Detection of chemometric-guided biomarkers associated with alcohol consumption in mice liver tissue using infrared spectroscopy

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Abstract

Among the leading contributors to fetal alcohol spectrum disorder (FASD), perinatal alcohol usage is characterized by neurodevelopmental dysfunction and growth retardation. This study aims to determine spectroscopic biomarkers referring to the results of early postnatal exposure to alcohol in the liver of newborn mice. The samples were divided into three groups: 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). After the decapitation of mice, liver tissues were removed and infrared data were obtained using Fourier Transform Infrared (FTIR) Spectroscopy. Spectral data was analyzed and evaluated using normalization, principal component analysis (PCA), and hierarchical cluster analysis (HCA). 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}. According to the results of this study, it can be proposed that FTIR is an effective tool for the detection of chemometric-guided biomarkers related to the consequences of early postnatal alcohol consumption on female rats.

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

INTRODUCTION

Fetal alcohol syndrome (FAS) is a condition in which the mother drinks alcohol before or during pregnancy, harming the baby and causing various anomalies in terms of development, cognitive abilities, facial deformities, the central nervous system, and internal organ damage. It is the most typical non-genetic cause of intellectual disability (Caprara et al., 2007; Dejong et al., 2019; Denny et al., 2017; Kaminen-Ahola, 2020). Perinatal (prenatal and early postnatal) alcohol consumption has been linked to behavioral problems, increased mental health problems, and worse academic achievement. Moreover, alcohol use during pregnancy can have a significant impact on the fetal brain, heart, lungs, metabolism, and placenta. It damages the brain, myelination rate, and memory of the develop- ing fetus, increases habitual stimuli in the fetus's body, and can change the weight of the placenta and fetus [8–10]. Continuing alcohol consumption in early postnatal period can also lead to long-lasting physical, behavioral, and cognitive issues in the baby, since alcohol can pass through breast milk from the mother to baby (Ehrhart et al., 2018; Mattson et al., 2019).

The liver is one of the most important organs to be impacted by alcohol misuse. Heavy drinkers can develop cirrhosis, the most severe stage of alcoholic liver disease in humans. The newborn liver is a crucial organ that goes through significant changes after birth, and alcohol dehydrogenase (ADH) is an enzyme that affects the liver development of the newborn (Arzu Koçkaya & Akay, 2006; Pikkarainen & RäIhä, 1967; Ponnappa & Rubin, 2000). According to animal studies (Degroat et al., 2018; Wagnerberger et al., 2013), female mice had higher levels of TNF- and IL- 6 as well as PAI−1, making them more vulnerable to the long-term effects of ethanol usage. The gonadal hormone estrogen may be involved in the early onset of chronic alcohol-induced liver damage (ALD), which affects female rats more frequently than male rodents (Wagnerberger et al., 2013). Additionally, early postnatal exposure to alcohol is also relevant for female sex since breast milk is the primary route of alcohol exposure in newborn.

Chemometrics is an exploratory data analysis method that uses Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA) to group samples based on how much they match multivariate pattern recognition algorithms. It is already being successfully adapted for different types of samples such as food samples (Gezek et al., 2019; Kalaycıoğlu et al., 2017; Kaygusuz et al., 2016) or bibliometric data (Kaygusuz, 2021). Unsupervised methods like PCA and HCA can be combined to analyze the data structure and spot patterns in a variety of samples including tissues and biofluids (Biancolillo & Marini, 2018; Elibol et al., 2022; Peris-Díaz & Krężel, 2021; Sharma et al., 2020). These techniques are efficient in terms of extracting significant information in complex spectroscopic data of biological samples, viz., biomarkers.

The relative molecular content and structural information of the biological systems including tissues can be examined and understood using Fourier transform infrared (FTIR) spectroscopy. FTIR spectroscopy with chemometric techniques can be used to determine spectral biomarkers of diseases, chemical agent- or drug-induced molecular changes in the tissues (Algburi et al., 2022; Elibol et al., 2022; Kucuk Baloglu et al., 2023; Ustaoglu et al., 2021) and biofluids (Yonar et al., 2018, 2022). Development of spectroscopic biomarkers are important for diagnosing and monitoring conditions like FAS and alcoholic liver disease. Based on the current knowledge, there are no spectroscopic biomarkers on liver tissue, therefore in the current study, it is aimed to find related biomarkers by FTIR spectroscopy and chemometric techniques especially in female mice samples.

