JOURNAL OF
EMERGING INVESTIGATORS

Article

Protein concentrations in cows' milk during the four stages of lactation

Madeleine Wilson[1], Robin Vaughn[1]
[1] North Bay Haven Charter Academy, Panama City, Florida

SUMMARY

Proteins play an essential role in the growth and development of young calves, and changes in the nutritional needs of these calves may be responsible for changes in milk protein concentrations across the four stages of bovine lactation. The purpose of this research was to analyze the fluctuations in the concentrations of casein and whey, which are the two primary sources of protein in cows' milk. Milk proteins provide essential nourishment for young calves, so we hypothesized that the total protein concentration of the milk would decline from the first stage of lactation to the fourth stage of lactation. Absorption spectroscopy was used to determine the concentration of the proteins in the milk, and five trials were conducted for each stage of lactation. A spectrometer was used to collect absorbance data for the whole milk, casein, and whey samples, and a standard curve was used to convert those absorbance readings into concentration values. The hypothesis for this research was supported in that the total protein concentration of the milk declined from the first stage of lactation to the fourth stage of lactation. More specifically, the concentration of casein proteins decreased as the concentration of whey proteins increased. These trends are important because they provide a foundation for future research to determine whether the observed fluctuations in the protein concentrations are significant enough to pose implications for individuals suffering from lactose intolerance.

INTRODUCTION

Proteins serve as the building blocks of cells, and they are responsible for regulating biological processes, repairing tissues, and fortifying the bones, muscles, cartilage, and blood. The proteins supplied by bovine milk are particularly important to the development of young calves. The primary milk proteins are casein and whey proteins, and they comprise about 3.3% of the total composition of milk (1). Casein proteins account for approximately 82% of the protein composition of milk, while whey proteins account for the remaining 18% (1). Casein proteins have amino acid structures specific to the nurturing of young calves, and they are easily digested in the human intestine (1). Whey proteins are also essential to the growth and development of young calves, and on the cellular level, they serve as growth factors and nutrient transporters (1).

Changes in the nutritional needs of growing calves may be responsible for fluctuations in milk protein concentrations across the stages of lactation (2). For a typical dairy cow, the first stage of lactation lasts from one to five days after the birth of a calf. The second stage lasts from two to four weeks after the birth of a calf. The third stage lasts from five to six weeks after the birth of a calf, and the last stage lasts from seven weeks to the end of lactation (3). In a 2014 study published in the *Czech Journal of Animal Science*, a team of researchers analyzed the effects of feed restrictions and tissue biopsies on protein yield in Holstein cows (4). The researchers recorded the concentrations of casein and whey subunits during the first 155 days of lactation to study how the concentrations were affected by diet changes and acute stress factors (4). The researchers concluded that acute stress factors should be avoided because they have a significant impact on protein composition, but the research did not identify a direct link between the stage of lactation and the corresponding protein concentration of the milk (4). The observed variations in milk protein composition can be attributed to the experimental changes induced during the research, but it is also likely that they can be attributed to changes in the nutritional needs of growing calves. Proteins are critical to the development of young calves, and we hypothesized that if a fluctuation exists in the concentration of milk protein, then higher protein concentrations are more likely to be found in milk produced at the beginning of lactation. During this stage, the newborn calf depends on milk protein for survival, but as time passes after the calf's birth, such high protein concentrations may not be as critical to its development.

Much of the existing literature on milk protein is speculative, and none of it directly addresses how the ratio of whey protein to casein protein changes throughout the stages of lactation. Studying the protein concentrations in milk from each stage of lactation addresses a gap in the existing research by allowing the ratio of whey protein to casein protein to be determined. The results of this experiment uncovered an increase in this ratio from the first stage of lactation to the fourth stage of lactation. The total protein concentration in the milk declined as hypothesized. The concentration of casein proteins also declined, but the concentration of whey proteins increased, producing an increase in the ratio of whey protein to casein protein. The increase in this ratio is significant because it may produce a change in the body's physiological response to the consumption of milk.

