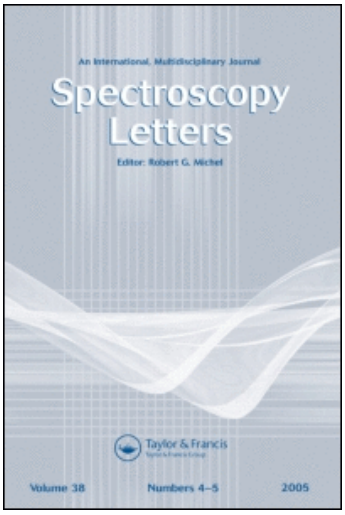

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Estimation of Postmortem Interval in Rat Liver and Spleen Using Fourier Transform Infrared Spectroscopy

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ABSTRACT The aim of this study was to investigate the changes of Fourier transform infrared (FT-IR) spectra of rat liver and spleen tissues from time zero to 144 h postmortem. The absorbance (A_x represents the infrared absorbance at wavenumber $x\,cm^{-1}$) ratios of major absorbance bands were examined. A_{3303}/A_{2925}, A_{1647}/A_{1541} (liver), A_{1238}/A_{1338}, A_{1153}/A_{1338} (liver), A_{1080}/A_{1338}, and $A_{1030–1050}/A_{1338}$ (liver) showed a decrease postmortem, whereas the A_{1396}/A_{1456} ratio (spleen) showed an increase. Furthermore, these absorbance ratios showed a strong linear relationship with increasing time postmortem, and the A_{1238}/A_{1338} ratio of spleen offered a stronger correlation ($R^2 = 0.88$) than did the others. A new absorbance band appeared at 1120 cm^{-1} for the liver and spleen at 120 h postmortem and another new band appeared at 1170 cm^{-1} for the liver, but the existing absorbance band at 1170 cm^{-1} disappeared in the spleen. Our initial results demonstrate that the quantitative analysis of FT-IR spectrum related to postmortem interval (PMI) shows a strong linear correlation between absorbance ratios and increasing time after death. The FT-IR changes of spectra and absorbance ratios can support further study for estimating short-term and long-term PMI. Upon future validation, FT-IR can offer advantages in combination with established methods to improve PMI estimation.

KEYWORDS fourier transform infrared spectroscopy, liver, postmortem interval, spleen

INTRODUCTION

Estimation of the postmortem interval (PMI) is one of the most important issues in forensic investigation. There are many studies on accurately and systematically estimating the time elapsed since death and examination of external physical characteristics of the body, internal body temperature, stomach contents, and chemical changes of body fluids.[1–3] Proton magnetic resonance spectroscopy has been used recently to estimate later postmortem interval.[4] Some molecular biology methods are also used, such as the analysis of postmortem DNA degradation by single-cell gel electrophoresis, the quantification of mRNA degradation by multiplex RT-PCR,

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and the detection of cardiac troponin changes,[5–7] but the accuracy of estimating the PMI is not high. In addition, these methods deal with the tissues using complicated chemical procedures. As a result, the substantial postmortem chemical changes are affected by these chemical procedures. Therefore, other reliable methods to estimate the time elapsed since death are still required.

Fourier transform infrared (FT-IR) spectroscopy relies on the absorption of radiation that excites molecular vibration. Various molecular components of the cell show a characteristic infrared spectrum, which is rich in structural and functional aspects. Biological molecules such as proteins, nucleic acids, glycogens, and lipids have specific, fingerprint-like infrared spectra. With the technical development of FT-IR spectroscopy, the quantitative FT-IR spectral method is widely used in the fields of biology and medical research due to its sensitivity and simplicity. Recently, FT-IR spectroscopy has been used as an effective tool for investigating chemical changes at the molecular level in the field of biomedical research.[8–11] FT-IR spectroscopy is used for the analysis of soil components, photocopy toners, plant fiber, and the examination of various other materials encountered in forensic investigation.[12–14]

There are many chemical processes after death that can result in changes of the quantity and the structure of biological molecules.[1] Therefore, in this study we have applied FT-IR spectroscopy to detect the changes of spectra quantitatively in rat liver and spleen and to evaluate its value for estimating PMI.

