"Influence of different storage conditions on the performance of spray-dried yogurt used as inoculum for milk fermentation"

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Short title: Spray-dried yogurt and milk fermentation

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Summary

In present study a commercial drinkable yogurt with and without 4% of added trehalose (as cell protectant) was spray-dried obtaining a powder with low water activity (a_w). Total bacterial count in the powder was between 8.48-8.90 log cfu/g. The dried yogurt was stored: i) at 38ºC and a_w = 0.33; ii) at 38ºC in hermetically sealed flasks (a_w = 0.21/0.22); iii) in a cyclic temperature chamber (10-20ºC) in hermetically sealed flasks (a_w = 0.21/0.22). Whole milk was then fermented by adding an inoculum of spray-dried yogurt after storage under the abovementioned conditions. The kinetics of acidification evidenced the presence of a *lag* time which was strongly dependent on storage conditions. The data was fitted with a logistic type equation from which the *lag* time was calculated. To evaluate structural differences among samples, Fourier Transform Infrared spectra (FTIR) were recorded. Partial Least Squares (PLS) models enabled a good correlation between *lag* time of fermentation and FTIR spectra. The *lag* time for yogurt powder stored at a_w about 0.21/0.22 and cyclic temperature 10-20 ºC remained approximately constant over the 12 weeks of storage, while all the other conditions resulted in a dramatic increase. The addition of trehalose had a small influence on *lag* time and, therefore, as a protectant of lactobacilli.

Keywords: Spray-drying; Yogurt; FTIR; acidification; *lag* time

Introduction

In response to consumer demand for foods with health benefits, the food industry began to diversify its products. Although most foods containing probiotic bacteria are dairy products, there is also a growing demand for incorporating probiotics in other food products. Spray-drying is widely used in the food, pharmaceutical and other industries because of its ability to efficiently transform a liquid feed into a dry powder (Huang, 2011). Recent references highlight that spray drying technique is a suitable long-term preservation method for liquid and semi-liquid food also used for the stabilization of matrices containing probiotic bacteria, namely yogurt and other dairy products (Barbosa et al., 2017; Reale et al., 2019).

The shelf-life of yogurt can be considerably changed by spray-drying resulting in a stable powder of high quality, without the need for refrigeration (Kumar and Mishra, 2004, Guergoletto et al., 2012). This yogurt powder can also be used as an ingredient for the manufacturing of several food products such as confectioneries, yogurt drinks, fruit mixes, etc, as well as for direct consumption after reconstitution (Kumar and Mishra, 2004). It is to be noted according to the Food and Agricultural Organization (FAO), the microorganisms in yogurt must be viable and in sufficient amount to promote health benefits (Guergoletto et al., 2012); this is usually greater than 6-7 log of colony forming units (cfu) of viable microorganisms per gram of product at the time of being consumed (Aquilina et al., 2013).

Survival of microorganisms in dried matrices or products depends on many factors including species and strain, drying conditions, the use of cell protective agents and storage conditions (*i.e.,* water activity (a_w), temperature and time). The decay of viable cell count is the main drawback when dehydrating probiotic formulations, and is associated to the severity of processing and storage conditions (Succi et al., 2014; Fiore et al., 2010). Cell protectants have been used to increase the survival of lactic acid bacteria after drying and storage. Among them, milk powder, whey, trehalose, sucrose, lactose and some oligo and polysaccharides can be mentioned (Schoug 2009, Meng et al, 2007). Very recently, Stefanello et al. (2018) reported on the role of trehalose as bioprotectant in freeze-drying.

The dried state limits the number of techniques that can be applied to study conformation and stability of intracellular biomolecules (Wolkers and Oldenhof, 2005). One of the few suitable techniques for *in situ* analysis of biomolecules in the dried state is Fourier Transform Infrared Spectroscopy (FTIR). On account of characteristic molecular vibrations that absorb in the infrared region, information can be derived on molecular conformation and intermolecular interactions of biomolecules in their native environment (Wolkers and Oldenhof, 2005).

