2020

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Recommended Citation

Richardson, Z. et al. (2019) Determining the Age of Spoiled Milk from Dried Films Using Attenuated Reflection Fourier Transform Infrared (ATR FT-IR) Spectroscopy, *Applied Spectroscopy,* 73(9):000370281984254 DOI: 10.1177/0003702819842548

DOI: 10.1177/0003702819842548

Determining the Age of Spoiled Milk from Dried Films Using Attenuated Reflection Fourier Transform Infrared (ATR FT-IR) Spectroscopy

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Abstract

Milk spoilage is an inevitable occurrence, which generates waste and can result in food poisoning. When milk spoils, the off-flavor and curdling are due to excessive proliferation of various bacteria which causes pH changes. Time, temperature, environment, and previous handling practice all affects the spoilage rate. There is a need for a fast reliable and accurate method that can identify in situ early spoilage of milk. Here we show the ability of attenuated total reflection Fourier transformed infrared spectroscopy (ATR FT-IR) in conjunction with multivariate data analysis to predict the age of milk. We found that dried films vastly increased the absorbance of important biomolecules within milk such as lipids, proteins, and sugars, compared to an unchanged milk sample. This allowed us to note the minor discrepancies that happened in spoilage. Spoilt milk was characterized by bands associated with increased lipids, proteins, lactic acid; and a decrease in carbohydrates. A semiquantitative prediction model for milk spoilage at room temperature demonstrated ATR FT-IR spectroscopy can predict milk age with a root mean square error of prediction of approximately 14 hours. The model showed poor performance in the first 40 hours but the predictions improved significantly after this time. The experimental procedure proposed for detecting biomolecules within milk has the potential to improve common practice. Furthermore, the model would be a starting point for a newer and improved methods to predict the spoilage date of milk, with potential commercial uses to reduce food waste and costs to the milk industry.

DOI: 10.1177/0003702819842548

Keywords: Milk, Fourier transform infrared spectroscopy, FT-IR, attenuated total reflection (ATR), partial least squares regression, PLSR, bacteria, milk spoilage

Introduction

Bovine milk has been a staple of many cultures throughout history because it is the easiest form of nutrition and minerals for the masses since domestication and is still widely used today. Due to its wide consumption, it is vital that every batch and bottle of milk upholds the same quality controls pertaining to the constituents of milk. Handling of milk post pasteurization requires great care to avoid any contamination because milk can be the perfect medium for the incubation of pathogens with its balanced levels of sugars, fats, pH, and minerals. Milk-born diseases are a constant concern for the industry, particularly considering the wide distribution and prevalence of the product. As a consequence, any outbreak of a milk-born disease has the potential to affect a large population.[1]

Bovine milk has many nutrients and minerals, which have been beneficial to the development and growth of infants and adults. The average bovine milk sample contains approximately 86.8% water, 4.8% lactose, 4.4% fat, 3.3% protein (of which 80% is casein and 20% whey) and 0.7% of minerals such as calcium, phosphorous and potassium.[2,3] The lipid composition of milk is unique and includes around 400 fatty acids, most of them short chained.[2] The overall chemical composition of milk does not vary significantly from country to country, with only minor percentage points variance in composition.[1]

A pathogenic outbreak of bacteria should not be confused with the process of milk spoilage, although both involve the proliferation of bacteria. Natural spoilage can cause food poisoning whereas a pathogenic outbreak of bacteria spreads disease past its prevalence baseline.[4] Spoilage occurs by proliferation of bacteria, including lactic acid fermenting bacteria (*Streptococcus*, *Lactococcus*, and *Lactobacillus*) and alkaline pathogens (yeasts and molds), which causes milk to spoil.[5,6] The olfactory and gustatory responses from spoiled milk is due to by-products from bacterial proliferation because they convert lactose sugar into lactic acid.[7] The lactic acid lowers the pH of the milk, causing triacyl glycerides and proteins to coagulate, forming a gel like substance.[7] Many factors contribute to the process of milk spoilage, each producing unique spoilage affects such as, e.g., acidic (bacterial spoilage) or chalky taste (denaturation of protein caused by excessive overheating).[8] Refrigeration has been proven to

DOI: 10.1177/0003702819842548

extend the consumption life of milk, due to the inhibitory effect of temperature decrease on bacteria's ability to proliferate. The influence of temperature on bacterial growth rates is related to the temperature affecting the rates of intracellular enzymatic reactions, crucial for cell viability,[9,10] as well as having a negative impact on the membrane fluidity and function.[11] Guidelines state that for the inhibition of bacterial growth milk should be kept at no higher temperature than 4 °C.[12] As stated above, not all bacteria are inhibited by the cold; psychrotrophs are bacteria that prefer cold environments (3–7 °C) and use proteins and lipids for growth.[4] Milk provides the physicochemical environment favorable for proliferation of a great variety of microorganisms (including psychrotrophs) and eventually all milk spoils as the proliferation of bacteria changes the composition of milk.[13] Even milk that has been treated to kill all bacteria present, such as ultra-high temperature (UHT), treated milk will eventually spoil because as soon as the carton is opened, bacteria will be introduced into the milk.

