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Light-assisted drying (LAD) for anhydrous preservation of biologics: Using Raman spectroscopy to assess the uniformity of drying in processed samples

Riley Q. McKeough[a], Daniel P. Furr[a], Fan Zhang[b], Yong Zhang[b] and Susan R. Trammell*[a]
[a] Dept. of Physics and Optical Science, Univ. of North Carolina at Charlotte, 9201 University City Blvd., Charlotte, NC USA 28223-0001
[b] Dept. of Electrical and Computer Engineering, Univ. of North Carolina at Charlotte, 9201 University City Blvd., Charlotte, NC USA 28223-0001

ABSTRACT

Protein-based therapeutics have been developed to treat a range of conditions and assays use immobilized capture proteins for the detection of diseases. A challenge in the development of protein-based products is maintaining the protein in the folded state during processing and storage. The most common method of stabilizing proteins for storage is lyophilization. However, the freeze-drying process remains expensive and many proteins that are lyophilized must be refrigerated or frozen to maintain functionality. Cold storage strategies can be challenging for the transportation of protein-based products and can be difficult or impossible in low resource settings. Recent research has demonstrated that anhydrous, or dry state, preservation in a trehalose amorphous solid matrix offers an alternative to freeze drying for the preservation of biologics. We have previously described a new processing technique, light assisted drying (LAD), to create trehalose preservation matrices. LAD uses illumination by near-infrared laser light to selectively heat water and speeds dehydration of small volume (40 μL) samples. Low end moisture contents (EMC's) are necessary for storage at supra-zero temperatures and this low water content must be uniform to insure successful long-term storage of embedded biologics. Our previous work has demonstrated the ability of LAD to reach EMCs necessary for storage at elevated temperatures. In this work, Raman spectroscopy is used to assess the trehalose distribution and water content across LAD processed samples. Results indicate that the water content of LAD process samples is uniform. LAD is a promising technique for processing biologics in preparation for anhydrous storage.

Keywords: anhydrous preservation, protein stabilization, optical processing, near IR irradiation, Raman spectroscopy

1. MOTIVATION AND BACKGROUND

Protein-based drugs have been developed to treat diseases ranging from arthritis to psoriasis [1, 2] and diagnostic assays use immobilized capture proteins for disease detection and monitoring protein levels.[3, 4] In addition, newly developed microfluidic devices require the use of protein indicators.[5] A challenge in the development of protein-based products is maintaining the protein in the folded state during processing and storage, as the three-dimensional structure of the protein is often responsible for its functional activity. The most common method of stabilizing proteins for storage and later use is lyophilization (freeze drying). However, the freeze-drying process is not effective for all proteins, remains expensive and requires lengthy processing times. In addition, many proteins that are lyophilized still must be refrigerated or frozen to maintain functionality.[6-9] Cold storage strategies can be challenging for the transportation of protein-based products and can be difficult or impossible in low resource settings due to a lack of available infrastructure.

Recent research has demonstrated that anhydrous, or dry state, preservation in a trehalose amorphous (non-crystalline) solid matrix may be an alternative to freeze drying for the preservation of biological samples.[10-12] An amorphous solid restricts molecular motion to a small volume over a finite time period, which can prevent the degradation of biologics, such as proteins, embedded in the matrix. Disaccharide trehalose can form a low mobility amorphous solid at room

*srtramme@uncc.edu

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temperature and can also act as a bioprotectant, making trehalose an attractive option as a preservation matrix for embedded biologics.

We have previously described a new processing technique, light assisted drying (LAD), to create trehalose amorphous solids for the preservation of biologics.[13] LAD uses illumination by near-infrared laser light to assist in the formation of trehalose preservation matrices. LAD selectively heats water to overcome cooling due to evaporation and speeds dehydration of the samples. As water is removed from the sample, the remaining sugars and salts become concentrated, and, as long as the solutes do not crystallize, the viscosity increases with progressive water loss until an amorphous solid is achieved. Because a substantial reduction of molecular mobility is necessary to ensure an extended shelf life, samples generally need to be stored below the glass transition temperature, T_g, of the trehalose matrix to prevent degradation.[14] The glass transition temperature for an amorphous trehalose solid formed by dehydration depends on the amount of water remaining in the sample after processing. The more water that remains in the sample, the lower the glass transition temperature. Low end moisture contents (EMC's) are necessary for storage at supra-zero temperatures and this low water content must be uniform to insure successful long-term storage of embedded biologics. Our previous work has demonstrated the ability of LAD to reach EMCs necessary for storage at elevated temperatures.[13] In this work, Raman spectroscopy is used to assess the distribution of trehalose and water as a function of position in LAD processed samples.

