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Evaluation of the Development of Crystals on Smear Ripened Cheese through Polarized Light Microscopy and X-ray Diffraction

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Honors College Thesis

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Abstract

Cheese crystals are a very polarizing topic for consumers; some people enjoy these crunchy constituents whereas others dislike their gritty texture. The presence of these crystals, or lack thereof, often dictates consumer's demand for cheese products. The purpose of this experiment was to look at the change in crystal composition over time on smear ripened cheese with the aim of gaining a better understanding of the crystals. The cheese samples were analyzed under a polarized light microscope to find the angle of extinction of the crystals as well as to gather morphological data on them. This study showed that there are nine different apparent crystal subtypes present on this cheese type. Three were definitively identified using powder X-ray diffractometry as calcite, ikaite, and struvite. Struvite and ikaite were able to be differentiated from the other subtypes based on single crystal X-ray diffractometry work. This study also revealed that the average area of the crystals present on the cheese tended to decrease during weeks 7, 8, and 9 post manufacture. The crystals then showed a significant increase in area by week 12. This work showed that the crystals on the bacterial smear of smear ripened cheese are very complex and varied. Single crystal X-ray diffraction work would greatly aid in the definitive identification of these different crystal subtypes.

Introduction

Significance

The consumption of cheese in the United States is consistently rising. In fact, by the turn of the century Americans ate nearly two and a half times the amount of cheese as they did in 1970 (Gallo, 1999). This increase in the demand for cheese has led to an increase in cheese production and the development of artisanal cheese producers. Cheese and dairy products have an increasing impact on regional economies. In Vermont, dairy makes up 70—80% of the state's annual agricultural sales (Parsons, 2010). Vermont's artisan cheese sector makes up more than 40 cheesemakers producing over 130 cheeses in the state (Parsons, 2010). Because there is so much reliance on this product for consumers and producers alike, many New England state legislators and activists herald artisanal cheeses as value—added products, which "might save small dairies from collapse or consolidation" (Wilk, 2006).

With an increasing demand for dairy and increasing dairy production, producing cheese that appeals to consumers is an increasingly important area for cheese manufacturers. Cheese crystals are a polarizing area for many consumers. Traditionally crystals were viewed with distaste by consumers, who often mistook them as spoilage (Singh, Drake, & Cadwallader, 2003). In one study mapping consumer perception of sharp cheddar cheese, it was found that Midwest consumers "do not like grainy texture (crystals) in Cheddar cheese" (Drake et al., 2009). Thus the presence of crystals may detrimentally affect the profit gained by cheese producers. More recently however, there has been a trend towards valuing the crystals as a mark of the artisanal quality (P. Kindstedt, 2012). Some consumers enjoy and value the crunchy constituents of cheese and seek them out (Carpenter, 2014).

Previous Work

Different cheeses have different crystal composition. In Cheddar cheese, the crystals are composed of calcium lactate (Agarwal, Sharma, Swanson, Yüksel, & Clark, 2006). In Roquefort (Dox, 1911) and Parmiagiano Reggiano (Noël, Zannoni, & Hunter, 1996) the crystals are tyrosine crystals. The crystal composition of smear ripened cheese however is more ambiguous. Previous work showed that the two major crystal constituents present in this cheese type were ikaite ($CaCO_3 \cdot 6H_2O$) (Tansman, Kindstedt, & Hughes, 2015) and struvite ($NH_4MgPO_4 \cdot 6H_2O$) (Tansman, Kindstedt, & Hughes, 2016). The transition to these crystals has not yet been studied extensively. The crystals on the rind surface of smear ripened cheeses can contribute to a gritty texture which may not be desirable for all consumers making them an important area of research. It is not yet understood how the crystals form on the surface of these cheeses. It is believed that the crystals serve as an outcome of the zonal ripening of these cheeses (P. S. Kindstedt, 2013). As the cheese ripens from the surface inward, the changing pH can lead to crystallization on the surface. It is believed that studying the crystals' structure and composition may lead to a better understanding of the ripening process of smear ripened cheese. The goal of this experiment is to observe the crystal transition over time in order to better understand the maturation process of smear ripened cheese.

Analysis Methods

Powder X-ray diffraction is a method of crystal analysis commonly used in, but not limited to, the field of geology. This method is growing in popularity among food scientists because it gives the definitive identity of crystals and it is being used increasingly in determining the identity of crystals in cheese. Most of this analysis has been done with Cheddar cheese (Agarwal et al., 2006). But this method has been used in other types of cheeses too, specifically Parmigiano Reggiano, Gouda, and soft washed-rind (smear ripened) cheeses (Tansman et al., 2015). Most of the cheese analysis done by this method has been done on mature cheese samples, but in this project, cheeses early on in their maturation were analyzed.

Petrographic polarized light microscopy is another useful cheese crystal tool. This method can be used to analyze morphology as well as extinction characteristics of the crystals under polarized light. The angle of extinction indicates the angle at which the crystal seems to disappear (go dark indicating the absence of light being transmitted), as shown by the figure below (Figure 1). This method has been used successfully for crystal analysis in other food products such as ice cream (Keller, Reid, Arbuckle, & Decker, 1943). Crystals have characteristic angle of extinction values that are indicative of their structure, which can be used to help identify the crystals. Using a polarized light microscope, angle of extinction values can be obtained for different crystal types. For example, previous studies have found the angle of extinction for ikaite to be 17° (Hesse, Küppers, & Suess, 1983), struvite exhibits parallel extinction of 0° (Ellis, 1962), and calcite ($CaCO_3$) exhibits symmetrical extinction of 45° ("Petrographic Data File: Calcite,").

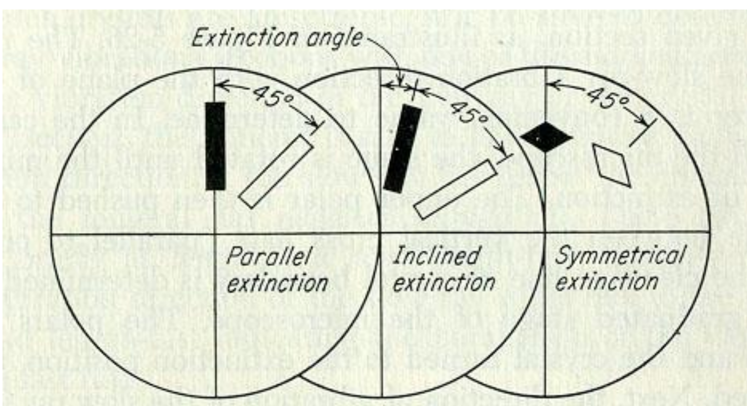


Figure 1: Extinction Angles [from ("Extinction Angle,")]

Crystal Morphologies

Recent findings have shown that brushite ($CaHPO_4 \cdot 2H_2O$) first appears on the cheese surface, then calcite, then ikaite, which may also be present with struvite (Tansman et al. unpublished data). Brushite has varying structure depending on the environment it is grown in as shown in Figure 2 below (Abbona, Christensson, Angela, & Madsen, 1993).