The contamination event of milk occurs either during or after the milking process, arising from dirtied equipment, improper handling or a bacterial infection of a cow's udder.[14] Pasteurized milk was collected and used for this study because it was the generic form of milk sold in the country where the research was performed. Pasteurization is the process where each milk batch is heated thoroughly to a temperature of 72 °C for 15 seconds where it aims to diminish the number of bacterial colony forming units (cfu), therefore reducing the likelihood to cause serious disease.[15] As pasteurization does not kill 100% of bacteria contained within a sample, there is always a small dormant number of bacteria remaining that leads to the eventual spoilage of milk, which is associated with the inability of pasteurization to destroy bacterial spores.[16-19] Bacterial spores are the most common cause for spoilage post pasteurization as the spore conformation allows the bacteria to resist the heat shock from pasteurization, in fact the heat shock activates the spores to divide and reproduce within the favorable growth media of milk.[4] There are other methods of treating milk such as UHT treated milk which destroys most bacterial spores in milk, resulting in a much longer shelf life than that of pasteurized milk.[4] However, even though UHT treatment of milk is more efficient in elimination of bacteria than pasteurization, it also causes milk to have an undesirable burnt taste.[15]

Infrared (IR) spectroscopy measures the absorption of infrared radiation due to molecular vibrations. The IR absorption is directly related to the concentration of the molecule at a particular wavenumber value according to the Beer–Lambert law. Attenuated total reflection

DOI: 10.1177/0003702819842548

(ATR) Fourier transform infrared (FT-IR) has the advantage of being portable and simple to use. In an ATR spectrometer, the light is internally reflected through a crystal with a higher refractive index than the sample (e.g. diamond or germanium crystal). The light in the form of an evanescent wave penetrates a few microns into the sample and is then reflected back through the crystal to the detector where the interferogram is Fourier transformed to give the resulting spectrum.[20] Therefore, ATR FT-IR spectra contain information about all the chemical compounds present in the sample. The complexity of the spectral data necessitates the application of multivariate methods to generate models that have predictive capacity. In this study partial least squares regression (PLSR) and support vector machine (SVM) was used to correlate the age of milk with the spectral profile and a predictive model was developed to determine milk age. This methodology establishes a linear relationship between a set of predictive variables (*X*) and the predicted variable (*Y*), being the *X* block the spectra and the *Y* vector the time in the case of this study. In PLSR, a set of latent variables (LVs), which explain the sources of variation in the X block correlated to the y vector are computed, and the LVs are used to calculate a regression vector b which relates the variable to predict with the predictive variables:[21]

$$Y = Xb^T + e \quad (1)$$

where *b* is the regression vector obtained from the LVs and *e* is the error vector (i.e., residuals).

The simplicity of IR spectroscopy has led to a number of studies investigating milk including differentiation between the type of milk based on lipid and sugar levels,[22–24] detection of adulteration in milk,[25–27] and detection of bacterial counts related to spoilage in milk.[28] One of the major components of milk is water. Water absorbs strongly in the infrared region and the spectra of milk are dominated by –OH stretching mode at ~3500 cm^{-1} and the –OH bending mode at 1640 cm^{-1}. Nicolaou and Goodacre[28] monitored the spectra of milk as it spoiled using samples of milk that were kept in a rotational incubator at 15 °C for 104 hours. This study focused on aqueous samples of milk kept out at room temperature (19–21 °C) for 102 hours. Nicolaou and Goodacre[28] found that by using FT-IR techniques the bacteria counts increased overtime in milk. They also found that the intensity of absorbance bands due to water obscured peaks associated with lipids.[28] In this work, we have increased the sensitivity of the ATR to detect milk components by allowing the sample to air dry directly onto the ATR crystal and measuring the resulting dry film. The measurement of dry films eliminates the contribution of

DOI: 10.1177/0003702819842548

water, but the measurement time is slower than the direct wet measurement. However, the drying process increases the signal-to-noise (S/N) of the non-aqueous components because they have better contact with the ATR crystal. This approach has been employed extensively in whole serum,[29] filtered serum[30] and organic extracts from serum[31] for predicting clinical parameters.

The aim of our work was to first identify FT-IR ATR spectral changes associated with milk spoilage and correlate these changes to macromolecular changes occurring within milk over time. Secondly, a PLSR and SVM models were built to predict the state and rate of spoilage of milk at room temperature. The model has potential to be used in the milk industry as a simple method to determine the age and the degree of spoilage in milk products.