MATERIALS AND METHODS

Animal Specimens

Eight male Sprague-Dawley adult rats (weight 240–260 g) provided by the Animal Center of Xi'an Jiaotong Universtiy School of Medicine were sacrificed by cervical dislocation. The bodies were kept in a controlled environment chamber set at $20 \pm 2°C$. The 50 mg liver and spleen were subsampled from the same rat at time zero, 12, 24, 48, 72, 96, 120, and 144 hours (8 readings for each rats). The liver and spleen were selected because the enzymes in these tissues tend to be more active and autolysis occurs early. The tissues were placed into 2.5 mL cylindrical tubes and frozen immediately in liquid nitrogen. All of the animal experiments in the current study were performed in accordance with the principles of the Care and Use of Laboratory Animal Committee of Xi'an Jiaotong University.

Sample Preparation and Infrared Spectral Measurement

The tissues were freeze-dried *in vacuo* at –50°C for 12 h to dehydrate them. About 2 mg of freeze-dried tissue was mixed with 200 mg of KBr, ground with an agate mortar and pestle for 5 min, and then pressed into a pellet die for 1 min using a force of 10 tons. The measured thickness of the pellets was 0.4 mm and the diameter was 12 mm. The FT-IR spectra were recorded quantitatively at room temperature in the range 4000–400 cm^{-1} on a Shimadzu 8400S spectrometer. The spectrometer was kept on continuously to minimize warm-up instability and was purged continuously with dry air to eliminate interference by atmospheric water vapor. Interferograms were averaged for 20 scans at 4 cm^{-1} resolution. IR solution 1.10 software (Shimadzu, Tokyo, Japan) was used for analysis of FT-IR spectra and for recording the data from the spectra. Baseline correction was done for all the spectra, and A_x represents the infrared absorbance at wavenumber x cm^{-1}.

Criteria of the Absorbance Bands Selected

The FT-IR spectra of rat liver and spleen tissues were quite complex, and there were many bands that appeared in the 4000–400 cm^{-1} region. Some of these bands need special care for data analysis, because they consist of several unresolved bands. For this reason, only the major bands were selected for this study and were 3303 cm^{-1}, 2925 cm^{-1}, 1647 cm^{-1} (liver), 1541 cm^{-1} (liver), 1456 cm^{-1} (spleen), 1396 cm^{-1} (spleen), 1338 cm^{-1}, 1238 cm^{-1}, 1153 cm^{-1} (liver), 1080 cm^{-1}, and 1030–1050 cm^{-1} (liver). The chemical assignment of these absorbance bands is given in Table 1.[8,15–25] Because FT-IR band absorbance varied with thickness of the sample, absorbance ratio was used

TABLE 1 Major Absorption Bands in the FT-IR Spectra of Rat Liver and Spleen

Frequency (cm^{-1})	Preliminary assignments
3303	Amide A (N–H stretching)
2925	CH_2 asymmetric stretching
1647	Amide I (C=O stretching)
1541	Amide II (C–N stretching)
1456	CH_3 asymmetric deformation stretching
1396	COO^- symmetric stretching
1338	CH_2 wagging stretching
1238	PO_2^- asymmetric phosphate stretching
1153	C–OH stretching
1080	PO_2^- symmetric phosphate stretching
1030–1050	CH_2OH groups and C–O stretching

to exclude the thickness effect. By the observation of all spectra, two bands with obvious absorbance changes were selected. There were A_{3303}/A_{2925}, A_{1647}/A_{1541} (liver), A_{1396}/A_{1456} (spleen), A_{1238}/A_{1338}, A_{1080}/A_{1338}, $A_{1030–1050}/A_{1338}$ (liver), and A_{1153}/A_{1338} (liver). For statistics, experimental replicates were averaged and the mean ± standard deviation was calculated for each time point. A linear relationship is achieved between PMI and absorbance ratio.

