Application of Fourier transform infrared spectroscopy with chemometrics on postmortem interval estimation based on pericardial fluids

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Postmortem interval (PMI) evaluation remains a challenge in the forensic community due to the lack of efficient methods. Studies have focused on chemical analysis of biofluids for PMI estimation; however, no reports using spectroscopic methods in pericardial fluid (PF) are available. In this study, Fourier transform infrared (FTIR) spectroscopy with attenuated total reflectance (ATR) accessory was applied to collect comprehensive biochemical information from rabbit PF at different PMIs. The PMI-dependent spectral signature was determined by two-dimensional (2D) correlation analysis. The partial least square (PLS) and \( \nu \)-support vector machine (\( \nu \)-SVM) models were then established based on the acquired spectral dataset. Spectral variables associated with amide I, amide II, COO\(^{-}\), C-H bending, and C-O or C-OH vibrations arising from proteins, polypeptides, amino acids and carbohydrates, respectively, were susceptible to PMI in 2D correlation analysis. Moreover, the \( \nu \)-SVM model appeared to achieve a more satisfactory prediction than the PLS model in calibration; the reliability of both models was determined in an external validation set. The study shows the possibility of application of ATR-FTIR methods in postmortem interval estimation using PF samples.

Postmortem interval (PMI) evaluation remains a challenge in the forensic community because routine methods rely on subjective evaluation of body signs alone during the early phase (usually within 24 h postmortem), including algor mortis, livor mortis, rigor mortis distribution, and corneal turbidity1. In recent years, an increasing number of investigations have focused on postmortem chemical changes in biofluids, especially the vitreous humor and blood1,2, to identify biomarkers for PMI estimation, since they are readily available at crime scenes or during autopsy. There is ample evidence that multiple components in pericardial fluid (PF), including heart-specific proteins (cardiac troponin and creatine kinase MB), mRNAs, and electrolytes (Ca\(^{2+}\) and Mg\(^{2+}\)), may be used to determine specific causes of death and elucidate the underlying mechanisms3–5. However, the potential of PF as a medium for PMI determination has not been documented sufficiently. Only a few studies indicate that electrolytes in PF tend to be used as parameters for PMI estimation. For instances, Balasooriya et al. showed that changes in K\(^{+}\), phosphates, and Na\(^{+}\) concentrations are significantly correlated with PMI6. Subsequently, Dalbir et al. established mathematical models based on electrolytic parameters for PMI prediction in independent samples7. Nevertheless, limited variables in PF are taken into account in the above studies; in addition, there is no evidence that other substances could contribute to sequential postmortem changes.

Fourier transform infrared (FTIR) spectroscopy is a powerful analytical tool for identifying chemical constituents and elucidating compound structures in various forms in real-world samples according to the vibrational

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modes of their molecular functional groups. FTIR has the capacity to perform global assessment of components found in samples with no need of sample preparation, which is practically impossible with other routine analytical approaches. In forensic investigations, FTIR has been extensively utilized in multiple evidence-based cases at a crime scene, including questioned documents, banknotes, paints, fibers, hair and gunshot residues. Alternatively, the feasibility of FTIR for chemically analyzing biological specimens has been demonstrated by other studies; indeed, multiple macromolecules, such as proteins, lipids, carbohydrates, and nucleic acids, can be monitored simultaneously in an FTIR spectrum based on their unique infrared absorption frequencies. However, due to the complexity and heterogeneity of biological systems, a variety of data processing methods have emerged to interpret and select spectral features. In this context, two-dimensional (2D) correlation analysis is commonly used to uncover overlapped bands and discriminate very complex mixtures under the conditions of external perturbations, such as time, temperature, concentration and oxidation. Moreover, a combination of FTIR spectroscopy and chemometric methods, including partial least square (PLS) and support vector machine (SVM) models, can convert the characteristic spectral pattern into a classifier or discriminator for automatic classification and prediction among different sample categories.