Materials and Methods

Sample Preparation

Ten cartons of milk with the same use-by date, each the same type of milk (whole milk) were purchased from the local convenience store (Pura original milk). Local store milk was only viable option as this was a preliminary investigation into the potential of using ATR for milk spoilage. The analysis of store samples has a more real-world application because this is what customers actually purchase, and subsequently spoil. The spoilage variables being considered is what a customer would experience such as opening the bottle as well as leaving the milk out at sub-optimal temperatures. A portion from each carton was transferred separately into falcon tubes (10 mL) resulting in ten tubes (M1–M10) corresponding to each carton with eight tubes (M1–M3, M5–M9) left out at room temperature (19–21 °C) for 102 hours. The remaining samples, M4 and M10 were placed in a fridge set at 4 °C and taken out every six hours for spectral acquisition over 102 hours (control). Each sample, prior to measurement, underwent 30–60 seconds of vortexing to homogenize the solution. This approach was necessary during the latter stages of the experiment, as a side effect of spoilage was the congealment of lipids separating out from solution.

ATR FT-IR Spectroscopy

ATR FT-IR spectra were collected using a Bruker Alpha (Ettlingen, Germany) instrument equipped with sampling device containing a single bounce diamond internal reflection element (IRE) All spectra were collected in the range 4000–600 cm^{-1}, with spectra resolution of 6 cm^{-1} by co-averaging 64 interferograms. A background spectrum was collected prior to each

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measurement (128 scans) and ratioed against the sample spectrum. After vortexing, 0.5 µL aliquots were pipetted from the sample tube onto the ATR crystal and air dried for five minutes, until the water bands in the IR spectrum was minimized. For each sample (n=10) three replicates were collected (n=30) every six hours within the time frame of 102 hours (total number of spectra, n = 540).

Hydrated Milk Samples

Samples were placed onto the ATR crystal with spectra taken immediately.

Dehydrated Milk Samples

The 5 µL samples were placed onto the ATR crystal and a Supalite Speedy hair dryer (model number SP4000P) with low airflow and cool temperature settings was used to dry the milk samples. Spectra were recorded after a dried film was observed and the live view spectra showed reduced water absorbance and increased biomolecular absorbance.

Data Analysis

Data analysis was performed using Matlab (The Mathworks, Inc.) using in house written functions and the graphical user interface and chemometrical functions from Partial Least Squares Toolbox from Eigenvector Research Inc. (Wenatchee, WA, USA). The spectral preprocessing for all models and including Figures 2–5 had data excluded from 4000–3750, 2790–1900, and 825–600 cm^{-1} regions. The order of spectral pre-processing was as follows, Savitzky–Golay second-derivative polynomial order 3 and 15 points of smoothing, mean centering and standard normal variate (SNV). For the PLSR models, the number of latent variables was selected considering the minimum root mean squared cross-validation error and simplicity of the model (less than 10 latent variables).

pH Testing

The pH of an independent sample of milk was tested at time point 60 hours and 24 hours later at time point 78 hours. The sample was vortexed for 45 seconds to ensure adequate homogenization

DOI: 10.1177/0003702819842548

and similarity to samples M1–4. The pH meter was calibrated by using standard solutions at pH 7.0 and 4.0 and was washed in ultrapure prior to usage.

Bacterial Plating

A set of nutrient agar plates were used to culture the microbial species changed due to stage of spoilage. A wire loop was used to collect a sample of milk from the same tube used in the pH test, which was then streak plated onto the nutrient agar plate under aseptic conditions. The plates were then incubated at 32 °C for 48 hours in O_2. A gram stain was performed on the isolated colonies and then identified through light microscopy.

Results and Discussion

We first optimized spectral parameters and investigated sample hydration. Figure 1 shows a comparison between the FT-IR spectra of hydrated and dehydrated milk samples. Hydrated sample spectra were naturally dominated by the broad O-H stretching band between 3700–3000 cm^{-1} and the band at 1640 cm^{-1} assigned to the O–H bending mode. Bands from macromolecules in the milk were barely detectable in the hydrated samples with small contributions from lipids (2942 and 2852 cm^{-1}), proteins (1640 cm^{-1}) and sugars (~1000 cm^{-1}). This method would presumably have led to poor differentiation between samples, with differentiation based mainly on differences in lipids and water and is thus a less than ideal method to determine the spoilage of milk over time. To this end we developed a drying approach that enhanced the signal of important biomolecules within the sample of milk and reduced the water spectral contributions. Figure 1, shows the spectrum of a dried milk sample yielding stronger absorbance of the major macromolecules of interest. The major difference is the dramatic intensity of sugar bands around 1000 cm^{-1} in the dehydrated milk sample, which is an important maker band region for spoiled milk as will be discussed.

Although it was clear that the dry method increased the sensitivity by eliminating the water contribution, the drying process could also induce irreproducibility. The standard deviation for all dried samples at 1630 cm^{-1}, (assigned to the amide I band) was calculated to be 0.0186 with the average standard deviation of spectra between 3750–2900 cm^{-1} and 1900–825 cm^{-1} being 0.0217 (relative standard deviation below 10% in all cases). These values indicate that the

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problems with irreproducibility were minimized. However, the absorbance can change due to the contact of the dry film with the ATR crystal. These differences affect the path length but do not affect the relative absorbance of the different IR bands, and thus can be corrected using normalization techniques.