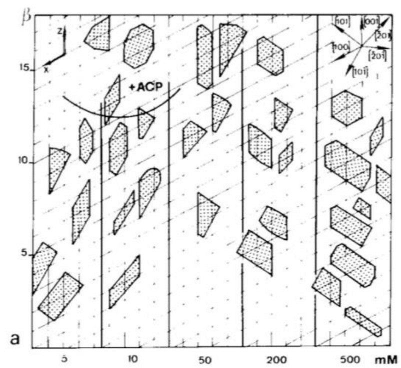


Figure 2: Brushite crystals at different concentrations of calcium phosphate [from (Abbona, Christensson, Angela, & Madsen, 1993)].

Calcite can also appear in many different forms shown below in Figure 3 below ("The Mineral Calcite,").

Figure 3: 3D representations of calcite crystals. The most common forms of calcite are rhombohedral and scalenohedral, shown on top left side of the image [from ("The Mineral Calcite,")].

Ikaite has a characteristic oval morphology shown in Figure 4 below (Rysgaard et al., 2012).

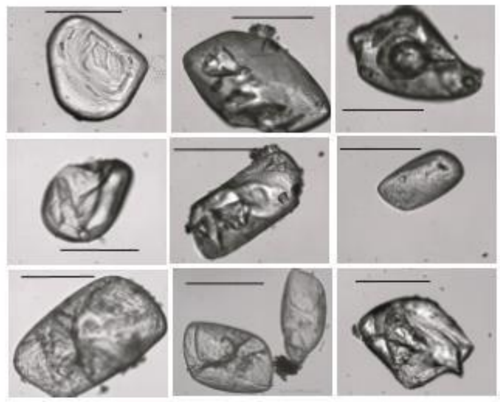


Figure 4: Examples of ikaite crystals found in the sea ice. Scale bar is 100 µm [from (Rysgaard et al., 2012).

Struvite also has a unique morphology, easily distinguished by visual inspection from that of ikaite, as shown in Figure 5 below (Wilsenach, Schuurbiers, & Van Loosdrecht, 2007) characterized by a rectangular oblong shape.

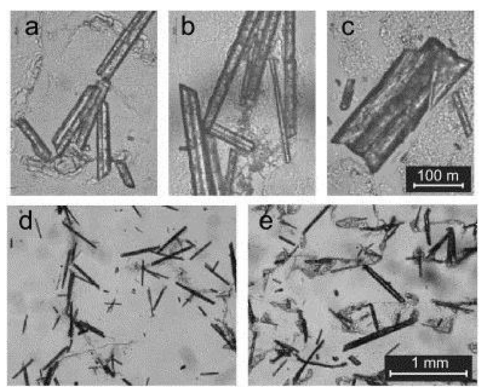


Figure 5: Struvite; Images (a)–(c) are 400 times magnified. Images (d) and (e) are 50 times magnified [from (Wilsenach, Schuurbiers, & Van Loosdrecht, 2007)].

The variable shape values in conjunction with varying angle of extinction values were the basis for differentiating crystals in this experiment. The purpose of this experiment was to observe the crystal transition over time through a variety of methods in order to better understand the maturation process of smear ripened cheese. In order to better understand the crystal composition, measurements of crystal morphology by image analysis as well as extinction values were obtained from the crystals in order to develop quantitative metrics on the crystals. X-ray (powder and single crystal) diffraction were also taken of the smears in order to definitively find the identity of the crystals.

Materials and Methods

Cheese Manufacture and Ripening

Four cheese samples each from two different cheese batches manufactured on the same day at The Cellars at Jasper Hill Farm, a Vermont cheese producer, were obtained. The eight samples were manufactured the same day from the same vat. They were ripened under commercial conditions at the Cellars until the end of week six post manufacture, at which point they were sent to the University of Vermont in an insulated shipping container. The samples were stored in an incubator at 4.5 °C until use during weeks 7, 8, 9, and 12 after manufacturing. During these four weeks, two cheese wheels, one from each batch, were obtained and analyzed, representing duplicates at each time point.

Cheese Composition

At each time point, moisture and fat contents of the cheese samples were determined using half of the grated cheese wheel. In order to determine the moisture content, weighing dishes were obtained from a 100 °C forced draft oven and cooled in a desiccator for 30 minutes. Cheese samples (two to three grams) were placed in the dishes and oven dried at 100 °C for 24 hours. The dishes were cooled in a desiccator for 30 minutes and reweighed (AOAC, 1990). Moisture analysis was done in triplicate.

The fat content was determined using the Babcock method (AOAC, 1990). Grated cheese samples (9.00 grams) was placed into 50% Paley test bottles. Hot (60 °C) distilled water (10 mL) was added to each bottle and they were stoppered. Babcock sulfuric acid (17.5 mL) was added in three equal portions, shaking between additions, until the curds were completely digested. The bottles were placed in a heated (60 °C) Babcock centrifuge and centrifuged for five minutes. Hot (60 °C) water was added to bring the liquid level up to the base of the bottle neck and the bottles were centrifuged for an additional 2 minutes. Hot water was then added to bring the fat column well up to into the calibrated bottle neck and it was centrifuged for one more minute. The bottles were transferred to a 60 °C tempering bath and allowed to set for five minutes. A few drops of glymol was added to the surface of the fat column. Fat analysis was done in duplicate.

Powder X-ray Diffraction (PXRD)

Smear samples were collected on weeks 7 and 12 and analyzed using PXRD. The bacterial smear surface was scraped and homogenized using a mortar and pestle. This mixture was loaded on a diffraction slide and a few drops of mineral oil were added to the surface to delay the onset of crystal dehydration. Diffractograms were obtained using a MiniFlex II powder XRay diffractometer (Rigaku, The Woodlands, TX) at a speed of 2°2θ/minute between 5 and 50°2θ. The results were compared to existing reference cards on the ICDD database.