RESULTS AND DISCUSSION

General Findings

Rat Liver FT-IR Spectra

The absorbance ratios were seen to decrease from time zero to 144 h postmortem. The quantitative analysis indicated a strong linear correlation between absorbance ratio and increasing time after death, and the A_{1080}/A_{1338} ratio provided a stronger correlation ($R^2 = 0.85$) than did the others (Fig. 1). From time zero to 144 h postmortem, the absorbance at 1238 cm^{-1}, 1080 cm^{-1}, and 1030–1050 cm^{-1} decreased, the absorbance at 2925 cm^{-1}, and 1541 cm^{-1} increased, and the absorbance at 3303 cm^{-1}, 1338 cm^{-1}, and 1647 cm^{-1} remained stable in the FT-IR spectra (Fig. 2 and Fig. 3). As demonstrated in Fig. 2, four full complete FT-IR spectra showed the changes of the major bands at time zero, 24, 72, and 144 h postmortem. The A_{1153}/A_{1338} ratio decreased rapidly at 12 h then remained stable until 120 h, and finally decreased suddenly at 144 h. Furthermore, the band at 1153 cm^{-1} disappeared in 7 of 16 samples after 120 h postmortem (Fig. 3c; Table 2). In addition, two new absorption bands appeared at 1170 cm^{-1} and 1120 cm^{-1} after 120 h postmortem.

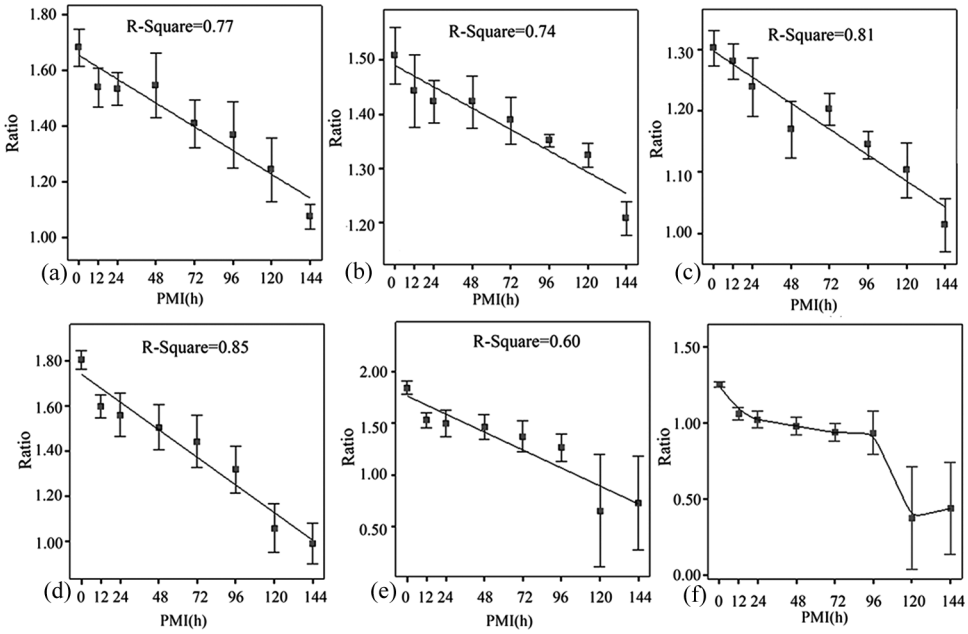

FIGURE 1 Temporal FT-IR spectral absorbance ratio of the bands (a) A_{3303}/A_{2925}, (b) A_{1647}/A_{1541}, (c) A_{1238}/A_{1338}, (d) A_{1080}/A_{1338}, (e) $A_{1030–1050}/A_{1338}$, (f) A_{1153}/A_{1338} in rat liver from time zero to 144 h after death. (a)–(e) The linear regressions are between the absorbance ratio of the bands and PMI.