Figure 2 shows the average spectrum of bovine milk at $t = 0$, dried directly onto the ATR crystal. Bands are assigned to carbohydrates (1067, 1024 cm^{-1}), protein (1640, 1546 cm^{-1}) and lipids (2942, 2852, 1745 cm^{-1}). In the study by Nicolaou and Goodacre[28] an advanced stage of milk spoilage was not achieved at temperature. The use of the dry film technique increased the S/N for a number of key bands and hence small molecular changes that occurred during milk during spoilage could be detected. A strong band between 3600–3000 cm^{-1} is still observed (Figure 2) after drying, which would have small contributions from water molecules bound to lipids and proteins. However, the major contribution to this band is the OH stretching modes from mainly lactose, which constitutes 36.4% of dried milk. Each lactose molecule has 8 hydroxyl groups, which would contribute to the broad OH stretching band in this region. In addition, a shoulder band from the N–H stretching vibration of amide A from proteins, (constituting up to 33.3% of the dried sample) is also observed. The NH stretching band at 3284 cm^{-1} can only be observed in the dried state demonstrating the improved S/N that can be achieved by drying the sample. Table I shows the basic components (disregarding water) that constitute milk with C–O and O–H bands associated with sugars, CH_2 and CH_3 bands mainly associated with lipids and aliphatic amino acid side chains from proteins. The small peaks within the large band at 1040 cm^{-1} are from a variety of different C–O environments associated with carbohydrates, predominantly lactose, as well as traces of galactose and glucose. Figure 2 and Table I highlight the spectral characteristics of unspoiled milk (t=0).

The spectral differences between “fresh” (t=0) and “spoiled” (t=102) milk are clearly noticeable (Figure 1). The visible differences include absorbance increases in bands associated with lipids, (2922 and 2962 cm^{-1}) and a decrease in bands associated with sugars (broad stretch from 3500-3000, broad band from 1200-1000 cm^{-1}). Over time there was a blue shift in the bands (2942–2922 cm^{-1}) due to a reduction in H-bonding between the lipid hydroxy groups and is a clear marker of spoilage. The decrease in the content of sugars was due to their usage as a source of energy for bacterial proliferation, converting lactose into lactic acid.

DOI: 10.1177/0003702819842548

Linear and nonlinear spectral changes were modelled for milk left outside of the fridge. A calibration dataset of milk samples was left without refrigeration and measured at different times. By modelling the spectra in an X matrix and time as a Y matrix, we can predict the spoiling age an unknown sample, relatively to a sample left in the fridge.

The results of PLS performed on the data collected over 102 hours is presented in Figure 3. The linearity results from an increase in lipid levels and a corresponding decrease in carbohydrates (Figure 3) over time. Whilst bacterial proliferation increases exponentially until a limit is reached (at a large colony forming unit count), the actual mass of bacteria remains quite small, in the picogram range. Due to the limitations in sensitivity of the ATR FT-IR technique this mass of bacteria on the crystal is undetectable, especially as there were relatively substantial amounts of milk components. Therefore, any changes in the spectra over time would be due to secondary effects from bacterial presence, such as conversion of carbohydrates (used as an energy source) to acidic by-products. The acid, in turn, lowers the pH of the sample, causing the formation of large micelles of lipids. The increase in lipids over time would not be correlated to an overall increase in lipid content within the sample, but rather to formation of larger micelles.

The refrigerated samples, M4 and M10 showed little to no change (Figure 3 and 4) over the 5 days of testing because refrigeration has shown to decrease the rate of spoilage, effectively extending the consumable life of milk.[9,13,32] The inversely proportional correlation between absorbance of lipid bands (2942, 2852 cm^{-1}) and the pH value was observed over the entire experiment. It is also important to underline here that different types of microbes are associated with the stage of spoilage. In general, *Streptococci*, *Lactobacilli*, *Pseudomonas*, yeast, molds, and *Bacillus* species[4,33,34] are all found in various stages of milk spoilage, with *Bacillus* species found to be the most common associated with spoilage due to some species being spore forming bacteria.[4] All of the above mentioned bacteria cause a decrease in pH due to secondary effects from by-products.[14] Yeast and molds, on the other hand, cause an increase in pH.

Furthermore, we tested the ability of a model built on the basis of ATR spectra to predict the of state of spoilage. Nicolaou and Goodacre[28] reported a PLS model based on ATR spectra to predict the bacterial content in the milk sample but not the degree of spoilage as a function of time.

Figure 3a shows that the samples follow a rough linear prediction line with no outliers. There is large variance associated with the prediction line, especially around t=84 with there being a

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large distance between sample M6 (predicted at *t*=117) and sample M3 (predicted at *t*=42). Regardless of the variance, the model overall follows a linear regression and thus has the potential to be used as a prediction model as shown in Figure 4. The loadings plot (Figures 3b, 4b, 5b) indicated that the PLS model was predicting samples based on bands associated with lipid, sugar and acid. The loadings show an increase in the absorbance of bands associated with lipids and acid, which correlated to a decrease in absorbance of bands associated with sugars. The SVM model produced better linearity than the PLSR model, as evinced by the lower RMSECV value. In general, the models were able to predict with more accuracy spoilage times above 40 hours. Below this time, predicted and actual times are not significantly correlated. This limits the use of the technique in the 0–40 hour range.