2. METHODS

A schematic of the LAD processing system is shown in Figure 1. An IPG Photonics continuous wave (CW) Nd:YAG at 1064 nm (YLR-5-1064) laser source was used for LAD processing (maximum power output of 5 W). The laser has a factory collimated Gaussian beam with a FWHM spot size of ~4.5 mm which was measured using a BeamTrack 10A-PPS thermal sensor (Ophir Photonics). A FLIR SC655 mid-IR camera was used to record the temperature of samples during processing. All studies were performed in a humidity-controlled environment that was kept at approximately 11% RH. This was achieved by pumping dry air into a chamber containing the experimental setup and monitoring the RH with a temperature and RH logger (ONSET UX100-011). Maintaining a low relative humidity expedited the drying process.

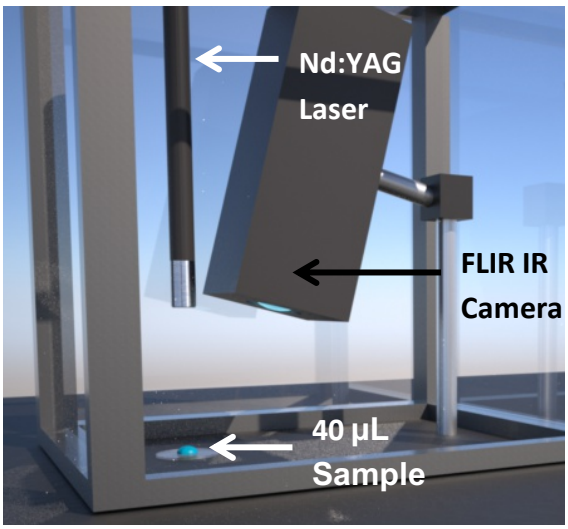

Figure 1: Experimental set-up of light-assisted drying (LAD) technique within a controlled low relative humidity chamber.

Samples consisted of 40µl droplets containing a model protein, egg white lysozyme (Worthington Biochemical LS002933), dissolved in drying solution (DS) at a concentration of 0.5 mg/ml. This concentration was verified using the absorption of light at 280 nm with a microplate spectrophotometer (Bio-Tek Synergy HT). The DS consisted of 0.2M disaccharide trehalose in 0.33 x phosphate buffer solution (PBS). The dry weight of the DS was determined through bake out method to be 7.01% the mass of a sample.

For each test, a 40 µL droplet of the protein/drying solution was deposited onto an 18 mm diameter borosilicate glass coverslip (Fisherbrand 12-546) substrate. The glass coverslips allow for easy recovery and rehydration of the proteins after

LAD processing. On the glass coverslips, the samples were droplets roughly 2 mm in thickness with a diameter of approximately 7 mm. The initial mass was determined gravimetrically using a 0.01mg readability balance (RADWAG AS 82/220.R2). The sample was then moved into the humidity chamber for laser irradiation. Samples were processed for 60 minutes at 5 W (26.9 W/cm^2). The temperature of the sample was monitored during processing using the thermal camera. Samples reached a maximum sample temperature of 44.4 ± 0.7 ºC during LAD processing. After irradiation, the sample was removed from the humidity chamber and immediately massed again. End moisture content (EMC), which is a measure of the amount of water relative to the dry mass of a sample was calculated as

$$\text{EMC} = \frac{m_f - m_s - m_{dw}}{m_{dw}} \quad (1)$$

where m_f is the mass of the final sample including the mass of the substrate, m_s, and m_{dw} is the measured dry weight of the initial sample.