The objective of this work was to obtain basic information for the development of a powdered drinkable "yoghurt". For this reason, we utilized as raw material a commercial drinkable formulation (see Materials and Methods) containing not only *L. bulgaricus* and *S. thermophilus*, but also, *L. casei* and *Bifidobacterium spp*. In studies of the stability of yogurt powder it is customary to determine the survival of lactic acid bacteria during storage (Kumar and Mishra, 2004). In the present study a different approach was selected. Instead of enumerating lactic bacteria during storage, whole milk was fermented using an inoculum of spray-dried yogurt previously stored for different times and under different conditions. The measured acidification kinetics by the lactobacilli present in dried yogurt was used as an indicator of the injuries caused by the different conditions of a_w, time and temperature during storage. A FTIR analysis of the stored powder samples was also performed to evaluate the spectral differences among samples, arising from the different storage conditions assayed. The influence of the addition of trehalose, a well-known cell protectant, was also studied.

Materials & Methods

Drinkable Yogurt

A commercial, drinkable yogurt without added sugar ("*SER*", Danone, Argentina) was used. The yogurt had the following composition: 8.15% (± 0.02) dried extract, 4.45 % carbohydrates, 3.15 % protein, 0% fat and pH 4.40 (± 0.02).

The list of ingredients included: defatted milk, lactic ferments (*Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium* spp., *Lactobacillus casei*), modified starch, stabilizers (gelatin, gellan gum), intensive sweeteners and artificial strawberry flavor.

Water activity, moisture content and pH

Water activity (a_w) was measured using a dew point hygrometer "Aqualab" Series 3 (Decagon Devices, USA) previously calibrated in the range of interest with standard saturated salt solutions. Moisture content was determined gravimetrically (2g sample) in a forced convection constant temperature oven at 90ºC during 6 hours. The pH of fresh yogurt and inoculated milk were measured with a pH meter (Hanna Instruments, Italy) previously calibrated with appropriate buffers. All measurements were carried out in triplicate.

Reagents

Salts for relative humidity control (reagent grade) were obtained from Biopack (Buenos Aires, Argentina). Food grade trehalose (dehydrate) was obtained from Hayashibara Co. Ltd. (Japan).

Spray-drying

The drinkable yogurt, as such or after the addition of 4 % trehalose, was spray-dried in a mini spray-dryer Büchi model B-290 (Büchi Laboratoriums Technik, Switzerland). The main operating conditions were selected so that the air outlet temperature did not exceed 70ºC, being this an important condition to obtain an adequate survival of lactobacilli (Zhang et al., 2016). A combination of an inlet air temperature of 140ºC and a feed (yogurt) flow rate of 500 g/h allowed to obtain an outlet temperature of 68-70ºC as well as a low a_w (about 0.20). The spray-drying runs were performed in duplicate and the yield was around 50%.

Plate counting

Bacterial viability was determined before and after spray-drying and in the presence (4%) or absence (0%) of trehalose. For each determination, samples were rehydrated in 1ml 0.85% w/w NaCl. Bacterial suspensions were serially diluted, plated on MRS agar (de Man et al., 1960) and incubated at 37ºC for 48 hours in aerobic conditions. Results were expressed in log cfu/ml and are the average of three independent determinations.

Storage conditions

Spray-dried yogurt powders (0% and 4% trehalose) were stored in the following conditions:

(a) hermetically sealed flasks to preserve its initial $a_{w.}$ Some were kept in a constant temperature oven at 38ºC and others in a cyclical temperature chamber where the temperature was maintained at 10ºC for 12 hours and then 12 hours at 20ºC. This cyclical variation of temperature adequately represents the daily variation of temperature in Buenos Aires between the months of August through November. The temperature of 38 ºC is often utilized for studies of accelerated storage of foods (Labuza, 1982).

Total storage time was 12 weeks; one sample from each condition was removed each week and kept in a freezer at -72ºC until the different analyses were carried out. **Table 1** shows abbreviated codes used for stored samples of dried yogurt.

