ACCELERATED RIPENING BY ENZYME MODIFIED APPLICATION IN SWISS CHEESE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

The objectives of this study were to utilize enzymes, previously used in Cheddar cheese, to accelerate the ripening of Swiss cheese; to determine the chemical nature associated with the acceleration of ripening; to do descriptive sensory screening to identify the most promising enzyme; and to monitor the chemical changes associated with the application of heat to the samples.

Initially, enzyme screening was done in Swiss cheeses of 150g and looking at a limited number of enzymes. Accelerzyme® CPG, peptidase enzymes, and Lipomod™ 621P – L621P, a mixed esterase, protease, and peptidase enzymes were used at different concentrations. Both enzymes increased bitterness in the cheese and Lipomod produced a rancid flavor. Both bitterness and rancidity were reduced by heating the cheeses in a microwave. Since Lipomod gave the stronger flavors, further study was made with the enzyme at two concentrations in 5 pound cheeses to confirm the findings in the screening experiment.

Gas Chromatography was used to measure the production of short chain free fatty acids. Acetic (C2:0) acid was the acid produced in largest amount. Butyric (C4:0), and n-caproic (C6:0) acids were produced in significantly greater amounts in the high enzyme concentration treatment, which could be associated with the rancid flavor. Propionic (C3:0) and isovaleric (i-C5:0) acid concentrations were independent of the addition of enzyme.

Swiss cheeses were analyzed by Fourier Transform Infrared (FTIR) spectroscopy to determine if they could be differentiated on the basis of enzyme addition. All samples
were differentiated based on the infrared spectra with major differentiation based on short
chain fatty acids, organic acids, and ester flavor compounds. Cheese made on different
days gave different patterns, but still were differentiated on the basis of enzyme
treatments.

After 60 days of ripening, the overall flavor was intensified, and the rancid and bitter
flavors were reduced by controlled heating the samples at 65°C for 5 minutes without
affecting the nutty and sweet flavors associated with Swiss cheese.

The results of this study suggest that enzyme treatment may provide promise to meet the
needs of the institutional markets desire for a Swiss cheese that provides better flavor
after cooking.
Further work is needed to develop a process for accelerating Swiss cheese flavor on a
commercial basis.
DEDICATION

I want to dedicate this work to my adorable son, David Jesús; to my wife, Virgilia Altagracia; and to Arlyn. They gave me strength and courage in my more adverse times during my studies at The Ohio State University. They were always there supporting me in those times of desperation. This dedication is also extended to all my relatives: my mom, Mirella Miladys; my father, Dante Jesús; my grandma, Gloria; my brothers, Eduardo Dario and Eduard; my sisters, Dania Flor, Daris Miladys, Laritsa, Karina, and Marlene; and to my uncles, aunts, nieces, nephews, and cousins.
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CHAPTER 1
INTRODUCTION

1.1 Background

There is an increasing desire to accelerate the ripening of Swiss cheese, as well as requests from the food service industry for a cheese with a flavor that will carry through in cooked dishes. Ohio State University pioneered in the acceleration of flavor in Swiss cheese slurries, and some of these principles may be applicable to Swiss cheese. Lipases, proteases and peptidases have been used in Cheddar cheese to accelerate ripening. Different enzymes will be used in the manufacture of Swiss cheese to accelerate flavor production.

1.2 Hypotheses

1. Addition of selected enzymes can accelerate the ripening of Swiss cheese by providing substrates that will stimulate growth and biochemical activity of Propionibacterium freudenreichii, which is known to be the most important source of flavor in Swiss cheese during ripening (Thierry et al., 2004; and Piveteau, 1999).

2. Addition of selected enzymes can modify the flavor of Swiss cheese to enhance the “carry over” of flavor, similar to that has been achieved in Cheddar cheese.
1.3 The objectives of this study are:

1. To enzyme modify Swiss cheese to accelerate and modify flavor development

2. To determine the chemical nature associated with the acceleration of ripening, including:
   i. Lower molecular weight fatty acids
   ii. Use of FTIR to differentiate cheese samples, giving attention to the functional groups that provide differentiation

3. To do descriptive sensory screening to identify the most promising enzymes

4. To monitor the chemical changes associated with the application of heat to the cheese samples

1.4 General Plan

The approach in this study was to accelerate Swiss cheese flavor production by screening different enzymes, especially lipases/esterases, proteases, and peptidases. The enzyme screening was done at the beginning by the use of mini cheese preparation and looking at a limited number of enzymes. After the enzyme screening was completed in the mini cheeses, 5 pound Swiss cheeses were produced to confirm the findings of the screening experiment. FTIR was used to analyze the cheese samples by grouping them according to their chemical similarities and/or differences. Gas Chromatography (GC) was used to analyze short chain fatty acid content of the 5 pound Swiss cheese samples as well. Cheese samples were also heated up and analyzed with the FTIR to monitor the chemical changes associated with the heat application. To test the acceptability of the enzyme
modified Swiss cheeses, descriptive sensory screening was done in the mini Swiss cheese samples by a panel of three Food Scientists who are familiar with the Swiss cheese product.
2.1 The cheese ripening process

Cheeses are usually ripened from 5 weeks to two years, depending upon the desired flavor intensity. During the ripening process, cheeses undergo different biochemical reactions that are important for the development of appropriate aroma, flavor, and texture.

Ripening involves three basic processes, which are glycolysis, lipolysis, and proteolysis. Each one of these processes takes particular importance depending on the cheese variety. The ripening of cheese has been widely reviewed by a number of researchers, including (Kristoffersen and others, 1967; Fox and others, 1994; Law, 1999; and Law, 2001).

2.1.1 Proteolysis during cheese ripening process

During the ripening period, cheeses experience a number of biochemical reactions including proteolysis. Proteolysis is the route in which proteins are degraded into large, medium, and small polypeptides, and into free amino acids.
The milk, coagulants, starter cultures, non-starter bacteria, adjunct and non-starter lactic acid microorganisms are the agents that contribute to the proteolitic procedure during cheese ripening, through the action of the associated enzymes.

The proteolysis process of most cheeses can be summarized as having an initial hydrolysis of milk caseins mainly due to coagulant residuals, then due to plasmin and probably cathepsin D. This result in the production of different peptides that are further degraded by the coagulant, the starter culture, and the non-starter bacteria enzymes. Fox and others, (1994) have indicated that the proteolic procedure may vary greatly depending on the manufacturing practices followed in the production of different cheese varieties.

Chymosin, a proteinase of gastric origin, is the most important coagulant traditionally used in cheese making. This is produced by mammalian species. It initially coagulates the milk and further then performs initial caseins proteolysis in a number of cheeses (Fox and others, 1994; Law, 2001).

As indicated by (Law, 2001), after casein micelle is attacked by rennet, polypeptides are produced. Casein derived polypeptides are further degraded, and they may produce bitter or tasteless peptides important for the texture development. Bitter peptides produce savory peptides that affect the flavor once they are degraded into amino acids. Amino acids like cysteine, methionine, isoleucine, valine, and phenylalanine act as precursor products during flavor and aroma development while proline, and glutamic acid have direct impact on cheese flavor profile (Law, 2001).
2.1.2 Enzymes

In cheese processing, enzymes from the milk, coagulants, starter cultures, non-starter bacteria, adjunct, and non-starter lactic acid microorganisms are very important since they are active factor in the proteolitic process during cheese ripening. Proteolitic enzymes have been found in non-starter lactic acid bacteria (NSLAB), Peterson and Marshall, (1990). Fox and others, (1994) have suggested that those enzymes may be important in the proteolysis process of cheese ripening.

McSweeney and others, (1993) have indicated that non-starter lactobacilli made a difference in short peptides and free amino acids formation in Cheddar cheese production from raw milk.

Exogenous enzymes have also been used to accelerate the cheese ripening process.

Lipases have been shown to have positive impact on Swiss cheese flavor (Vangtal and Hammond, 1986; Biede and others, 1979)

2.1.3 Swiss cheese

Swiss cheese is characterized by its eyes and its sweet, nutty flavor. Although number of methods has been developed to accelerate cheese ripening, including the addition of enzymes (Law, 2001; Roberts and others, 1995); there’s no literature on accelerated ripening of Swiss cheese using enzyme addition.
2.1.4 Flavor compounds in Swiss cheese

Ney, (1981) indicated that the most important chemical compounds determining the flavor of Emmental type Swiss cheese were acetic acid, propionic acid, alpha-keto acids, aldehydes, methyl ketones, and amines. Later on (Bosset and Liardon, 1985; Kowalewska and others, 1985; Griffith and Hammond, 1989); among others, have indicated an additional number of compounds which have implications in the flavor profile of Swiss cheese types, including alkylpyrazines, indole, benzothiazole, alcohols, ketoalcohols, ketones, aldehydes, esters, lactones, and phenols. Most recently, (Harper, 2007, personal communication) have indicated the construction of a library database of Swiss cheese flavor compounds for application in electronic nose differentiation. Butyric acid, isobutyric acid, diacetyl, acetic acid, acetaldehyde, and propionic acid are among the compounds reported.

2.2 Accelerated cheese ripening

The acceleration of the ripening of cheese has been reviewed by (El-Soda, 1986; Fox, 1989; Law, 2001; Roberts and others, 1995).

The methods to accelerate the cheese ripening process include the use of elevated ripening temperature, high-pressure processing and storage, attenuated heat or freeze shocked starter cultures, culture adjuncts addition, genetically modified cells or lactic cultures, addition of enzymes, slurry systems, and combinations of all the above methods.
In this review, specific attention is given to the application of enzymes for the acceleration of flavor.

### 2.2.1 Exogenous Enzymes used to accelerate cheese ripening

The principal approaches to accelerate cheese ripening by the addition of enzymes are to add lipases and proteinases. Until now, accelerated cheese ripening research has focused on the identification of potential enzymes to accomplish proteolysis or lipolysis acceleration.

Lipases are used in enzyme modified cheese applications to enhance flavor intensity and aroma notes.

Peptidases and proteinases contribute directly or indirectly to accelerated cheese ripening producing low-molecular weight non-volatile peptides and free amino acids (Aston and Dulley, 1982; Aston and Creamer, 1986). Some proteinases may produce bitter flavor notes so they need to be used with peptidases with debittering properties. Most commercial enzymes used for accelerated ripening are a blend of proteinase, peptidase and lipase.

### 2.2.2 Other enzymes that have been suggested to accelerate cheese ripening

Other enzymes that have been suggested to accelerate cheese ripening includes cell extracts homogenates of microbial origin, (Vema and Anand, 1989; Braun and Olson, 1986; El-Soda and others, 1982; Law and Wigmore, 1983; Law and Wigmore, 1985; demethiolase, (Lindsay and Rippe, 1986).
2.3 The use of FTIR analysis to differentiate enzyme modified Swiss cheese

2.3.1 Infrared Spectroscopy

Infrared (IR) spectroscopy is an important tool used to identify organic functional groups. IR is widely used in the food industry for the analysis of food and food ingredients quantitatively and qualitatively. IR radiation is divided into near infrared, from 0.8 to 2.5 µm; mid infrared, from 2.5 to 15 µm; and far infrared, from 15 to 100 µm. For food analysis, the near and mid infrared regions are more useful (Wehling, 1998).

2.3.1.1 Mid-Infrared Spectroscopy

The mid-infrared methodology has been reviewed by (Wehling, 1998; Carey, 2003; Coates, 2000; among others). This region of the Infrared spectroscopy, deals with the absorption of light by a sample in the 4000 to 625 cm⁻¹ wavenumbers or 2.5 to 15 µm wavelength range. Mid-infrared is particularly important as a tool for qualitative analysis. The identification of specific functional groups at specific frequencies allows the qualitative analysis. Milk is the most common food item analyzed quantitatively with mid-IR spectroscopy (Wehling, 1998).

