# Studying the degradation of chlorophyll compounds and production of carotenoids in dairy silage: the role of anaerobic fermentation

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## ABSTRACT

## INTRODUCTION

There has been an increasing demand for *organic* dairy products among the food industry because of the general conscience that they are healthier compared to their *non-organic* counterpart. Many of the dairy companies label their products as *grass-fed* as a signature of organic products. Our current investigation stemmed off a previous project focusing on authenticating the *grass-fed* label by analyzing chlorophyll metabolite[13] content in the milk and correlating that to the cattle-diet, specifically those which are either grass or derived from grass. After the grazing season is over the cattle are fed with fermented silage products such as that from alfalfa and corn. It has been shown[1], however, that the degradation of chlorophyll compounds occurs as the color of the silage turns from green to brown-yellow with time. This color change occurs during the fermentation process as the amount of lactic acid increases, degrading the plant pigments and decreasing the pH.[2,8,24] The degradation of chlorophyll compounds can be monitored by measuring the fluorescence and absorption spectrum of the fermented silage in solution. In addition to that, we used more sensitive fluorescence spectroscopy to determine the concentration of chlorophyll compounds in the silage solution. A typical fermentation process for silage will take about three weeks to complete, but the literature indicates that the chlorophyll content decreases rapidly within the first week of ensiling.[1,2]

## MATERIALS AND METHODS

Chopped corn that was freshly harvested was collected from the Iowa State dairy and tightly packed in glass jars to ensure an anaerobic environment. A portion of the chopped materials was placed in a zip-lock plastic bag and frozen immediately. This sample was labeled as the zero-time-point sample. All other samples were kept in a dark room at room temperature to ferment. Fermented samples were removed from their jars in three-day intervals with respect to a zero time-point sample. The content of each jar was preserved at $-20\ °C$ in a plastic zip-lock bag to stop the fermentation process further. Two of these samples were allowed to ferment for 70 to 80 days with respect to the zero time-point, in order to confirm the conclusion of the fermentation process. The well-preserved silage displayed a brownish-yellow color due to acidic degradation of the plant pigments. As we discuss below, the color of the silage is related to the concentration of chlorophyll metabolites present following fermentation, which has been investigated by fluorescence and absorption spectroscopy.

In the second part of the preparation process, the frozen sample was thawed and wet-blended to a homogenous mixture. The blended silage was extracted with acetone for the absorption and fluorescent emission measurement, and mixed with water for the pH measurement. This ratio was used to correct for the overall concentration of the solutions. The wet-mass ratio is assumed to have a random distribution. Each solution was placed in a dark room for 24 hours to ensure the full extraction of the silage material to the solvent. This is to ensure that the chlorophyll in the solution does not undergo photodegradation.[13] In all the solutions, the concentrations of silage to solvent were kept at 5 grams/100 mL. Three replicates were prepared for each acetone and water solution to estimate errors in the measurements. In order to correct for variable water content in the frozen silage, three

portions of the frozen sample were ground to a homogeneous consistency and then placed in an oven to dry for 24 hours in order to obtain dry to wet mass ratio. This ratio was used to correct for the overall concentration of the solutions. During the measurement, 400 $\mu$L of the silage in acetone solution was transferred to a quartz cuvette and diluted by the addition of 3000 $\mu$L of acetone. This is to ensure the signal of the absorption spectra was in a region in which the error is minimized. Absorption spectra were recorded using an Agilent 8453 UV-visible spectrometer with 1-nm resolution. Fluorescence emission spectra were measured on a Fluoromax-4 spectrometer (Horiba Scientific) at an excitation wavelength of 420 nm. The slit bandwidths were kept at 5 nm for both excitation and emission. Fluorescence spectra were corrected for lamp spectral intensity and detector response. The pH was measured using AB 15 pH meter (Fischer Scientific) by dipping the probe halfway into the jar after all the components had settled. Before measurement, the water and silage solution jars were shaken vigorously and then allowed to settle for an hour to ensure none of the silage interfered with the probe.