- Insert Table 1 about here -

Kinetics of milk fermentation using yogurt powder as inocula

Pasteurized whole milk was fermented by adding an inoculum of the spray-dried yogurt (previously stored at different conditions as explained in section 2.6) and the kinetics of fermentation were evaluated by determining their rate of acidification (pH decrease). The following procedure was used: 1) sterilization of glass jars, 2) preheating of milk at 43 ºC, 3) inoculation of 20 ml of milk in the jars with 1.0g of yogurt powder without trehalose, or 1.45g of yogurt powder with 4% trehalose, and 4) glass jars were kept in a constant temperature bath at 43ºC and pH was measured every 30 minutes during a 6 hours period; pH measurements were made in triplicate. An excess of inoculum (1.45 g instead or 1.0 g) was used in the yogurt powder runs with trehalose to compensate for the dilution of yogurt caused by the addition of trehalose.

Fourier Transform Infrared Spectra (FTIR)

About 5mg of dried yogurt powder stored under the different conditions (as explained in section 2.6) were used to register the FTIR spectra. Samples were placed on the sample holder of an ATR-FTIR Thermo Nicolet iS10 spectrometer (Thermo Scientific, MA, USA). Spectra were registered in the 4000-500 cm^{-1} range by co-adding 100 scans with 4cm^{-1} spectral resolution, using OMNIC software (version 8.3, Thermo Scientific, MA, USA). At least three spectra were recorded.

Data Analysis

Multivariate analysis and data pre-processing as mean centering and extended multiplicative scatter correction were performed on the FTIR spectra, using The Unscrambler software (version 10.2, CAMO, Norway). To evaluate the spectral differences among samples, arising from the different storage conditions assayed, a principal component analysis (PCA) was performed on the FTIR spectra. Taking into account the spectral differences in the PCA scores plot, PLS models were calibrated to determine the *lag* time (Esbensen, 2005). FTIR spectra covering the whole range of values were used to define the models. To set-up the PLS models, carried out on the raw spectra, 54 spectra were registered for calibration. Leave-one-out-cross validation method (LOOCV) was used for validation, and 7 PLS-factors were used for prediction. *Lag* times as results of experiments described in Section 2.7 were used as reference values. The reliability and robustness of the calibrated models were determined as a function of their correlation values, R-square, BIAS and their calibration errors (RMSEC).

Results & Discussion

Moisture content, a_w and microbiological analysis of spray-dried yogurt

Fresh drinkable yogurt with (4%) and without added trehalose was spray-dried using the aforementioned operating conditions. The outlet air temperature not only influences the survival of lactic bacteria but also the a_w of the powder, so a compromise value was taken since a decreased outlet temperature improves survival but also increases final a_w. The yogurt powder spray-dried with 140ºC air inlet temperature and 68/70ºC outlet air temperature had the following a_w and moisture content (dry basis): 0.206 ± 0.005 and 5.32 ± 0.15, respectively for yogurt without trehalose, and 0.225 ± 0.001 and 5.16 ± 0.15, respectively for yogurt with 4% trehalose. Kumar and Mishra (2004) suggested that for a better survival of *Streptococcus*, *Lactobacillus* and *Bifidobacterium* spp. during storage the a_w of yogurt powder should be about 0.20, which is in good agreement with the present values. Microbiological analysis indicated that fresh yogurt had a total bacterial count of 8.51 log cfu/ml, plain dried yogurt had 8.48 log cfu/g and dried yogurt added with trehalose (4%) had 8.89 log cfu/g. Reconstituted (to their initial solids content) spray-dried yogurt was also analyzed and total bacterial counts were of 7.81 log cfu/g for 0TR, and 7.96 log cfu/g for 4TR. These values indicate satisfactory survival of yogurt cultures (Zhang et al. 2016, Bielecka and Majkowska, 2000) and are very similar to those reported by Koc et al (2010) for spray-dried yogurt.

Kinetics of milk fermentation using inoculum of yogurt powder

Whole milk was fermented using inocula of spray-dried yogurt previously stored for different times and conditions. **Figure 1** shows the measured kinetics of acidification using yogurt powders inocula; for the purposes of clarity only eight curves were displayed. The standard deviation for pH values ranged between 0.04 to 0.06. The eight conditions plotted in Figure 1 adequately illustrate the general shape of pH curves obtained after mild or drastic storage conditions. However, as will be shown later, a much larger number of conditions (several combinations of a_w, temperature, presence of trehalose and time) will be utilized for further calculations. The measured kinetics of fermentation was used as an indication of the metabolic activity. Golowczyc et al. (2010) also measured the acidification kinetics in milk by fresh and dried lactobacilli to evaluate the injuries caused by spray-drying on lactobacilli isolated from kefir grains.