Six samples were LAD processed for 1 hour (standard processing time used in previous studies), resulting in an average EMC of 0.22 ± 0.02 gH_2O/gDryWeight. For comparison, two samples were LAD processed for shorter times resulting in higher EMCs (see Table 1). After LAD processing, samples were stored individually in small volume containers above a saturated salt solution of lithium chloride (LiCl) (ChemCenter, La Jolla, CA). The RH of the LiCl saturated salt was 14.3±0.5 RH (measured with a RH probe, HH314A, Omega). Our previous studied have shown that this method of storage keeps the amorphous trehalose matrix stable and embedded proteins undamaged.[15]

Table 1. Samples for which Raman spectroscopy was obtained.

Processing Technique	**Resulting EMC (gH_2O/gDryWeight)**
LAD processed for 1 hour (N=6)	0.22 ± 0.02
LAD processed for 40 minutes (N=1)	0.29
LAD processed for 42 minutes (N=1)	0.43

Raman spectroscopy was performed within 24 hours of LAD processing. Raman spectroscopy was obtained using a Horiba LabRAM HR800 confocal microscope with 1200 g/mm grating and 532 nm excitation laser. A 100× (NA = 0.9) objective resulting in a spot size of 0.72μm was used for all samples with a laser power of 11 mW after the objective, and integration time of 10 seconds (with 3 accumulations) was used to obtain all spectra. Raman spectra were obtained in 5 locations in each sample (see Figure 2), approximately 20μm beneath the surface of the sample.

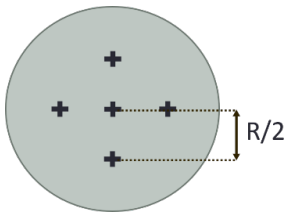

Figure. 2. Schematic of sample showing the five locations at which Raman spectroscopy was obtained. The radius of each sample was 3.5 mm. Raman spectra were obtained 20 μm below the surface of the sample at each location.

3. RESULTS AND ANALYSIS

Figure 3 shows a representative Raman spectrum of a LAD processed sample. Peak 1 (near 850 cm^{-1}) is from a C-O-C skeletal structure and peak 2 (near 900 cm^{-1}) is from a C-C stretch. In our samples, these features are produced by trehalose. Peak 3 (near 3400 cm^{-1}) is an O-H stretch feature that has contributions from both water and trehalose.[16] Line intensities for these three features were measured by removing a baseline (determined in the 1550-2500 cm^{-1} region) and integrating under the features. The intensity ratio of feature 2 to feature 1 was used to evaluate the trehalose distribution in samples.

Ratios of features 3 to 1 and 3 to 2 were used to probe the water distribution in the samples. Average intensity ratios (averaged over 5 positions) for each sample are reported in Table 2.

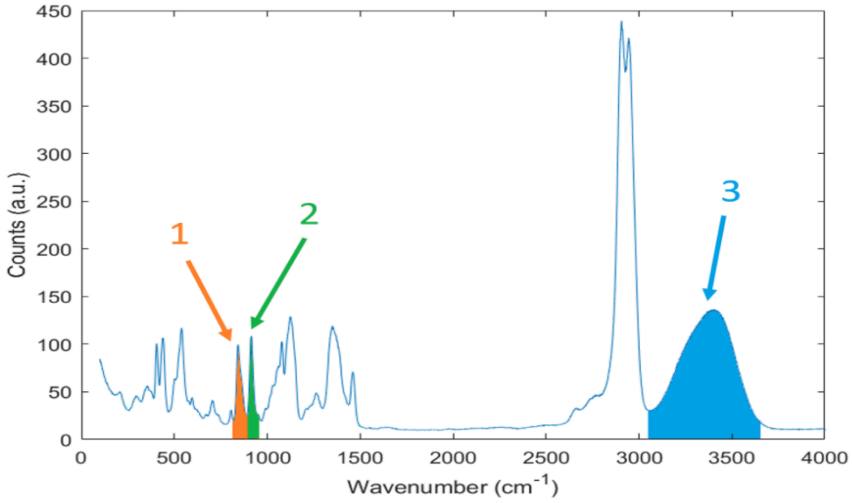

Figure 3. Representative Raman spectrum of a LAD processed sample. Peaks 1 and 2 are produced by trehalose. Peak 3 has contributions for both water and trehalose.