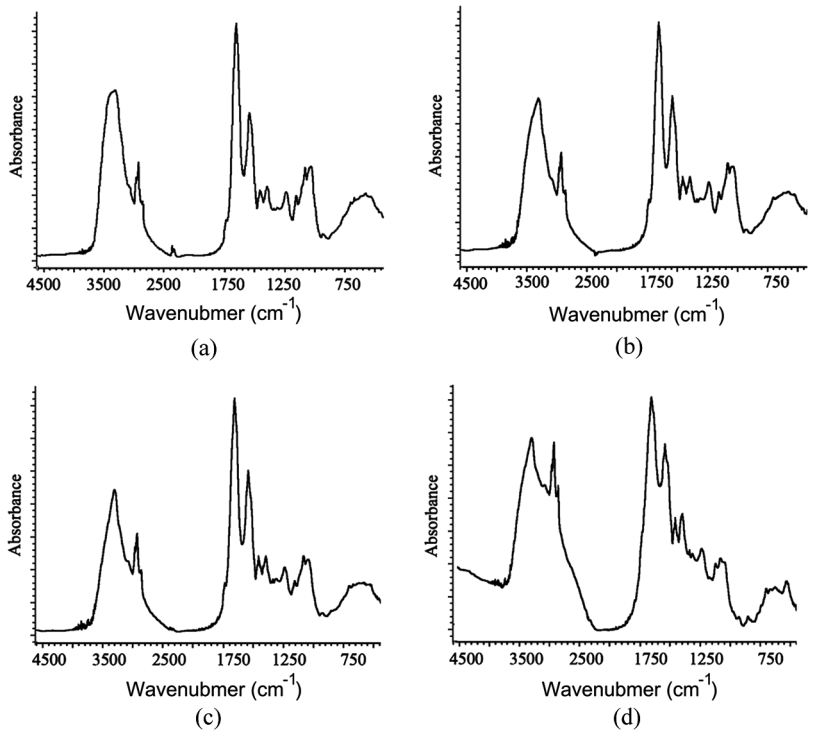

FIGURE 2 **Four complete FT-IR spectra: (a) time zero, (b) 24 h, (c) 72 h, and (d) 144 h in rat liver.**

The new bands were found in 11 of 16 samples for 1170 cm^{-1} and in 15 of 16 samples for 1120 cm^{-1} after 120 h postmortem. The bands at 1030–1050 cm^{-1} disappeared in 5 of 16 specimens after 120 h postmortem (Fig. 3c; Table 2).

Rat Spleen FT-IR Spectra

The absorbance ratios of A_{3303}/A_{2925}, A_{1238}/A_{1338}, and A_{1080}/A_{1338} decreased with increasing time elapsed postmortem, and the A_{1396}/A_{1456} ratio increased from time zero to144 h since death. Four absorbance ratios showed strong linear relationships with PMI. The correlation of A_{1238}/A_{1338} ratio ($R^2 = 0.88$) was stronger than those of the others (Fig. 4). From time zero to144 h after death, the band absorbance at 1238 cm^{-1} and 1080 cm^{-1} was seen to decrease, whereas the absorbance at 1396 cm^{-1} and 2925 cm^{-1} increased. The absorbance at 3303 cm^{-1},