## RESULTS AND DISCUSSION

### Pheophorbide *a* Degradative Pathway

The Pheophorbide *a* Oxygenase (PAO) pathway (**Figure 1**) is the mechanism for the degradation of fluorescent molecule, pheophorbide *a*. [7,12] Louda et al. shows that the conversion of chlorophyll to pheophorbide *a* occurs by fermentation in vitro[20,23], which characterizes the step previous to the PAO pathway. The fluorescence signal from pheophorbide *a* dropped by about a third as the porphyrin ring of pheophorbide *a* is opened. In an aerobic environment, the porphyrin ring for pheophorbide *a* oxidized and opened into

the intermediate red chlorophyll catabolite (RCC).[10,12] In this experiment the aerobic environment was provided by the trapped oxygen in the jars during the collection time. Since this is the first step to the PAO mechanism, the available oxygen during the fermentation process is rate limiting. During the anaerobic conversion of RCC into a primary fluorescent chlorophyll catabolite (pFCC-1)[10,12] the reaction is mainly influenced by the acidic environment induced by lactic acid. This step is catalyzed by the enzyme RCC reductase which is in its active form when protons are readily available. The final step is the non-enzymatic thermodynamically driven isomerization of pFCC-1 into a non-fluorescent chlorophyll catabolite (NCC)[11,10,12], a non-conjugated molecule which characterizes the completion of senescence.

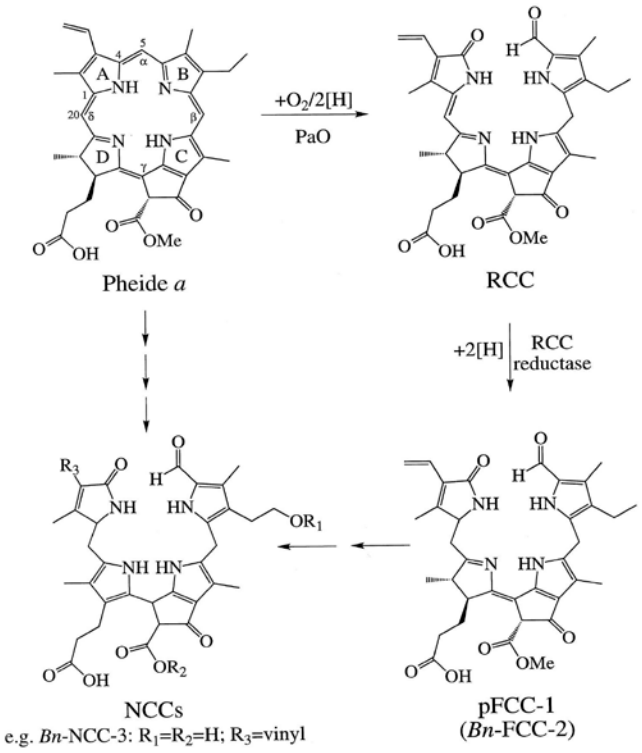
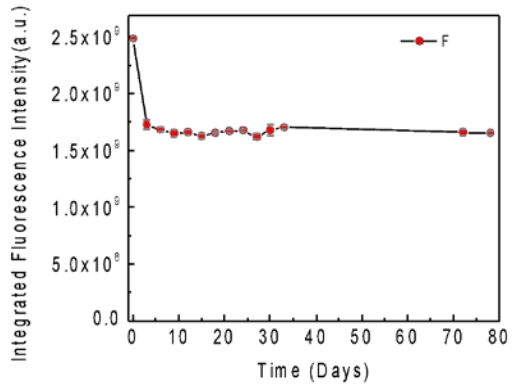



**Figure 1**. PAO pathway, the degradation of pheophorbide *a* into a non-fluorescent chlorophyll catabolite.[7]

**Pheophorbide *a* Degradation Analysis**

The presence of chlorophyll catabolite, pheophorbide *a*, was monitored by the absorption peak at 663 nm and the integrated fluorescence intensity in the 650-750 nm region. Upon fermentation, there is a substantial signal drop between the S1 point (zero time-point sample) and the S2 point (sample fermented for three days) for the fluorescence and pH. Qualitatively, we can see that the unfermented silage, sample S1, displayed a bright healthy green color, while S2 displayed a dramatically reduced green color in which a brown tint was beginning to be established. In the fluorescence spectra of the solutions for the following two samples (S3 and S4) we see a small decrease in the integrated fluorescence intensity. By this time, samples following S3 already display a uniform brown-yellow color. The samples following S3 display a plateau in which the signal fluctuates by 1 percent (Figure 2a) of the mean fluorescence signal at the region.