Polarized Light Microscopy

During each time point, the bacterial crystal smear was scraped from each face of the cheese and the circumference. This smear was dabbed on to a glass slide and overlaid with mineral oil. Images were obtained using a Nikon E200POL petrographic microscope with a rotating stage

(Nikon Corporation, Tokyo, Japan) and a SPOT Idea 1.3 Mp color camera (SPOT Imaging Solutions, Sterling Heights, MI). The extinction angles of the crystals were obtained using both the darkfield setting as well as the quarter wave filter on the microscope.

Single Crystal X-ray Diffraction (SCXRD)

Smears of washed rind cheese were analyzed by polarized light microscopy (see above) and two presumed crystals each of ikaite and struvite were chosen based on morphology, birefringence, and angle of extinction. The crystals were pulled from the slide and analyzed using a Bruker Apex II CCD single-crystal diffractometer using graphite-monochromated Mo K_a radiation. The information was collected and the unit cell parameters were determined using the Bruker Apex2 package programs.

Image Analysis

The images obtained from the polarized light microscopy were analyzed using MetaMorph Image Analysis Software (Molecular Devices, LLC). The shape factor, length, breadth, circumference, and total area of each crystal was determined on the software.

Results and Discussion

Characterization of Ikaite and Struvite

Two crystals each of ikaite (Figure 6) and struvite (Figure 7) were definitively identified using SCXRD and a scorecard was developed for them shown below.

Figure 6: Example of ikaite crystal pulled for SCXRD analysis with typical angle of extinction and shape factor values.

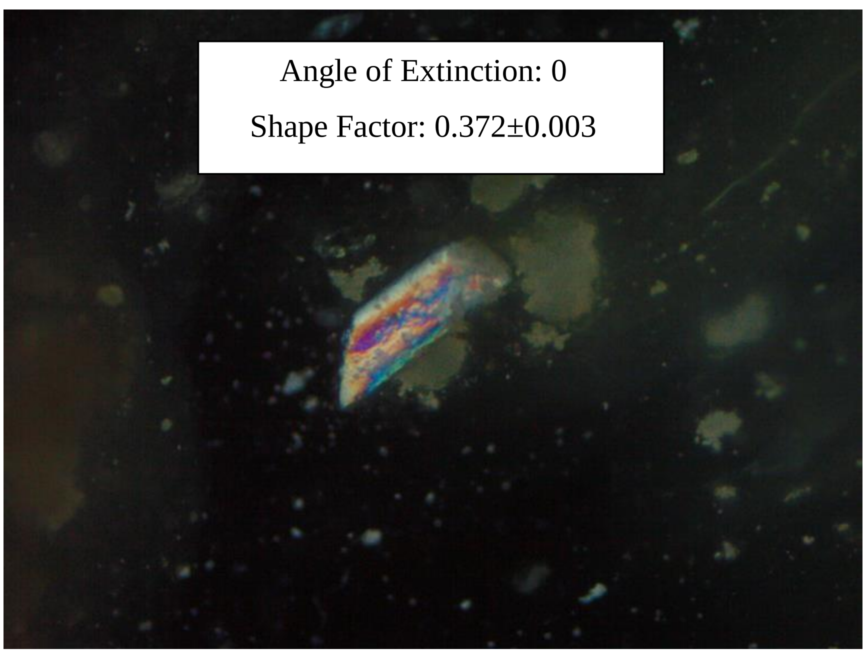


Figure 7: Example of struvite crystal pulled for SCXRD analysis with typical angle of extinction and shape factor values.

Cheese Composition

The moisture and fat analysis showed that the percent moisture in both cheeses went down and the percent fat went up during the time points analyzed in this study (weeks 7, 8, 9 and 12 post manufacture). Both cheeses came from the same vat, but unknown to us, one batch was treated normally with brine wash while the other one was beer—washed. This complicated our ability to use them as duplicates. Subsequent composition testing showed that despite different washing, the cheeses showed comparable characteristics. MNFS, moisture in the nonfat substance, is an important determinant of microbial, enzymatic, and ripening behavior so this value was determined to assess the similarity between the cheeses. The change in percent moisture, percent fat, and MNFS are shown below in Figures 8-10.

$$MNFS = \frac{\%\, moisture}{(100 - \%\, fat)} x\ 100$$

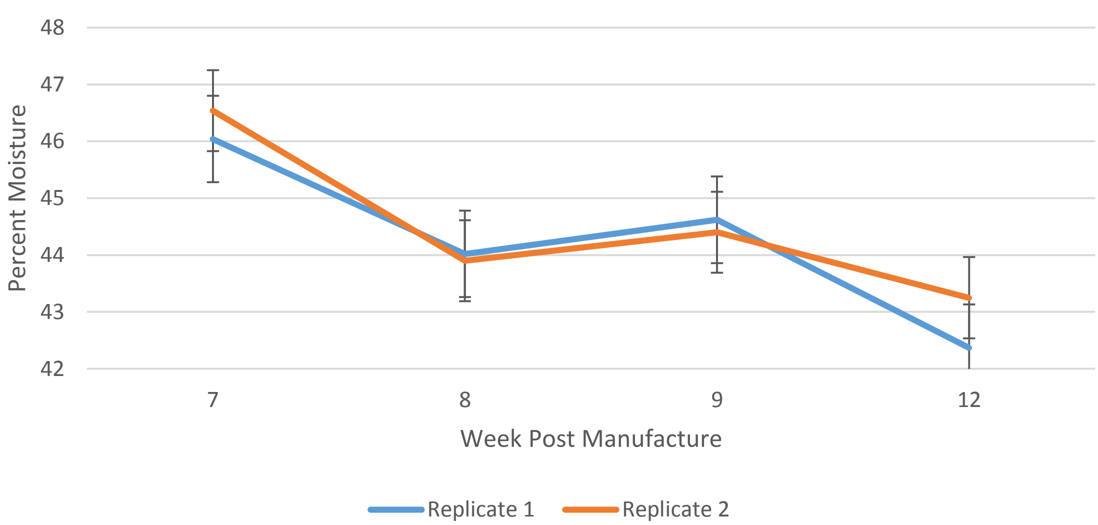


Figure 8: Change in percent moisture over time. Replicate 1: brine washed cheese; Replicate 2: beer washed cheese. Replicate 2 started and ended with more moisture. Despite the slight

differences, both replicates (brine washed and beer washed cheeses) were comparable and showed similar changes in moisture content.