RESULTS

To study the fluctuations in milk protein concentrations, we collected absorbance readings for the whole milk, casein, and whey samples using a wireless PASCO spectrometer. The first step in the analysis of the absorbance data was the construction of four standard curves, one for each set

of data collected from the four stages of lactation (**Figures 1-4**). We used a set of seven Bovine Serum Albumin (BSA) protein standards with known concentrations to construct the calibration curves of absorbance versus concentration. Next, linear regression was performed on each standard curve to generate a line of best fit passing through the data points, and R-squared values were calculated to provide a goodness-of-fit measure for the linear regression models. The R-squared value for the standard curve for the first stage of lactation was 0.905. The R-squared value for the second stage of lactation was 0.923, the value for the third stage of lactation was 0.900, and the value for the fourth stage of lactation was

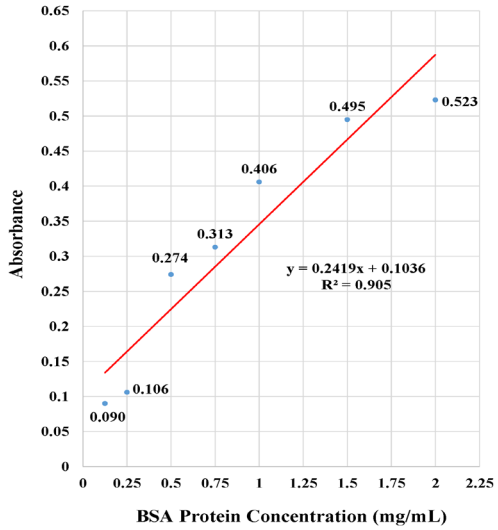

Figure 1. Standard Curve for the First Stage of Lactation. Calibration curve of absorbance vs. concentration constructed using BSA protein standards. The line of best fit was generated using Excel software and was used to calculate protein concentrations for the first stage of lactation.

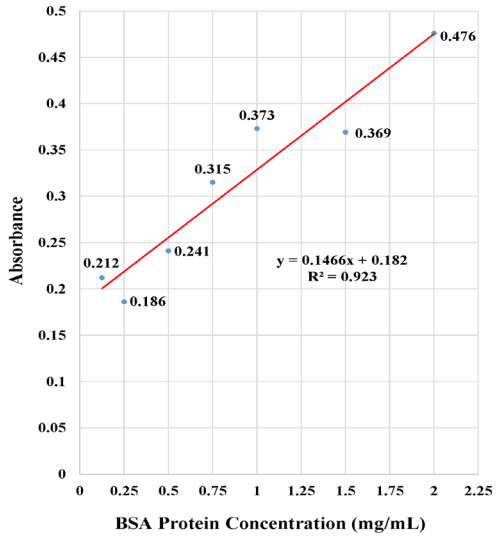

Figure 2. Standard Curve for the Second Stage of Lactation. Calibration curve of absorbance vs. concentration constructed using BSA protein standards. The line of best fit was generated using Excel software and was used to calculate protein concentrations for the second stage of lactation.

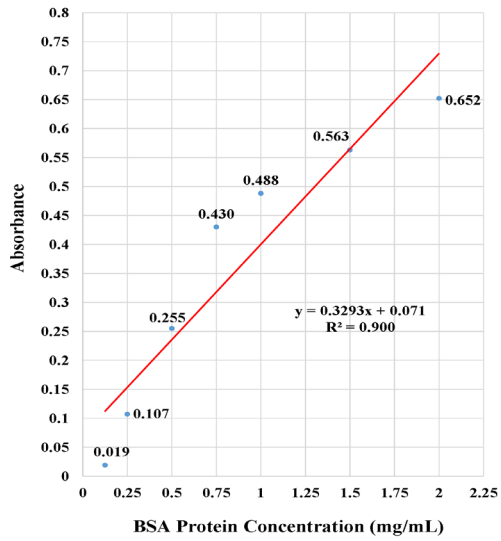

Figure 3. Standard Curve for the Third Stage of Lactation. Calibration curve of absorbance vs. concentration constructed using BSA protein standards. The line of best fit was generated using Excel software and was used to calculate protein concentrations for the third stage of lactation.