In our research team, much efforts have been devoted to characterizing postmortem changes in biological samples by FTIR spectroscopy. We found that some spectral parameters, e.g. peak intensities and areas, are correlated with PMI in different tissues. Recently, PMI groups of the rabbit plasma are successfully distinguished by FTIR spectroscopy coupled with PLS models. The present study primarily focused on PF due to its advantages. For instance, large amounts of PF are easily obtained in contrast to VH; meanwhile, PF is less susceptible to microbial contamination and bacterial degradation compared with blood samples. To the best of our knowledge, this is the first study of PMI estimation based on infrared spectroscopic analysis of PF.

Materials and Methods

Animal model. A total of 99 male Japanese rabbits (6 months; 2.5–2.8 kg) were purchased from the animal center of Xian Jiaotong University. They were socially housed under a 12 h light/dark cycle with food and water ad libitum. The animal experiments were approved by the Committee of Laboratory Care and Use of Xian Jiaotong University. All methods were performed in accordance with the relevant guidelines and regulations outlined by the Committee of Laboratory Care and Use of Xian Jiaotong University. The rabbits were sacrificed by air injection through the ear-rim vein, and carcasses were placed in isolated chambers at a constant temperature of 25 °C. PF samples were harvested from the pericardium using sterile syringe needles within 48 h postmortem at 6 h intervals (11 rabbits per time point). The samples were then immediately submitted to centrifugation at 14000 rpm for 10 minutes to eliminate particle matters, which may cause Mie-type scattering. The supernatants were obtained and snap frozen in liquid nitrogen until use for FTIR analysis. The animals were randomly divided into calibration (8 rabbits per group) and validation (3 rabbits per group) groups.

FTIR measurements. Spectroscopic measurements were performed on a Nicolet IS 50 FTIR spectrometer coupled with an ATR accessory (Smart Orbit Diamond, Thermo Scientific Fisher, USA). When an infrared beam is directed onto the ATR diamond crystal with a high refractive index, the generated evanescent waves penetrate a few microns on the sample surface and are subsequently attenuated or altered due to energy absorption. ATR-FTIR measures such energy variation for selected wavelengths, and produces corresponding infrared spectra. Peak intensity and position in an infrared spectrum are primarily dependent upon global vibrational modes of molecular functional groups in a given sample. In this study, the laboratory environment was kept at a temperature of 37 °C, with a relative humidity below 20%, in order to remove atmospheric water vapor as much as possible. Before FTIR measurements, approximately 100 μL of the thawed sample was shaken on a vortex mixer for 30 s and mixed with a micropipettor. Next, a sample aliquot (1 μL) was carefully deposited on the ATR diamond window and sufficiently dried with an air dryer. Spectra were collected at frequencies ranging from 1800 to 900 cm⁻¹, with a resolution of 4 cm⁻¹ and 32 scans. Background spectra collected on blank ATR spectra were automatically subtracted. For each sample, nine replicates were automatically averaged to produce a spectrum in order to eliminate loading errors.

Two-dimensional (2D) correlation analysis. All FTIR spectra in each PMI group were averaged. Average spectra in all groups were normalized by SNV and analyzed by the 2Dshige software package (Shigeaki Morita, Osaka Electro-Communication University, Japan; version 1.3).

