symmetric stretching-mainly protein with minor contribution from lipids, carbohydrates, nucleic acids) and 2960 cm^{-1} (CH_3 antisymmetric stretching-protein and lipid).

The detected markers which are found in this study are the bands which correspond to the lipid region, which indicate that alcohol consumption affected the lipid metabolism.

Findings of this study indicate that mentioned bands at the lipid region in the FTIR spectra can be used as biomarkers for the effect of alcohol consumption in mice. These values can be efficiently used for detection of alcohol consumption in similar samples. Further studies can reveal the exact mechanism for this observation of mice samples.

Drinking alcohol while pregnant can have negative effects on the development of the fetus as well as long-term neurological and behavioral effects on the mother. The effects of FASD are felt in all areas of functioning, including general intelligence, motor function, attention and activity levels, language development, visual perception and creation, learning and memory, adaptive functioning, and executive functioning, a neurobehavioral condition. Neuropsychological and physical tests may be useful in identifying people with FASD, according to study, but further studies are needed to hone this profile. To better identify those impacted by prenatal alcohol consumption and offer ongoing monitoring of prevalence rates, further research is required. Diagnostic criteria also need to be improved. Accurate identification and diagnosis will be made easier with the discovery of biomarkers. While little is now known regarding neurobehavioral deficits throughout adulthood, further research is required to understand the long-term effects of prenatal alcohol consumption. Affected people, their families, the community, and society will all benefit in the end from improved identification, diagnosis, and intervention efforts (Mattson et al., 2019)

The social, adaptive, and cognitive aspects of the patient are impacted by FASD. Assessment challenges are caused by its low frequency and challenging diagnosis, particularly in the context of speech treatment. Alcohol intake during pregnancy poses hazards for children with FASD, as do unfavorable developmental circumstances. For children with complex clinical profiles that include linguistic, cognitive, behavioral, and

socialization difficulties, speech-language pathologists must be a part of interdisciplinary diagnostic teams. To assess a patient's language and communication skills, clinicians can consider more inclusive and dynamic activities in natural settings, such as role plays, narrative tasks, table games, and monitoring peer interactions (Vega-Rodríguez, Garayzabal-Heinze, & Moraleda-Sepúlveda, 2020)

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