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# ATR based Infrared Spectroscopy for the Diagnosis of Neonatal Respiratory Distress Syndrome

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## ABSTRACT

Optical spectroscopy offers a potential non-invasive, label free and rapid method to assist clinicians to diagnose diseases for which biomarkers are known. Neonatal respiratory distress syndrome (nRDS) diagnosis in preterm infants is known to be correlated with the lecithin/sphingomyelin ratio (LS ratio) in gastric aspirates, with a ratio less than 2.2 indicating that surfactant replacement therapy is needed. Currently no widespread method exists that can give clinically relevant answers in less than 2 hours from the point of sample collection as it is difficult to identify those who could benefit from prompt surfactant treatment. Various LS ratios were generated using pure dipalmitoylphosphatidylcholine (DPPC) and sphingomyelin (SM) dissolved in dichloromethane and infrared spectra generated using Attenuated Total Reflection (ATR) assisted Fourier Transform InfraRed spectrometry (FTIR). Subsequent analysis obtained the LS ratio using the spectra alone. Further, we demonstrate the application of principal component regression (PCR) and partial least squares (PLS) fits to measured spectra to assist in the determination of the LS ratio using a model trained with multiple runs of the different batches of the same concentration.

**Keywords:** Mid-IR spectroscopy, Biomedical diagnostics, ATR-FTIR, nRDS, Biomarker diagnosis

## 1. INTRODUCTION

Diagnosis of disease is a prerequisite to therapeutic intervention and shortening the time between the presentation of symptoms to treatment is associated with better clinical outcomes. Diseases such as cancer, or uncontrolled diabetes, exhibit symptoms which require rapid diagnosis to enable therapeutic decisions, increasing the damage in the intervening period when prolonging the time to effective treatment. Point of care testing is a key enabling technology that can reduce patient wait times, and even bring the provision of such care to a primary setting, as opposed to requiring secondary care by default.

A simple diagnostic point of care device rapidly detecting the specific biomarkers of a disease in their natural form from a human derived sample without any need for labelling or sending the sample to centrally located laboratories for analysis can shorten the time to treatment and are especially vital for critically ill patients. Neonatal respiratory distress syndrome (nRDS) is one such disease that can benefit from a rapid point-of-care diagnostic device. Pre-term neonates suffering with nRDS are born with under-developed lungs typified by insufficient quantity and quality of lung surfactant<sup>1,2</sup>. Surfactant is required to reduce the effort required to breathe, and without it the very act of breathing causes damage to their lungs. Untreated the prognosis was usually death, but exogenous surfactant replacement therapy has decreased the mortality rate associated with the disease<sup>3</sup>. Clinically, better outcomes are known to be associated with early treatment<sup>4</sup>, however, the symptoms of nRDS are similar to other diseases which also present at birth with different underlying causes and requiring different treatment. To differentiate between these cases no point of care test currently exists.

nRDS can be diagnosed by assessment of lung maturity, for which biomarkers are known<sup>5</sup>. The lecithin/sphingomyelin (LS) ratio of biological samples taken from gastric aspirate (GA), bronchoalveolar lavage (BAL) or amniotic fluid (AF) is known to increase with increasing gestational age and has been used to diagnose nRDS for ratios below a cut-off

value. Since the LS ratio has traditionally been measured using thin layer chromatography (TLC) along with densitometry, the robustness of the measurement technique could explain the variability observed. Other measurement methods have been reported but these methods share the same limitation that they are not suitable as point of care diagnosis tool either owing to a long process time, or complex analysis. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) has also been used to measure the LS ratio of dried patient samples<sup>6</sup>, with a reported cut-off LS ratio of 2.2, but its use as a point of care test is confounded by difficulty associated with cleaning the ATR crystal and the artefacts associated with producing a dried sample<sup>7,8</sup>.

In order to measure the concentrations present, in absolute terms, the optical path length should be identical; by drying the sample this information is lost. Drying the sample introduces further complications into the spectra unrelated to the concentration of the analytes because the thickness of the dried sample is uncontrolled and the process can cause separation of the components present, violating the assumption that the sample on the ATR crystal is homogenous. In addition, the process of drying can cause formation of cracks which result in scattering effects being present in the spectra requiring pre-processing to remove these effects and complicating the analysis<sup>9</sup>. Therefore, in our approach we are using liquid samples (Lecithin and Sphingomyelin dissolved in dichloromethane, DCM) presented to the ATR crystals for the ATR-FTIR analysis.