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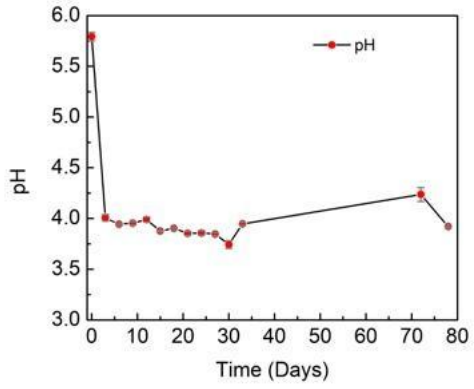
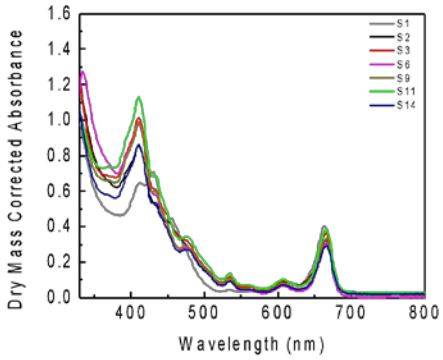



**(b)**

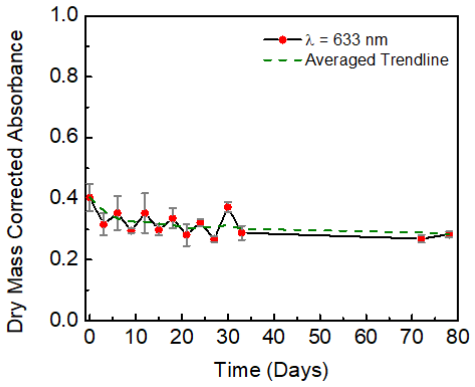
**Figure 2**. (a). Integrated fluorescence intensity in the region of 650-750 nm as a function of fermentation time for the samples in acetone; (b). pH of silage solution in water as a function of fermentation time.

After 6 days of fermentation, the pH drops from 5.79 to 4.01, and then begins to plateau for samples with fermentation times longer than 6 days. The pH data (Figure 2b) are in sync with the activity of the acidic step of the PAO pathway[11, 12] (Figure 1) corresponds to the lactic acid fermentation process. Savard et al. have shown that the pKa of lactic acid is 3.86.[21] Therefore, the enzyme RCC reductase which requires an acidic environment[10] is in its active protonated state at 9 days of ensiling (S3) with a recorded pH of 3.89. We can assess this qualitatively, by observing the brown color in the full fermented samples versus the green color of the zero-time sample (S1). Interestingly, in the period from 30 days to 70 days we see a slight increase in pH. This is possibly due to the increase in mold and yeast activity, which can be inferred from the visible growth of mold in the silage, because of the storage jars leaking air during the fermentation period[3,6,11].

When observing the absorbance of the solution at 663 nm, which corresponds to the porphyrin ring, we see about a 0.1 total decline from day zero to day thirty. Even though the points fluctuate every other sample, the trend-line shows a consistent decline from day zero to day thirty.




**(a)**

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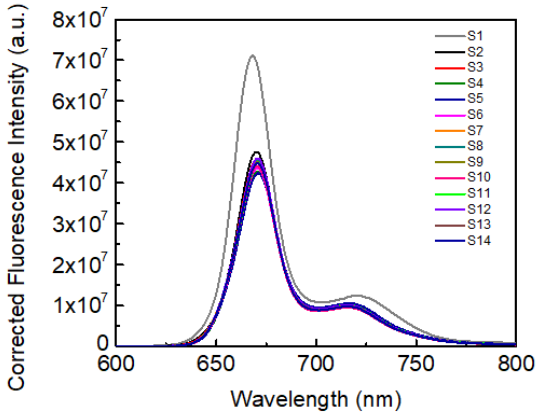



**(c)**

**Figure 3**. (a). Dry mass corrected absorbance spectra of 7 fermented silage solution samples; (b). dry-mass-corrected absorbance of the samples in acetone at 663 nm peak; (c) Fluorescence intensity spectra of 14 fermented silage solution samples.