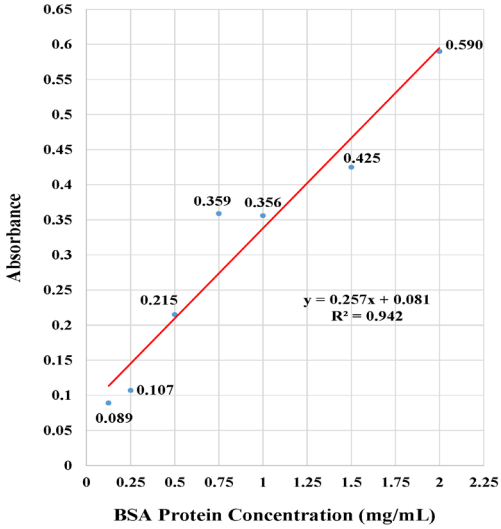

Figure 4. Standard Curve for the Fourth Stage of Lactation. Calibration curve of absorbance vs. concentration constructed using BSA protein standards. The line of best fit was generated using Excel software and was used to calculate protein concentrations for the fourth stage of lactation.

0.942. When all of the absorbance data had been collected for the whole milk, whey, and casein samples, we converted the absorbance values into concentration values using each line of best fit.

Following the proposed hypothesis, the average total protein concentration declined from 2.5±0.1 mg/mL to 1.66±0.03 mg/mL from the first stage of lactation to the fourth stage of lactation (**Figure 5**). The average casein concentration also declined from the first stage of lactation to the fourth stage of lactation, falling from 2.3±0.2 mg/mL to 1.26±0.01 mg/mL (**Figure 5**). In contrast to the trend observed for the casein protein, the average concentration of whey

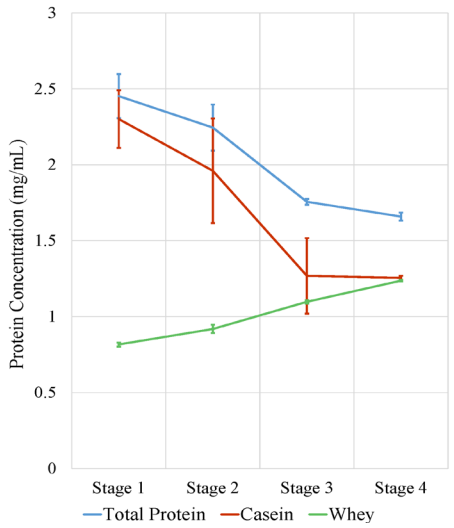

Figure 5. Concentration Measurement Averages. This graph highlights the decline in the total protein concentration as well as the increase in the ratio of whey protein to casein protein. The error bars measure one standard deviation from the mean.

proteins increased from 0.82±0.01 mg/mL to 1.237±0.005 mg/mL from the first stage of lactation to the fourth stage of lactation (**Figure 5**), resulting in an increased ratio of whey protein to casein protein (**Figure 6**).

DISCUSSION

For the milk produced by the single Jersey cow used in this experiment, both the total protein concentration and the casein concentration declined from the first stage of lactation to the fourth stage of lactation. However, the concentration of the whey proteins increased from 0.82±0.01 mg/mL to 1.237±0.005 mg/mL instead of staying consistent as

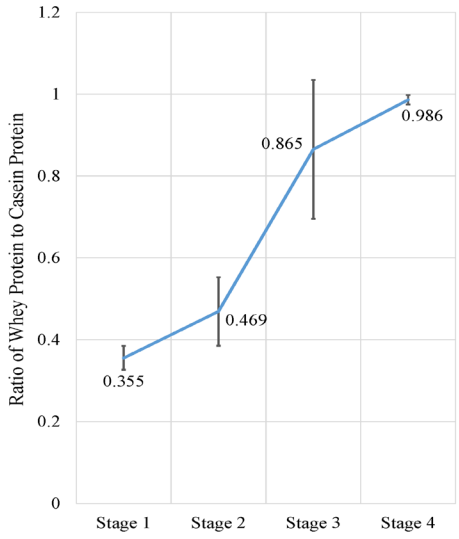

Figure 6. Average Ratio of Whey Protein to Casein Protein. From the first stage of lactation to the fourth stage of lactation, the average ratio of whey protein to casein protein increased from 0.36±0.03 to 0.99±0.01. The error bars were calculated by propagating the uncertainties of the casein and whey concentrations. These uncertainties measure one standard deviation from the mean.

predicted (**Figure 5**). One possible explanation for this trend is that when a cow reaches the end of lactation, the secretion of lactoferrin, a whey protein, increases to confer disease resistance to the cow (3). The fluctuations in milk protein concentration uncovered by this study address a gap in the existing literature by identifying a direct link between the stage of lactation and the corresponding protein concentration of milk.