Chemometric analysis uses mathematics and statistical techniques to extract information from collected experimental data. This can be used to build a regression model in order that the concentration of analytes present in a spectrum can be predicted on the basis of the spectra alone. Such an approach, which is quick to perform, would allow a point of care measurement of the LS ratio.

In this study, we demonstrate the potential of ATR-FTIR spectroscopy for the diagnosis of nRDS by analysing the biomarkers in liquid form (dipalmitoylphosphatidylcholine (DPPC) and sphingomyelin (SM) dissolved in DCM) and applying chemometric techniques to predict the LS ratio.

## 2. MATERIALS AND METHODS

Solutions containing DPPC (Sigma Aldrich) and SM (Sigma Aldrich) in DCM (Fisher UK) in different ratios ranging from 1.0 to 3.4 were prepared. To obtain a homogenous solution, we heated them in a water bath at 63 °C for 30 s followed by vortex mixing for a further 30 s. This was repeated until the solutions were visibly homogenous and then repeated thrice more. Left to stand, the solutions showed a tendency to form precipitates. To mitigate against this, the solutions were heated for 1 min and vortex mixed for 30 s prior to placing on the ATR crystal. It was also observed that bubbles formed within the lipid solutions approximately 10 s post pipetting the sample onto the ATR crystal, these being removed by agitation of the solution using the pipette tip.

The ATR apparatus was a 10-bounce zinc selenide (ZnSe) horizontal ATR accessory (Pike) coupled to a Cary 670 FTIR machine with a deuterated triglycine sulphate (DTGS) detector. The beam splitter was made from potassium bromide, and the whole setup controlled using Resolutions Pro software. Nitrogen purging was set to 4 litres per minute, and scan settings were 4 cm<sup>-1</sup> resolution with 32 scans. Each measurement was performed on 0.5 ml of analyte.

To clean the ATR crystal 1ml dichloromethane was added to the well, and a cotton bud used to mix the solution. The solution was removed by wicking it away using a Kimwipe. The process was repeated thrice, and then the well refilled with dichloromethane and an FTIR spectrum recorded. Observations of the peak heights at locations known to pertain to the lipid of interest near 2900 cm<sup>-1</sup> were observed to be less than 0.0004 absorbance units (A.U.) when the cleaning was deemed adequate, as further cleaning steps beyond this point did not yield any further reduction in these peaks. The testing was performed from the lowest concentration up to the highest concentration in order to prevent this source of error featuring in the results.

The processing of the spectra was performed initially in EssentialFTIR (Operant LLC) by baselining at a single point (2500 cm<sup>-1</sup>), truncation of the spectral regions from 850 to 3500 cm<sup>-1</sup> and removal of regions containing carbon dioxide or strong dichloromethane absorbances (1190 to 1310 cm<sup>-1</sup>). The spectra were then further processed in a Python based Jupyter Notebook using the Scikit-Learn library.

### 3. RESULTS AND DISCUSSION

The initial scans were performed on each of the lipids in isolation, as a serial dilution from 1mM to 0.0001 mM. The purpose of these scans was to identify the lowest concentration of lipids that could show detectable absorption peaks in an ATR-FTIR spectrum. These scans were also useful in showing the similarities and differences in spectra between the DPPC and SM.