\- Insert **Figure 1** about here -

In all acidification curves, a period during which the pH remains approximately constant (*lag* time) was observed; in most of them this was followed by a rapid fall in the pH values. The *lag* time is associated with the time for repair sublethal injuries experienced by lactobacilli stored under various stress conditions. In cases of extreme storage conditions (38ºC and a_w=0.33), the *lag* time was very long and even 400 minutes was not enough for obtaining the final pH of around 4.5. In order to better understand the relationship between storage condition, time and addition of trehalose, all acidification curves were fitted using a logistic type equation (eqn.1) (Romano et al. (2016).

$$pH(t) = \frac{pH_0 - pH_f}{1+\frac{t}{c}^{p}} + pH_f \qquad \text{(Eqn. 1)}$$

where,

pH(t): is the pH at different fermentations times (every 30 minutes)

pH_0: is pH at the beginning of milk fermentation

t: time (minutes)

The obtained variables were,

c: time at which the inflection point occurs in the fitted function

p: exponential adjustment factor

pH_f: pH at the end of the fermentation

The *lag* time was calculated as the intersection between the tangent line at $t= c$ and pH_0. **Figure 2** shows the effect of storage time of yogurt powder (inocula) on the *lag* time during milk acidification.

- Insert **Figure 2** about here -

It can be seen that the *lag* time for yogurt powder of both samples stored at the most gentle condition, which was 10/20ºC and a_w=0.22, remains approximately constant over the 12 weeks of storage (samples codes 0TR-10/20-Ai-Wn and 4TR-10/20-Ai-Wn). For these samples, trehalose showed a modest decrease in the *lag* period, suggesting a small effect as cell protector; this was only perceived under this gentle storage condition. At all other conditions of a_w and temperature, the *lag* time increased rapidly with storage time up to a point (8 weeks) at which all samples either leveled off or tended to vary in a more or less erratic way (data not shown). It is clear that both the a_w and the temperature of storage are key factors determining bacterial viability during storage; even small variations in a_w (0.21/0.22→0.33) and temperature (10/20ºC→38ºC) were highly detrimental of cell functioning.

FTIR

FTIR spectroscopy was used to monitor the changes in *lag* period experimented by spray-dried yogurt stored under different conditions. In order to discriminate spectra according to the physical conditions used for storage, and to avoid the interference of trehalose, strongly absorbing in the infrared region of the electromagnetic spectrum, two different PCA was carried out on spray-dried yogurt without (**Figure 3A**) and with the protective compound trehalose (**Figure 3B**). Samples of yogurt powders spray-dried without trehalose, were mainly distributed along PC2, which explained 25% of the variance. In turn, yogurt powders containing trehalose were mainly grouped along PC1, which explained 58% of the variance. However, regardless of the presence of trehalose, sample grouping was associated to differences in the *lag* times. FTIR spectroscopy was used by Kher et al. (2007) to examine the conformation of proteins in spray-dried milk protein concentrate prepared from a range of processing conditions and after storing for 4 weeks at 21 ºC. They reported that FTIR spectroscopy could be used to predict protein behavior since the obtained data correlated well with changes in solubility of the dry powders on storage.

- Insert **Figure 3** about here -

According to the loading plots in PC1 (**Figure 4**), the main differences among samples were observed in the 1200-900 cm^{-1} region, associated to the C-O-C glycosidic linkage, the δCOH and the νC-C vibrational modes (Santos et al., 2014a). For example, 0TR-38-33 samples stored for 12 weeks showed the largest *lag* times and were observed at the highest PC1 values (**Figure 3A**, open circles). On the contrary, sample 0TR-10/20-Ai stored for shorter times (squares-open, gray or black in **Figure 3A**) were observed in the other extreme of the plot. Samples with *lag* times within these two extremes were observed at intermediate positions. A similar behavior was observed along PC1 for yogurt powders containing trehalose (**Figure 3B**).