METHODOLOGY

Sample Classification and Measurement

There were three main groups in the experiment: Negative control group (n=5), gavage control group (positive control) (n=7) and group with gavage + alcohol (n=5), 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). The raw data was based on our previous study which investigates moleculer content and antioxidant capacity of mice liver tissue (Algburi et al., 2022). The identical method used for the alcohol group intubations was used twice daily for the control group. After the decapitation of mice, liver tissues were removed and stored at -80 ºC for spectroscopic investigation. IR spectra were generated from the liver tissue samples by putting them on the Diamond/ZnSe (Di/ZnSe) Zinc Selenide crystal plate of the Universal ATR using the Bruker Alpha II 100 FTIR spectrometer (Bruker, Berlin, Germany). The details of the animal and FTIR studies were reported in (Algburi et al., 2022). Experimental protocol of this study was approved by the local scientific ethical committee of Bezmialem Vakıf University,

Istanbul, Turkey (2017 / 99).

The FTIR data were grouped with the following codes attached to them: Negative control (FCN) (n=5), positive control (FCG) (n=7) and alcohol (FA) (n=5). For these groups, at least five different samples were measured using FTIR spectra, and in statistical analysis these 17 spectral data was evaluated.

Chemometric Analysis and Visualization

Spectral data of the samples were processed using SpectraGryph software (Menges, 2022). After the baseline correction, the data was normalized using standard normal variate (SNV) technique which is a common method for normalization (Peris-Díaz & Krężel, 2021). For the statistical analysis step, principal component analysis (PCA) was evaluated using R statistical language with FactoMineR package (Lê et al., 2008; R Core Team, 2018). With 17 total number of samples and 14 different wavenumbers, a 14x17 matrix was calculated. Similar studies were conducted for different datasets (Gezek et al., 2019; Kalaycıoğlu et al., 2017). Clustering analysis was done using a hierarchical cluster analysis (HCA) in R language. HCA was applied to the corresponding principal components calculated in PCA step, preferably the first two principal components. Plots of the results are also generated in R.

RESULTS

Infrared region contains a high number of information on the biological samples, and a careful multivariate statistical approach is useful for obtaining useful information (Rusak et al., 2003). These processes require a normalization step after the baseline correction (Kepenek et al., 2020). Figure 1 shows the vector normalized spectra in the range of 3900 – 900 cm^{-1}. Significant differences among the samples and sample groups can be visible especially in the fingerprint region, which requires further investigation. In order to evaluate the spectrum in detail, three regions were studied: 1800 – 900 cm^{-1}, 3030 – 2800 cm^{-1} and 3800 – 3030 cm^{-1}. Within these regions, the following peaks were evaluated: 1022, 1080, 1150, 1207, 1238, 1398, 1454, 1547, 1638, 2853, 2873, 2925, 2960 and 3282 cm^{-1}. Each region and peak is corresponding to a specific bond and/or presence of a biochemical group in FTIR.