Infrared is the radiation between visible light and microwave. The most useful section of the IR region to determine chemical structures is from 2.5 µm to 16 µm wavelength. The reciprocal of wavelength is the wavenumbers and it is expressed in centimeters, cm⁻¹. The IR wavelength region, 2.5 µm to 16 µm is the same as 4000 to 625 cm⁻¹ when expressed in wavenumbers.
The IR electromagnetic radiation excites a molecule from its ground or lowest vibrational energy state to a higher energy state by causing vibration in the modes of stretching, bending, scissoring, rocking and/or wagging.

2.3.2 FTIR as a tool to analyze cheese

Fourier transform infrared spectroscopy FTIR has been used with a MIRacle 3-reflection diamond attenuated total reflectance (ATR) accessory as a successful tool for analysis of Swiss cheese at Ohio State University, (Rodriguez-Saona and others, 2006). The technique is reproducible, rapid, simple and convenient for rapid screening of Cheese chemical composition. It has also been used to predict the shelf life of Swiss cheese (Cattaneo and others, 2005); in cheese quality control (Giardina and others, 2003); monitoring the geographic origin of Cheeses (Karoui and others, 2005); and to predict the chemical composition, volatile fatty acids, and volatile compounds in Montasio Cheese (Sqorlon and others, 2003).

2.4 Gas chromatography as a tool to measure short chain free fatty acids present in Swiss cheese

2.4.1 Gas chromatography

Gas chromatography (GC) is a separation technique that has been in used since the 1950’s (Reineccius, 1998). The separation is conducted by passing a sample through a stationary phase with the help of a carrier gas or mobile phase. The sample is injected in the injection port, it passes all the way through the column or stationary phase, and then
reaches the detector. The higher the affinity of the sample for the stationary phase, the longer the retention time or the time the sample will take to go from the injection port to the detector. Different chemical compounds present in the sample and showing different affinity for the stationary phase, make possible the separation. Gas chromatography is used to analyze a number of compounds being the identification and quantification of fatty acids one of the most important analyses conducted by the used of GC (Reineccius, 1998).

2.5.2 Short chain fatty acids present in Swiss cheese

Short chain free fatty acids (FFAs) such as propionic (C\textsubscript{3}), acetic (C\textsubscript{2}), propionic (C\textsubscript{3:0}), and butyric (C\textsubscript{4}) are important compounds in cheese flavor profiles. During cheese production, lactose is degraded into lactic acid within the first 24 hours, then, during warm room treatment at 21\degree C; lactic acid is metabolized by propionic bacteria to propionic and acetic acids (Thierry et al. 2004).

The amount and relative proportions of propionic and acetic acids are important factors in Swiss cheese flavor, (Piveteau, 1999). The theoretical ratio of propionic to acetic acid is about 2:1. However, recent analysis of 15 commercial Swiss cheeses showed that about 1/3\textsuperscript{rd} had a ratio of propionic to acetic acid of less than 1:1 (Harper, 2006, personal communication).

Isovaleric acid is also important to the flavor of Swiss cheese (Thierry et al., 2004) and is produced by propionic bacteria concomitantly with acetic and propionic acids (Thierry et al., 2004).
Lipolysis has also been associated with Swiss cheese, especially in Europe, and there is a relationship between free fatty acids associated with lipolysis and Swiss cheese flavor (Biede and others, 1978; Vanttrol and Harnond, 1986).
Initially, selected commercial enzymes used in the accelerated ripening of Cheddar cheese were screened with the purpose to accelerate the ripening in Swiss cheese. The screening was done in mini cheeses (150g), taking advantages of the fact that a number of mini cheeses could be made to test enzymes in pairs at different concentrations at the same time with minimum of effort.

Commercial enzymes supplied by different companies were tested during the preliminary screening of this study.

3.1 Approach

The approach followed to screen the enzymes in miniature Swiss cheese was as shown below:

Mini Swiss cheeses were processed using commercial enzymes to accelerate the ripening. The following commercial enzymes were used at concentrations recommended by the manufacturers. a) Accelerzyme® CPG at concentrations of 0.002% and 0.01% v/v. b) Lipomod™ 621P – L621P at concentrations of 0.05% and 0.15% w/w based on milk solids
3.1.1 Treatments:
Control mini cheeses produced with no enzyme addition, and
Enzyme modified mini Swiss cheeses produced with different enzymes at different
concentrations as recommended.

3.1.2 Enzymes and concentrations:

3.1.2.1 Enzymes
The following commercial enzymes were utilized in the acceleration of ripening in the
Swiss cheese study:

1. Accelerzyme® CPG from DMS Food Specialties USA, Inc. Accelerzyme is a
   food grade fungal peptidase that has shown it highest activity in the amino acids
   Phenylalanine, Isoleucine, Leucine, Methionine, and Valine. This product comes
   as a brown liquid. (For more information about this product contact DMS Food
   Specialties Dairy ingredients at P.O. Box 1 2600 MA Delft, The Netherlands.
   Phone 31-15-2792355, Fax 31-15-2793200 or visit www.dsm-
   foodspecialties.com )
2. Lipomod™ 621P – L621P from BIOCATALYSTS. L621P is a food grade mixed fungal esterase, protease and peptidase that comes as a white to off-white powder. The esterase activity is on tributyrin as substrate and the protease activity is against casein as substrate. (For more information about this product contact BIOCATALYSTS at Cefn Coed, Parc Nantgarw, Cardiff, CF 15 7QQ, Wales, UK. Phone 44 (0)1443 843712, Fax 44 (0)1443 846500 or visit www.biocatalysts.com)

3.1.2.2 Concentrations

The concentrations of enzymes used was as follows:

Accelerzyme at 0.002% and 0.01% v/v, and

Lipomod at 0.05 and 0.15% weight/weight or weight of enzyme as percent of milk solids.

3.1.3 Evaluations:

The mini Swiss cheeses were evaluated for:

1. pH before pressing, before brining, and after the warm room treatment
2. FTIR analysis at day 60 after production to investigate chemical profiles
3. Sensory screening to evaluate the flavor
4. Selection of the most promising enzyme(s) to produce normal size enzyme modified Swiss cheese.
3.2 Procedure

3.2.1 Mini cheese manufacture

3.2.1.1 Materials:

Fresh milk from Waterman Dairy Center ¹ was used; The starter cultures used were:

*Streptococcus thermophilus* STC-6, *Lactobacillus helveticus* LH-32, and

*Propionibacterium freudenreichii* PS-4 supplied by Chr Hansen Inc. ²;

The commercial coagulant, CI-IY-MAX R, rennet or chymosin provided by Chr Hansen Inc. as well; Enzymes: Accelerzyme R CPG supplied by DMS Food Specialties USA, Inc. ³; and Lipomod TM 621P – L621P provided by BIOCATALYSTS ⁴.

¹ Waterman Dairy Center, 2433 Carmack Rd., Columbus OH 43221-3586

² Chr Hansen Inc., 9015 West Maple Street, Milwaukee, WI 53214-4296, USA. Phone 414-607-5700 www.chr-hansen.com

³ DMS Food Specialties Dairy ingredients, P.O. Box 1 2600 MA Delft, The Netherlands. Phone 31-15-2792355, Fax 31-15-2793200 or visit www.dsm-foodspecialties.com

⁴ BIOCATALYSTS, Cefn Coed, Parc Nantgarw, Cardiff, CF 15 7QQ, Wales, UK. Phone 44 (0)1443 843712, Fax 44 (0)1443 846500 or visit www.biocatalysts.com
3.2.1.2 Method for cheese making:

The mini-cheese procedure previously developed was modified to permit evaluation of multiple enzymes at the same time and making a comparison to the control which was Swiss cheese produced without adding enzyme;

The procedure is outlined as follows:

1. Cheese was made by the standard pilot plant procedure, using 5 gallons of milk.

2. When the cheese was ready to be put into form, about one gallon of cheese and whey were transferred into 3 gallon milk cans and treated as follows:

   - Control (with no enzyme addition)
   - Enzyme at various concentrations

3. Next, the samples were placed in 18 Oz. Whirl Pak bags for centrifugation.

4. They were centrifuged at 350 x g for 10 minutes, at 1400 x g for 30 minutes; then, inverted and centrifuged again at 1400 x g for 30 extra minutes.

5. Then, they were pressed (0.07 psi) until reaching a pH ~5.2 or 5.3, placed in 23% brine at 4°C for 20 minutes. Then, vacuum packed in nylon/polyethylene vacuum pouches.
(Thompson Equipment & Supply, 3249 East Kemper Rd., Cinti, OH 45241), and placed in a cold room at about $4^0C$ for five days.

6. Next, the mini cheeses were placed in a warm room at $21^0C$ for 5 weeks.

7. They were transferred back to cold room at about $4^0C$ until they were 60 days old.

3.2.2 Evaluation:

3.2.2.1 pH evaluation:
The pH was measured and recorded for raw milk, before pressing the mini cheeses, before brining, and at the end of the 5 weeks in the warm room at $21^0C$.

3.2.2.2 FTIR analysis
Samples were screened with the FTIR for chemical differences and/or similarities at 60 days after production.

An Excalibur 3100 FTIR spectrometer (Varian Inc., 68 Mazzeo Drive, Randolf, MA 02368, USA) was used with MIRacle ATR™ or attenuated total reflectance clamp, and ATR diamond crystal and selenide accessories.
3.2.2.2.1 Method:

The Swiss cheese samples were analyzed in the FTIR in three different ways:

a) Solid cheese samples of about 0.5 g were placed on the ATR diamond crystal for the FTIR analysis. A clamp was used to apply pressure to bring the solid cheese sample into close contact with the ATR diamond crystal.

b) The cheese samples were also analyzed as a nitrogen powder. The solid cheeses were mixed with liquid nitrogen and ground into a powder as follow: 15-20 g of cheese sample was taken, cut into small pieces, and put into a blender 7012 (Waring Commercial Blender, New Hartford, Connecticut 06057). About 15 ml of liquid nitrogen were added to freeze the cheese sample, and immediately mixed to produced a fine powder. The powder was stored at -40 °C until it was used for the FTIR analysis. For the FTIR analysis, the cheese and nitrogen powder was placed on the ATR diamond crystal. A clamp was used to apply pressure to bring the powder sample into contact with the ATR diamond crystal.

c) A solvent extraction procedure previously developed in the Department for Cheddar cheese analysis was utilized. Water, chloroform, and ethanol were used as solvents to make an extraction out of a nitrogen powder previously made. The extraction was prepared as follows:
One hundred to one hundred and five (100-105) micrograms of the powder were placed into a 1.5 ml vial tube. Five hundred microliters of deionized water were added, shake it
for few seconds; and then it was mixed using a sonic demembrator model D100 (Fisher Scientific, Pittsburgh, PA) until getting a uniform mixture. Five hundred microliters of chloroform were then added, shake it, and the mixture was centrifuged at 16,060 x g centrifugal force or 13,000 rpm for 3.5 minutes. Two hundred microliters from the supernatant were taken and transferred into a 0.6 ml vial tube, 200 microliters of 100% ethanol added, shake it, and centrifuged again at 16,060 x g centrifugal force for 3.5 minutes. Then, 200 microliters from the top area were taken as the sample to be analyzed.

For the three sample preparation methods, FTIR data was collected using Varian Resolutions Pro, Varian, Inc. program, Version 4.0.5.009; 679 Springvale, Rd. Mulgrave, Victoria 3170, Australia. Six FTIR spectra or replicates per mini cheese sample were taken between 4000 to 700 cm⁻¹ wavenumbers and 4 cm⁻¹ resolution. Prior taking the FTIR spectra of each sample, a background was collected. After taking the spectra, data were analyzed using Pirouette multivariate data analysis package, version 3.11, from Infometrix Inc., P.O. Box 1528, Woodinville, WA 98072-1528. Soft Independent Modeling of Class Analogy, SIMCA, was used as a classification method to classify samples according to their chemical similarities or differences. SIMCA was run with second derivative and divide by as selected transforms.