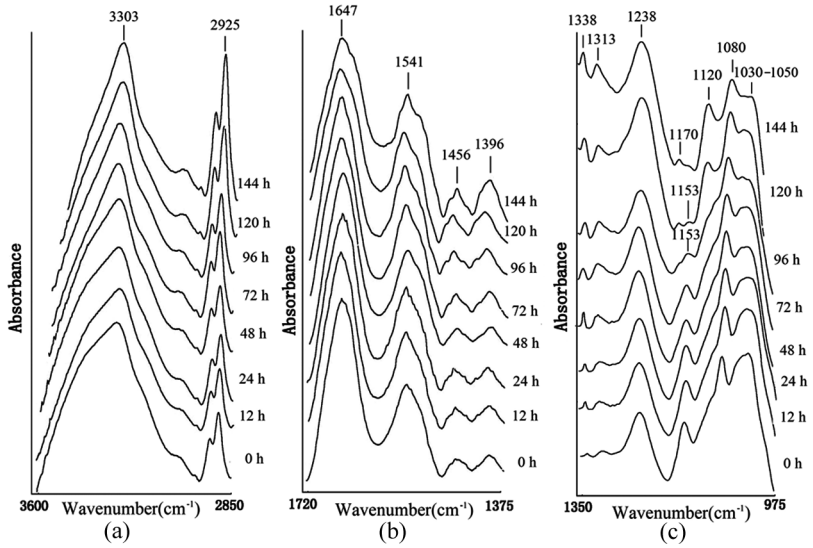

FIGURE 3 **Time course of FT-IR spectra from time zero to 144 h postmortem in rat liver: (a) 3600–2850 cm^{-1}, (b) 1720–1375 cm^{-1}, (c) 1350–975 cm^{-1}.**

TABLE 2 **Frequency of Appearance of New Bands and Disappearance of Existing Bands in FTIR Spectra for Rat Liver and Rat Spleen**

PMI (h)	No. samples	New bands at 1170 cm^{-1}	1153 cm^{-1}	1120 cm^{-1}	1030–1050 cm^{-1}
Liver					
0–96	48	0	48	0	48
120	8	5	4	7	5
144	8	6	5	8	6
Spleen					
0–96	48	48	—	0	—
120	8	0	—	8	—
144 h	8	3	—	8	—

1456 cm^{-1}, and 1338 cm^{-1} remained stable in the spleen FT-IR spectra (Fig. 5 and Fig. 6). In addition, the FT-IR spectra in all samples showed an absorption band at 1170 cm^{-1} in the 0–96 h postmortem period, whereas this band was found in only 3 of 16 samples after 120 h postmortem. A new absorbance band at 1120 cm^{-1} was found in all samples taken at 120 h postmortem (Fig. 6c; Table 2).

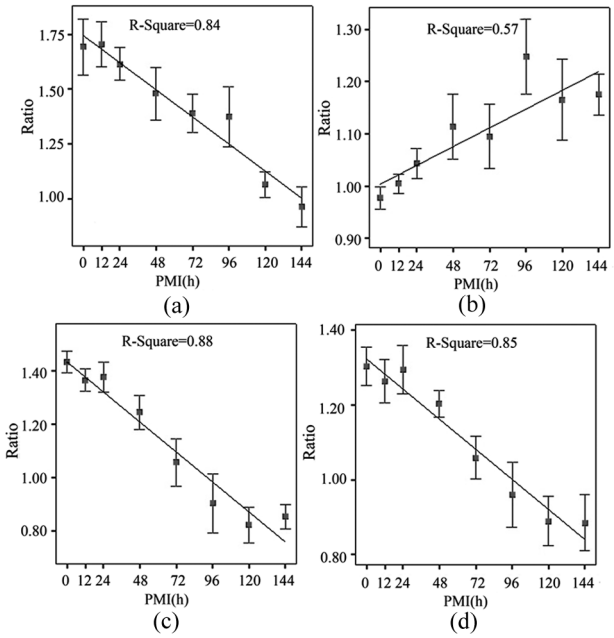

FIGURE 4 **Temporal FT-IR spectral absorbance ratio of the bands (a) A_{3303}/A_{2925}, (b) A_{1396}/A_{1456}, (c) A_{1238}/A_{1338}, (d) A_{1080}/A_{1338} from time zero to 144 h after death in rat spleen. The linear regressions are achieved between the absorbance ratio of the bands and PMI.**