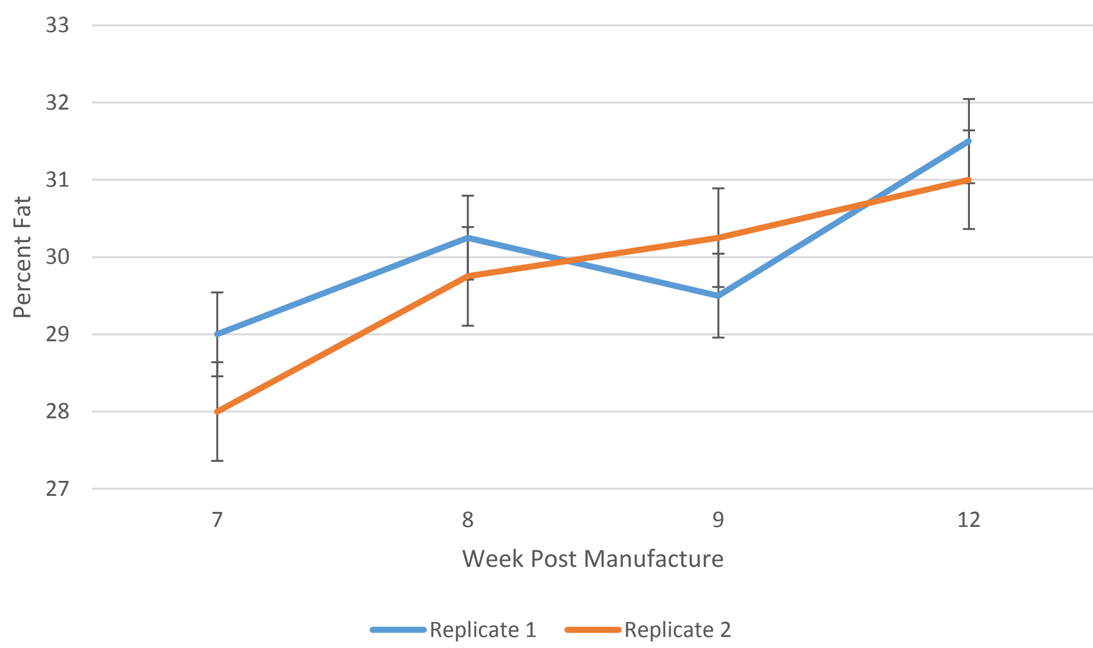


Figure 9: Change in percent fat over time. Replicate 1: brine washed cheese; Replicate 2: beer washed cheese. The brine washed cheese, replicate 1, started and ended with a higher overall fat percentage. Despite slight fluctuations, the percent fat in both replicates (brine washed and beer washed cheeses) were comparable and showed a similar upward trend over time.

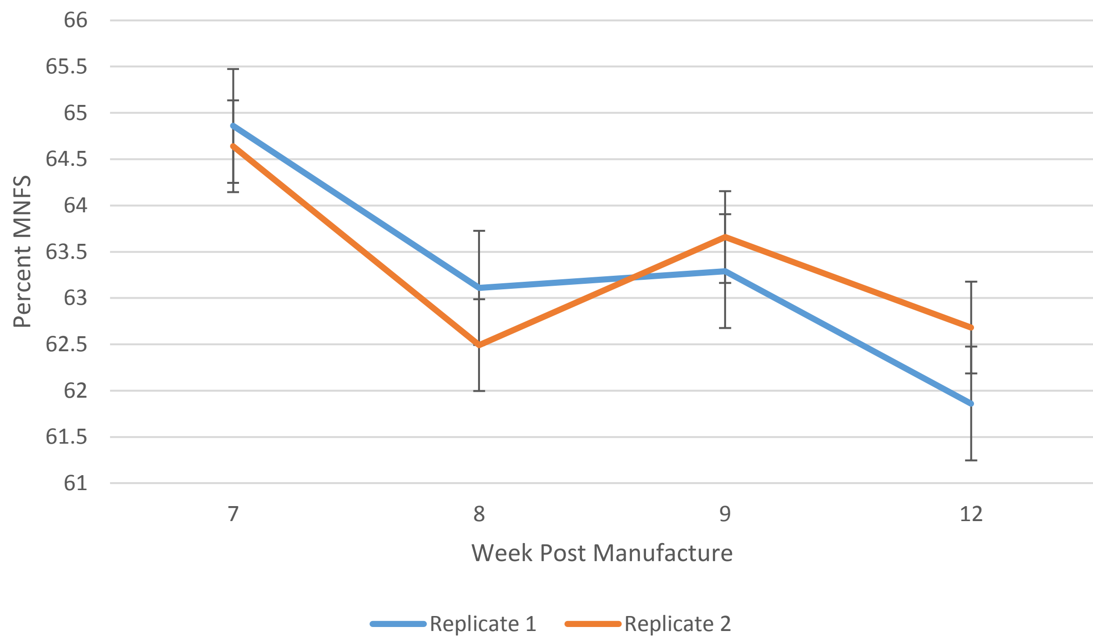
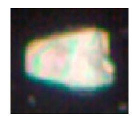
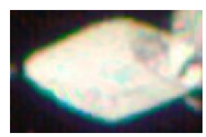
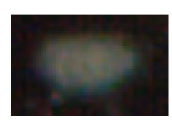


Figure 10: Change in percent MNFS over time. Replicate 1: brine washed cheese; Replicate 2: beer washed cheese. The percent MNFS trended downwards for both cheese samples. Despite slight fluctuations, the percent MNFS in both replicates (brine washed and beer washed cheeses) was very comparable and showed similar change, indicating that, despite slightly different treatments, there was no apparent effect on key chemical factors such as MNFS. Therefore, data from the two replicates were pooled and the results presented below are based on the pooled data set.

Crystal Results

This study presumptively found 9 unique crystal types which were grouped together based on angle, shape, and color similarities (see Figure 11). This finding is complicated by many factors though. Many of the crystals fragmented during the analysis process, skewing their shape factor values. There was also room for error in the angle of extinction measurements. It was difficult to visualize the longitudinal grains of many of the crystals, complicating the accuracy of reliable angle of extinction measurements. Some crystals also show variable birefringence. Despite these complications, the crystals were grouped into best fit groups. SCXRD and other methods are required in order to definitively classify these crystals. The average shape factor and angle of extinction values for each crystal type is shown in the table below. The individual values for each crystal are represented below in Figure 12.

Crystal Type	Average Shape Factor	Average Angle of Extinction	Sample Image
Presumptive Ikaite (n=42)	0.568	24.9	
Presumptive Calcite (n=8)	0.759	44.6	
Small Angle Large Shape Factor (n=13)	0.773	12.6	
Diamond Shaped (n=13)	0.642	35.3	
Struvite Lookalike (n=2)	0.548	0	
Pink and Teal Colored (n=15)	0.556	25.3	
Orange/Blue Colored (n=4)	0.777	17.6	

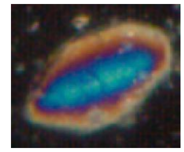
Presumptive Struvite (n=3)	0.412	0	
Super Colorful Rainbow (n=8)	0.587	0	

Figure 11: Selected characteristics of nine unique crystal types observed on the surface of washed rind cheeses during aging.