### 3.2.2.3 Flavor evaluation

Samples were screened for sensory evaluation at day 60 after production. The flavor of 60 days old mini Swiss cheeses was evaluated by a panel of 3 Food Scientists familiar
with Swiss cheese. The panel was provided with the mini Swiss cheese samples, and water, and they were asked to evaluate them by answering the questions in appendix G. A 1 to 9 Hedonic scale was used to evaluate the sensory attributes of interest, where 1 indicates low perception of the attributes and 9 indicates high or strong perception of the attributes. The questionnaire applied was a variation of standard procedures (Hootman, 1992; Lawless and Heymann, 1999; Meilgaard and others, 1991).

3.3 Results and discussion:

3.3.1 Effect of treatment on pH of the mini cheeses:

The pH of the cheeses during pressing, before brining, and after warm room are presented in table 3.1. Just before brining, the pH values were within the pH range of Swiss cheese, and they were from 5.13 to 5.22 and showed no effect of treatment. After the warm room, the pH values range from 5.24 to 5.5. In all cases except for the Lipomod at the low concentration, showed the expected increase in pH.

<table>
<thead>
<tr>
<th>Date Made</th>
<th>Enzyme and concentration</th>
<th>Raw Milk Ph</th>
<th>pH before pressing</th>
<th>pH before brining</th>
<th>pH after warm room</th>
</tr>
</thead>
<tbody>
<tr>
<td>July 20</td>
<td>Control</td>
<td>6.70</td>
<td>6.43</td>
<td>5.15</td>
<td>5.44</td>
</tr>
<tr>
<td></td>
<td>Accelerzyme 0.002% v/v</td>
<td>6.03</td>
<td>5.20</td>
<td>5.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Accelerzyme 0.01% v/v</td>
<td>6.03</td>
<td>5.13</td>
<td>5.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipomod 0.05% w/w</td>
<td>6.01</td>
<td>5.22</td>
<td>5.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipomod 0.15% w/w</td>
<td>6.02</td>
<td>5.17</td>
<td>5.41</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1: pH measurement of mini Swiss cheese samples at different times during the ripening process.
3.3.2 FTIR analysis results:

The SIMCA plots for the three different approaches are shown in the following figures. Figures 3.1, 3.2, and 3.3 show the class projections of the three methods of analysis. The analyses were run at the time that the samples were 60 days old.

Figure 3.1 shows the direct method FTIR class projection of mini Swiss cheese samples produced on July 20, 2006. It shows two clustering areas: on one side, clustered together the treatments with Lipomod at 0.5% and Lipomod at 0.15% w/w of milk solids. At the other side, the control and the treatments with Accelerzyme at 0.002% and Accelerzyme at 0.01% on a v/v basis clustered together and could not be differentiated.

![Figure 3.1: Direct method FTIR class projection of 60 days old mini Swiss cheeses produced on July 20, 2006. Each oval represents 95% probability clouds.](image-url)
Next, figure 3.2 shows the nitrogen powder FTIR class projection of the same mini Swiss cheese samples produced on July 20, 2006. As figure 3.1 it also shows two clustering areas: on one side, clustered together the treatments with Lipomod at 0.5% and Lipomod at 0.15% w/w of milk solids. At the other side, the control and the treatments with Accelerzyme at 0.002% and Accelerzyme at 0.01% on a v/v basis clustered together.

Figure 3.2: Nitrogen powder method FTIR class projection of 60 days old mini Swiss cheeses produced on July 20, 2006. Each oval represents 95% probability clouds.
Next, figure 3.3 shows the FTIR class projection of the water-chloroform-ethanol solvent method of analysis run in the same mini Swiss cheese samples produced on July 20, 2006. This method allowed a good separation among control, enzyme modified mini Swiss cheese samples with both enzymes Accelerzyme and Lipomod at low and high concentrations.

Figure 3.3: FTIR class projection of the water-chloroform-ethanol solvent method of 60 days old mini Swiss cheese produced on July 20, 2006. Each oval represents 95% probability clouds.

After analyzing the miniature Swiss cheese samples with the three approaches, it can be concluded that:

1. The method of analysis makes a difference.
2. Making the nitrogen powder does not make a big improvement over the direct method.
3. The water-chloroform-ethanol solvent method gave the best results; consequently, this method was used for following FTIR analyses.

Next, the discriminating power region having greater effects on the mini Swiss cheese samples separation is presented. Figures 3.4, 3.5, and 3.6 show the discriminating powers of the direct, the nitrogen powder, and the water-chloroform-ethanol solvent methods respectively.

Figure 3.4: Discriminating power from direct method FTIR analysis of 60 days old mini Swiss cheeses produced on July 20, 2006.
Figure 3.5: Discriminating power of nitrogen powder method of FTIR analysis of 60 days old mini cheeses produced on July 20, 2006.

Figures 3.4 and 3.5 show similar discriminating wave numbers in both methods of analysis except for a small shift. There were two important bands in both cases. These peaks belong to the fingerprint region.

Figure 3.6 shows the discriminating power of the water, chloroform, and ethanol solvent method of FTIR analysis for the same cheese samples.
The discriminating power of the extraction method showed a number of bands that allowed the differentiation for all the treatments. The method gave a clear separation based on different groups that have flavor potential. The wave numbers associated with functional groups are in tables 3.2, 3.3, and 3.4. The wave numbers 1537, 1549, 1574, and 1576, associated with proteins and high molecular weight peptides, were not significant for differentiation of the water-chloroform-ethanol solvent extract. This was because proteins and fats were removed by extraction procedures, thus permitting a better evaluation of compounds associated with cheese flavor. The groups represented in the
extraction method can be associated with C-O containing compounds like carboxylic acids, ketones, alcohols, organic acids, ethers, esters, and fatty acids. They can also be associated with amides and amines of peptides; and aromatic nitrogen compounds.

Then, tables 3.2, 3.3, and 3.4 present the peaks, peaks’ wave numbers, and their most related functional groups for the three FTIR approaches respectively.

Table 3.2 shows the peaks, peaks’ wave numbers, and the related functional groups for the direct method of FTIR analysis. The table shows two discriminating peaks, which belong to the fingerprint region (1500-600 cm\(^{-1}\)).

<table>
<thead>
<tr>
<th>Peak(s)</th>
<th>Wave number(s) or frequency (cm(^{-1}))</th>
<th>Functional group(s)</th>
<th>References</th>
</tr>
</thead>
</table>

Table 3.2: Peaks, wave numbers, their assigned functional groups, and references of the most discriminating region of the direct method of FTIR analysis. Analysis was conducted to the mini Swiss cheese samples produced on July 20, 2006 and sampled after they were 60 days old.
Table 3.3 presents the peaks, peaks’ wave numbers, and the related functional groups for the nitrogen powder method of FTIR analysis. The table shows two discriminating peaks, which belong to the fingerprint region (1500-600 cm\(^{-1}\)). The wave numbers suggest that separation of the cheeses in direct method and nitrogen powder analyses are primarily in the protein and peptide region.

<table>
<thead>
<tr>
<th>Peak(s)</th>
<th>Wave number(s) or frequency (cm(^{-1}))</th>
<th>Functional group(s)</th>
<th>References</th>
</tr>
</thead>
</table>

Table 3.3: Peaks, wave numbers, their assigned functional groups, and references of the most discriminating region of the nitrogen powder method of FTIR analysis. Analysis was conducted to the mini Swiss cheese samples produced on July 20, 2006 and sampled after they were 60 days old.

Table 3.4 shows the peaks, peaks’ wave numbers and the related functional groups for the water, chloroform, ethanol solvent method of FTIR analysis. The table shows a number of discriminating peaks within the wave numbers range of 1000 to 1400 cm\(^{-1}\).
<table>
<thead>
<tr>
<th>Peak(s)</th>
<th>Wave number(s) or frequency (cm(^{-1}))</th>
<th>Functional group(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1298</td>
<td>C-N stretch from tertiary amine, and aromatic amino; C-H deformation vibration from primary &amp; secondary alcohols, and aromatic ethers.</td>
<td>Coates, 2000; &amp; Spectroscopy tools web site, 2007.</td>
</tr>
<tr>
<td>E</td>
<td>1379</td>
<td>C-H deformation vibration from R-CH(_3), acetates, aldehydes; ketones; &amp; tertiary alcohols.</td>
<td>Coates, 2000; Spectroscopy tools web site, 2007.</td>
</tr>
</tbody>
</table>

Table 3.4: Peaks, wave numbers, their assigned functional groups, and references of the most discriminating region of the water, chloroform, ethanol method of FTIR analysis. Analysis was conducted to the mini Swiss cheese samples produced on July 20, 2006 and sampled after they were 60 days old.
The discriminating bands belong to the fingerprint region, 1500-600 cm\(^{-1}\) with the most important groups having flavor potential. These groups include carboxylic acids, ketones, alcohols, ethers, and amides II and they are associated with fatty and amino acid families.

### 3.3.3 Results of the flavor evaluation

Mini Swiss cheese samples were screened for sensory evaluation. Table 3.5 shows the sensory screening data expressed as the average ± the standard deviation of the mini Swiss samples evaluated in a 1 to 9 Hedonic scale.

<table>
<thead>
<tr>
<th>Samples Attributes</th>
<th>Control</th>
<th>0.002% v/v Accelerzyme</th>
<th>0.01% v/v Accelerzyme</th>
<th>0.05% w/w Lipomod</th>
<th>0.15% w/w Lipomod</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Flavor</td>
<td>3 ± 2 a</td>
<td>5 ± 0 ab</td>
<td>7 ± 0 bc</td>
<td>8 ± 1 c</td>
<td>9 ± 0 c</td>
</tr>
<tr>
<td>Nutty</td>
<td>6 ± 1 a</td>
<td>6 ± 2 a</td>
<td>7 ± 1 a</td>
<td>5 ± 2 a</td>
<td>4 ± 2 a</td>
</tr>
<tr>
<td>Sweetness</td>
<td>4 ± 2 a</td>
<td>4 ± 0 a</td>
<td>4 ± 0 a</td>
<td>3 ± 1 a</td>
<td>3 ± 1 a</td>
</tr>
<tr>
<td>Rancid</td>
<td>1 ± 0 a</td>
<td>1 ± 0 a</td>
<td>1 ± 0 a</td>
<td>4 ± 2 b</td>
<td>7 ± 1 c</td>
</tr>
<tr>
<td>Bitter</td>
<td>2 ± 1 a</td>
<td>4 ± 1 ab</td>
<td>5 ± 2 ab</td>
<td>4 ± 1 ab</td>
<td>6 ± 2 b</td>
</tr>
<tr>
<td>Overall texture</td>
<td>7 ± 1 a</td>
<td>7 ± 1 a</td>
<td>7 ± 1 a</td>
<td>7 ± 1 a</td>
<td>7 ± 1 a</td>
</tr>
</tbody>
</table>

Table 3.5: Sensory screening of 60 days old mini cheese samples produced on July 20/2006. Values in the same row not sharing a common letter are different at the 5% level of significance by Tukey’s HSD. (n=3).

There was a significant difference in the overall flavor profile between the control and the enzyme modified mini Swiss cheeses, except for the 0.002% Accelerzyme. The mini
cheeses produced with Lipomod showed a tendency to produce a higher flavor characteristic than those produced with Accelerzyme, but this was associated with a strong rancid flavor.

The nuttiness was statistically the same for all the treatments; however, it tends to be lower for the enzyme modified mini cheese produced with Lipomod.

The sweetness was statistically the same for all the treatments. Even though it tends to be a little lower for the enzyme modified mini cheese produced with Lipomod, the difference was not significant.

The rancidity was statistically the same for the control and the enzyme modified mini cheese produced with Accelerzyme; however, it was significantly higher for the enzyme modified mini cheese produced with Lipomod which showed further differences due to different concentrations.

The bitterness was statistically similar in all the treatments except between the control and the enzyme modified mini cheeses with Lipomod at higher concentration.

Mini cheese samples were then heated at the microwave for 25 seconds and right after that they were sensory screened again by the same panel. Enzyme addition increased the melting properties of the cheeses as compared to the control, especially cheeses made with Lipomod.
Figure 3.7 shows mini Swiss cheese samples after being heated for 25 seconds in the microwave.