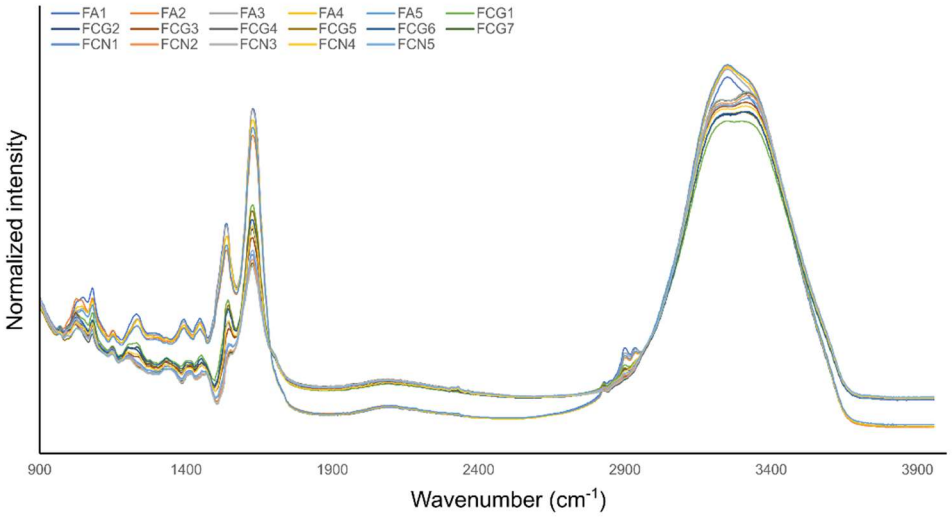

Figure 1. SNV Normalized FTIR spectra of the samples

Initially, PCA was applied to the full spectrum. Preliminary PCA results showed that the first two principal components (PC's) dominate the total variance (82.45% and 11.83%), which

means they explain 94.28% of the total variance. As seen from Figure 2, alcohol groups are totally separated from the control and gavage groups in the score plot. All alcohol group samples are located in the right side of the score plot, where control and gavage groups are located on the opposite side. According to the loadings plot, the major peak contribute to this observation is 2873 cm^{-1}. Multiple other bands also contribute to this observation, and further detailed analysis is needed.

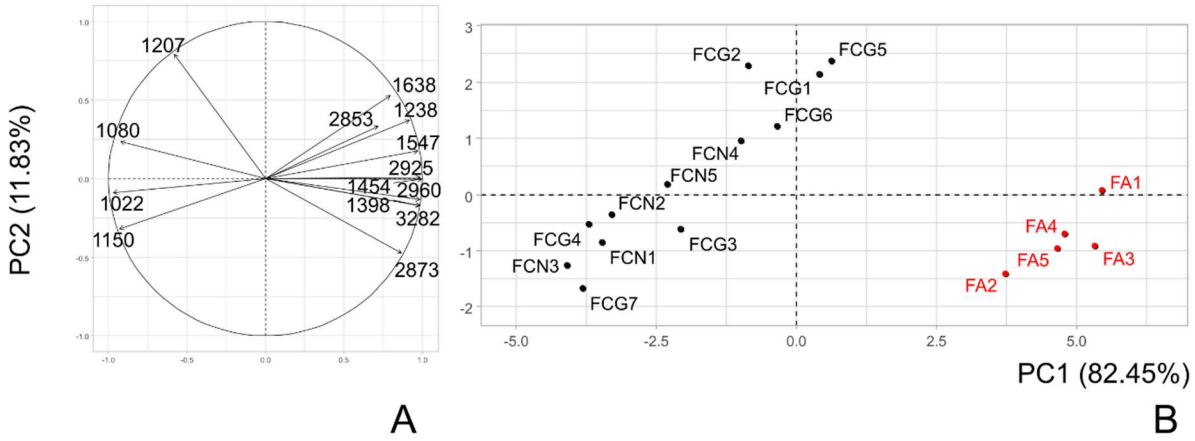

Figure 2. PCA loadings and score plot for the full spectrum

To detect the biomarkers, PCA was applied more specifically and in a narrower spectrum range. When applied to the fingerprint region, similar to the full-spectrum results, two first PC's explain the majority of the total variance (83.26% and 13.88%, respectively). Results of the PCA in the fingerprint region is shown in Figure 3.