Chemometrics. PLS and nu-SVM regression models were established with MATLAB R2014a (MathWorks, USA). Spectral datasets were preprocessed by SNV and second derivatives (25 points smoothing) within a frequency window of 1800–900 cm⁻¹. The predictor X corresponded to the matrix of spectral intensity while the response variable Y was associated with PMI values. To reduce computational complexity in establishing a nu-SVM model, the dimensions of preprocessed spectra were reduced to 8 latent factors by principal component analysis (PCA). This method can transform a high dimensional dataset into a lower dimensional orthogonal feature set while retaining maximum information from the original high dimension dataset. In this study, these 8 latent factors explained rough 98% of the variance. The calibration dataset was used to establish mathematical models. Their reliability was evaluated by 8-fold cross-validation and a permutation test to avoid overfitting, which usually renders models impractical in predicting independent samples accurately. In 8-fold cross-validation, the calibration dataset was divided into 8 equal sized sub-datasets, each of which contained spectra from 9 PMI groups. Of the 8 sub-datasets, one was retained as the test dataset, and spectral categories in this sub-dataset were predicted by the model established using the remaining sub-datasets. This process was repeated 8 times, and the determination coefficient (R²) and root-mean-square error of cross-validation (RMSECV) were assessed each time; these parameters represented the goodness of fitting between actual and
predictive PMI values, and the global predictive error, respectively. Performances of the PLS and nu-SVM models were compared by unpaired t-test based on R² and RMSECV, using Prizm 5.0 (GraphPad Software Inc., La Jolla, CA). P < 0.05 was considered statistically significant. Data were expressed as mean ± standard deviation (SD). In the next step, the established PLS and nu-SVM models were used to estimate PMI values in the validation group. Determination coefficient (Q²) and root-mean-square of prediction (RMSEP) values were also calculated to evaluate the generalization of the above models.

Results and Discussion
Figure 1 shows a comparison of average spectra with SNV normalization among PMI groups from 0 to 48 h postmortem.

Table 1. FTIR frequencies of measured range and their peak assignment.

| Position [cm⁻¹] | Assignment                                                                 |
|-----------------|-----------------------------------------------------------------------------|
| 1650            | Amide I: C=O stretching of the peptide back bone                             |
| 1540            | Amide II: N-H bending coupled to C-N stretching                              |
| 1453            | Asymmetric and symmetric C-H bending from CH₂ and CH₃ on proteins           |
| 1398            | C=O vibrations of COO⁻ from free fatty acids, free amino acids and polypeptides |
| 1324            | Amide III from proteins                                                     |
| 1078            | Symmetric stretching of P-O from nucleic acids and phospholipids; C-H or C-OH vibrations from saccharides. |
| 1033            | C-O or C-OH vibrations from glucose, polysaccharides                        |
| 926             | C-O or C-OH vibrations from carbohydrates                                    |

Table 1. FTIR frequencies of measured range and their peak assignment.

Figure 1. A comparison of average spectra with SNV normalization among PMI groups from 0 to 48 h postmortem.
changes. The cross-peaks at the non-diagonal line provided information on relative correlations between pairs of spectral variables; positive features (red) were in the same direction, and negative (blue) ones in the opposite direction. In contrast, asynchronous spectra (Fig. 2B) showed the sequence of kinetic changes, with cross-peaks corresponding to counterparts in the synchronous spectral map. According to Noda’s rules[35], when cross-peak signals are the same for both synchronous and asynchronous maps, intensity changes of spectral variables on the x-axis occur before those on the y-axis, and vice versa.

Table 2. Signs of the synchronous (Φ) and asynchronous (Ψ) cross peaks4. “n” means no cross peaks in the synchronous and asynchronous maps. Greater-than and less-than signs represent that ν1 occurs before (>) or after (<) ν2 respectively. The equal sign means that ν1 coincides with ν2.

| [νν1, νν2] | Φ | Ψ | Sequential order |
|-----------|---|---|-----------------|
| (1089, 1656) | n | + | no correlation |
| (1324, 1656) | + | n | 1324 = 1656 |
| (1517, 1656) | − | n | 1517 = 1656 |
| (1581, 1656) | n | + | no correlation |
| (1089, 1581) | + | n | 1089 = 1581 |
| (1324, 1581) | n | + | no correlation |
| (1517, 1581) | − | − | 1517 > 1581 |
| (1089, 1517) | − | + | 1089 < 1517 |
| (1324, 1517) | − | n | 1324 = 1517 |
| (1089, 1324) | n | − | no correlation |