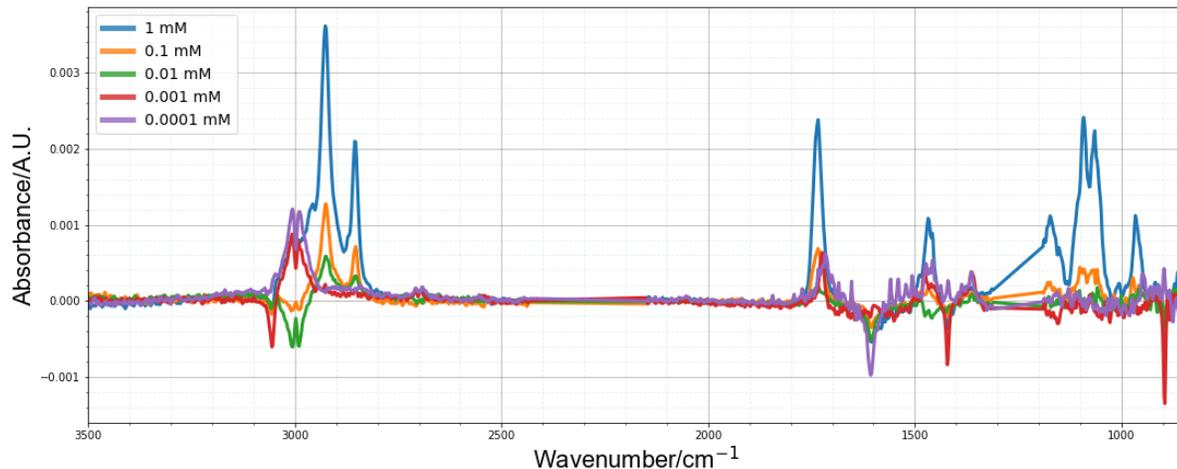


Figure 1: ATR-FTIR spectra obtained from DPPC serial dilutions (one sample spectrum for each concentration shown)

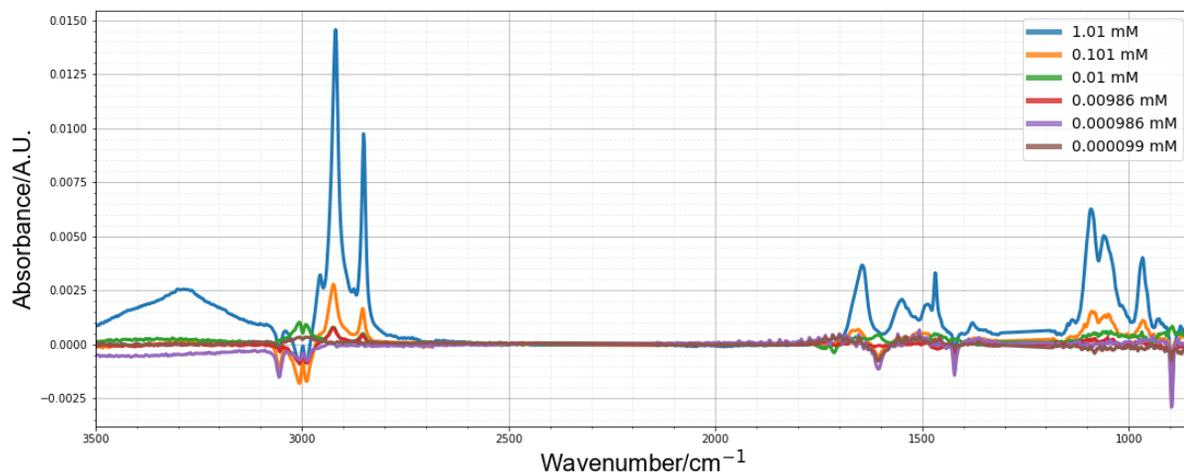


Figure 2: ATR-FTIR spectra obtained from SM serial dilutions (one sample spectrum for each concentration shown)

The LS ratio tests were performed by varying the DPPC concentration in relation to 1 mM SM within the solution. Nine different ratios were tested, with the more closely spaced ratios centring around a ratio of 2.2. This was done in order to challenge the model to be able to discern small differences in ratio and to see whether this would act to confound it. The effects of increasing L are plainly visible when observing the peak height at 1734 cm<sup>-1</sup> increasing with increasing LS ratio.