Figure 3.7: Mini Swiss cheese samples produced on July 20, 2006 and heated in the microwave for 25 seconds; 480 is the control with no enzyme added, 410 is enzyme modified with Accelerzyme at concentration of 0.01% v/v, 415 is enzyme modified with Lipomod at a concentration of 0.15% w/w on milk solids, and 345 is enzyme modified with Lipomod at a concentration of 0.05% w/w on milk solids.

Table 3.6 shows the sensory screening results of the mini Swiss cheese samples produced on July 20, 2006 after they were microwaved for 25 seconds.
Table 3.6: Sensory screening of 60 days old mini cheese samples produced on July 20 and after they were heated for 25 seconds in the microwave. Values for each lot were average for statistical analysis. Values in the same row not sharing a common letter are different at the 5% level of significance by Tukey’s HSD. (n=3).

After the heat treatment for 25 seconds in the microwave, there were some significant changes in some of the attributes which are shown in figures 3.8 through 3.12.

Figure 3.8 shows the overall flavor comparison before and after heating the mini Swiss cheese samples.
Figure 3.8: Overall flavor comparison before and after 25 seconds in the microwave, mini Swiss cheese samples produced on July 20th, 2006.

The figure shows minor changes in the overall flavor after the heat application, but the changes were not significant statistically.

Figure 3.9 shows the nuttiness comparison before and after heating the mini Swiss cheese samples.
Figure 3.9 shows the nuttiness comparison before and after 25 seconds in the micro wave, mini Swiss cheese samples produced on July 20th, 2006.

The figure shows minor changes in nuttiness after the heat application. Changes were not significant.

Figure 3.10 shows the sweetness comparison before and after heating the mini Swiss cheese samples.
Figure 3.10: Sweetness comparison before and after 25 seconds in the micro wave, mini Swiss cheese samples produced on July 20th, 2006.

Figure shows some changes in sweetness after the heat application treatment; however, changes were not statistically significant.

Figure 3.11 shows the rancidity comparison before and after heating the mini Swiss cheese samples.
Figure 3.11: Rancidity comparison before and after 25 seconds in the microwave, mini Swiss cheese samples produced on July 20th, 2006.

The figure shows significant reduction in the rancidity for Lipomod enzyme modified mini Swiss cheese treatments at both concentrations after heat application.

Figure 3.12 shows the Bitterness comparison before and after heating the mini Swiss cheese samples.
Figure 3.12: Bitterness comparison before and after 25 seconds in the microwave, mini Swiss cheese samples produced on July 20th, 2006.

The figure shows that after heat application, the bitterness decreased in all treatments. Bitterness was significantly reduced in the enzyme modified mini Swiss cheeses produced with both enzymes Accelerzyme and Lipomod.

From the initial enzyme screening study, the conclusions are:

1) Enzyme addition increased the melting properties of the cheeses as compared to the control, (based on visual appreciation of the samples).

2) After the heat application, rancidity and bitterness perception were significantly reduced in the enzyme modified mini Swiss cheese samples.
3) Heat treatment did not affect the overall flavor of the Swiss cheese samples.

4) Enzyme modified mini Swiss cheeses produced stronger flavor profile compared to the control samples. They also developed rancid and nutty flavors, but they disappear on heating without major effects in nuttiness.

5) All these findings suggest that the application of commercial enzymes could provide the food industry service with a Swiss cheese with carry through flavor in cooked dishes.
CHAPTER 4

FINAL EVALUATION OF THE ACCELERATED RIPENING IN SWISS CHEESE USING ENZYME MODIFIED APPLICATION

After accomplishment of the enzyme screening stage in miniature Swiss cheese samples, detailed evaluation was conducted in 5 pounds size Swiss cheeses to confirm the findings. Based on the screening evaluations, only Lipomod was used in these further experiments. The 5 lb size Swiss cheeses were produced using Lipomod enzyme at 0.05% and 0.15% w/w and control with no addition of enzyme.

4.1 Approach

The approach for the final stage of this study included the following evaluations:

1. FTIR chemical differentiation among the enzyme modified Swiss cheeses as compared to their corresponding controls.

2. Monitor the chemical changes occurring in the Swiss cheese samples as a result of applying heat.
3. Descriptive sensory screening to compare enzyme modified Swiss cheeses with control samples, and

4. Fatty acid determination by Gas Chromatography

4.2 Evaluation of 5 pound Enzyme modified Swiss cheeses

After the initial enzyme screening was done in mini Swiss cheese, investigation was extended to 5 pound cheeses, using the enzyme with most promising flavor carrier profile. The previous work showed that Lipomod has flavor carrier potential when the enzyme modified Swiss cheeses were submitted to heat treatment. Based on this finding, Lipomod at 0.05% and 0.15% w/w of milk solids was used for further analyses. Control Swiss cheeses with no enzyme were also produced to serve as references in the study.

4.3 Production of 5 pound Swiss cheeses:

Swiss cheeses of 5 pounds were produced as control (with no enzyme application), and enzyme modified cheese samples using Lipomod at 0.05% and 0.15% w/w of milk solids. Three separate lots of cheese were made from the same milk supply over a 4 weeks period.

4.3.1 Materials:

Fresh milk from Waterman Dairy Center (2433 Carmack Rd., Columbus OH 43221-3586) was used to produce the Swiss cheese samples. The starter cultures: *Streptococcus*
thermophilus STC-6, *Lactobacillus helveticus* LH-32, and *Propionibacterium freudenreichii* PS-4 were supplied by Chr Hansen Inc. (9015 West Maple Street, Milwaukee, WI 53214-4296, USA); The commercial coagulant CI-IY-MAX R, rennet or chymosin, was also provided by Chr Hansen Inc. (9015 West Maple Street, Milwaukee, WI 53214-4296, USA). Lipomod™ 621P enzyme was provided by BIOCATALYSTS (Cefn Coed, Parc Nantgarw, Cardiff, CF 15 7QQ, Wales, UK).

4.3.2 Method for cheese making:

Swiss cheese was made by the standard pilot plant procedure which is outlined as follows:

1. Milk was pasteurized at 63 °C for 30 minutes, then cooled down to 34 °C, and transferred to 5 gallon vats

2. Starter cultures (STC-6, LH-32, and PS-4) were added at 0.06g, 0.12g, and 0.30g per 100 liters of milk respectively; then stirred for 1 minute, and then left undisturbed for 20 minutes

3. Rennet was added, stirred for 1 minute, and then left undisturbed for 25 to 30 minutes

4. The formed curd was cut for 10 minutes with 3/8 square inch knife, and then left to rest for 5 minutes
5. After cutting, it was stirred continuously for 30 minutes

6. Then, warm water (at 34 °C) equivalent to ~10% of cheese milk was added

7. Lipomod enzyme was added at two different levels and one of the vats did not receive any enzyme to serve as control

8. Then, while stirring continuously, the temperature was raised at 2-4 °C per every 5 minutes until reaching 53 °C within approximately 30 minutes

9. Next, stirring continuously, the curd was cooked at 53 °C for 30 to 60 minutes, or until the curd pH was about 6.45

10. Next, keeping the curd within the whey, the curd was pressed at 0.30 psi for 15 minutes

11. Then, the whey was drained and the cheese curd transferred to the incubator at 40 °C to press it over night or until the pH reached 5.15 to 5.30. The first hour press in the incubator was at 0.36 psi and after the first hour at 0.55 psi.

12. Next, cheeses were placed at 23% brine at 4 °C for 4 hours, (2 hours one side and then flipped over for 2 more hours). Then, they were vacuum packed with Nylon/polyethylene vacuum pouches, (Thompson Equipments & Supplies, 3249 East Kemper Rd., Cinti, OH 45241), and placed in cool room at 4°C for 6 days.
13. They then were transferred to warm room at 21\textdegree{}C for 3 weeks, after which they were ripened at 4 \textdegree{}C until they were 2 months old.

4.4 Evaluation

4.4.1 FTIR chemical differentiation between enzyme modified Swiss cheeses and control samples

For FTIR analysis, samples were collected from each production batch after aging 60 days.

4.4.1.1 Sample preparation for FTIR analysis

Sampling preparation was done following a water-chloroform-ethanol solvent method which is summarized as follows:

1) 15-20 g of cheese sample were taken, cut into small pieces, and put into a blender 7012 (Waring Commercial Blender, New Hartford, Connecticut 06057)

2) About 5 ml of liquid nitrogen were added and after the sample was frozen, it was mixed until a fine powder was obtained

3) The sample was stored at -40 \textdegree{}C until used.
4.4.1.2 The Extraction

1) 100-105 micrograms of the powder sample was placed in a 1.5 ml vial tube

2) 500 microliters of deionized water were added, shaken for few seconds, and then mixed using a sonic demembrator model D100 (Fisher Scientific, Pittsburg, PA)

3) Then, 500 microliters of chloroform were added, shaken, and the mixture centrifuged at 16,060 x g (13000 rpm) for 3.5 minutes

4) Next, 200 microliters of the supernatant were taken, transferred into a new 0.6 ml vial, and mixed with 200 microliters of 100% ethanol

5) Then, the vial was centrifuged at 16,060 x g (13000 rpm) for 3.5 minutes, and 200 microliters from the top of the vial were taken to be analyzed in the FTIR

4.4.1.3 The spectra collection

Infrared spectra were collected between 4,000 and 700 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\) using an Excalibur 3100 FTIR spectrometer (Varian Inc., 68 Mazzeo Drive, Randolph, MA 02368, USA) mounted with a three reflection zinc selenide MIRacle attenuated total reflectance, ATR, crystal (Pike Technologies, Madison, WI). The FTIR has a computer-controlled dual source assembly with PERMAGLOW\(^{TM}\) mid-IR source and extended range KBr beam splitter. Spectra were ratioed against the background to obtain the
absorbance spectra. In order to improve the signal to noise ratio, 128 scans were co-added for each spectrum.

The spectra collection was as follow:

a) About 10 microliters of the extraction sample were taken; placed in the center of the zinc selenide sampling cell

b) The sample extract was then dried under vacuum conditions until it reaches a thin film

c) Then, the sampling cell was mounted in the sample holder for spectrum collection

d) Three spectra were collected from each cheese sample extraction for a total of nine spectra per cheese sample

4.4.1.4 The data analysis

After collecting the spectra, data was analyzed using Pirouette multivariate data analysis package, (Version 3.11 for Windows NT, Infometrix, Inc., P.O. Box 1528, Woodinville, WA 98072-1528). Classification models were developed using Soft Independent Modeling of Class Analogy (SIMCA). Samples were classified according to their chemical similarities and/or differences using the original data scores plot for the visualization of clustering among samples. The spectral regions influencing the classification of the different cheese samples was determined from the measure of variable importance or discriminating power.
4.4.2 Monitoring the chemical changes occurring in the Swiss cheese samples as a result of applying heat

During preliminary work, it was found that Lipomod enzyme modified mini Swiss cheese had middle to high rancid and bitter flavor defects. However, when heat was applied by putting them 25 seconds in the microwave at high power, there was a significant reduction in those two flavor defects. Based on these findings, the study was extended to the analysis of the chemical changes associated with application of heat in the Swiss cheese samples.

4.4.2.1 Heat application

A force convection oven was used instead of microwave to provide a better control of the heating process. To analyze the chemical changes in Swiss cheese, as a result of heat application, Swiss cheese samples of about 5 g were placed in the oven at 65°C for 2 and 5 minutes. Then, after applying heat to the samples; the sample preparation for FTIR analysis, the extraction, spectra collection, and the data analysis were done following the normal procedure described previously for the FTIR analysis. This analysis could help to monitor chemical changes occurring to the cheese samples as a result of heat application.

4.4.2.2 The experimental design

Two replicates of each Swiss cheese sample were heated at 65°C for 2 minutes and two replicates were heated at 65°C for 5 minutes. The treatments were control Swiss cheese with no enzyme application, and Lipomod enzyme modified Swiss cheeses at two
different levels: a) at 0.05% w/w and b) at 0.15% w/w of milk solids. Swiss cheeses of 60 days of aging were tested from three separate lots or batches.