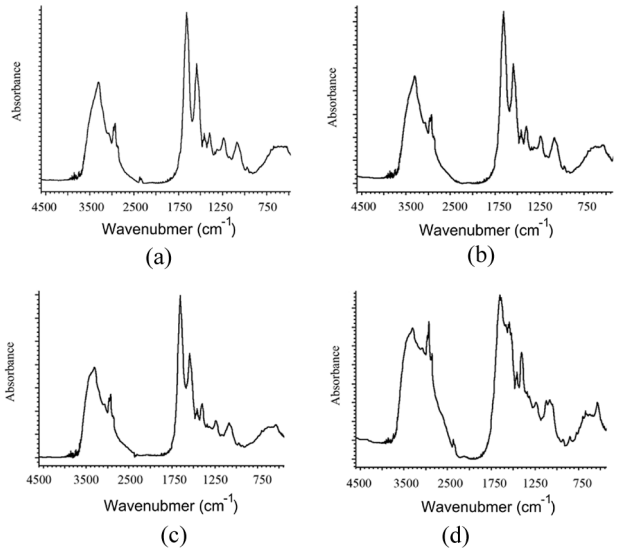

FIGURE 5 **Four complete FT-IR spectra: (a) time zero, (b) 24 h (c) 72 h, and (d) 144 h in rat spleen.**

FT-IR Spectroscopy Analysis and Valuation of Estimating PMI

In the current study, the rat liver and spleen FT-IR spectra was complex, because the liver and spleen have a lot of different kinds of components including the parenchymal, mesenchymal, and blood cells. FT-IR spectra of the liver and spleen can deliver these components. The changes of band absorbance ratio are related to the increase or decrease of each functional group within postmortem tissues. According to our study results, the absorbance changes suggested that some existing functional groups may be degraded and some new groups may be produced by chemical reactions. However, some functional groups are either not influenced by enzymes or they reach a balance between production and degradation within the tissues.

The degradation of proteins in different tissues postmortem has been studied for many years, such as cardiac troponin, calmodulin binding protein in muscle and lung, as well as calpain in brain.[7,26,27] Although FT-IR spectral technique is not able to analyze the special proteins in the tissues, the basic groups assigned to proteins and peptides may be shown in the spectrum. The absorbance band at 3303 cm^{-1} in the FT-IR spectra is mainly responsible for the stretching of the N–H and O–H in H_2O and proteins.[25,26] In the current study, water was removed during the sample preparation, so its

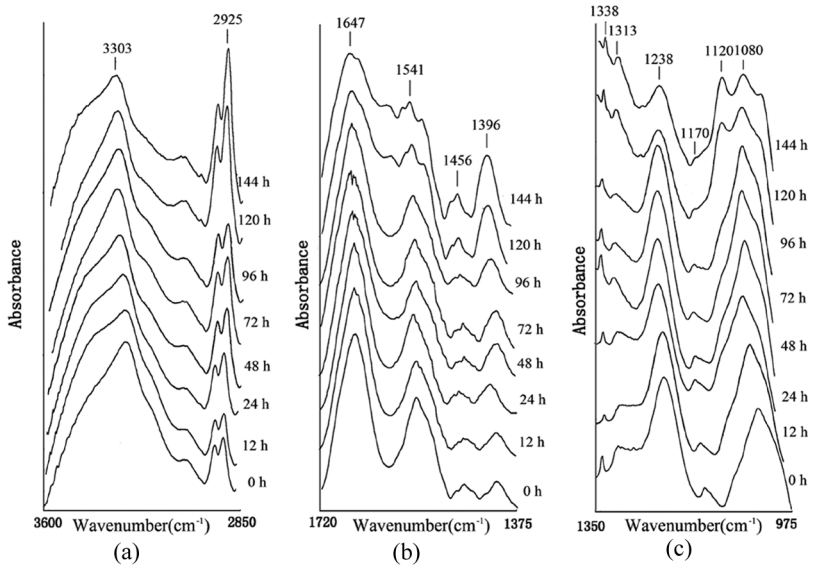

FIGURE 6 **Time course of FT-IR spectra from time zero to 144 h postmortem in rat spleen: (a) 3600–2850 cm^{-1}, (b) 1720–1375 cm^{-1}, (c) 1358–975 cm^{-1}.**