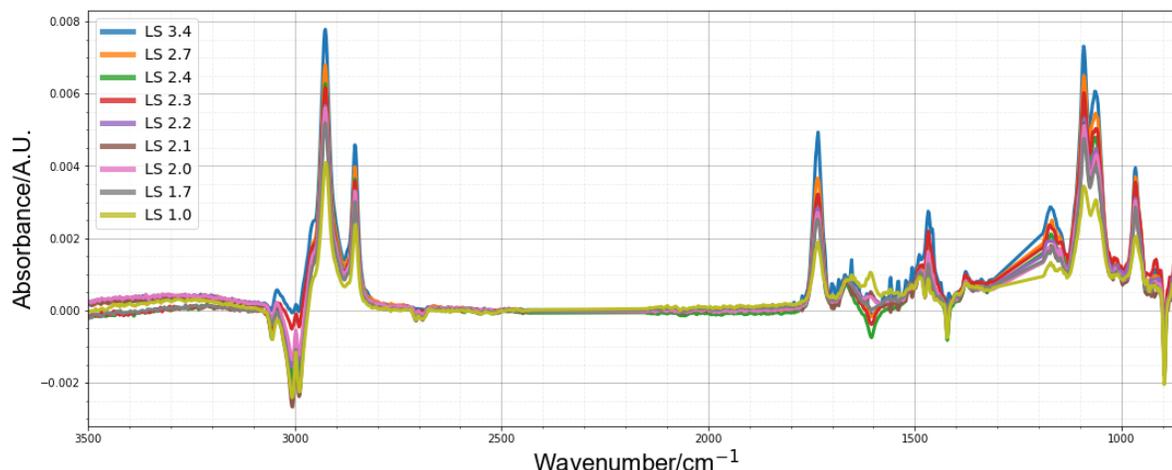


Figure 3: ATR-FTIR spectra obtained from generated LS ratio solutions (one sample spectrum for each concentration shown)

In addition to the raw spectra, the modelling was also performed on second derivative spectra. The advantage of doing so is to remove the effects of trends or offsets in the baseline providing a better basis to compare spectra from different runs at the expense of decreasing the signal to noise ratio.

The search for an appropriate model measured the performance of the PCR (Principal Components Regression) and PLSR (Partial Least Squares Regression) algorithms as applied to the original dataset and the second derivative spectra by each increasing the factor by one, and measuring the calibration, cross-validation and prediction  $R^2$  (Pearson's moment of correlation/goodness of fit) and mean squared errors (MSE). This was combined with cumulative explained variance in both the PCR and PLSR models, to decide the appropriate number of components/latent variables: four components were chosen for the PCR models while two latent variables were chosen for the PLSR models.

Prior to testing the ability of the models to finally predict the LS ratio on the basis of the spectra, the spectra pertaining to a 0mM concentration of SM were removed, in order that meaningless LS ratios did not skew an assessment of the model performance. The LS ratio prediction ability was tested by obtaining the ratio of the predicted DPPC/SM concentrations.