4.4.3 Descriptive sensory evaluation to compare enzyme modified Swiss cheeses with control samples

The purpose of the sensory evaluation was to have a basic descriptive sensory test of the enzyme modified Swiss cheeses compared to the control. The evaluated attributes were: overall flavor, nuttiness, sweetness, rancidity, bitterness and texture.

Descriptive sensory evaluation was done by a panel of three Food Scientists familiar with Swiss cheese. The questionnaire used for sensory evaluation is in appendix G. The questionnaire used is a variation of standard procedures (Hootman, 1992; Lawless and Heymann, 1999; Meilgaard and others, 1991). The most important Swiss cheese attributes concerning to this research were taken into consideration. The panel was provided with water, and coded Swiss cheese samples to be evaluated. Samples were evaluated at 60 days based on industry practice.

Descriptive sensory test was done for all the treatments and to the three lots of cheese productions. The evaluation was also extended to heated Swiss cheese samples. All Swiss cheese samples were heated for 5 minutes at 65°C and sensory tested to measure the sensory changes associated with the application of heat.
4.4.4 Fatty acid determination by Gas Chromatography

Gas Chromatography was used to measure short chain fatty acids in the Swiss cheese samples. Analyses were made for acetic (C2:0), propionic (C3:0), butyric (C4:0), isovaleric (i-C5:0), and n-caproic (C6:0) acids.

4.4.4.1 Sample extraction for FFA analysis

Water soluble short chain fatty acids were extracted by a modification of extraction methods reported by Kuchroo and Fox, 1982; Kleinhenz and Harper, 1997; and Ji and others, 2004. Cheese samples were grated; ten grams were measured and placed into a Stomacher bag with twenty ml of double deionized water (DD H2O). To compensate for losses during the preparation of the water extract, 20 µL of n-heptanoic acid (minimum 97% pure, Sigma-Aldrich Co., St. Louis, MO) was added to the Stomacher bag prior mixing as internal standard. The mixture was homogenized for 10 minutes in a stomacher (STO-400, Tekmar Co., Cincinnati, OH), and held for 1 hour in a water bath at 40ºC. Then, it was centrifuged (Sorvall SS34, Kendro Laboratory Products, Newtown, CT) in 50 mL polypropylene screw-capped centrifuge bottles for 30 minutes at 11,180 x g and 8ºC. Then, samples were kept in ice for 1 hour to help the fat solidification, and then filtered through Whatman # 4 (150 mm, Whatman ®, Cat No 1004 150) to separate the supernatant from solidified solids. The supernatant was warmed to 25ºC in water bath and then pH adjusted to 4.6 with 85% concentrated phosphoric acid (Mallinckrodt, Inc., St. Louis, MO) to precipitate the casein. The pH adjusted supernatant was stored overnight at refrigeration temperature (4ºC), and centrifuged the next morning in 50 mL polypropylene screw-capped centrifuge bottles for 30 minutes at 16,099 x g and 4ºC.
Then, the supernatant was filtered through a 0.4 µm and a 0.2 µm syringe filters respectively. Then the extract was injected in the GC for the FFA analysis.

4.4.4.2 GC settings for the analysis of Free Fatty Acids (FFA)

A Gas Chromatograph (HP 5890 Series II, Avondale, PA) equipped with a flame ionization detector was used for the analysis. A 25 m length x 0.32 mm internal diameter x 0.52 µm film thickness HP Crosslinked FFAP column was used as well for the quantification of free fatty acid in the Swiss cheese samples. Volumes of 0.5 µL of water extracts were manually injected using a 10 µL capacity syringe (Gastight ® # 1701, Hamilton Co., Reno, Nevada). The injection port temperature was set at 220°C and the flame ionization detector was 260°C. The oven temperature was programmed to start at 90°C and stay at 90°C for 1 minute after the injection, and then increase at a rate of 10°C per minute until reaching 185°C and kept there for 0.5 minutes. Then, the temperature was raised again at 20°C per minute until reaching 230°C, and kept there for 15 minutes, to recondition the column before injecting a new sample.

The analysis was run in split injection mode, with split ratio of 1:1. Helium 99.99% pure was used as the carrier gas at 42.2 cm/sec velocity. HP Chemstation data analysis software (Hewlett Packard, G1202-90300, 6th ed., 1994, Avondale, PA) was used to integrate and analyze the FFA chromatograms. To quantify the amount of acetic (C_{2:0}), propionic (C_{3:0}), butyric (C_{4:0}), isovaleric (i-C_{5:0}), and n-caproic (C_{6:0}) acids in the Swiss cheese samples, the internal standard was used and calibration curves were prepared using three replicates from different levels of pure fatty acids (Sigma-Aldrich Co., St.
Louis, MO). Calibration curves of acetic, propionic, butyric, isovaleric, and n-caproic acids are shown in appendixes 2, 3, 4, 5, and 6 respectively.

### 4.4.4.3 Statistical Evaluation

The FFA concentrations were calculated as milligrams per 100 grams of Swiss cheese sample. Three separate lots of Swiss cheese were used for this study. Tukey HSD at the 5% significance level was used to compare the means of the treatments (n=3). Statistical computing was done with JMP 5 software package (SAS Institute Inc. 2002).
4.5 Results and discussion

4.5.1 Effects of treatments on pH and composition:

Overall the addition of Lipomod had no significant effect on pH or composition of the cheese.

Table 4.1 shows the effect of treatment on pH. pH of the milk range from 6.60 to 6.70, 6.46 to 6.53 at pressing, and 5.20 to 5.28 after brining. The addition of Lipomod had no significant effect on the pH of the cheese during manufacturing. As expected, the pH increased during ripening in most cases. At two months, the pH in cheese made with 0.15% Lipomod had pH about 0.1 less than the control, which could be related to the formation of free fatty acids to a greater increasing.

<table>
<thead>
<tr>
<th>Date Made</th>
<th>Treatment</th>
<th>Raw Milk pH</th>
<th>pH before pressing</th>
<th>pH after brining</th>
<th>pH at month two</th>
</tr>
</thead>
<tbody>
<tr>
<td>January 24</td>
<td>Control</td>
<td>6.60</td>
<td>6.53</td>
<td>5.27</td>
<td>5.34</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.05% w/w</td>
<td>6.60</td>
<td>6.53</td>
<td>5.20</td>
<td>5.38</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.15% w/w</td>
<td>6.60</td>
<td>6.52</td>
<td>5.20</td>
<td>5.27</td>
</tr>
<tr>
<td>January 31</td>
<td>Control</td>
<td>6.68</td>
<td>6.51</td>
<td>5.28</td>
<td>5.39</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.05% w/w</td>
<td>6.68</td>
<td>6.46</td>
<td>5.28</td>
<td>5.48</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.15% w/w</td>
<td>6.68</td>
<td>6.46</td>
<td>5.20</td>
<td>5.24</td>
</tr>
<tr>
<td>February 14</td>
<td>Control</td>
<td>6.70</td>
<td>6.49</td>
<td>5.21</td>
<td>5.36</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.05% w/w</td>
<td>6.70</td>
<td>6.49</td>
<td>5.23</td>
<td>5.36</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.15% w/w</td>
<td>6.70</td>
<td>6.53</td>
<td>5.28</td>
<td>5.21</td>
</tr>
</tbody>
</table>

Table 4.1: The effect of treatment on pH

There were small differences in pH within treatments from one lot to another.
The composition of the cheese samples is shown in tables 4.2 and 4.3. Table 4.2 shows that fat content was significantly different from one batch to another. It also shows that protein content is slightly higher with Lipomod addition.

<table>
<thead>
<tr>
<th>Date Made</th>
<th>Treatment</th>
<th>Fat (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>January 24</strong></td>
<td>Control</td>
<td>26.51 ± 0.07 a</td>
<td>28.92 ± 0.15 ab</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.05% w/w</td>
<td>26.48 ± 0.07 a</td>
<td>29.15 ± 0.14 ab</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.15% w/w</td>
<td>26.40 ± 0.38 a</td>
<td>31.65 ± 0.39 c</td>
</tr>
<tr>
<td><strong>January 31</strong></td>
<td>Control</td>
<td>30.79 ± 0.46 b</td>
<td>28.63 ± 0.21 b</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.05% w/w</td>
<td>32.68 ± 0.31 c</td>
<td>29.21 ± 0.22 ab</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.15% w/w</td>
<td>29.31 ± 0.07 d</td>
<td>31.73 ± 0.21 c</td>
</tr>
<tr>
<td><strong>February 14</strong></td>
<td>Control</td>
<td>31.57 ± 0.35 b</td>
<td>25.19 ± 0.08 d</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.05% w/w</td>
<td>27.86 ± 0.09 e</td>
<td>28.82 ± 0.03 b</td>
</tr>
<tr>
<td></td>
<td>Lipomod at 0.15% w/w</td>
<td>27.82 ± 0.03 e</td>
<td>29.66 ± 0.07 a</td>
</tr>
</tbody>
</table>

Table 4.2: Fat and protein composition of different batches of Swiss cheese samples. Values in the same column not sharing a common letter are different at the 5% level of significance by Tukey’s HSD. (n=3).
Table 4.3 shows that moisture content was variable in different lots (batches), but was not affected by enzyme addition. Salt in moisture, known to affect the ripening, varied from 0.75 to 0.84 and was slightly higher but not significantly different in any of the cheeses.

<table>
<thead>
<tr>
<th>Date Made</th>
<th>Treatment</th>
<th>Moisture (%)</th>
<th>Salt in moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>January 24</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>35.58 ± 0.18 a</td>
<td>0.75 ± 0.04 a</td>
<td></td>
</tr>
<tr>
<td>Lipomod at 0.05% w/w</td>
<td>34.43 ± 0.03 a</td>
<td>0.76 ± 0.03 a</td>
<td></td>
</tr>
<tr>
<td>Lipomod at 0.15% w/w</td>
<td>32.24 ± 0.67 b</td>
<td>0.80 ± 0.03 a</td>
<td></td>
</tr>
<tr>
<td><strong>January 31</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>26.03 ± 0.10 c</td>
<td>0.76 ± 0.06 a</td>
<td></td>
</tr>
<tr>
<td>Lipomod at 0.05% w/w</td>
<td>21.93 ± 0.27 d</td>
<td>0.77 ± 0.07 a</td>
<td></td>
</tr>
<tr>
<td>Lipomod at 0.15% w/w</td>
<td>26.64 ± 0.88 c</td>
<td>0.80 ± 0.03 a</td>
<td></td>
</tr>
<tr>
<td><strong>February 14</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>28.78 ± 0.20 e</td>
<td>0.76 ± 0.06 a</td>
<td></td>
</tr>
<tr>
<td>Lipomod at 0.05% w/w</td>
<td>32.19 ± 0.13 b</td>
<td>0.80 ± 0.07 a</td>
<td></td>
</tr>
<tr>
<td>Lipomod at 0.15% w/w</td>
<td>31.73 ± 0.26 b</td>
<td>0.84 ± 0.06 a</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3: Moisture and salt in moisture composition of different batches of Swiss cheeses samples. Values in the same column not sharing a common letter are different at the 5% level of significance by Tukey’s HSD. (n=3).
4.5.2 Results of the FTIR analysis of the Swiss cheese samples before the heat treatment:

FTIR analyses were made of the water, chloroform, and ethanol solvent extracts of the three lots of cheese after two months of aging. Optimized figures show the class projections of the FTIR analyses. Using the class projection figures with PC1, PC2, & PC3 in the same orientation, show similar separation of the treatments (data not shown). In each batch of cheese, control and Lipomod enzyme modified Swiss cheese at 0.05% and 0.15% w/w of milk were produced.