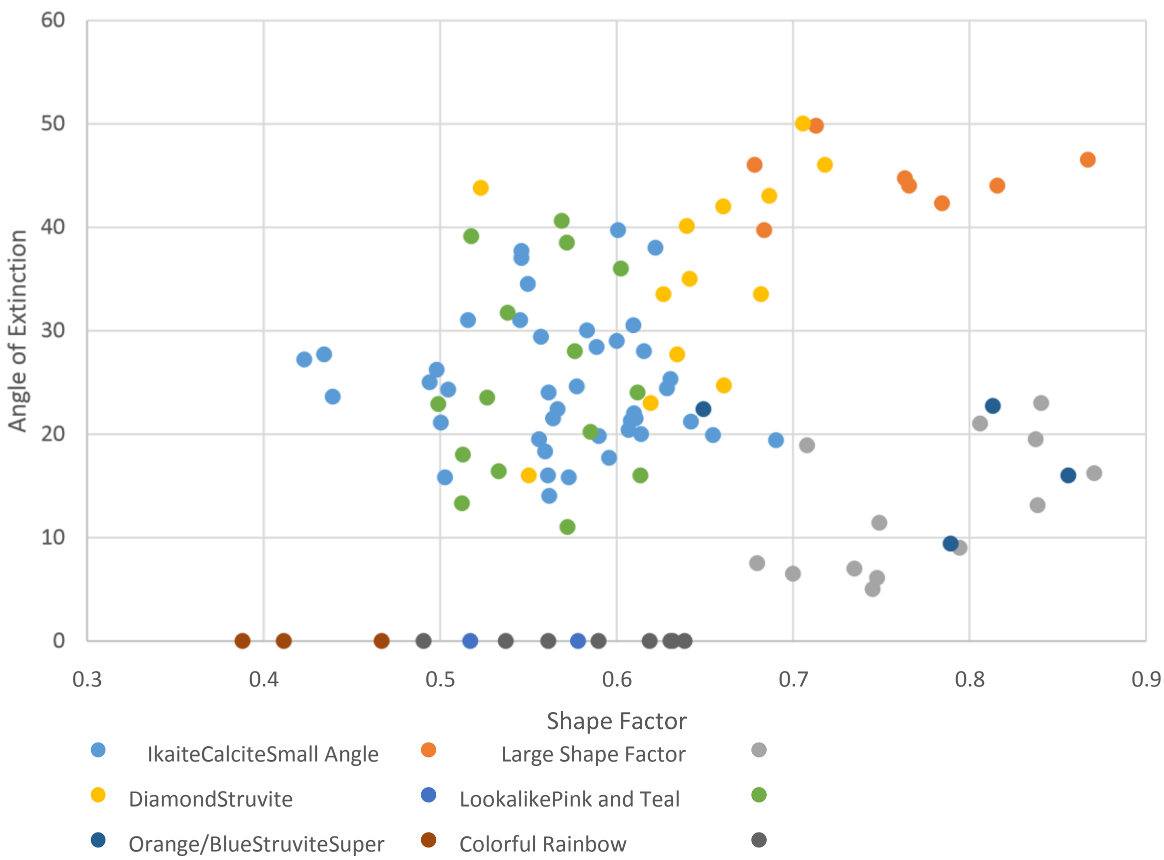


Figure 12: Individual crystal angles of extinction and shape values of nine unique crystal types observed on the surface of washed rind cheeses during aging. Figure 12 shows the following—

presumed ikaite crystals tend to group around 25 degrees, presumed calcite crystals around 45 degree, and presumed struvite crystals around 0 degrees. This figure also shows that there is overlap between crystals in both angles of extinction and shape factor illustrating the difficulty of grouping these crystals without definitive methods.

The wide array of crystals on the cheese suggest that brushite, calcite, struvite, and ikaite may not be the only crystals present on the cheese surface as previously found. There seems to be a wide variety and prevalence of different crystal subtypes. SCXRD would be required though in order to definitively identify each crystal subtype.