Figure 2. The results of 2D correlation analysis include synchronous (A) and asynchronous spectral maps (B).
In Fig. 4A, the nu-SVM model achieved a better prediction with higher R² value (0.98 ± 0.0082) and lower RMSECV (2.38 ± 0.42) compared with the PLS model (R², P < 0.05; RMSECV, P < 0.05). This may be due to the ability of the nu-SVM model to avoid difficulties of using linear functions in high dimensional feature space. Indeed, nu-SVM regression introduces the new penalty parameter nu, which was set to 0.02 in this study. This parameter controls the number of support vectors and training errors. In SVM regression, only support vectors were used for the final PMI estimation. A Radial Basis Function (RBF) kernel with parameter cost and gamma was selected for non-linear transformation that maps observations into a high-dimensional space. The parameter gamma is a regulation constant that affects the generalization performance of the nu-SVM model, while cost refers to the cost factor, which controls the balance between calibration errors and model complexity. The best combination of the parameters gamma and cost was determined according to the minimal predictive error of cross-validation in the form of a two-dimensional grid search (Fig. 4B).

Figure 5 shows the predicted results in the validation group using the PLS and nu-SVM models, respectively. The Q² and RMSEP values of both models were generally close to their R² and RMSCV, respectively. Moreover, a permutation test was performed to assess whether the established models were over-fitted by randomly permuting class labels and refitting new models with the same number of components as the original ones. Well fitted and meaningful models have significantly higher R² and Q² values than permuted data. In Fig. 6, the y-intercept values of the regression line were 0.08 and −0.1 for R² for Q², respectively, in the PLS model; 0.08 and −0.09, respectively, were obtained in the nu-SVM model. Both tests suggested that the PLS and nu-SVM models were reliable in predicting PMI in independent samples.

As reported previously, some metabolites and proteins in biological samples could be considered biomarkers for PMI estimation. For example, studies have shown that the concentrations of certain metabolites in plasma and muscle samples from rats are highly correlated with PMI. Pittner and colleagues have identified degradation profiles of candidate proteins in human muscle tissues for delimiting certain periods of time postmortem, even
under heterogeneous conditions such as variations in ambient temperature, age, sex, and cause of death. Along with PF advantages, the current spectroscopic study suggests that the PF may be a potential medium for PMI estimation in forensic practice. Given limitations of ATR-FTIR spectroscopy, omics approaches, such as metabolomics and proteomics, are invaluable in identifying specific substances in the PF, which can greatly contribute to the discovery of new biomarkers for PMI estimation.

Conclusion

In this work, ATR-FTIR spectroscopy was applied for the first time to acquire biochemical information in PF samples from rabbits within 48 h postmortem. Along with 2D correlation analysis, spectroscopic findings suggested that PMI-dependent changes in the PF almost solely derive from molecular vibrations of proteins, polypeptides, and amino acids, and are associated with time-ordered protein degradation. Moreover, the nu-SVM and PLS models were established to predict PMI, with the SVM model yielding a more satisfactory prediction according to 8-fold-cross-validation. Overall, the present findings demonstrate that ATIR-FTIR spectroscopy combined with chemometrics may be used to determine PMI, offering a promising new approach in the forensic field.

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Figure 5. The prediction results of the PLS (A) and nu-SVM (B) regression models in an independent dataset which is not included in the calibration group.

Figure 6. The PLS and nu-SVM models are validated by 50 random permutation tests as shown in (A) and (B) respectively, whose results indicate both models were acceptable.
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**Author Contributions**

Ji Zhang and Bing Li wrote the main manuscript and prepared all figures. Yijiu Chen, Ping Huang and Zhenyuan Wang oversaw the project and assisted with the writing of the manuscript. Other authors designed and performed the experiments. All authors read and approved the final manuscript.

**Additional Information**

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