Figure 4 and 5 depict the predictive models. Figure 3 confirms that the control samples, M10 and M4, were accurately predicted as controls (see Supplemental Material, Table S1), with the SVM model in Figure 4c clearly has an excellent prediction over the course of the experiment. The control refrigerated samples show some degree of spoilage that occurs within the 102 hours of the experiment as refrigeration only slows the process of spoilage but the changes are only small when the milk is refrigerated. Six samples, randomly chosen from samples M1–3 and M5–9 were used to validate and the remaining two samples, along with the controls, were loaded into the model as a predictive set. For Figure 4, the root mean square error of prediction (RMSEP) was large (33.07) as the model and predictive set was only needed to confirm the controls were set apart from the rest of the sample set. The RMSEP value was significant (14.31) in Figure 5 as the created model aimed to ascertain the spoilage state of the samples. RMSEP is the error in time that the model is able to predict, so the model created in Figure 5 can predict samples within an error of 14 hours. The SVM model was more accurate when compared to the PLSR model (fig. 5a and 5c) based on the RMSECV and RMESP values. A downside to SVM is that unlike PLSR, there was no loadings plot and thus one cannot ascertain what factors contribute to the prediction and building of the model. The loadings for all plots and models were similar as seen in Figure 3b, 4b and 5b, with variations only in the intensity of absorbance, indicating that the models were consistently separating on the same basis and thus can be compared to one another.

An additional experiment, which focused on the pH change and microbial growth, was performed after the extended milk spoilage experiment in order to confirm that different microbes were responsible for the observed spectral changes in milk over time. The milk sample

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was pH tested and plated at t=50 and t=74. The plates showed growth of bacteria, with gram positive *bacilli* organized in chains observed for both plates, although the *t*=74 plate contained many bacteria that had formed spores. The pH value dropped from 6.5 at *t*=50 to 6.0 at *t*=74, confirming that the decrease of pH during the stage of spoilage[14] resulting in coagulation of proteins and lipids within milk. Spore formation from *t*=74 indicated that within the 24 hours between plating, the bacteria underwent environmental stress due to lack of nutrients for proliferation and the presence of acid.

Conclusion

In this study we identified the major constituents of milk using ATR FT-IR spectroscopy with the dried samples. Bovine milk has a distinct FT-IR profile that enabled differentiation between "normal" and "spoiled" milk. The major differences observed were an increase in lipid bands and a decrease in sugar bands, around 2900 cm^{-1} and 1000 cm^{-1}, respectively. The spoilage process of milk is complex and occurs at different rates regardless of the controlled conditions. The microbial spoilage was, however, in each case correlated to coagulation of lipids and proteins caused by a drop in pH from bacteria converting lactose sugar to lactic acid. The PLS and SVM models enabled us to predict optimal spoilage of milk left out at room temperature with a RMSEP of 14 hours but the prediction performance was satisfactory only after the first 40 hours. The semiquantitative approach reported herein provides an invaluable tool to assessing the quality of milk near spoilage.

Supplemental Material

All supplemental material mentioned in the text, consisting of Table S1, is available in the online version of the journal.

Conflict of Interest

The authors report there are no conflicts of interest.

Funding

DOI: 10.1177/0003702819842548

BRW was financially supported by an Australian Discovery Council Future Fellowship grant FT120100926.

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Table I. Spectral band assignments of samples.

Wavenumber (cm^{-1})	Molecular mode of stretching	Associated compound
2942	ν_{as} (CH_2)	Lipids
2852	ν_s (CH_2)	Lipids
1745	ν (C=O)	Acids/Lipids
1640	Amide I	Proteins
1546	Amide II	Proteins
1377	δ (CH_2)	Lipids
1244	ν (C–O)	Esters, sugars, ethers, fatty acids, acids
1150	ν (C–O)	
1067	ν (C–O)	
1024	ν (C–O)	

Figure 1. Comparison of Dry method in blue and Wet method in red. Each class was made up of nine samples with wet samples obtained from a previous experiment, and dry samples chosen randomly from the 30 replicates in M1–M10 at time point zero. Significant bands are labelled in Table I.