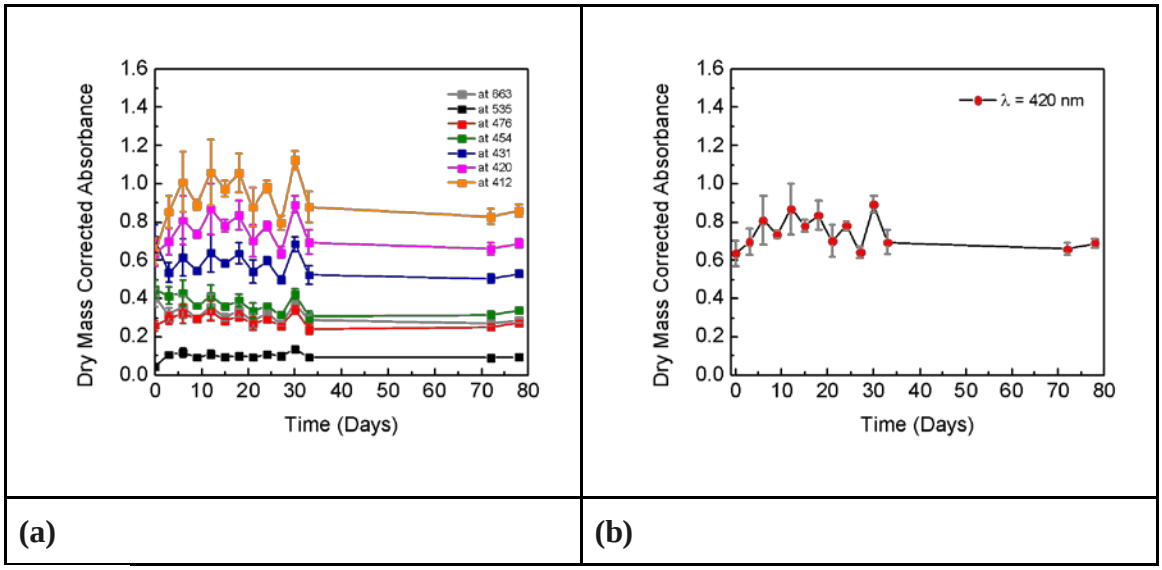
An interesting observation when comparing the trends of the fluorescence and absorbance signals is the inconsistency in their signal degradation. The fluorescence decreases very rapidly from S1 to S2, then by relatively small increments from S3 to S6 and then plateaus. The absorbance signal decreases at a smaller but consistent rate from S1 to S10 (Figure 3b).

This information shows that the same samples that have a degraded fluorescence signal due to the high presence of non-fluorescent chlorophyll catabolites are still able to absorb in the visible spectrum at 663 nm. The reasoning behind this could be that the conversion of pFCC-1 to NCC is the step that degrades the fluorophore[9] therefore eliminating the fluorescence at 420 nm excitation (Figure 3c). But the product NCC still absorbs significantly at 663 nm.

**Carotenoid Analysis**

Carotenoids are protective units for chlorophyll and synthesized from proteins during the fermentation of silage.[23] They absorb strongly in the 400 nm to 550 nm region,[18] thus giving them their red-orange appearance. Two important carotenoids that are present in silage are alpha-carotene and beta-carotene. Beta-carotene has an absorption maximum peak at 454 nm, a secondary peak at 476, and tertiary peak at 420 nm.[19] Alpha-carotene differs from beta-carotene by the placement of one double bond. The alpha carotene structure has one less double bond in the conjugated system, compared to beta-carotene. Therefore, alpha-carotene absorbs maximally at a peak left of beta-carotene. Alpha-carotene absorbs maximally at 445 nm, but also shares a secondary and tertiary peak with beta-carotene at 476 nm and 420 nm respectively.[19] Unfortunately, the maximum peak (445 nm) for alpha carotene was not observed in our spectra. When observing the maximum

peak (454 nm) for beta-carotene, we see that absorption signal decreases by about 0.1 arbitrary units within 30 days of fermentation (Figure 4a). The second (476 nm) and third (420 nm) peaks are of interest to us because they confirm the increase of carotenoid concentration by lactic acid fermentation in alpha and beta-carotene. The 476 nm peak shows an increase by about 0.1 by day 33 of fermentation (Figure 4a). The tertiary peak for both the carotenoids, located at 420 nm, displays an increase of 0.3 at 33 days of fermentation (Figure 4b).




**(a)** **(b)**

**Figure 4**. (a). Dry mass corrected absorbance of significant peaks of fermented silage solutions; (b). Dry mass corrected absorbance of 420 nm peak of fermented silage solutions.

An interesting question arises when comparing the decrease in absorption at the primary peak, versus an increase in absorption in the secondary and tertiary peaks. The decrease in the absorption at the primary peak (454 nm) is most likely due to the loss of chlorophyll catabolites and other factors that were integrated into the silage solution. The primary peak for both of these carotenoids are also not prominent in the overall absorption

spectra of the silage solutions (Figure 3a), they are mixed with many other peaks and are difficult to clearly decipher. We need to take into account that we are comparing to spectrums of pure carotenoid samples and therefore our maximum peaks are less prominent. The 420 nm peak, although it is the tertiary peak[19] in the spectra of pure substance, confirms in the increase of alpha and beta-carotene. The prominence of the peak in the raw spectra of the absorption peaks (Figure 2a) allows us to confirm this conclusion that has been explored in multiple literature[14,15,16,23].

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