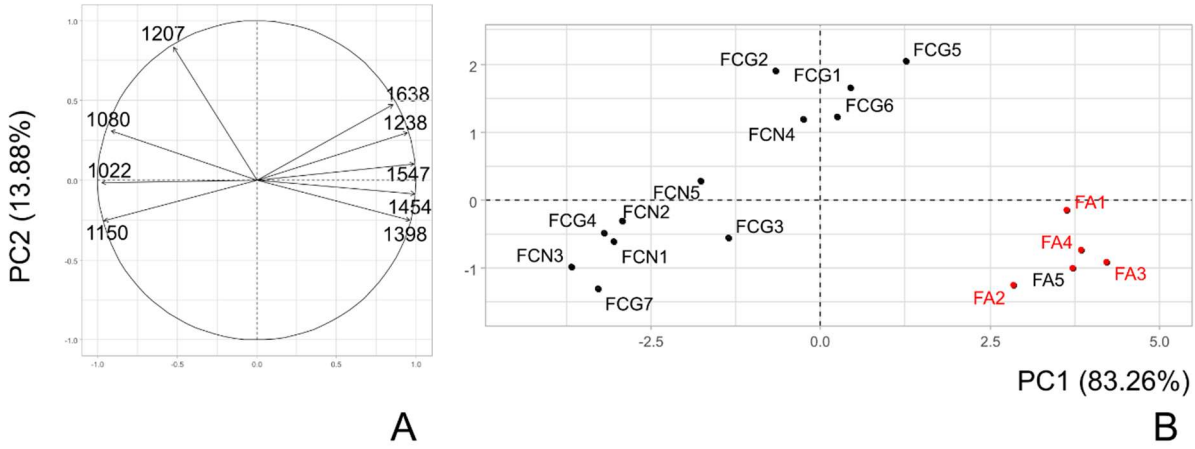

Figure 3. PCA loadings and score plot for the fingerprint region

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 also shown. 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.

For the lipid region, 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 PC's explain 98.33% of the total variance shown in Figure 4. 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. The loadings plot indicates the contribution of the wavelengths in this separation. For the 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. 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 (equal contribution of protein and lipid).

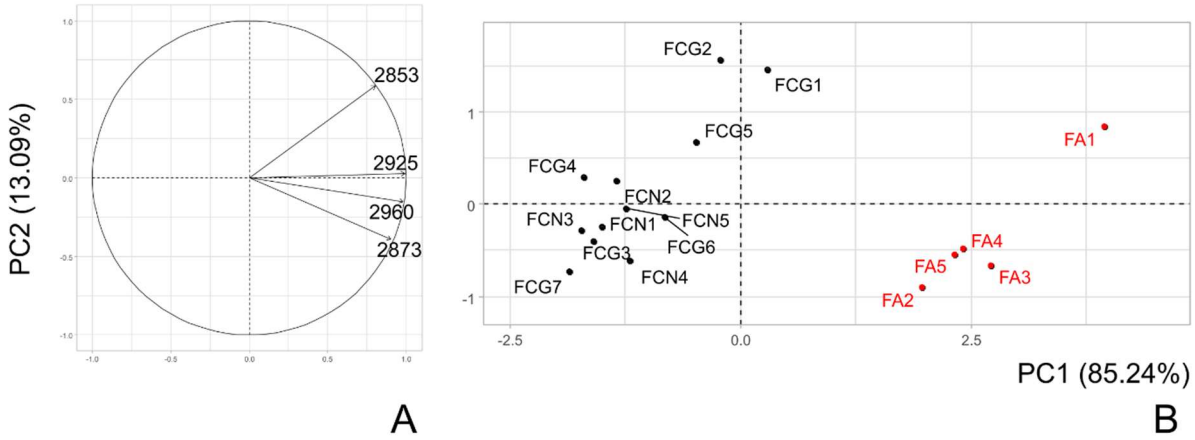

Figure 4. PCA loadings and score plot for the lipid region

For defining the similarities among the samples locating the unnoticed clusters within the spectral data, HCA was generated. 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, as seen in Figure 5.