The extraction of the casein protein from the centrifuge tubes was one technical limitation encountered during the experiment. The casein protein formed a solid pellet at the bottom of each tube as the milk samples were centrifuged, and a small amount of phosphate-buffered saline (PBS) had to be added to each tube to break up the pellet and allow it to be extracted. Despite this limitation, adding a small amount of PBS to break up the casein did not compromise the accuracy of the readings given by the spectrometer, and the range of concentration values recorded for both the casein and whey was narrow. We conducted five trials to minimize the effect of any possible outliers. Another limitation of the experiment was that milk samples were collected from only one cow. The dairy farm that supplied the milk samples was small, so only one cow gave birth within the time frame established for the experiment. Future research can be conducted to analyze milk samples collected from multiple cows, including cows of different breeds, to ensure that the fluctuations in the protein concentrations are due to a change in the stage of lactation and not due to variability in the milk produced by different cows.

Furthermore, the ambiguity in the time frames established for the stages of lactation of a typical dairy cow was a limitation of this research. The length of lactation largely depends on the frequency with which a dairy cow is bred, and the length of the dry period can be manipulated by the dairy farmer (3). The Jersey cow that produced the milk tested in this experiment was a first-time mother, and no protocols had yet been established for the length of her lactation period. A dairy cow can produce milk for up to 305 days, but the time constraints for this experiment made it unreasonable for milk samples to be collected over such a long period (3). As a result, the period of lactation studied in this research was restricted to 60 days. While multiple existing studies document fluctuations in milk protein concentrations, very few of them directly link those fluctuations to the stage of lactation in which the milk was produced, so no precedent exists for the length of lactation recommended for experimental research. As the experiment was designed, the uncertainty of the time constraints for the stages of lactation posed a challenge, but further research may help establish more clear observation parameters. As more extensive research is conducted, tests can be performed on milk samples collected every week or every day as opposed to only once during each stage of lactation. As more data is collected, the transition from high casein and low whey concentration to low casein and high whey concentration can be studied in more detail, and the critical points of this transition can be used to redefine the stages of lactation. Creating a second set of parameters for defining the stages of lactation could benefit future researchers by allowing them to compare and communicate the results of their experiments more effectively.

In addition to the limitations encountered when designing and conducting the experiment, a potential limitation of

the experimental results was the use of linear rather than nonlinear regression to generate the standard curves for each stage of lactation. For the standard curves for milk collected from lactation stages 1 and 3, logarithmic regression fits the recorded data better than linear regression. However, linear regression is a better fit than logarithmic regression for the standard curves generated for milk samples collected from lactation stages 2 and 4. At higher protein concentrations, the linearity of Beer's law is expected to no longer hold, but a 1:50 dilution was made for each BSA standard and each milk sample to minimize the effects of this breakdown. Apart from the use of linear rather than nonlinear regression, another limitation of the experimental results is the discrepancy between the average total protein concentration and the sum of the average casein and whey concentrations. For each stage of lactation, the average total protein concentration is greater than the sum of the average casein and whey concentrations. One possible explanation is that the casein and whey proteins may not have been completely separated in the centrifuge. When the whey proteins were extracted from the centrifuge tubes for testing, a small amount of casein may have also been extracted, and this may have caused the observed concentration of whey to overestimate the actual concentration of whey.

The fluctuations in milk protein concentrations discovered in this research are significant because they may impact individuals suffering from lactose intolerance. Lactose intolerance is characterized by a reduced ability to digest lactose, the sugar found in milk (5). Lactase is the enzyme that facilitates the breakdown of lactose, but in individuals with lactose intolerance, the small intestine does not secrete enough of the enzyme to sufficiently digest the sugars found in milk. This incomplete digestion is responsible for the symptoms of lactose intolerance, which include nausea, stomach cramps, and bloating (6).