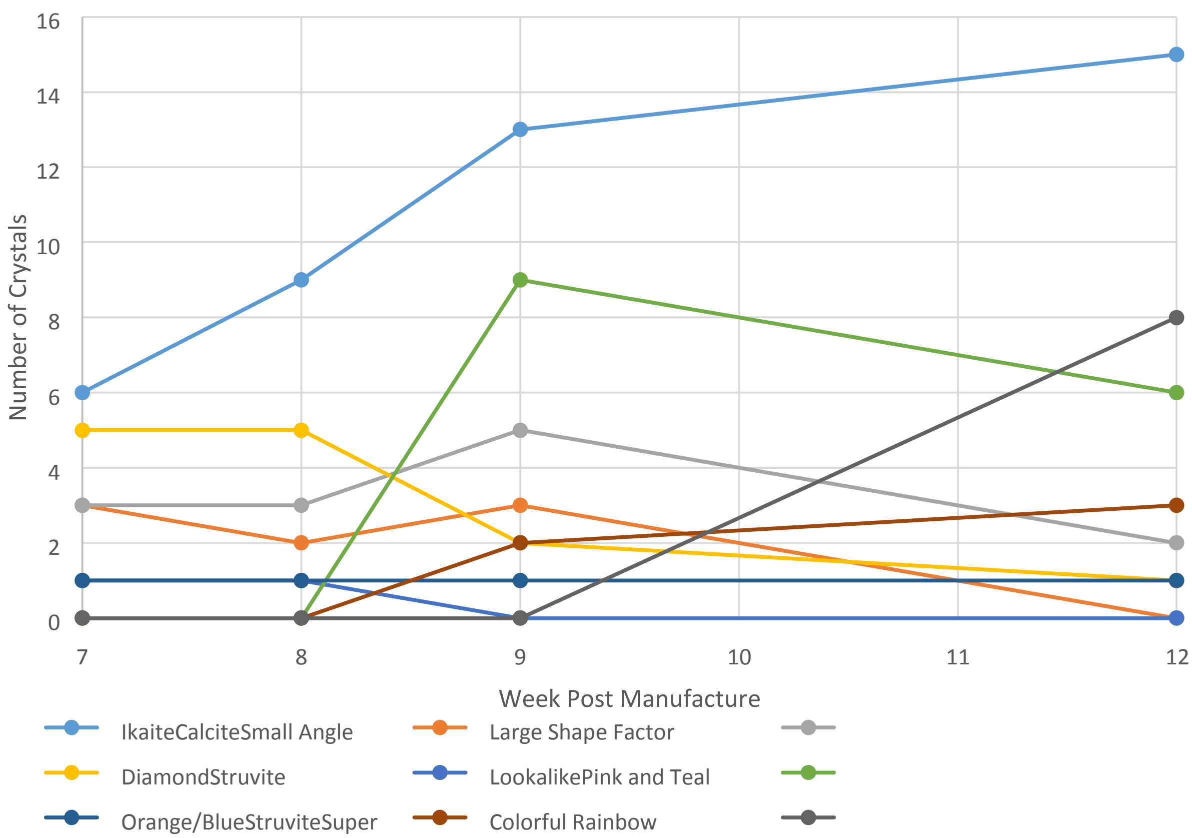


Figure 13: Change in the number of nine unique crystal types over time. Figure 13 shows that crystals like ikaite increased in number over time. Struvite didn't appear at the first time point, but increased in number over time. The super colorful rainbow crystals didn't appear

until the last time point. Other crystals such as the diamond shaped ones, small angle large shape factor, and calcite decreased over time.

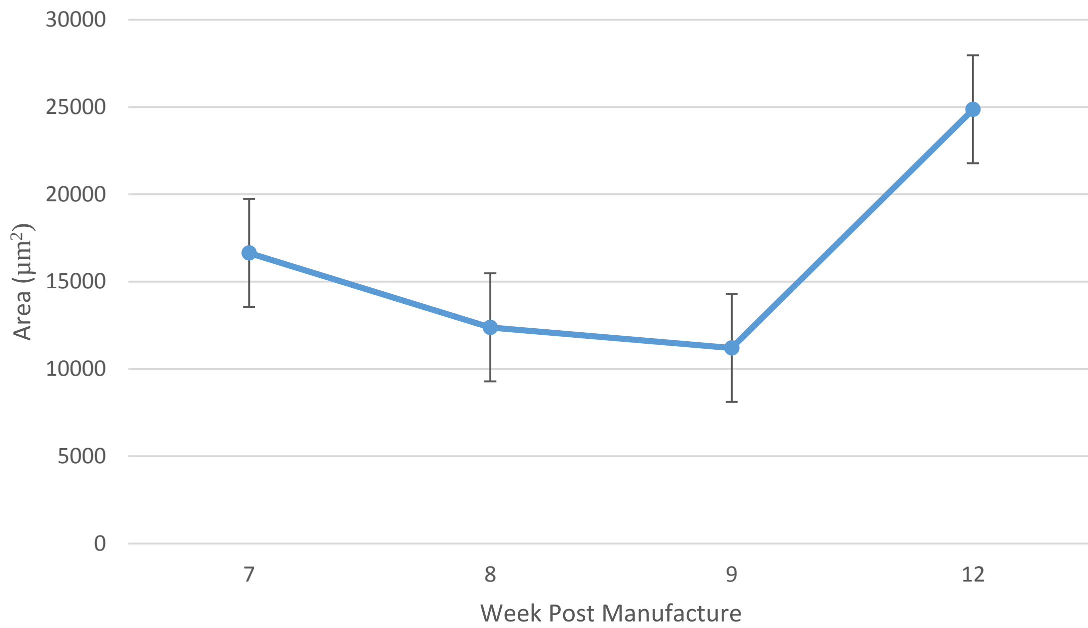


Figure 14: Change in average area of all crystals over time. Figure 14 shows that the overall average area of all crystals went down over the first three weeks analyzed (7, 8, and 9). The final week showed a significantly greater average area.

The average area of the crystals decreased in the first three weeks of this study which was not expected. Perhaps the crystals broke down and new ones were formed from the elements released by the broken down ones. The final time point though showed extremely large crystals. This is an interesting point for cheese manufactures and consumers who don't like the gritty texture of some of these cheeses. By week 12 the crystals are very large and could add to consumer distaste. This overall area trend was observed for presumed ikaite and calcite crystals as well, as evidenced in Figures 15 and 16.

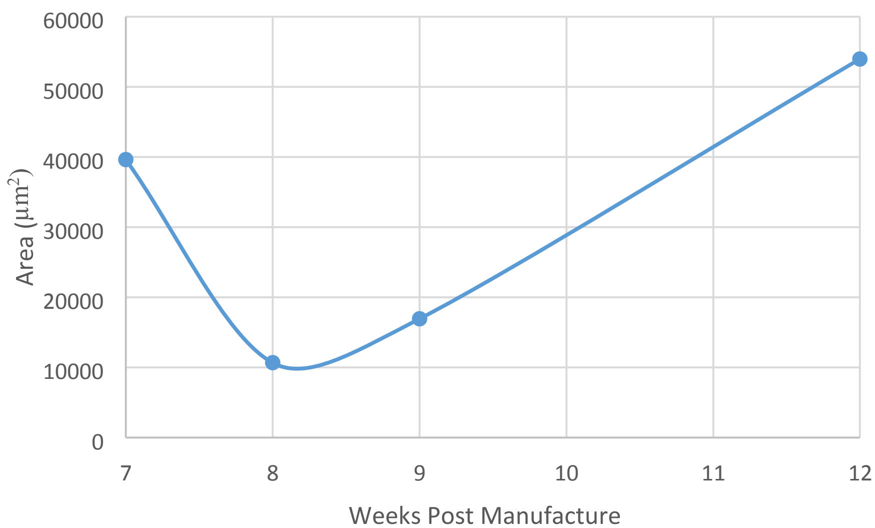


Figure 15: Change in average area over time for ikaite crystals. The average area went down then gradually increased.

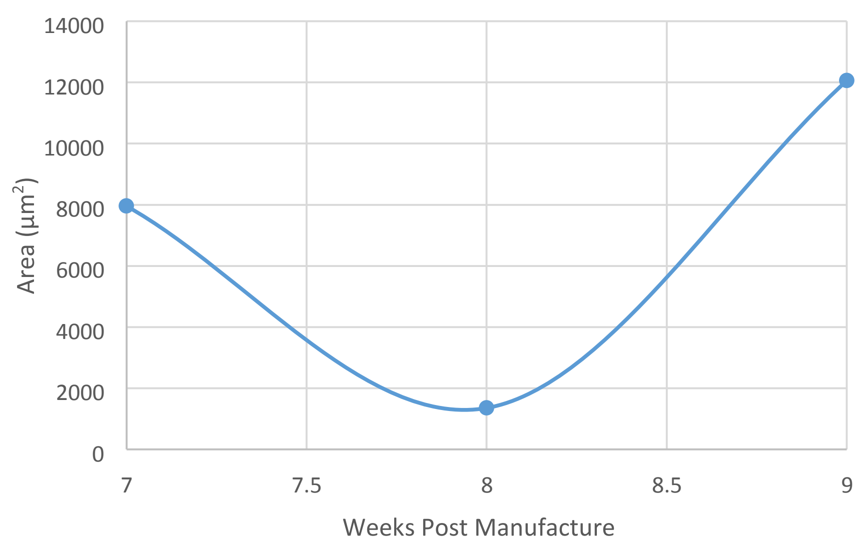


Figure 16: Change in average area over time for calcite crystals. Like ikaite, the crystal size decreased then gradually increased.