Figure 4.1 shows the optimized class projection of the FTIR analysis for the three batches of Swiss cheese samples when analyzed all together before heat treatment and after two months of aging. The figure shows separation among treatments and also within treatments from one lot to another. Except for the Lipomod 0.05% lot A, the cheeses with a given treatment clustered together. Additional data for cheese made on the same day are shown in appendix A.

The lot to lot differences could be caused by a number of factors that influence the ripening process. Factors affecting ripening include pasteurization, non-starter lactic acid bacteria, anything affecting the growth of non-starter lactic acid bacteria, ripening temperature, salt in moisture, moisture, fat, pH, and milk supply (Rosenthal, 1991; Scott and others, 1998; Fox, 1987; Fox, 1999). In this particular study, pasteurization was done at the same time & temperature combination (it was manipulated manually); microbiological aspects were out of the core of the project; ripening temperatures was kept similar for each lot, and salt in moisture was consistent enough in all three cheese
lots. However, there were significant variations in moisture and fat content from one lot to another. There were slight differences in pH as well. Milk supply was the same. Consequently, the separation within treatments from one batch to another may be related to the variation in moisture and fat content in the cheese composition over lots. It could be also related to the slight pH differences. Even though pH differences were small, that may be large enough to cause some separation from lot to lot since pH values are expressed in a log scale. In the case of the milk supply, supplier was the same but batches were produced over a 4 week period so there is a possibility for small differences in the milk quality over time.
Figure 4.1: FTIR class projection of the three batches of Swiss cheese samples analyzed all together before heat treatment and after two months of aging. Each oval represents 95% probability clouds.

All the treatments were separated by the FTIR analysis as shown by the interclass distance, table 4.4.
Table 4.4: Interclass distance of the three lots of Swiss cheese samples analyzed all together before heat treatment and after two months of aging. Contr means control and Lipo means Lipomod enzyme.

<table>
<thead>
<tr>
<th></th>
<th>Contr C</th>
<th>Lipo 0.05 C</th>
<th>Lipo 0.15 C</th>
<th>Contr A</th>
<th>Contr B</th>
<th>Lipo 0.05 A</th>
<th>Lipo 0.05 B</th>
<th>Lipo 0.15 A</th>
<th>Lipo 0.15 B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contr C</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo 0.05 C</td>
<td>4.90</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo 0.15 C</td>
<td>8.41</td>
<td>5.04</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contr A</td>
<td>6.34</td>
<td>6.55</td>
<td>9.02</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contr B</td>
<td>7.80</td>
<td>9.67</td>
<td>13.51</td>
<td>6.13</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo 0.05 A</td>
<td>9.39</td>
<td>7.26</td>
<td>7.16</td>
<td>4.93</td>
<td>10.18</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo 0.05 B</td>
<td>9.19</td>
<td>5.03</td>
<td>7.74</td>
<td>7.77</td>
<td>12.09</td>
<td>7.70</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo 0.15 A</td>
<td>9.01</td>
<td>7.70</td>
<td>5.79</td>
<td>7.61</td>
<td>11.47</td>
<td>4.40</td>
<td>8.00</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>
| Lipo 0.15 B | 7.99  | 4.57        | 5.47        | 5.04    | 9.68    | 4.03        | 4.76        | 6.35        | 0.00        

The interclass distance represents the distance between the clusters formed by each treatment. Greater distance between clusters indicates greater difference in the composition of the Swiss cheese samples. As a rule of thumb, when the distance between classes is 3 or more, the samples are well separated and consequently they are different.

Table 4.4 shows that the Swiss cheese samples were well separated among treatments. There was separation from lot to lot also.

Figure 4.2 shows the discriminating power region having more effect in the chemical differentiation among the cheese samples. The discriminating power is used to identify the spectral wavenumbers and the related functional groups that are responsible for the
differentiation among the Swiss cheese samples. Higher wavenumbers’ peaks mean a greater influence in the classification of the samples.

Figure 4.2: Discriminating power of the FTIR analysis of the three batches of Swiss cheese samples analyzed all together before heat treatment and after two months of aging.

Multiple wave numbers were associated with the discrimination. The most important wave numbers were found to be in the 1100-2000 cm\(^{-1}\) region. The most important bands were 1381, 1412, 1433, 1516, and 1651 cm\(^{-1}\).

Table 4.5 shows the functional groups that are associated with the above wave numbers and most related to Swiss cheese. The functional groups mostly represented with these bands are esters, alcohols, carboxyl groups, medium and short chain fatty acids, acetates,
amide II, amino acids, and polypeptides (Coates, 2000; Guillen et al., 1997; koca et al., 2007; Rodriguez-Saona et al., 2006).

<table>
<thead>
<tr>
<th>Peak(s)</th>
<th>Wave number(s) or frequency (cm(^{-1}))</th>
<th>Functional group(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1412</td>
<td>C-H from fatty acids</td>
<td>Koca et al., 2007; Guillen, 1997; Coates, 2000, 2007.</td>
</tr>
</tbody>
</table>

Table 4.5: Primary peaks, wave numbers, the assigned functional groups, and references of the FTIR analysis performed to the three batches of Swiss cheese samples all together before the heat application treatment.

* indicates major differentiating wave numbers.
The functional groups are mainly associated with C-O containing compounds and with amides and amines from peptides which are important flavor related compounds in Swiss cheese.

Swiss cheese samples were also analyzed by individual batches made on the same day. Appendix A, figures A.1, A.2, & A.3 show the optimized class projections of the FTIR analyses of the three batches of Swiss cheese samples analyzed individually. In all cases the cheeses were differentiated in the basis of enzyme addition.

The discriminating powers of the FTIR analyses of the three batches of Swiss cheese samples analyzed individually in appendix A, figures A.4, A.5, & A.6 showed difference in the differentiating wave numbers. This provides an understanding of why the lots do not superimpose when all cheeses are considered together (figure 4.1). The one wave number that is common to all three lots is 1516 cm⁻¹. This suggests that difference in the three lots relates in large part to differences in peptidase activity. This in turns may reflect the differences in moisture content. Differences in amides and amines from peptides and in fatty acids were the primary basis for the differences of the individual lots. Table A.1 in appendix A shows more detail about the wave numbers and related functional groups of the FTIR analyses of the individual lots.
4.5.3 Results of the FTIR analysis of the Swiss cheese samples after the heat treatment:

Swiss cheese samples were put in the oven for 2, and 5 minutes at $65^\circ$C to analyze the effect of the heat application in the samples.

Figures 4.3 and 4.4 show the optimized class projections of the FTIR analyses of the three lots of Swiss cheese samples after the heat application treatment for 2 and 5 minutes.

Figure 4.3 shows the optimized FTIR class projection of the three lots of Swiss cheese samples after application of heat for 2 minutes at $65^\circ$C in the oven.
Figure 4.3: Optimized FTIR class projection of the three lots of Swiss cheese samples after heat application for 2 minutes at 65°C in the oven. Each oval represents 95% probability clouds.

The FTIR class projection showed chemical differentiation among treatments after heating for 2 minutes. Separation pattern was similar to the separation before heat application. Similarly to before heating, the same treatment from different lots clustered close each others except for Lipomod at 0.05% w/w concentration lot A, which clustered apart from lots B and C.
Table 4.6 shows the interclass distance of the three lots of Swiss cheese samples analyzed all together after heat application for 2 minutes at 65\(^{\circ}\)C in the oven.

<table>
<thead>
<tr>
<th></th>
<th>Contr C</th>
<th>Lipo 0.05 C</th>
<th>Lipo 0.15 C</th>
<th>Contr A</th>
<th>Lipo 0.05 A</th>
<th>Lipo 0.05 B</th>
<th>Lipo 0.15 A</th>
<th>Lipo 0.15 B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contr C</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo 0.05 C</td>
<td>5.80</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo 0.15 C</td>
<td>10.08</td>
<td>5.26</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contr A</td>
<td>14.16</td>
<td>19.84</td>
<td>12.92</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contr B</td>
<td>4.69</td>
<td>9.16</td>
<td>13.88</td>
<td>14.13</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo 0.05 A</td>
<td>18.86</td>
<td>19.46</td>
<td>21.02</td>
<td>26.32</td>
<td>20.84</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipo 0.05 B</td>
<td>8.84</td>
<td>5.17</td>
<td>8.20</td>
<td>11.81</td>
<td>10.33</td>
<td>17.64</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Lipo 0.15 A</td>
<td>12.01</td>
<td>9.52</td>
<td>8.09</td>
<td>19.98</td>
<td>15.79</td>
<td>13.20</td>
<td>9.84</td>
<td>0.00</td>
</tr>
</tbody>
</table>
| Lipo 0.15 B | 9.99 | 4.91        | 6.52        | 17.15   | 11.87       | 19.77       | 4.95        | 10.03       | 0.00

Table 4.6: Interclass distance of the three lots of Swiss cheese samples analyzed all together and after application of heat for 2 minutes at 65\(^{\circ}\)C in the oven. Contr means control and Lipo means Lipomod enzyme.

The interclass distance indicates that after application of heat for 2 minutes at 65\(^{\circ}\)C in the oven, the treatments were chemically different from one another. They were also different from lot to lot as they were before the application of heat. As a rule of thumb, interclass distance of 3 or more indicates that the samples are well separated and consequently they are different.

Figure 4.4 shows the optimized FTIR class projection of the three lots of Swiss cheese samples analyzed all together after application of heat for 5 minutes at 65\(^{\circ}\)C in the oven.
Figure 4.4: Optimized FTIR class projection of the three lots of Swiss cheese samples analyzed all together and after heat application for 5 minutes at 65°C in the oven. Each oval represents 95% probability clouds.

The FTIR class projection in figure 4.4 showed separation among all the treatments after application of heat for 5 minutes at 65°C. There was separation from lot to lot similarly to the findings before heat application.

Table 4.7 shows the interclass distance of the three lots of Swiss cheese samples analyzed after heat application for 5 minutes at 65°C in the oven.
Table 4.7: Interclass distance of the three lots of Swiss cheese samples analyzed all together and after application of heat for 5 minutes at 65\(^\circ\)C in the oven. Contr means control and Lipo means Lipomod enzyme.

The interclass distance indicates that after application of heat for 5 minutes at 65\(^\circ\)C in the oven, the treatments were chemically different from one another. They were also different from lot to lot. These findings are similar to those found before the application of heat and after application of heat for 2 minutes.

Figures 4.5 and 4.6 show the discriminating powers of the FTIR analyses of the three lots of Swiss cheese samples after the heat application for 2 and 5 minutes respectively.
Figure 4.5: Discriminating power of the FTIR analysis of the three lots of Swiss cheese samples after heat application for 2 minutes at 65°C in the oven.

Figure 4.5 showed that there were important changes in the major peaks of the discriminating power after heat application treatment for 2 minutes.

Table 4.8 shows the functional groups that are associated with the above wave numbers and most related to Swiss cheese.
<table>
<thead>
<tr>
<th>Peak(s)</th>
<th>Wave number(s) or frequency (cm⁻¹)</th>
<th>Functional group(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1123</td>
<td>C-O from lipids</td>
<td>Coates, 2000; Spectroscopy tools web site, 2007.</td>
</tr>
<tr>
<td>B</td>
<td>1315</td>
<td>Carbonyl groups from fatty acids</td>
<td>Coates, 2000.</td>
</tr>
<tr>
<td>C</td>
<td>1412</td>
<td>C-H from fatty acids</td>
<td>Koca et al., 2007; Guillen, 1997; Coates, 2000.</td>
</tr>
<tr>
<td>F</td>
<td>1638</td>
<td>Primary amine, and primary and tertiary amides</td>
<td>Spectroscopic tools, web site, 2007.</td>
</tr>
</tbody>
</table>

Table 4.8: Primary peaks, wave numbers, the assigned functional groups, and references of the FTIR analysis performed to the three batches of Swiss cheese samples all together after heat treatment of 2 minutes at 65°C in the oven.

* indicates major differentiating wave number.