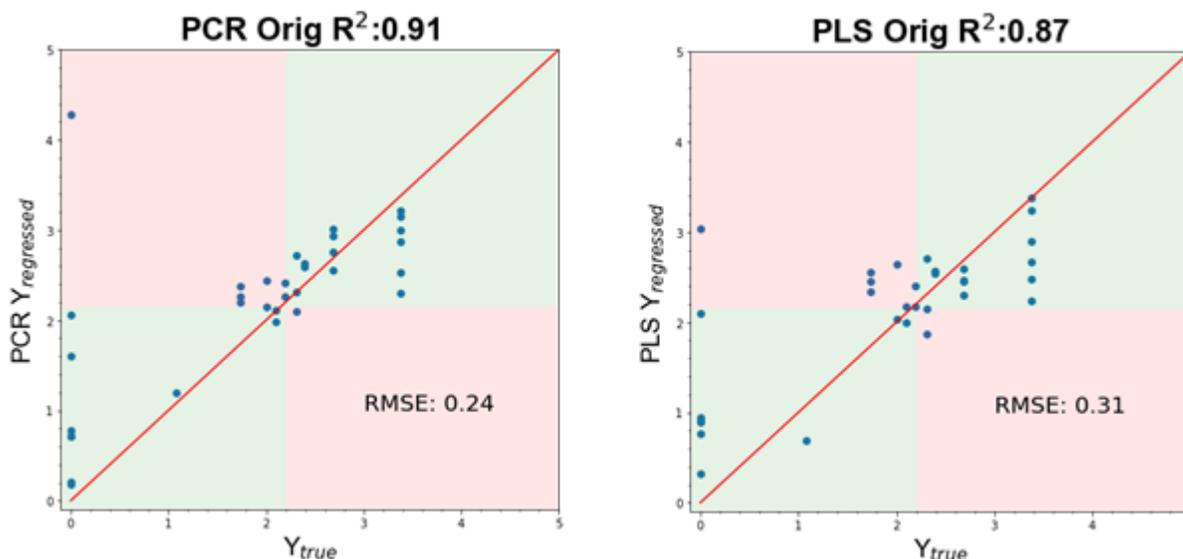


Figure 4 Predicted LS ratios on the basis of the original spectra

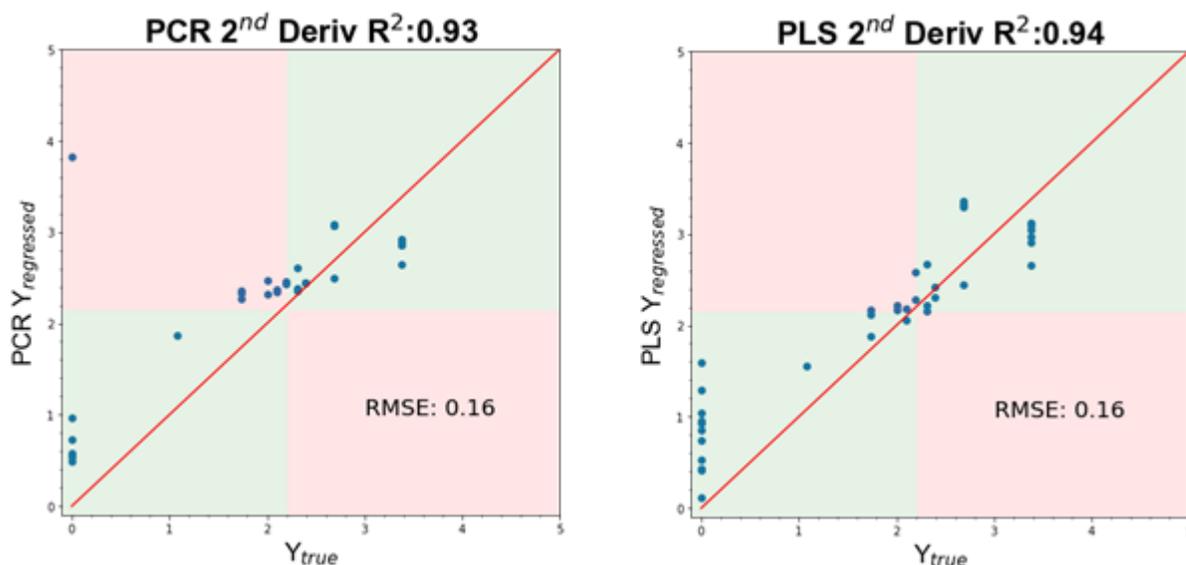


Figure 5 Predicted LS ratios on basis of second derivative spectra.

Figure 4 shows the predicted LS ratios obtained by the PCR and PLSR models as applied to the original spectra. Predictions relating to pure DPPC, with no SM present gave skewed LS ratio measurements, with the absolute predicted DPPC values remaining below 0.5 mM. In practice a calibration by performing measurements for DPPC and SM between 0.1 to 1mM would be expected to improve the model performance in this region. In this comparison, the PCR model performs better than the PLSR model, but as it requires a greater number of principal components, it would be expected to be less robust than the PLSR model. However, by using the second derivative spectra to build the model, a PLSR model (Figure 5, right) with two latent variables performs better than a PCR model with four principal components (Figure 5, left) to predict the concentrations in the test dataset, with better  $R^2$  and an equivalent root mean squared error (RMSE).

#### 4. CONCLUSIONS

This paper has shown that the quantification of the LS ratio is possible using only the mid-IR spectrum. The complete workflow from biomarker identification through to building the model, assessing which performed the best and finally showing what the ‘real-world’ performance of such a model to elucidate the LS ratio of a test dataset, not previously used to train the model, is also demonstrated. Developing models based on using second derivative spectra removes baseline trends or offsets and can be applied on each spectral measurement in isolation, so does not limit such a model to a specific set of conditions. Such a method is extendible to all cases in which a biomarker concentration needs to be measured to assist in the diagnosis of a disease. Further, since ATR-FTIR systems can be coupled to quantum cascade lasers we are working to realise the possibility of miniaturising such a system into an appropriate point of care device, offering a tantalising prospect for handheld diagnostic measurement systems able to provide evidence-based point of care medicine.

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