Powder X-ray Diffraction

PXRD was run on the samples at the beginning of the trials at week seven, but the results were inconclusive. Each time the samples were run, they showed different results. The crystals dehydrated too quickly and their structure began to break down. This is understandable because many of the crystals are hydrates. The only crystal consistently present was calcite, shown below in Figure 16.

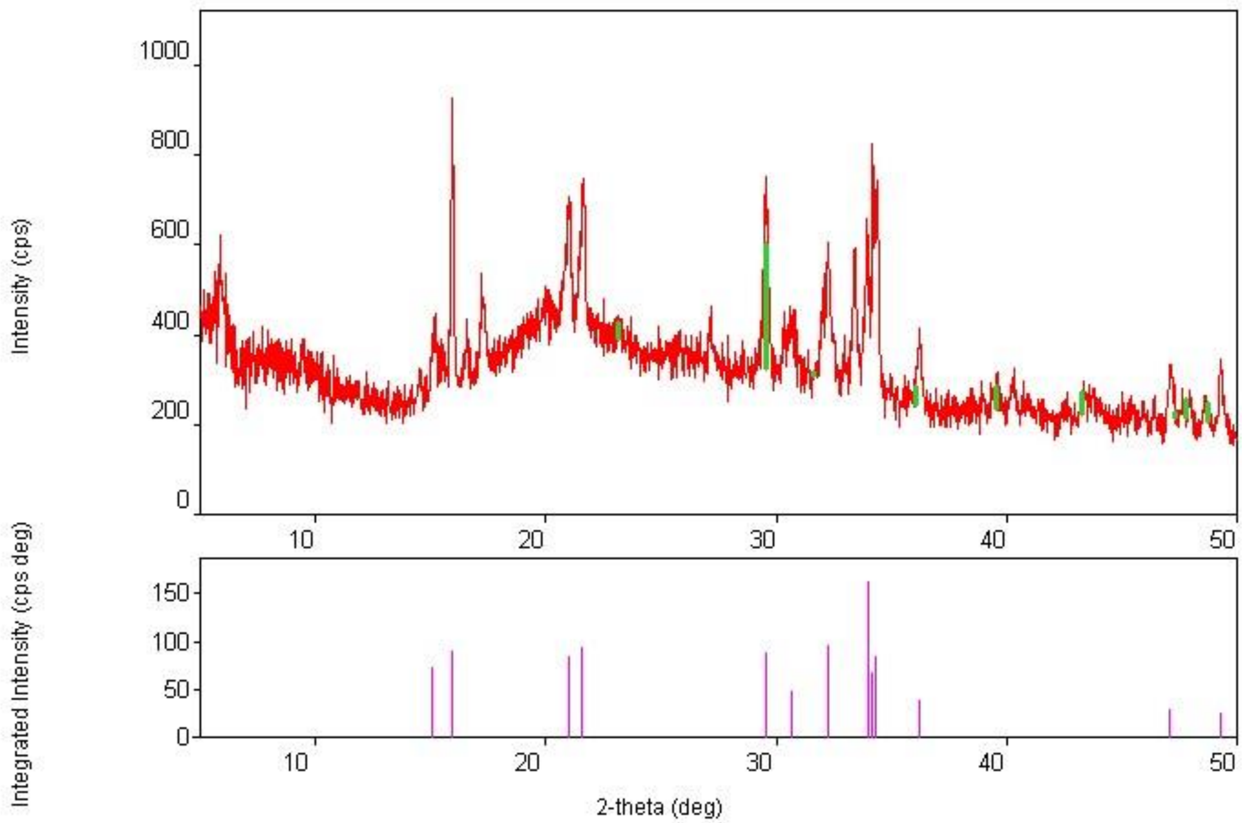


Figure 17: PXRD diffractogram of a young cheese sample at week seven post manufacture with a calcite card reference. The green overlapping peaks indicate the presence of calcite at week seven.