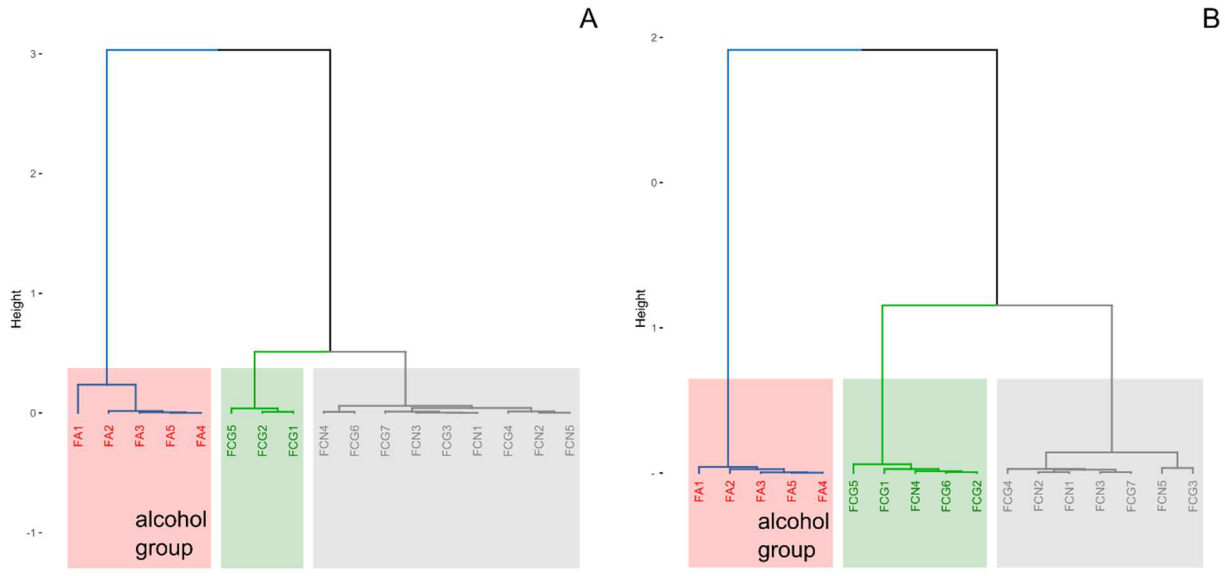

Figure 5. HCA results of the fingerprint (a) and lipid (b) regions, based on the first two PC's.

DISCUSSION

The liver plays an important role in many biological processes, and any damage to the liver tissue can have a significant impact on the metabolism, immune system and many other functionalities of the body. Excessive drinking during pregnancy can have negative effects on the developing baby. Early in pregnancy, pre-exposed mice exhibit regulatory changes that impact their ability to absorb glucose and how quickly their intracellular glucose disposal system reacts (Lee et al., 2020; Probyn et al., 2012). To avoid a possible fetal alcohol damage, it is important to evaluate the safety of vitamins C and E during pregnancy as well as the experimental use of antioxidants in alcohol-consuming mothers (Cohen-Kerem & Koren, 2003). FASD has significant social effects and plays a big role in birth anomalies (Fofana et al., 2010; Liu et al., 2016).

The goal of the study is to detect spectroscopic biomarkers in the liver of newborn mice that reflect the effects of prenatal early postnatal alcohol consumption. The findings of this work support the notion that FTIR is a useful technique for creating chemometric-guided biomarkers in liver tissue, particularly for observing the effects of alcohol consumption on female mice.

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 in different regions, 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. This result is in accordance with the existing literature. Liver proteins such as such as glutathione, mitochondrial proteins were found to be down-regulated in the exposure of prenatal alcohol (Addolorato et al., 1997; Ojeda et al., 2009; Reyes et al., 1993). Another study reported significant decrease in levels of alkaline phosphatase (ALP) and significantly higher levels of alanine transaminase (ALT), Gamma-glutamyl transferase (GGT) enzymes and bilirubin in the liver due to alcohol consumption (Alatalo et al., 2008). Those changes might be related to the lipid peroxidation and changes in protein conformation of the liver tissue (Algburi et al., 2022).

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. Another possible future study can be the detection of biomarkers in male liver tissue by the means of alcohol consumption.

CONCLUSIONS

Detection of biomarkers associated with alcohol consumption in the liver tissue is an important challenge due to many health effects of the alcohol-related syndromes. Results indicate that the four detected spectral biomarkers are related to the lipid metabolism of the liver and this

information is previously confirmed in the literature. This study concludes that coupling the infrared spectral data with chemometric analyses (PCA and HCA) can discriminate and cluster the complex spectra as a rapid, facile, and effective method.

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