Figure 4 shows the ratio of feature 2 to feature 1 as a function of EMC (a measure of the amount of water remaining in the sample after LAD processing). Each ratio shown in this figure is the average value (over all 5 positions) for each sample and the error bars are the standard deviation reported in Table 2. The coefficient of variation (standard deviation/mean) was calculated for each line ratio. For the 2:1 ratio the coefficient of variation is 0.01 indicating that the 2:1 intensity ratio varies from the mean by 1% across all EMCs. This suggests that the ratio of the trehalose features remains nearly constant with increasing EMC indicating that this ratio is minimally affected by water content.

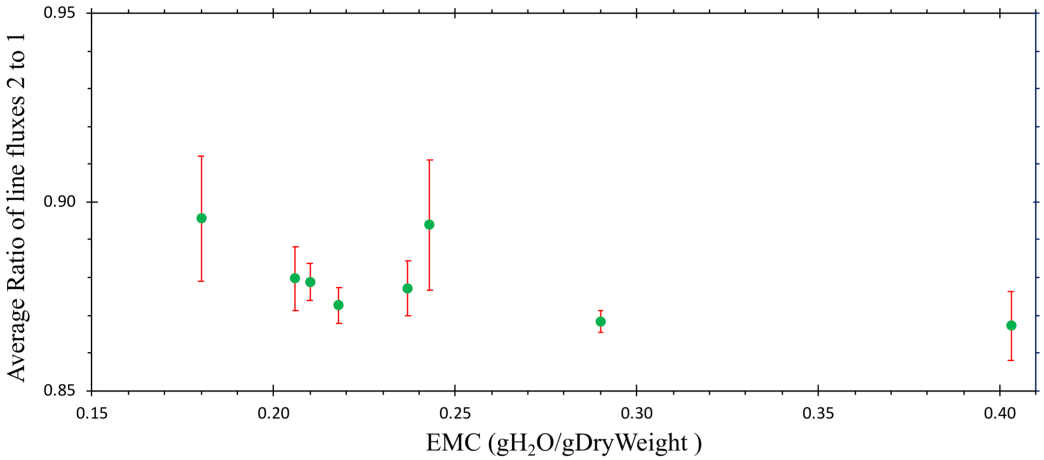

Figure 4. Average line ratio of features 2 to feature 1 as a function of EMC for LAD processed samples.

Table 2.Intensity Ratios, Average intensity Ratios, Standard Deviations and Coefficients of Variation for LAD Processed Samples

Sample EMC ($gH_2O/gDryWeight$)	2:1 ratio	3:1 ratio	3:2 ratio
0.180	0.90 ± 0.02	15.7 ± 0.4	17.6 ± 0.3
0.206	0.88 ± 0.01	14.3 ± 0.2	16.2 ± 0.3
0.210	0.88 ± 0.01	16.0 ± 0.8	18.3 ± 0.9
0.218	0.87 ± 0.01	14.5 ± 0.3	16.7 ± 0.4
0.237	0.88 ± 0.01	14.3 ± 0.3	16.3 ± 0.4
0.243	0.89 ± 0.01	15.8 ± 0.4	17.7 ± 0.3
0.290	0.87 ± 0.01	17.9 ± 1.6	20.6 ± 1.8
0.403	0.87 ± 0.01	17.4 ± 1.3	20.1 ± 1.7
Average	0.88	15.75	17.93
Standard Deviation	0.01	1.38	1.66
Coefficient of Variation	0.01	0.09	0.09

Figure 5 shows the ratio of feature 3 to feature 2 as a function of EMC (the 3:1 ratio exhibits similar behavior). Each ratio shown in this figure is an average value (over all 5 positions) for each sample and the error bars are the standard deviation reported in Table 2. As water content increases, the 3:2 and 3:1 ratios increase. The coefficient of variation for both the 3:1 and 3:2 ratios is 0.09 indicating that the intensity ratios vary from the mean by 9% across all EMCs. The strength of feature 3 is dependent on water content. The ratios of features 3 to 1 and 3 to 2 provide an indication of relative water content in LAD processed samples.