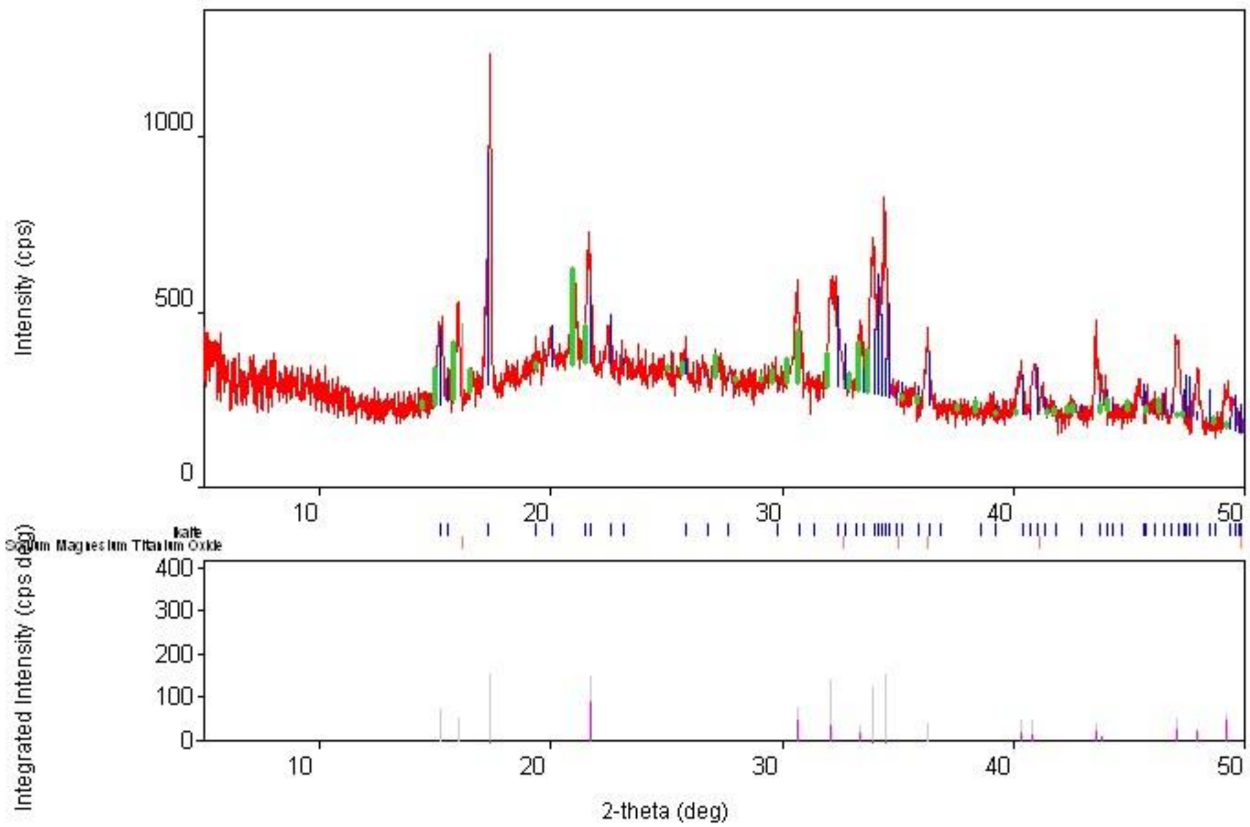


Figure 18: PXRD diffractogram of a mature cheese sample at week 12 post manufacture with a struvite card reference. The overlapping peaks indicate the presence of struvite by week 12.

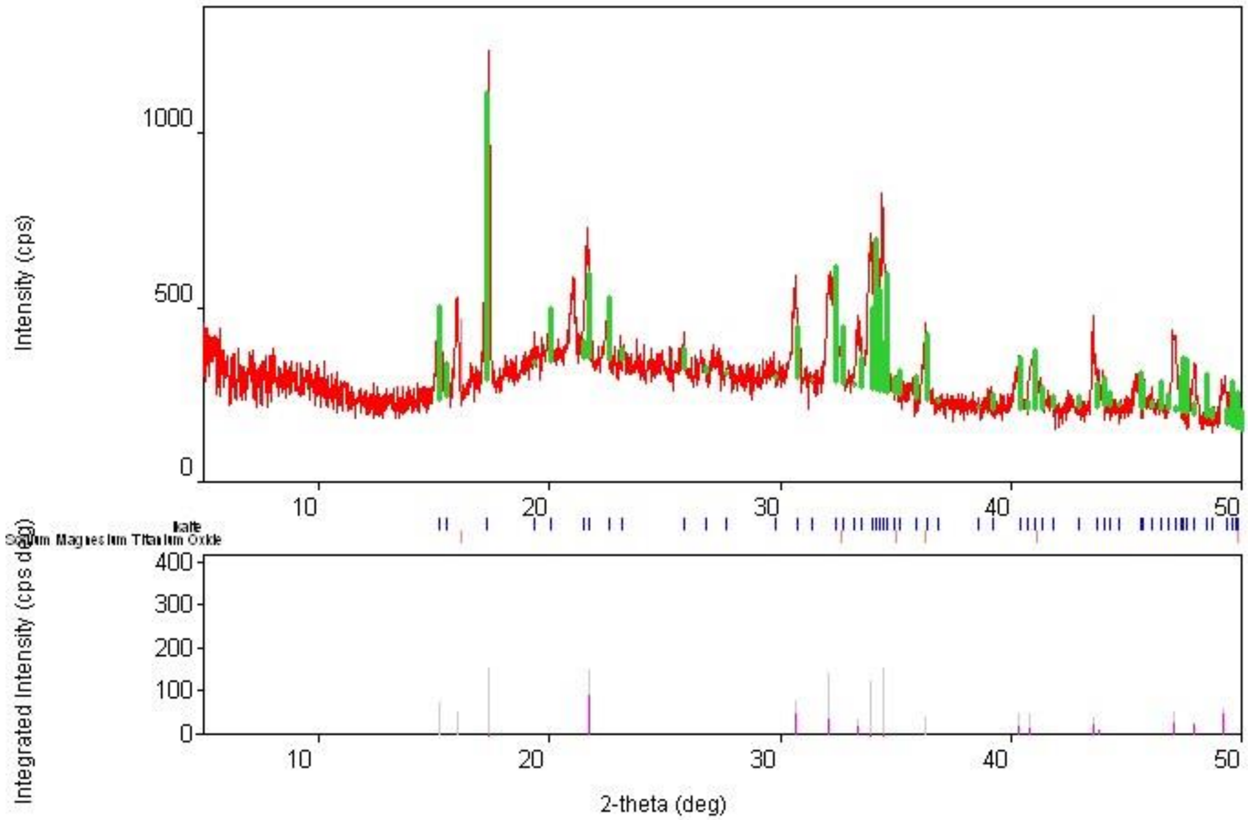


Figure 19: PXRD diffractogram of a mature cheese sample at week 12 post manufacture with an ikaite card reference. The overlapping peaks indicate the presence of ikaite at week 12.

This PXRD analysis revealed that the crystals present on young cheeses are often too unstable to be analyzed using PXRD, but it is a slightly better tool on mature cheese samples, in the case of smear ripened cheese. This method had been previously used in smear ripened cheese (Tansman et al., 2015, 2016) but the samples used in that study were fully ripened cheeses made by a different cheesemaker using a different method. The cheeses analyzed in this study were incompletely ripened and had a considerably more viscous smear. The variable age and composition of the cheeses might account for why PXRD wasn't an effective analysis tool in this study. SCXRD seems to be the better alternative in order to definitively identify crystals.

Conclusion

This study illustrated the complexity of the bacterial crystal smear on smear ripened cheese. There appear to be many different crystal types present on the cheese. This study found nine apparent types, but complications in study methodology might have led to this finding. Fractured crystals may have led to erroneous shape factor results. In order to definitively confirm these nine subtypes, further work is required. SCXRD will greatly aid in the identification of these crystals. This study illustrated limitations of PXRD especially with unstable crystals like those found early on on smear ripened cheese. This study also showed that total average area of the crystals gradually decreased during weeks 7, 8, and 9 post manufacture. The area of the crystals then showed a significantly greater average area during week 12. This may be a result of the ripening process. Perhaps the crystals broke down and the released elements gave rise to new crystal types. The major conclusion drawn from this work is the necessity of improved techniques.

Acknowledgements

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