DOI: 10.1177/0003702819842548

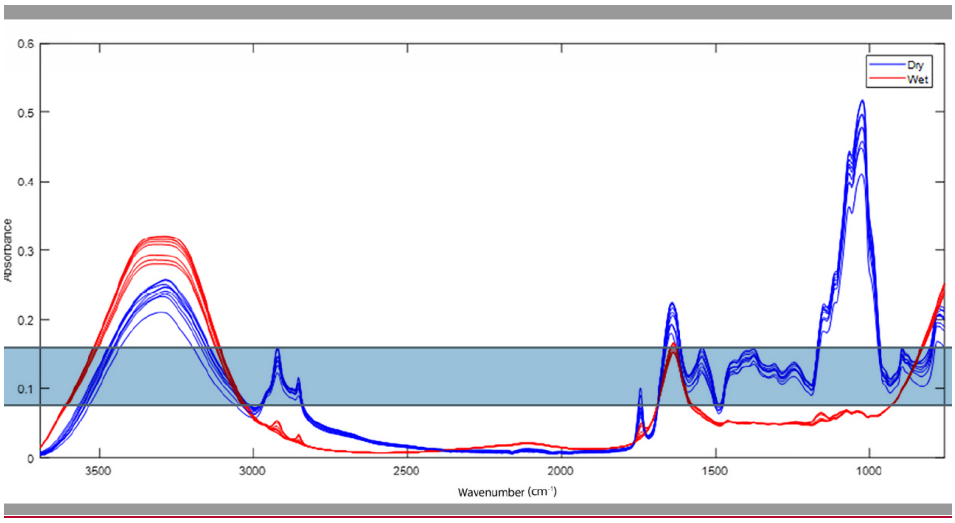


Figure 2. Averaged ATR FT-IR spectrum of dried milk at t = 0 (red) and t = 102 hrs (blue) with n = 24 spectra per time point and the standard deviation (SD) of each set. The second derivative spectra are shown in the inserted boxes. Controls, M4 and M10, have been excluded from the data as they were refrigerated and thus no observable change occurred over the 102 hours of the experiment. Significant bands are labelled in Table I.

DOI: 10.1177/0003702819842548

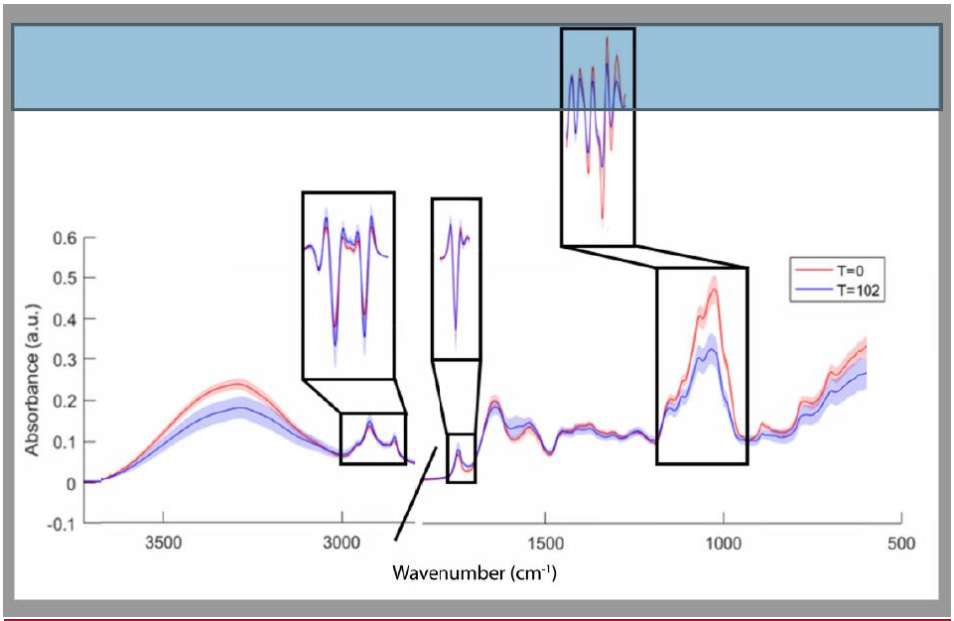


Figure 3. Mapping of spectral changes within milk over time against a linear and a sinusoidal regression with controls removed from the model. (a) Time predicted with the use of a PLS regression model (based on ATR FT-IR spectra) versus the actual Time. The RMESCV value for the PLS regression model was 16.80 with a R^2 value of 0.7442 (b) the loadings corresponding to the PLS model of Figure 2a, indicating that the model was based off changes in lipids and carbohydrates (c) Time predicted with the use of an SVM model (based on ATR FT-IR spectra) versus the actual time. The RMESCV value for the SVM model was 11.08 and the R^2 value was 0.8756.