The observed increase in the whey protein concentration from the first stage of lactation to the fourth stage of lactation may be associated with an increase in the severity of the symptoms of lactose intolerance. The two primary proteins found in whey are beta-lactoglobulin and alpha-lactalbumin (7). Alpha-lactalbumin comprises about 17% of the proteins found in whey, and it is important because it plays a critical role in the synthesis of lactose (7). In the mammary gland of both humans and cows, alpha-lactalbumin is a subunit of an enzyme known as lactose synthase, which catalyzes the conversion of glucose and galactose into lactose (7). Given that alpha-lactalbumin plays a critical role in the synthesis of lactose, an increase in its concentration may increase the severity of the symptoms of lactose intolerance by increasing the lactose content of bovine milk. On a similar note, the lower concentration of whey proteins in the first stage of lactation may be associated with less severe symptoms of lactose intolerance. To determine whether such a correlation exists, case studies will need to be performed on lactose intolerant individuals. While the high casein and low whey concentration of the first stage of lactation contrasts sharply with the low casein and high whey concentration of the fourth stage of lactation, additional experiments will need to be conducted to determine whether this difference is significant enough to produce variations in the symptoms of lactose intolerance.

If future research suggests that a low concentration of whey proteins helps alleviate the symptoms of lactose intolerance, the results of this experiment can provide a foundation for determining the most effective way to produce and market milk with a lower whey protein content. In addition, since this study indicates that whey protein concentration is the lowest in the first stage of lactation, future researchers can study the mechanisms by which cows restrict whey production at the beginning of lactation to provide direction for their own attempts to repress whey protein synthesis. Until future studies can identify effective procedures for suppressing whey production or reducing whey protein concentration after milk is collected, milk can be marketed according to the stage of lactation in which it was produced. Under the current system of marketing milk, consumers have no way of knowing from which stage of lactation the milk was collected. However, if dairy farms and grocery outlets work together to market milk according to the stage of lactation in which it was produced, lactose intolerant individuals may be able to safely consume milk from the first stage of lactation, which contains the lowest concentration of whey protein.

MATERIALS AND METHODS

Rationale

The use of absorption spectroscopy allowed us to determine the protein concentrations of the milk samples, which were collected from a single Jersey cow at four different times over the course of 60 days. A certified farmer from a local dairy farm collected the milk samples. The first samples were collected the day the calf was born, the second samples were collected 15 days after birth, the third samples were collected 43 days after birth, and the final samples were collected 59 days after birth. Five samples, each measuring 5 mL, were collected from each stage of lactation. The owner of the dairy farm, who was also the individual collecting the milk samples, signed a consent form permitting the use of the milk samples in research and acknowledging that the dairy farm could discontinue its participation in the study at any time.

Application of Absorption Spectroscopy

A wireless spectrometer (PASCO PS-2600) and its corresponding software were used to determine the protein concentration in the collected milk samples. The wavelength used to measure the absorbance of the milk samples was 595 nm. This wavelength of light was selected based on the absorption spectrum of a Bradford dye reagent. Proteins alone do not absorb enough light to be detected by the spectrometer, so a dye reagent had to be added to the milk samples to allow the spectrometer to quantify the difference between the amount of light entering and leaving the solution (8). The Bradford assay, or dye reagent, is the most sensitive method used to determine protein concentration, and the Bradford dye reagent used in this experiment was Coomassie G-250 (9). The absorption spectrum of Coomassie G-250 indicates that, when bound to protein, the dye exhibits maximum absorption of light at a wavelength of 595 nm (10). Maximizing the absorption of the dye was critical because it allowed smaller concentrations of protein to be detected and accurately measured by the spectrometer (11).