contribution can be considered to be from proteins. The bands at 1647 cm^{-1} and 1541 cm^{-1} are attributed to the vibration of amide I and amide II, respectively (Table 1). As shown by Fig. 2, Fig. 3a, Fig. 5, and Fig. 6a, the absorbance at 3303 cm^{-1} (liver) and 1647 cm^{-1} (spleen) was stable for up to 144 h after death. However, the absorbance at 1541 cm^{-1} (liver) showed increase with increasing time postmortem. A possible explanation might be the result of the different metabolic changes in different proteins. Some proteins probably are not degraded postmortem or they reach a balance of degradation and production postmortem. This stable status was also shown by others studied. For example, CaMKII protein levels did not change appreciably over 96 h postmortem in rat muscle and lung.[26] M-calpain did not significantly alter in rat brain during the earlier postmortem interval (0–24 h).[27] The absorbance at 1541 cm^{-1} increased postmortem, and the reason could be the increase of proteins synthesis by bacteria. Our FT-IR spectral results show the absorption bands due to proteins stretching were increased or stable after death. The specific reason for the increased proteins level needs to be further studied.

The band at 2925 cm^{-1} by streching mode of the CH_2, which was mainly from lipid, obvious increased. It was also supported by the band at 1396 cm^{-1} from the stretching of COO^- in fatty acids.[24,25] The increase in the two bands indicated the postmortem metabolism could result from the increase of free fatty acids by lipid mobilization after death.

The degradation of nucleic acids has been examined for estimating PMI. There is a correlation between the nuclear degeneration of white blood cells and PMI.[28] Johnson et al.[5] demonstrated that nuclear DNA is fragmented from 3 to 56 h postmortem in porcine skeletal muscle tissue, but the method is not able to detect DNA fragments after 56 h, and DNA cannot be detected at all in the liver and spleen tissues. The nucleic acids, especially RNA, are degraded rapidly by decomposing of phosphodiester bond due to nuclease releasing after death. The bands at 1238 cm^{-1} and 1080 cm^{-1} in the FT-IR spectra are mainly responsible for the symmetric and asymmetric PO_2^- stretching vibrations of nucleic acids (Table 1). The A_{1238}/A_{1338} and A_{1080}/A_{1338} ratios give an estimation of the level of nucleic acids. Our results showed that the level of nucleic acids decreased significantly after death. The A_{1238}/A_{1338} and A_{1080}/A_{1338} ratios in liver and rat spleen demonstrated a strong linear relationship with increasing time postmortem (Fig. 1c, d; Fig. 4c, d). Furthermore, FT-IR spectroscopy is able to detect accurately changes of nucleic acids with longer postmortem interval.

The band at 1030–1050 cm^{-1} is due to the vibration mode of CH_2OH groups and C–O stretching of glycogen and glucose[11,15,24,25]. Our results from FT-IR spectra showed the band at 1030–1050 cm^{-1}

appeared only in liver but not in spleen, and the band absorbance decreased with increasing time after death until it disappeared (Fig. 2 and Fig. 3c). The disappearance of the bands is due to the glycogen degradation reaching an extreme low level that FT-IR spectroscopy cannot detect. There was also a decrease in the absorbance of the asymmetric CO–O–C and C–OH stretching band at 1153 cm^{-1}, which is mainly glycogen in tissues. As shown by Fig. 2 and Fig. 3c, the band absorbance at 1153 cm^{-1} (liver) decreased after death and, in some specimens, disappeared after 120 h (Fig. 3c). The degradation of glycogen was also supported by other studies.[29–31]

The appearance of new bands or the disappearance of existing bands after 120 h suggests that the changes may differentiate the various PMI. For both liver and spleen, a new band appeared at 1120 cm^{-1} at 120 h postmortem. The band at 1170 cm^{-1}, however, appeared in rat liver and disappeared from spleen after 120 h (Fig. 3c and Fig. 6c). This result implied that the chemical components and reactions in the liver and spleen may be different. In addition to major bands, we observed many weak bands as postmortem time increased. The absorbance of these weak bands has not been assigned to particular functional groups, but we consider that the increase of the amount of weak bands is closely related to increasing metabolites after death. In addition, the frequency of major bands selected from time zero to 144 h postmortem remained stable. The stability could demonstrate that the structure of these functional groups was not affected by the chemical reaction.