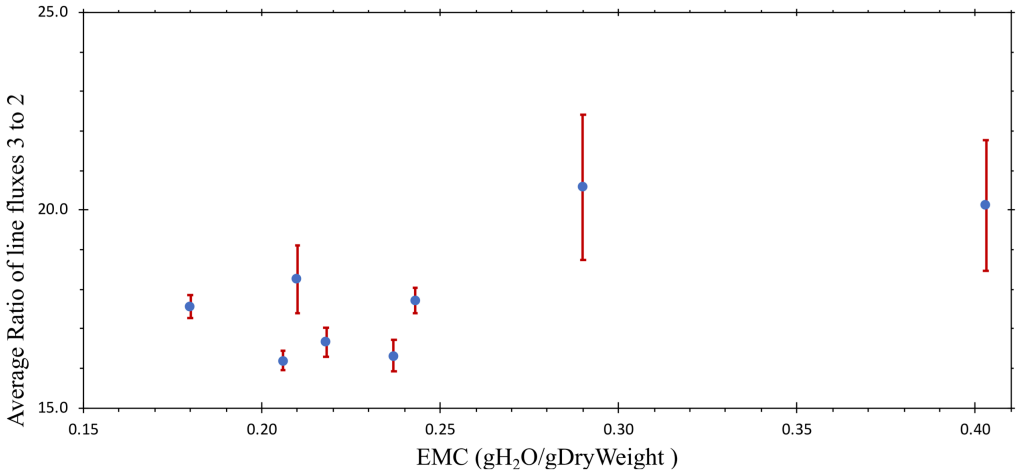

Figure 5. Average line ratio of features 3 to feature 2 as a function of EMC for LAD processed samples.

Figure 6 shows the 2:1 and 3:2 intensity ratios as a function of position within each LAD processed sample (the behavior of the 3:1 ratio is the same as the 3:2 ratio and is not shown in the figure). Panels a-f display the intensity ratios as a function of position for samples processed for 1 hour to an average EMC of 0.22 ± 0.02 $gH_2O/gDryWeight$ and panels g-h show results for higher EMC samples. Position 3 is the center of the sample and positions 1, 2, 4 and 5 are locations at half a radius from the center (see Figure 1). The samples with larger EMC have larger 3:2 and 3:1 ratios at all positions compared to the samples with lower EMC, confirming that these large EMC samples have a larger water content than the lower EMC samples. The coefficients of variation for the intensity ratios as a function of position for each sample are reported in Table 3. The coefficient of variation for the 2:1 ratio is 1-2% in all samples, regardless of EMC. The trehalose is distributed uniformly across the samples regardless of EMC. For the 3:2 and 3:1 ratios, lower EMC samples have smaller coefficients of variation than the higher EMC samples. This suggests that the lower EMC samples are more uniformly dry than the high EMC samples.