Construction of the Standard Curves

Another principle of absorption spectroscopy, Beer's law, was used to convert the absorbance readings given by the spectrometer into concentration values. Beer's law

states that the absorbance of a substance in a solution is directly proportional to its concentration (12). To illustrate this proportional relationship, four standard curves were constructed to relate the absorbance readings from the spectrometer to a set of known protein concentrations. Seven Bovine Serum Albumin (BSA) protein standards were prepared and placed in the spectrometer to yield absorbance readings that could be plotted on a graph of absorbance versus concentration. BSA, derived from bovine blood, is commonly used as a standard for spectroscopy experiments because of its low cost and low reactivity in biochemical reactions (13). The seven standard concentrations were 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1.0 mg/mL, 1.5 mg/mL, and 2.0 mg/mL. A 50 µL syringe was used to add 50 µL of each BSA protein standard to seven different cuvettes, which are small test tubes placed in the sample chamber of a spectrometer. Following the instruction manual for the Quick Start Bradford Protein Assay, 2.5 mL of Bradford dye reagent was added to each cuvette. A blank solution was then prepared to calibrate the spectrometer. The blank solution consisted of 2.5 mL of Bradford dye reagent and 50 µL of phosphate-buffered saline (PBS). The blank contained no protein because it was used as an experimental control.

Before the blank solution and the prepared samples were placed in the spectrometer, the spectrometer was connected via Bluetooth to a laptop with compatible PASCO spectroscopy software. The spectrometer was turned on and allowed to warm up for 10 minutes before the blank solution was placed inside the sample chamber. The transmittance was set to 100% to calibrate the spectrometer. The blank solution was carefully removed and set aside when the software indicated that the calibration was complete. The mode was then switched from transmittance to absorbance, and the 0.125 mg/mL BSA standard was placed in the sample chamber. The start button in the digital application was selected for each sample to allow the spectrometer to begin collecting absorbance data. When the absorbance stabilized, the stop button was selected, and the absorbance was recorded. This process was repeated until absorbance readings had been obtained and recorded for each of the seven prepared protein standards. The experiment was conducted in four stages. In the first round of testing, only the milk samples from the first stage of lactation were analyzed. For consistency, a standard curve was constructed for each round of testing to yield a total of four standard curves. Excel software was used to generate the line of best fit for each standard curve.

Separation into Casein and Whey Proteins

To test the concentrations of the individual casein and whey proteins, a centrifuge was used to separate the milk into its components. First, five milk samples, each 5 mL in volume, were collected from each stage of lactation. 1 mL of each 5 mL sample was reserved for testing the total protein concentration, while the remaining 4 mL were reduced to a pH of 4.6 by the addition of hydrochloric acid (HCl). The 4 mL was poured into a 250 mL beaker, and a volumetric pipette with a rubber bulb was used to slowly add drops of 0.1 M HCl. Next, a wireless pH meter (PASCO, SparkVue software) was used to monitor the pH of the samples as drops of HCl were added. As the pH was monitored, a glass stirring rod was used to stir the milk samples. The research was conducted in a well-ventilated laboratory, and gloves, goggles, and an apron were worn for the entirety of the experiment.

Of the 4 mL of milk in each of the five beakers, 1 mL was extracted and transferred to one of five centrifuge tubes. The samples were placed in the centrifuge, and a sixth tube was filled with 1 mL of distilled water to maintain balance while the centrifuge was operating. The centrifuge was set to a speed of 3200 rpm for 15 minutes. If the layers of milk required further separation, the samples were centrifuged in additional five-minute increments.

Preparation of the Samples

While the centrifuge was operating, the whole milk samples were prepared. Because these samples were used to test the total protein concentration of the milk, they were not separated into their components and no centrifuging was required. A pipette was used to transfer 1 mL of milk from each sample into a 250 mL beaker. For each sample, 4 µL of milk, 196 µL of PBS, and 2.5 mL of Bradford dye reagent was added to a cuvette. Using 4 µL of milk and 196 µL of PBS created a 1:50 dilution that allowed the protein concentrations to fit into the linear range of the standard curve. If the samples had not been diluted, the protein concentration in the milk would have been too high to be detected by the spectrometer.