DOI: 10.1177/0003702819842548

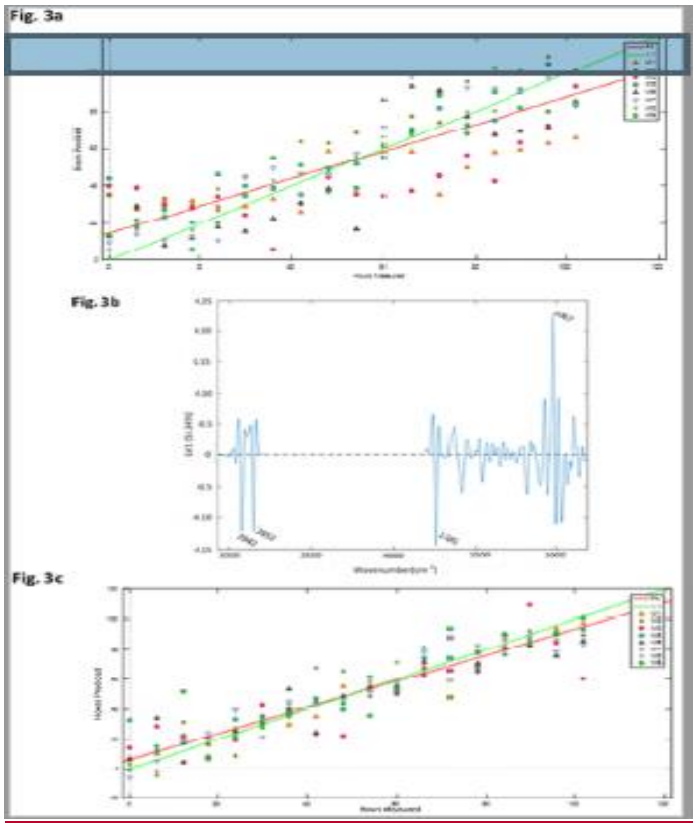


Figure 4. Mapping of spectral changes within milk over time against a linear and a sinusoidal regression. Six samples were used to create and validate the model whilst four samples, M2, 4, 8, and 10 were applied to the model for prediction. (a) Time predicted with the use of a PLS regression model (based on ATR FT-IR spectra) versus the actual Time. The RMESCV value for the PLS regression model was 17.43 and the R^2 value was 0.6896, (b) the loadings corresponding to the PLS model of Figure 3a, indicating that the model was based off changes in lipids and carbohydrates, and (c) time predicted with the use of an SVM model (based on ATR FT-IR spectra) versus the actual time. The RMESCV value for the SVM model was 15.22 and the R^2 value was 0.8308.

DOI: 10.1177/0003702819842548

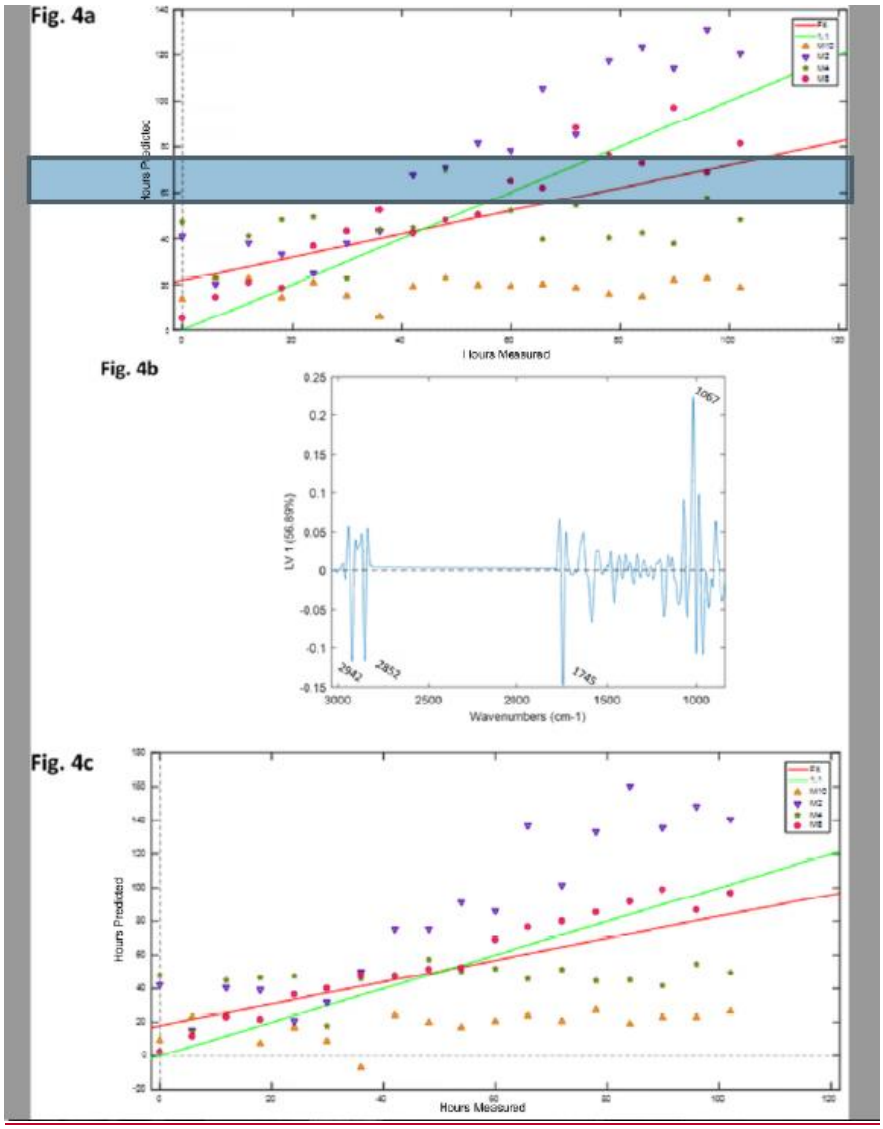
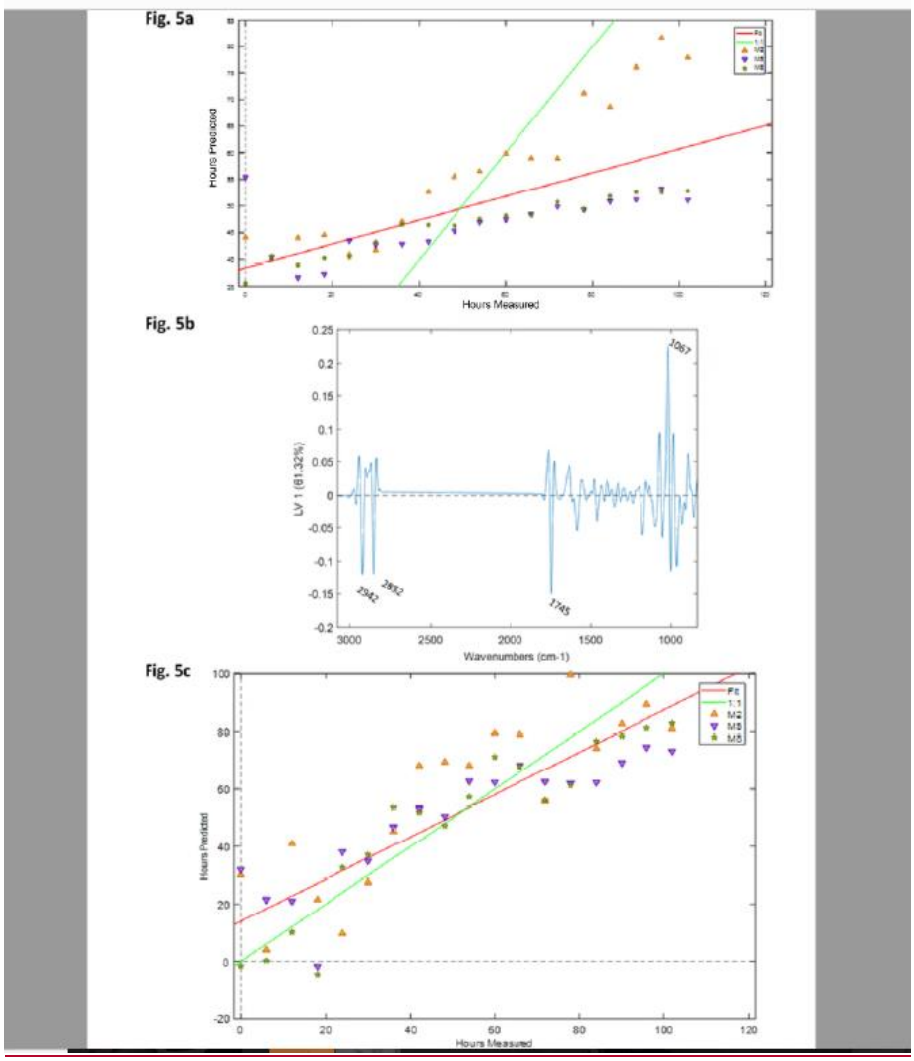