- Insert **Figure 4** about here –

A PLS model was defined to determine the *lag* time directly from the FTIR spectra**.** In the definition of such model, results obtained in section 3.2 were used as references. The PLS model was calibrated using 54 spectra (correlation of 0.989; R^2, 0.977; Root Mean Square Error of Calibration (RMSEC), 15.95; Standard Error of Calibration (SEC), 16.101; BIAS, -1.907×10^{-6}), and cross-validated (**Figure 5**). Using spectra covering a broad range of *lag* times strengthened the predictive capacity of the model. The mean of the predicted values fitted nicely to those shown in **Figure 2** and were not dependent on the presence (or absence) of trehalose in the samples. This broadens the application of the obtained model, supporting its use to investigate unknown samples. Considering that the *lag* time is closely related to the functionality of yogurt powders, using the defined PLS model, would be valuable to determine this parameter just by registering FTIR spectra.

\- Insert **Figure 5** about here –

Conclusion

Whole milk was fermented using inocula of spray-dried yogurt (with and without added trehalose) previously stored for up to 12 weeks at different a_w and temperature. The *lag* time in the kinetics of milk acidification was associated with the impact of the storage condition and the presence of trehalose on the repair sublethal injuries experienced by lactobacilli. *Lag* times for yogurt powder of samples stored at the most gentle condition, which was 10/20ºC and a_w=0.22, remained approximately constant over the 12 weeks of storage. For these samples, trehalose showed only a modest decrease in the *lag* time, suggesting a small effect as cell protector which was only perceived under this gentle storage condition. At the other conditions of a_w and temperature, the *lag* time increased rapidly with storage time and the presence of trehalose could not counterbalance the negative effect of temperature and a_w. Both, a_w and temperature of storage were key factors determining sublethal injuries during storage; even small variations in a_w (0.21/0.22$\rightarrow$0.33) and temperature (10/20ºC$\rightarrow$38ºC) were highly detrimental of cell functioning.

Partial Least Squares (PLS) models enabled a fairly good correlation between *lag* time of fermentation and FTIR spectra. Using the defined PLS model, would be valuable to predict *lag* time just by registering FTIR spectra.

Acknowledments

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Figure legends:

Figure 1:

Kinetics of milk fermentation using yogurt powder inocula for some of the storage conditions for samples with (black reference) and without (grey reference) trehalose. Data shown are the average of three independent determinations. Error bars for pH are not observed because they overlap with data symbols.

Figure 2:

Effect of storage time of yogurt powder on the *lag* time during milk acidification. Data shown are the average of three independent determinations.

Figure 3:

PCA performed on the spray-dried yogurt samples, stored at different a_w and temperatures. **A:** without trehalose; **B:** with trehalose. Squares: 0TR-10/20-Ai or 4TR-10/20-Ai; circles: 0TR-38-33 or 4TR-38-33; triangles: 0TR-38-Ai or 4TR-38-Ai. Full black symbols (squares, circles and triangles): samples stored for one week; full gray symbols: samples stored for 6 weeks; empty symbols: samples stored for 12 weeks.

Figure 4:

1-D Loading plots in PC1 and PC2, performed on the FTIR spectra of powder yogurts without (A) and with (B) trehalose.

Figure 5:

Predicted *vs* reference values for *lag* time.

Table 1- Description of abbreviated codes used for stored samples of dried yogurt

Code	Trehalose (%)	Storage temperature (ºC)	a_w of dried yogurt during storage
0TR*	0	-	-
4TR(**)	4	-	-
0TR-10/20-Ai-Wn	0	10 ←→20 (***)	0.21
4TR-10/20-Ai-Wn	4	10 ←→ 20	0.22
0TR-38-Ai-Wn	0	38	0.21
4TR-38-Ai-Wn	4	38	0.22
0TR-38-33-Wn	0	38	0.33
4TR-38-33-Wn	4	38	0.33

(*) 0TR= 0% of trehalose addition

(**) 4TR= 4% of trehalose addition

(***) Cyclic temperatures: 12 hours cycles (20°C and 10°C) resembling the daily variation of temperature in a temperate climate region.