Analysis of Other Factors

Many factors can influence the rate of a decomposition of a dead body, especially the environment temperature. In this study, we chose 20°C which is generally considered a room temperature environment. In addition to room temperature, our research group also monitored the spectral changes of liver and spleen at 4°C and 30°C. We found these are similar changes compared with 20°C, and the spectral changes are most dramatic at 30°C (Fig. 7). These results indicate temperature dependency of postmortem metabolic changes based on FT-IR spectra. The increase in temperature causes stronger changes in the absorbance, but there were no significant changes for these band frequencies.

There were some overlaps of the absorbance ratios at some time points postmortem (Fig. 1 and Fig. 4). At some continuous time points postmortem, single absorbance ratio possibly showed some

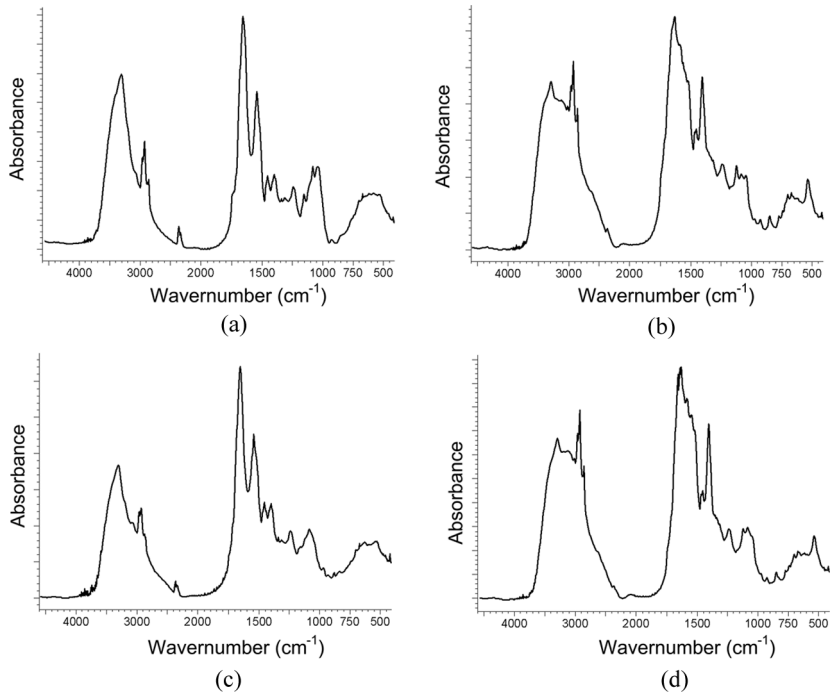

FIGURE 7 FT-IR original spectra of the liver and spleen at 72 h after death: (a) 4°C liver, (b) 30°C liver, (c) 4°C spleen, (d) 30°C spleen.

overlaps, however, the various absorbance ratios within the liver and spleen all indicated there was obviously increase or decrease tendency after the rats died from time zero to 144 h. Thus the full complicated analysis of different ratios of liver and spleen will resolve the problem of overlaps efficiently, and a more detailed analysis of FT-IR spectra should be studied at different postmortem time points.

By carefully controlling the sample extraction and experimental condition, this study discovered a strong relationship between PMI and absorbance ratio. Although this study is based on animal data, the result is ensured and consistent at each time point. It will provide important information for PMI estimation in forensic practice. Further studies with human tissues will be developed to confirm that the relationship is similar between different species.

CONCLUSIONS

The results showed that FT-IR spectroscopic analysis can monitor the changes of functional group from time zero to 144 h postmortem. In rat liver and spleen, there were three different types of metabolic changes after death based on the spectral results: increase, decrease, and stable. The various ratios of band absorbance within rat liver and spleen show great increase and decrease in linear regression relationships. Increase in temperature causes more changes in the bands' absorbance. Besides using other classic methods, the use of FT-IR spectroscopy may have a potential for estimating PMI in the field of forensic medicine.

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