Figure 5. Mapping of spectral changes within milk over time against a linear and a sinusoidal regression. Five samples were used to create and validate the model whilst four samples, M2,4,8 and 10 were applied to the model for prediction. (a) Time predicted with the use of a PLS

DOI: 10.1177/0003702819842548

regression model (based on ATR FT-IR spectra) versus the actual time. The RMESCV value for the PLS regression model was 16.951, the RMSEP was 20.28 and the R^2 value was 0.7264 (b) the loadings corresponding to the PLS model of Figure 4a, indicating that the model was based off changes in lipids and carbohydrates (c) Time predicted with the use of an SVM model (based on ATR FT-IR spectra) versus the actual time. The RMESCV value for the SVM model was 11.25, the RMSEP was 14.31, and the R^2 value was 0.8462.

DOI: 10.1177/0003702819842548



Supplemental Material

DOI: 10.1177/0003702819842548

Determining the Age of Spoiled Milk from Dried Films Using Attenuated Reflection Fourier Transform Infrared (ATR FT-IR) Spectroscopy

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Table S1. Actual versus predicted spoilage time for the controls.

	PLS-Predicted		SVM -Predicted	
Actual Time (hours)	M4 (hours)	M10 (hours)	M4 (hours)	M10 (hours)
0	42.7	9.9	41.5	9.8
6	26.5	3.3	42.7	0.7
12	35.2	14.4	34.0	9.4
18	44.1	7.4	41.5	8.0
30	23.6	15.3	31.5	8.8
24	48.6	15.5	43.5	9.0
36	40.8	9.3	42.5	6.9
42	37.6	15.3	38.4	18.9
54	44.7	12.7	41.1	9.5
48	57.3	3.2	62.0	5.0
60	46.7	9.6	51.4	10.2
66	32.4	7.1	40.1	10.7
78	31.9	5.4	37.7	4.7
72	45.9	10.2	40.2	10.4
84	38.8	1.6	44.6	16.1
90	32.9	12.9	37.6	19.0
96	53.8	2.5	49.1	10.9
102	42.8	12.8	39.4	17.7