When the centrifuge finished operating, the centrifuge tubes were removed and placed in a test tube rack. Three distinct layers were present in the milk from each centrifuge tube: cream at the top, whey in the middle, and casein at the bottom. A disposable transfer pipette was used to extract and discard the cream. A 50 µL glass syringe was then used to extract 4 µL of whey from the first centrifuge tube. The whey was transferred to the first labeled cuvette, and 196 µL of PBS was then added. After the syringe was cleaned with distilled water, the process was repeated for the four remaining centrifuge tubes. Then, 2.5 mL of Bradford dye reagent was added to each of the five cuvettes. A second disposable transfer pipette was used to extract and discard the whey remaining in each centrifuge tube, leaving only the casein at the bottom. Because the casein protein formed a solid pellet in the centrifuge, a small amount of PBS had to be added to the centrifuge tubes to extract the casein with the syringe. Following the same procedure for the whole milk and whey samples, each casein sample was prepared with 4 µL of casein, 196 µL of PBS, and 2.5 mL of Bradford dye reagent.

Testing the Samples

The same procedure used to collect absorbance readings for the standard curve was used to collect absorbance readings for the whole milk, whey, and casein samples, and the same blank solution was used to calibrate the spectrometer. Once the spectrometer had been calibrated, the mode was set to absorbance, and the first whole milk sample was placed in the sample chamber. The absorbance readings for each sample were recorded, and the spectrometer was calibrated again to test the whey samples. When the data for the whey samples had been collected and recorded, the spectrometer was calibrated to test the casein samples. The collection of absorbance data for the casein samples concluded the data collection for the first stage of lactation. The entire process was repeated for the second, third, and fourth stages of lactation.

ACKNOWLEDGEMENTS

I would like to thank North Bay Haven Charter Academy for their lab equipment and Ocheesee Creamery for supplying the milk samples analyzed in this research.

Received: June 14, 2021
Accepted: August 22, 2021
Published: January 07, 2022

REFERENCES

1. "Milk Protein." *Milk Facts,* milkfacts.info/Milk%20Composition/Protein.htm.
2. Heinrichs, Jud, and Coleen M. Jones. "Milk Components: Understanding Milk Fat and Protein Variation in Your Dairy Herd." *Penn State Extension,* 5 May 2016, extension.psu.edu /milk-components-understanding-milk-fat-and-protein-variation-in-your-dairy-herd.
3. "Milking and Lactation". *Holstein Foundation*, Nov. 2017, www.holsteinfoundation.org/education/workbooks.html.
4. Gellrich, Katharina, *et al*. "Composition of Major Proteins in Cow Milk Differing in Mean Protein Concentration during the First 155 Days of Lactation and the Influence of Season as well as Short-Term Restricted Feeding in Early and Mid-Lactation." *Czech Journal of Animal Science,* vol. 59, no. 3, 2014, pp. 97-106., doi:10.17221/7289-cjas.
5. "Lactose Intolerance: MedlinePlus Genetics." *MedlinePlus,* 18 August 2020, medlineplus.gov/genetics/condition/lactose-intolerance/.
6. "Symptoms and Causes of Lactose Intolerance." *Mayo Clinic,* 7 April 2020, www.mayoclinic.org/diseases-conditions/lactose-intolerance/symptoms-causes/syc-2037423.
7. Layman, Donald K., *et al*. "Applications for α-lactalbumin in human nutrition." *Nutrition Reviews,* vol. 76, no. 6, 2018, pp. 444-460., doi: 10.1093/nutrit/nuy004.
8. Bower, Julie. "Spectrometer." *Chemistry LibreTexts,* Libretexts, 15 August 2020, chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_(Analytical_Chemistry)/Instrumental_Analysis/Spectrometer.
9. "Measuring Protein Concentration through Absorption Spectrophotometry." *Marietta College,* 23 April 2018.
10. "Testing protein content of common foods using the Quick Start Bradford Protein Assay." *Got Protein? Instruction Manual,* Bio-Rad Laboratories, 2019.
11. He, Fanglian. "Bradford Protein Assay." *BIO-PROTOCOL,* vol. 1, no. 6, 20 March 2011, doi:10.21769/bioprotoc.45.
12. Ball, David W. *Field Guide to Spectroscopy.* SPIE Press, 2006.
13. "Quick Start Bradford Protein Assay." *Instruction Manual,* Bio-Rad Laboratories.