Important changes occurred in the differentiating power after the heat application treatment. Among the changes, the wave number 1123 cm⁻¹ appeared as a primary peak.
after 2 minutes of heat treatment. This peak mainly represents C-O stretching from lipids (Coates, 2000). Another important change was an important decrease in the 1412 cm\(^{-1}\) wave number and this peak is associated with C-H symmetric bending vibrations of medium and short chain fatty acids (Koca et al., 2007). Another important change was a significant reduction in the 1433 cm\(^{-1}\) wave number which was the highest peak before heat treatment. This peak is also related to stretching vibration of carboxyl groups from fatty acids (Coates, 2000). The band 1516 cm\(^{-1}\) which represents aromatic nitro compounds was also decreased significantly. And the band 1651 cm\(^{-1}\) which is a major peak before heat had a shift to 1655 cm\(^{-1}\). Table 4.8 shows in detail the primary peaks and wave numbers found in the discriminating power of figure 4.5 and the corresponding functional groups most related to Swiss cheese.
Figure 4.6 shows the discriminating power of the FTIR analysis of the three lots of Swiss cheese samples after heat application for 5 minutes at 65\(^{\circ}\)C in the oven.

![Figure 4.6: Discriminating power of the FTIR analysis of the three lots of Swiss cheese samples after heat application for 5 minutes at 65\(^{\circ}\)C in the oven.](image)

Table 4.9 shows the functional groups that are associated with the above wave numbers and most related to Swiss cheese.

Figure 4.6 shows important changes in the major peaks of the discriminating power after heat application treatment for 5 minutes.
Table 4.9: Primary peaks, wave numbers, the assigned functional groups, and references of the FTIR analysis performed to the three batches of Swiss cheese samples all together after heat treatment of 5 minutes at 65°C in the oven.

* indicates major differentiating wave numbers.

Important changes occurred in the differentiating power after the heat application treatment. Among the changes, the wave number 1123 cm\(^{-1}\) which was not a major peak before heating and appeared after heating for 2 minutes, got a significant increase after 5 minutes of heat. This wave number mainly represents C-O stretching from lipids (Coates,
The wave number 1315 cm\(^{-1}\) which started to appear after 2 minutes of heat got a significant increase after the 5 minutes of heat. This wave number is also associated with stretching vibration of carboxyl groups from fatty acids (Coates, 2000). Other important changes were the significant increase of the bands 1699 and 1716 cm\(^{-1}\). These bands are associated with amide I of peptides and with carboxylic acids (Coates, 2000).

From the application of heat treatment can be concluded that significant changes in the FTIR analyses occurred. Important changes were seen in the influence of functional groups from fatty acids and peptides in the classification of the Swiss cheese samples. The discriminating power showed important changes in the primary peaks compared to the FTIR analyses before the heat treatment.
4.5.4 Results of the descriptive sensory evaluation

The Swiss cheese sensory attributes were evaluated by a panel of three people who are familiar with Swiss cheese.

Table 4.10 shows the mean of the descriptive sensory evaluation of the three lots of Swiss cheese samples made by a panel of Food Scientists before the application of heat.

<table>
<thead>
<tr>
<th>Samples Attributes</th>
<th>Control</th>
<th>0.05% w/w Lipomod</th>
<th>0.15% w/w Lipomod</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Flavor</td>
<td>3.6 ± 0.2 a</td>
<td>4.5 ± 0.5 ab</td>
<td>5.7 ± 0.7 b</td>
</tr>
<tr>
<td>Nutty</td>
<td>2.8 ± 2.0 a</td>
<td>3.6 ± 1.9 a</td>
<td>2.7 ± 1.4 a</td>
</tr>
<tr>
<td>Sweetness</td>
<td>2.4 ± 0.8 a</td>
<td>2.7 ± 0.4 a</td>
<td>1.7 ± 0.4 a</td>
</tr>
<tr>
<td>Rancid</td>
<td>0.5 ± 0.4 a</td>
<td>1.0 ± 0.5 a</td>
<td>4.8 ± 0.6 b</td>
</tr>
<tr>
<td>Bitter</td>
<td>0.8 ± 0.3 a</td>
<td>1.2 ± 0.4 a</td>
<td>3.0 ± 0.3 b</td>
</tr>
</tbody>
</table>

Table 4.10: Sensory evaluation of all three lots of Swiss cheese samples before application of heat. Values in the same row not sharing a common letter are different at the 5% level of significance by Tukey’s HSD. (n=3).

The enzyme modified Swiss cheese treatments produced stronger overall flavor compared to the control samples which were produced without application of enzyme. The results also showed that the higher the enzyme concentration, the stronger the flavor profile, and that can be related to the high bitter and rancid flavors in the Lipomod samples.

Nuttiness and sweetness are statistically similar for all the treatments. Lipomod at 0.15% was slightly higher in rancid and bitter flavors.
The development of rancid flavor is related to the development of lower molecular weight fatty acids in the samples. The bitterness is believed to be related to the presence of medium and small size bitter peptide compounds, (Bartels and others, 1987; Rosenthal, 1991). Bitter peptides can be produced from casein derived polypeptides, (Law, 2001).

Then, Swiss cheese sensory attributes were also evaluated by the same panel after putting them in the oven for 5 minutes at 65°C to evaluate the effects of heat application.

Table 4.11 shows the results of the descriptive sensory evaluation by the panel for the Swiss cheese samples after putting them in the oven for 5 minutes at 65°C.

<table>
<thead>
<tr>
<th>Samples Attributes</th>
<th>Control</th>
<th>0.05% w/w Lipomod</th>
<th>0.15% w/w Lipomod</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Flavor</td>
<td>3.9 ± 0.7 a</td>
<td>5.2 ± 0.9 ab</td>
<td>6.3 ± 0.4 b</td>
</tr>
<tr>
<td>Nutty</td>
<td>3.3 ± 1.5 a</td>
<td>5.0 ± 1.2 a</td>
<td>2.7 ± 1.4 a</td>
</tr>
<tr>
<td>Sweetness</td>
<td>2.9 ± 0.5 a</td>
<td>3.2 ± 0.4 a</td>
<td>2.3 ± 1.2 a</td>
</tr>
<tr>
<td>Rancid</td>
<td>0.2 ± 0.2 a</td>
<td>0.7 ± 0.4 a</td>
<td>3.3 ± 0.4 b</td>
</tr>
<tr>
<td>Bitter</td>
<td>0.4 ± 0.2 a</td>
<td>0.5 ± 0.2 a</td>
<td>2.1 ± 0.2 b</td>
</tr>
</tbody>
</table>

Table 4.11: Sensory evaluation of the Swiss cheese samples after putting them in the oven for 5 minutes at 65°C. Values in the same row not sharing a common letter are different at the 5% level of significance by Tukey’s HSD. (n=3).
After the heat treatment, 5 minutes in the oven at 65°C, there was significant reduction in rancid and bitter flavors. Statistically, there was no difference in nutty or sweetness in the three groups.

Figure 4.7 shows the overall flavor comparison before and after heating the Swiss cheese samples.

Figure 4.7: Overall flavor comparison of the Swiss cheese samples before and after heat treatment.

The graph suggests a slight tendency for an increase the perception of the overall flavor after the application of heat; but this was not statistically significant at an α level of 5% by Tukey’s HSD.
Figure 4.8 shows the nuttiness comparison before and after heating the Swiss cheese samples.

![Nuttiness comparison of the Swiss cheese samples before and after heat treatment.](image)

The variation in nuttiness was not statistically significant at an $\alpha$ level of 5% by Tukey’s HSD.
Figure 4.9 shows the sweetness comparison before and after heating the Swiss cheese samples.

![Sweetness Comparison](image)

Figure 4.9: Sweetness comparison of the Swiss cheese samples before and after heat treatment.

The data suggests the tendency for little increment in the perception of the sweetness after the application of heat. Changes within treatments were not statistically significant at an α level of 5% by Tukey’s HSD.

Figure 4.10 shows the rancidity comparison before and after heating the Swiss cheese samples.
Figure 4.10: rancidity comparison of the Swiss cheese samples before and after heat treatment.

The results showed a decrease in the rancidity perception within treatments after the heat application. This reduction was not significant for the control and the low enzyme concentration treatments but it was significant at an $\alpha$ level of 5% by Tukey’s HSD only in the case of the high enzyme concentration treatment.

The development of rancid flavor is related to the development of lower molecular weight fatty acids in the samples. The decrease in rancidity on heating may be due to some degree of volatilization of the short chain fatty acids or interactions with other components.
Figure 4.11 shows the bitterness comparison before and after heating the Swiss cheese samples.

![Bitterness Comparison Graph](image)

Figure 4.11: Bitterness comparison of the Swiss cheese samples before and after heat treatment.

The results showed a decrease in the bitterness perception within treatments after the heat application. This reduction was not significant for the control but it was significant at an $\alpha$ level of 5% by Tukey’s HSD in the case of the enzyme modified cheese samples at both low and high concentrations.

The bitterness is believed to be related to the presence of medium and small size bitter peptide compounds derived from casein, (Bartels and others, 1987; Rosenthal, 1991;
Law, 2001). Consequently, Lipomod enzyme produces some bitter compounds from casein during cheese ripening. A possible reason for the reduction in bitterness is due to bitter peptide aggregation. (Bartels and others, 1987) found that degradation of peptides caused by peptidases from heat-shocked added cells reduced bitterness intensity in Gouda cheese. From these finding, it could be thought that another reason for the decrease in bitterness after heat treatment in this study, may be related to some degree of degradation of the bitter peptides due to heating. Another possibility could be Strecker degradation through Millard reaction where amino groups from amino acids react with an α-dicarbonyl like a reducing sugar and produce flavor compounds (Smit and others, 2005).

4.5.5 Results of the free fatty acids analysis

Table 4.12 shows the free fatty acid content of the Swiss cheese samples. Acetic (C\textsubscript{2:0}) acid was the one produced in larger amount in all the treatments. Propionic (C\textsubscript{3:0}) acid production was relatively low which is a normal situation that occurs when propionic bacteria of low acid production is used as starter culture.

On the other hand, the total free fatty acid content had a tendency to be higher in the enzyme modified Swiss cheeses. A higher free fatty acids content was observed at the highest enzyme concentration except for propionic (C\textsubscript{3:0}) and isovaleric (i-C\textsubscript{5:0}) acids. C\textsubscript{3:0} and C\textsubscript{5:0} concentrations were independent of the addition of enzyme. Both propionic and isovaleric cids are produced by Propionibacterium freudenreichii which was added as starter culture (Thierry and others, 2004). There was a significant production of free fatty acids with even number of carbons [acetic (C\textsubscript{2:0}), butyric (C\textsubscript{4:0}), and n-caproic (C\textsubscript{6:0}) acids] in the enzyme modified Swiss cheeses with the highest concentration. The increase
in acetic acid associated with the enzyme would be considered to be undesirable. Butyric acid was produced significantly highest in the Lipomod at 0.15% treatment and its production within a treatment showed the same pattern as the rancidity.

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>* Control</th>
<th>* Lipomod at 0.05%</th>
<th>* Lipomod at 0.15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2</td>
<td>285.56 ± 17.99 a</td>
<td>303.85 ± 13.51 ab</td>
<td>335.04 ± 9.62 b</td>
</tr>
<tr>
<td>C3</td>
<td>19.47 ± 16.06 a</td>
<td>27.58 ± 20.03 a</td>
<td>11.91 ± 5.72 a</td>
</tr>
<tr>
<td>C4</td>
<td>9.86 ± 2.18 a</td>
<td>28.34 ± 18.52 a</td>
<td>117.93 ± 16.20 b</td>
</tr>
<tr>
<td>C5</td>
<td>5.41 ± 0.79 a</td>
<td>4.91 ± 0.49 a</td>
<td>8.62 ± 4.20 a</td>
</tr>
<tr>
<td>C6</td>
<td>8.55 ± 1.08 a</td>
<td>8.92 ± 2.24 a</td>
<td>48.02 ± 15.94 b</td>
</tr>
</tbody>
</table>

Table 4.12: Short Chain Free Fatty Acids Analysis of the Swiss cheese samples. Values in the same row not sharing a common letter are different at the 5% level of significance by Tukey’s HSD. (n=3).