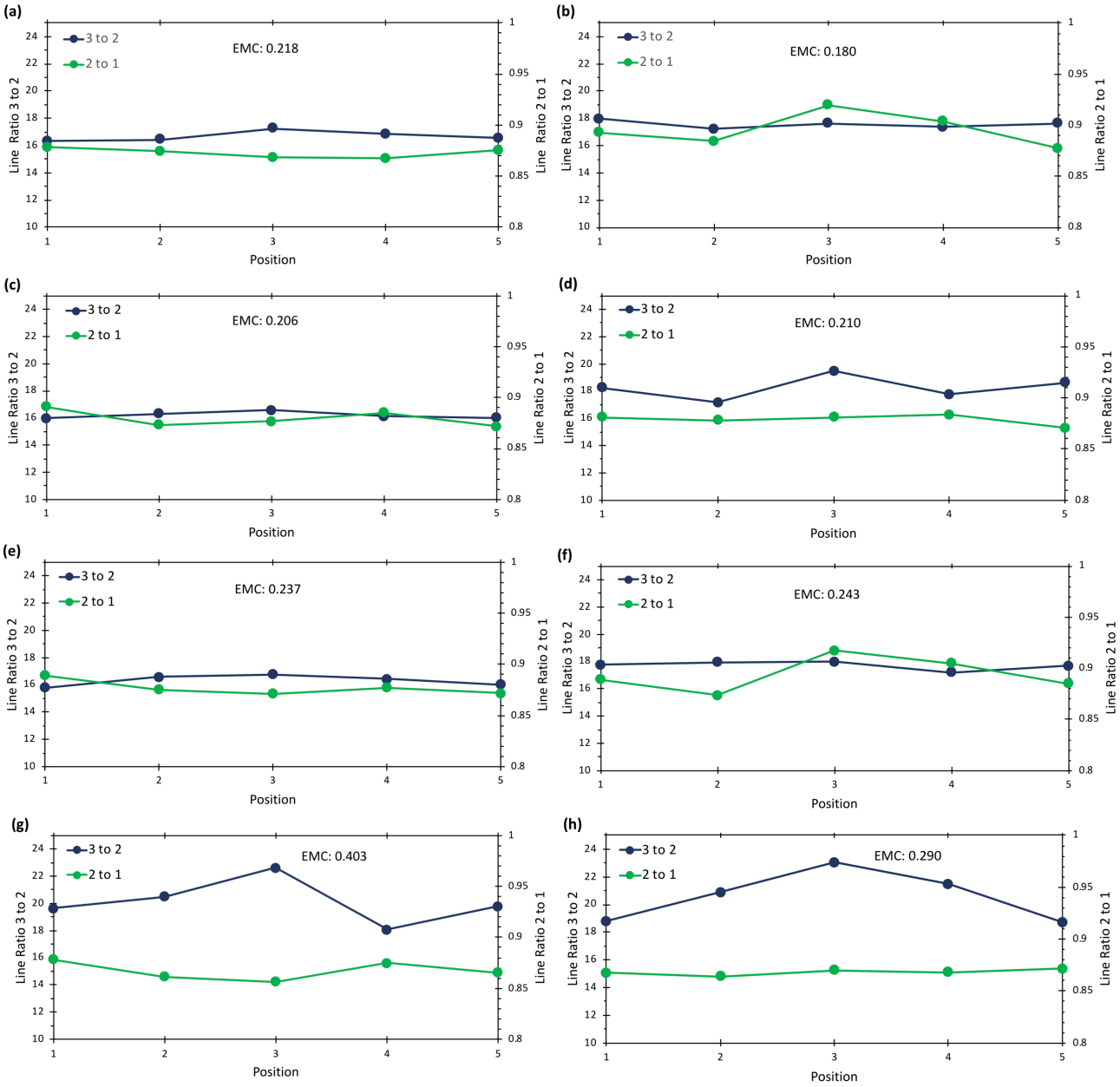

Figure 6. Line ratios as a function of position for LAD processed samples.

Table 3. Coefficients of Variation for Line Ratios as a Function of Position for LAD Processed Samples.

Sample EMC ($gH_2O/gDryWeight$)	Coefficient of Variation for 2:1 ratio	Coefficient of Variation for 3:1 ratio	Coefficient of Variation for 3:2 ratio
0.180	0.02	0.03	0.02
0.206	0.01	0.01	0.02
0.210	0.01	0.05	0.05
0.218	0.01	0.02	0.02
0.237	0.01	0.02	0.02
0.243	0.02	0.02	0.02
0.290	0.01	0.09	0.09
0.403	0.01	0.07	0.08

4. DISCUSSION

The goal of this project was to determine if LAD processed samples were uniformly dried. The intensity ratio 2:1 is not dependent on EMC and is uniform across samples. This indicates that trehalose is uniformly distribute in the LAD processed samples regardless of EMC. The 3:1 and 3:2 ratios indicate the water content in the samples. These ratios get larger as the EMC increases, consistent with the idea that that larger EMC samples contain more water. These ratios showed more variation as a function of positions in large EMC samples compared to low EMC samples. The lower EMC samples were more uniformly dry. Future work will include acquiring more data on LAD processed samples as well as making Raman measurements in more locations on each sample.

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