Ai= *As is*; in relation to the water activity of the powder

Wn: weeks stored, n = 1 to 12

Figure 1:

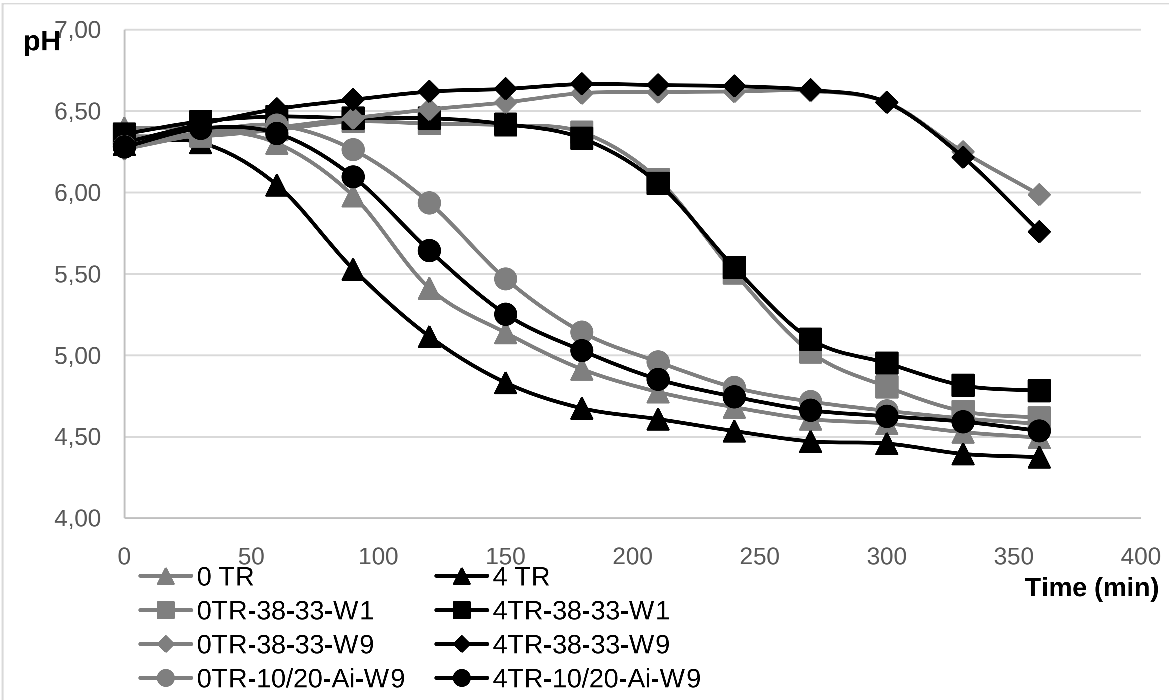

pH
7,00
6,50
6,00
5,50
5,00
4,50
4,00
0
50
100
150
200
250
300
350
400
Time (min)
0 TR
4 TR
0TR-38-33-W1
4TR-38-33-W1
0TR-38-33-W9
4TR-38-33-W9
0TR-10/20-Ai-W9
4TR-10/20-Ai-W9

Figure 2:

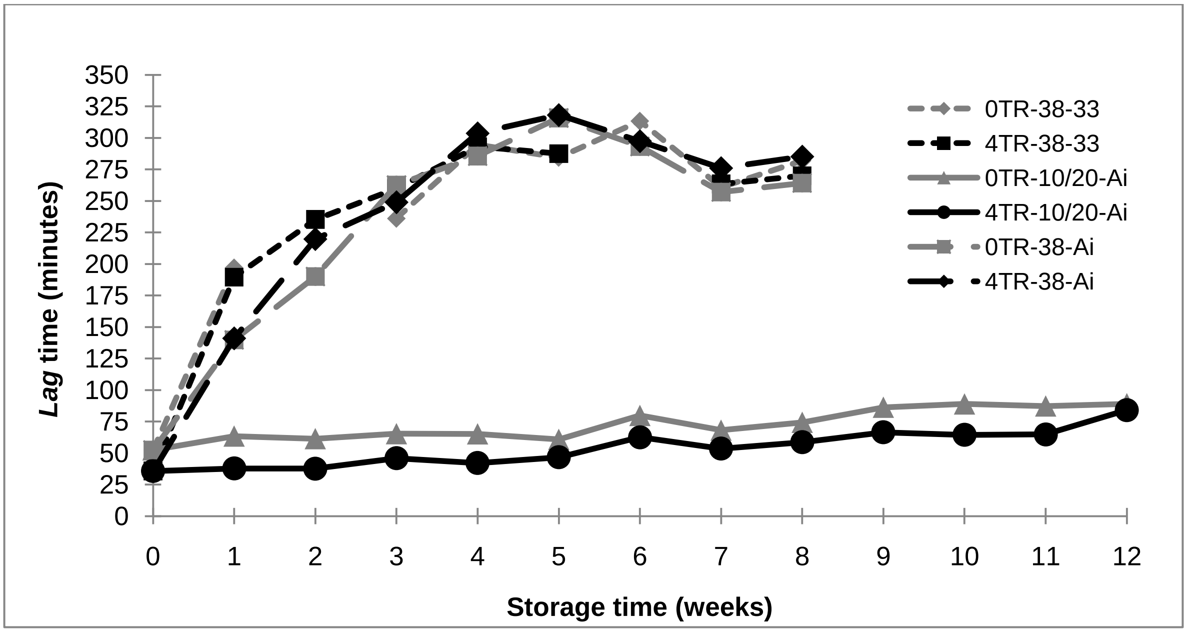
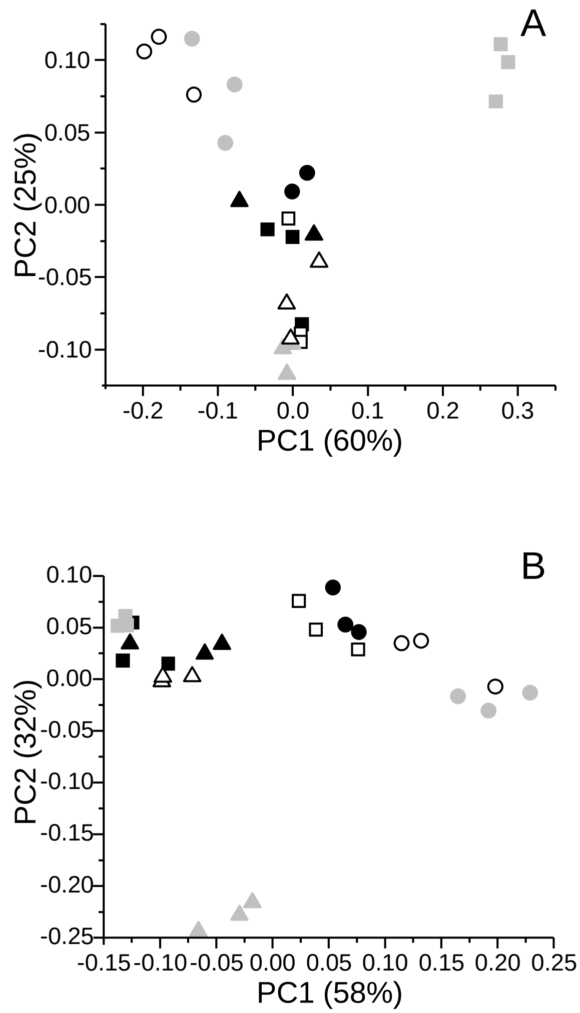

350
325
300
275
250
225
200
175
150
125
100
75
50
25
0
Lag time (minutes)
0
1
2
3
4
5
6
7
8
9
10
11
12
Storage time (weeks)
0TR-38-33
4TR-38-33
0TR-10/20-Ai
4TR-10/20-Ai
0TR-38-Ai
4TR-38-Ai

Figure 3:


A
PC2 (25%)
0.10
0.05
0.00
-0.05
-0.10
-0.2
-0.1
0.0
0.1
0.2
0.3
PC1 (60%)
B
PC2 (32%)
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
-0.15
-0.10
-0.05
0.00
0.05
0.10
0.15
0.20
0.25
PC1 (58%)

-

Figure 4:

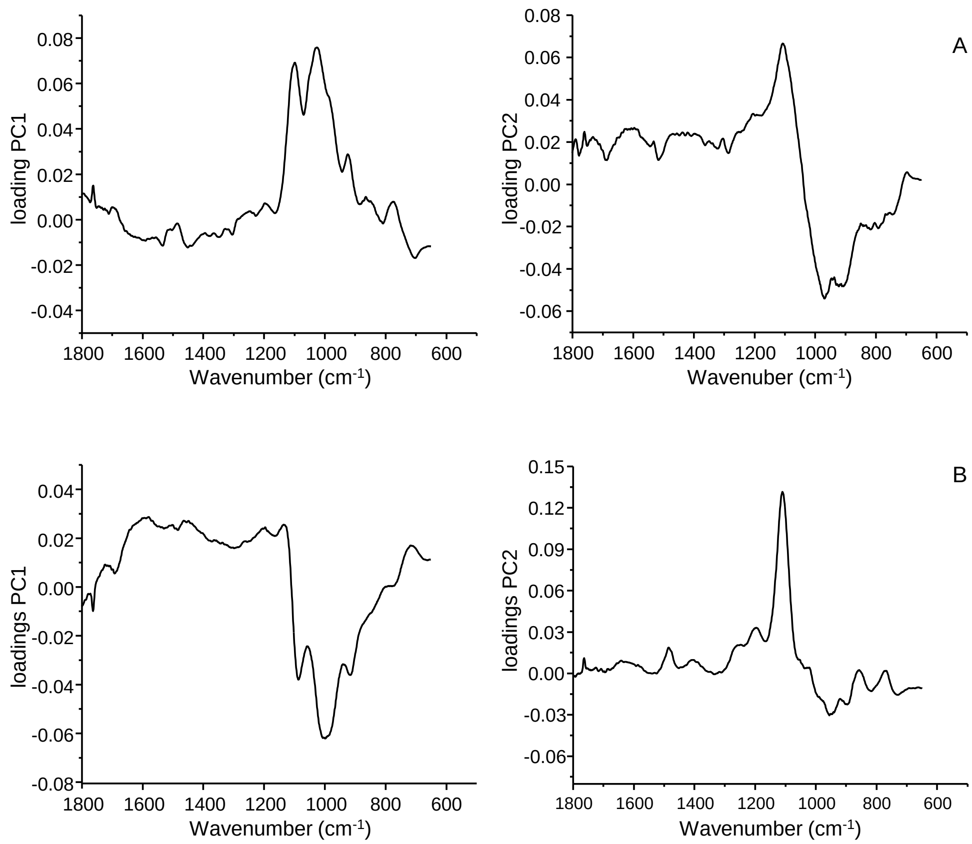
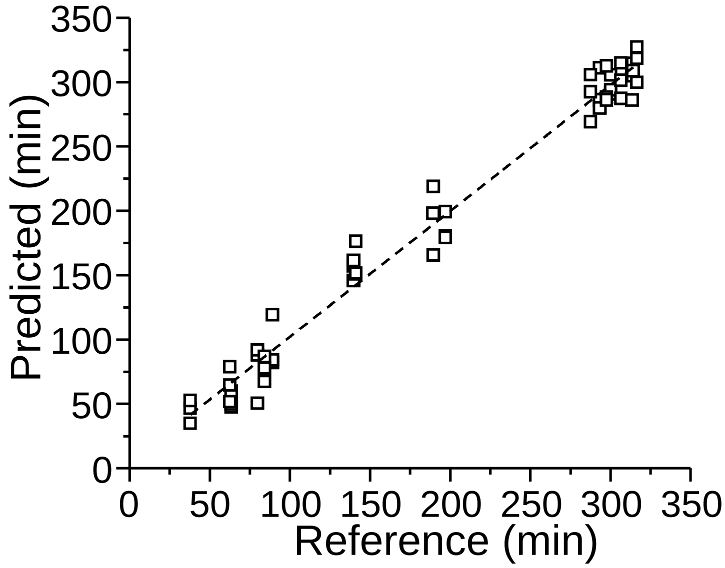

A
B
loading PC1
loading PC2
loadings PC1
loadings PC2
Wavenumber (cm-1)
Wavenuber (cm-1)
1800 1600 1400 1200 1000 800 600

Figure 5:


350
300
250
200
150
100
50
0
Predicted (min)
0
50
100
150
200
250
300
350
Reference (min)