* Fatty acid concentration is expressed as milligram per 100 grams of cheese (mg/100g of cheese).

* Each value is the mean of three replicates and expressed as the average ± standard deviation (Std Dev).

* The concentration of the Lipomod enzyme is weight of enzyme as % of milk solids.
Figure 4.12 shows a graphical perspective of the free fatty acid content in the Swiss cheese samples.

Figure 4.12: Short chain free fatty acids measure in the Swiss cheese samples and expressed as milligrams per 100 grams of cheese. Each value was measured as the mean of three replicates.
4.5.6 Discussion

Investigation of the possibility of using enzymes to accelerate and modify Swiss cheese has not been reported previously. Enzyme modified Cheddar cheese is known to be strong flavored with little relation to standard Cheddar cheese and has found wide use in the institutional segment of the food industry (Hannon and others, 2006). This study has shown that the addition of lipase, proteases, and peptidases all produce a strong flavor with rancid and bitter notes. What has not been reported previously is that heating the enzyme modified cheese at 65°C still provides a stronger flavor than the control, but reduces the bitter and rancid flavor notes and suggests that such cheese will have the potential to the institutional customers desired to provide a flavor that carries through the cooking process. Considerable further research is needed before the process will be ready for commercialization.

The application of FTIR to a protein/lipid free water solvent extract has been shown to be a valuable method for differentiating the flavor chemistry of the enzyme modified cheeses. FTIR results have clearly shown the ability to differentiate enzyme modified cheese from control for cheeses made on the same day, as well as differentiating the cheeses before and after heat treatment. Lot to lot differences are clearly shown by FTIR, most likely related to the small scale of manufactures resulting in day to day variability. Much of this variability can be related to moisture differences that would influence the enzymatic activity of the culture organisms most related to flavor production (Scott and others, 1998; Fox, 1987; Fox, 1999).
The data support the probability that heat treatment causes the bitter peptides to aggregate and thus reduce bitter flavor. The two most probable causes of reduction of rancidity is either fatty acid volatilization or conversion to esters. The FTIR data does not appear to support the later mechanism.
CHAPTER 5
CONCLUSIONS

In the first stage of this study, commercial enzymes: Accelerzyme R CPG, and Lipomod TM 621-L621P showed to have the potential to increase the overall Swiss cheese flavor intensity after 60 days of ripening of miniature Swiss cheese samples. Both enzymes intensified the flavor, but Lipomod showed to have stronger flavor profile. Lipomod also caused distinct bitter and rancid flavors that decreased on heating without major changes in nuttiness and sweetness.

The FTIR analyses demonstrated that the method of analysis makes a difference when analyzing the chemical nature of the Swiss cheese samples. The water, chloroform, ethanol solvents extraction method showed to be the best method to differentiate the cheese samples. With this method, all the cheese samples were differentiated based on short chain fatty acids, organic acids, and ester flavor compounds; with major discriminating power found in the 1000-1500 cm⁻¹ region.

The preliminary results indicated that the use of commercial enzymes could be used to produce stronger cheese flavor in relatively short period of ripening. Lipomod showed to have the ability to produce stronger cheese flavor and maintain it after application of heat without affecting nuttiness or sweetness. This finding suggested that Lipomod
could be an option to assist the food service industry with a carry through flavor cheese in cooked dishes.

During the 5 pound Swiss cheeses study, Lipomod produced Swiss cheeses with stronger flavor and the flavor intensity increased with enzyme concentration. There was an increase in rancidity and bitterness as well, but these defects decreased significantly when heat was applied to the cheese samples.

After heat application to the Swiss cheese samples, the overall flavor of the enzyme modified Swiss cheeses was significantly higher than the control. There was not significant change in nuttiness and sweetness, but there was a significant reduction in rancidity and bitterness.

The FTIR analysis could differentiate all the cheese samples based on chemical differences. The discriminating power was most important in the 1400-1600 cm\(^{-1}\) region with the most predominant peak in the 1516 cm\(^{-1}\) wave number which may be related to fatty acid and amino acid families.

There was FTIR chemical differentiation within a treatment from one batch to another. There was significant variation in moisture and fat content from one lot of cheese to another as well. Consequently, differentiation could be related to moisture and fat variations.
The FTIR analysis could also differentiate the Swiss cheese samples after the heat application. Differentiation was clear among treatments and also within treatments heated for different times (2 and 5 minutes).

In general, acetic (C\textsubscript{2:0}) acid was the acid that was produced in larger amount compared to the other acids. Free fatty acid (FFA) analysis showed that free fatty acids with even number of carbons [acetic (C\textsubscript{2:0}), butyric (C\textsubscript{4:0}), and n-caproic (C\textsubscript{6:0}) acids] were produced in significantly large amounts in the high enzyme concentration treatment. Beside acetic acid, butyric acid was produced in significantly large amount in the high enzyme concentration treatment. The high production of butyric acid appeared to be associated with the rancid flavor development for that particular treatment.

Based on this study, it could be concluded that the use of Lipomod TM 621P could be an option to assist the food service industry with a cheese with a better carry through flavor in cooked dishes. Lipomod could assist to accomplish shorter ripening time as well. At the higher enzyme concentration used, the cheese would not be suitable for table use because of the strong rancid flavor that developed. The rancid and bitter flavors developed, can be significantly reduced by the application of heat in cooked dishes.

The optimum amount of enzyme to use would require further investigation to get a positive profile without a strong residual rancidity. Further research is needed to commercialize the process of enzyme modification of Swiss cheese.
LIST OF REFERENCES


Harper WJ. 2007. Professor. The Ohio State University. Columbus, Ohio 43210.


APPENDIX A: FTIR ANALYSES
Figure A.1: Optimized FTIR class projection of batch A of the Swiss cheese samples before the heat application treatment. Each oval represents 95% probability clouds.
Figure A.2: Optimized FTIR class projection of batch B of the Swiss cheese samples before the heat application treatment. Each oval represents 95% probability clouds.
Figure A.3: Optimized FTIR class projection of batch C of the Swiss cheese samples before the heat application treatment. Each oval represents 95% probability clouds.
Figure A.4: FTIR discriminating power of batch A of the Swiss cheese samples before the heat application treatment.
Figure A.5: FTIR discriminating power of batch B of the Swiss cheese samples before the heat application treatment.
Figure A.6: FTIR discriminating power of batch C of the Swiss cheese samples before the heat application treatment.
<table>
<thead>
<tr>
<th>Peak(s)</th>
<th>Wave number(s) or frequency (cm⁻¹)</th>
<th>Functional group(s)</th>
<th>References</th>
</tr>
</thead>
</table>

Table A.1: Primary peaks, wave numbers, the assigned functional groups, and references of the FTIR analyses performed to batches A, B, and C individually and before the heat application treatment.

* Primary peaks found only in batch A. ** Primary peak found in all 3 lots.
Figure A.7: Optimized FTIR class projection of batch A of the Swiss cheese samples after heat treatment for 0, 2, and 5 minutes at 65°C in the oven. Each oval represents 95% probability clouds.
Figure A.8: Optimized FTIR class projection of batch B of the Swiss cheese samples after heat treatment for 0, 2, and 5 minutes at 65°C in the oven. Each oval represents 95% probability clouds.
Figure A.9: Optimized FTIR class projection of batch C of the Swiss cheese samples after heat treatment for 0, 2, and 5 minutes at 65°C in the oven. Each oval represents 95% probability clouds.
Figure A.10: FTIR discriminating power of batch A of the Swiss cheese samples after heat treatment for 0, 2, and 5 minutes at 65°C in the oven.
Figure A.11: FTIR discriminating power of batch B of the Swiss cheese samples after heat treatment for 0, 2, and 5 minutes at $65^\circ$C in the oven.
Figure A.12: FTIR discriminating power of batch C of the Swiss cheese samples after heat treatment for 0, 2, and 5 minutes at 65°C in the oven.
Table A.2: Primary peaks, wave numbers, the assigned functional groups, and references of the FTIR analysis performed to batch A of Swiss cheese samples after heat treatment for 0, 2, and 5 minutes at 65°C in the oven.

<table>
<thead>
<tr>
<th>Peak(s)</th>
<th>Wave number(s) or frequency (cm(^{-1}))</th>
<th>Functional group(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1315*</td>
<td>C-N stretch from aromatic primary, secondary &amp; tertiary amines, alcohols, and phenols.</td>
<td>Coates, 2000; Spectroscopic tools web site, 2007.</td>
</tr>
<tr>
<td>C</td>
<td>1506</td>
<td>Aromatic nitro compounds, C=O and C=C stretching from ketones, &amp; Amide II.</td>
<td>Coates, 2000; Spectroscopic tools web site, 2007.</td>
</tr>
<tr>
<td>D</td>
<td>1558</td>
<td>Aromatic nitro compounds, C=O and C=C stretching from ketones, acetates, and amide II.</td>
<td>Coates, 2000; Spectroscopic tools web site, 2007.</td>
</tr>
<tr>
<td>F</td>
<td>1716</td>
<td>C=N stretching from guanidine, C=O stretching from ketones &amp; aldehydes, and R-COOH.</td>
<td>Coates, 2000; Spectroscopic tools, web site, 2007.</td>
</tr>
</tbody>
</table>

* indicates major differentiating wave number.
<table>
<thead>
<tr>
<th>Peak(s)</th>
<th>Wave number(s) or frequency (cm(^{-1}))</th>
<th>Functional group(s)</th>
<th>References</th>
</tr>
</thead>
</table>

Table A.3: Primary peaks, wave numbers, the assigned functional groups, and references of the FTIR analysis performed to batch B of Swiss cheese samples after heat treatment for 0, 2, and 5 minutes at 65\(^{\circ}\)C in the oven.

* indicates major differentiating wave number.
<table>
<thead>
<tr>
<th>Peak(s)</th>
<th>Wave number(s) or frequency (cm(^{-1}))</th>
<th>Functional group(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1364</td>
<td>Acetates, ketones, acetonates, tertiary alcohol, phenols, aromatic amines, and -COOH.</td>
<td>Coates, 2000; Spectroscopic tools, web site, 2007.</td>
</tr>
<tr>
<td>B</td>
<td>1431</td>
<td>Ethers, esters, acetates, ketones; S-CH(_2)-R,</td>
<td>Coates, 2000; Spectroscopic tools web site, 2007.</td>
</tr>
</tbody>
</table>

Table A.4: Primary peaks, wave numbers, the assigned functional groups, and references of the FTIR analysis performed to batch C of Swiss cheese samples after heat treatment for 0, 2, and 5 minutes at 65\(^\circ\)C in the oven.

* indicates major differentiating wave number.
Please rinse your mouth twice between samples and feel free to resample as needed

Please, for the samples below use a 1 to 9 scale to score the indicated sensory attributes.

For the attributes below, use the 1 to 9 scale as follow:
   a) Overall flavor: use 1 for mild, 5 for medium and 9 for sharp;
   b) Nutty, sweetness, rancid, and bitter: use 1 for the low and 9 for the high;
   c) Overall texture: 1 for soft and 9 for hard;
   d) Overall eye formation: 1 for blind, 5 for good, and 9 for overset;
   e) Overall appearance: 1 liking the least and 9 liking the most.

<table>
<thead>
<tr>
<th>Samples Attributes</th>
<th>220</th>
<th>432</th>
<th>346</th>
<th>448</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Flavor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutty</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweetness</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rancid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bitter</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall texture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Please indicate any comment below

<table>
<thead>
<tr>
<th>Comments</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>
APPENDIX C: FATTY ACIDS CALIBRATION CURVES
Figure C.1: Calibration curve of acetic acid

\[ y = 8 \times 10^{-5}x \]

\[ R^2 = 0.9616 \]
Figure C.2: Calibration curve of propionic acid.
Figure C.3: Calibration curve of butyric acid
Figure C.4: Calibration curve of isovaleric acid
Figure C.5: Calibration curve of n-caproic acid