Application of High Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared (FTIR) Spectroscopy to Swiss-type Cheese Split Defects

THESIS

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Abstract

Splits, slits and cracks are a continuing problem in the US Swiss Cheese industry and cause downgrading of the cheese, bringing economic loss. The defects generally occur during secondary fermentation in cold storage, which is known to be an issue caused by many factors. Many chemical and physical changes can be indicators to help predicting the defects. Various techniques have been applied to test different compounds in cheese. HPLC is one of the reference methods which have been applied in cheese chemistry for the past decades. Its application involves in the qualification and quantification of organic acids, amino acids and peptides, and sugars in dairy products. However, limited research has been conducted on a rapid and easy testing regimen to investigate split defects in Swiss-type cheese. For over fifty years, researchers have investigated the application of Fourier Transform Infrared (FTIR) Spectroscopy in various food samples, including cheeses and other dairy products because FTIR spectroscopy gives fast, simple and comprehensive detection of chemical compounds. There is great potential for applying FTIR spectroscopy in developing a rapid test method to detect and predict the occurrence of split defects.

In this study, initially four pairs of cheese samples were analyzed using a standard HPLC methodology, to assess the capability of this method in chemical detection addressing the defects, and to provide a reference to later FTIR results. Four pairs of
cheeses, each pair from its own vat, were chosen for this analysis. Each pair of cheeses contained one defective item and one non-defective item. Three major organic acids (oxalic, pyruvic and lactic acid) in the Swiss cheese extract were detected. Oxalic Acid concentration remained constant over the samples and the concentrations of the two remaining acids were determined by standard curves. The HPLC results showed a lower level of lactic acid concentration and also a higher ratio of pyruvic acid to lactic acid in cheeses with split defects than cheeses with good grades. At the same time, Fourier Transform Infrared (FTIR) supported HPLC results. The spectra information showed significant difference between the pair members in the wavenumbers range corresponding to these organic acids. Modification of the sampling method was applied, with the FTIR analysis being used on a further nine individual cheese samples. The sampling was done from different areas of split, “eye” and “blind” from each cheese. This allowed FTIR spectra to give more significant differentiation between defective and non-defective samples. Better differentiation was attained following modification of the sampling method, with a shift of significant wavenumber ranges, from organic acid bands to amino acid bands. Results showed the application of FTIR in Swiss-type Cheese has great potential, targeting different compounds to address split defects.
Dedication

To my family and my fiancé,

for your love and always believing in me.
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1. Chapter 1: Introduction

Cheese is a major fermented dairy product in the United States with a per capita consumption of around 34 pounds per year. This number keeps increasing with the need for healthy diets. Swiss is one of the major cheese types in the United States (NASS, 2011). Several grading factors determine the price and application of Swiss-type cheese, and the most common grading criteria include split and/or secondary fermentation defects. These defects are manifested as undesirable slits or cracks that always lead to downgrading of cheese (Daly et al., 2009). Splits, slits and cracks are a persistent problem in the US Swiss cheese industry. The defects generally occur during secondary fermentation in cold room storage after the desirable propionic acid fermentation has taken place in the warm room, and in this process two or more different factors may be involved (Daly et al., 2009). Analytical methods which can rapidly determine the contributing factors of split defects, such as pyruvate, certain free fatty acids and organic acids, can greatly benefit studies on Swiss-type cheese quality assurance (Weinrichter et al, 2001).

The most common technique for the analysis of free fatty acids is gas chromatography (GC). In addition, application of GC-MS (mass spectroscopy) and liquid chromatography mass spectroscopy were expanded to free fatty acid analysis for cheese.
High performance liquid chromatography (HPLC) is commonly used to monitor carbohydrates, organic acid and various other compounds in cheese (Zeppa et al, 2001). However, these methods generally are time-consuming, tedious, commonly involve extensive chemical use, and sometime require pretreatment of samples. These disadvantages have prompted the evaluation and adoption of new, rapid and simple methods based on Fourier-transform infrared (FTIR) spectroscopy.

Several researchers (Chen, 1998; Kansiz et al, 2007; Koca, 2007; Martin-del-Campo, 2009; Subramanian, 2009 a &b) have recommended FTIR spectroscopy for applications in dairy products and the Association of Analytical Communities (AOAC) International has approved an FTIR method to determine fat, protein, lactose and total solid contents in some dairy products (AOAC, 2005). Most of the applications of FTIR spectroscopy in cheese have been focused on determination of chemical parameters and composition of cheese, and cheddar cheese was usually used as the model due to its great market value. Applications of FTIR spectroscopy on assessment of Swiss-type cheese and prediction of defects in cheese are limited.

Developing a rapid testing procedure to detect or predict the probability of formation of split defects in Swiss-type Cheese is necessary and possible. There is great potential for applying FTIR spectroscopy towards this objective. Further research is needed to address the problem and examine the possibility of rapid determination of contributing factors in split defects by using FTIR spectroscopy.
The objective of this study were to investigate the capacity of HPLC in chemical change detection addressing the split defects in Swiss-type cheese, and to evaluate the potential of applying FTIR spectroscopy to assessment of Swiss-type cheese and to prediction of defects. Cheese samples were analyzed using the FTIR method combined with multivariate analysis for classification, and the reference HPLC method was applied for comparison.
2. Chapter 2: Literature Review

2.1 Swiss-type cheese

Swiss-type cheese is a generic name for a variety of hard or semi-hard cheeses with characteristic regular eye formation. They were manufactured originally in the Emmen valley in Switzerland. Emmental cheese is probably the best-known Swiss-type cheese and is frequently referred to as “Swiss cheese” (Fox et al., 2003). Although there is no internationally recognized definition of Swiss-type cheeses, they are characterized by propionic acid fermentation following common lactic acid fermentation.

Lactic acid fermentation occurs early in production of Swiss-type cheese, where lactose from milk is converted into lactic acid by starter cultures. In Swiss-type cheese these include mainly thermophilic species such as Streptococcus thermophilus, Lactobacillus helveticus and Lactobacillus delbrueckii subsp. lactis, and may also contain mesophilic species such as L. lactis subsp. lactis and L. lactis subsp. cremoris. The mixed culture renders homofermentative catabolism in which lactose is mostly converted into lactate (Fröhlich-Wyder and Bachmann, 2004). Lactic acid fermentation provides the foundation for cheese manufacturing and it affects pH, syneresis, the inhibition of other bacterial growth and the texture development due to removal of calcium. In addition,
lactic acid fermentation provides lactate as a substrate for propionic fermentation. (Daly et al., 2009)

The propionic fermentation mostly involves one species, Propionibacterium freudenreichii, which produces propionic acid, acetic acid and carbon dioxide. Generally, the two acids contribute to the characteristic nutty-sweet flavor of Swiss-type cheese and the CO₂ produced is responsible for eye formation (Fröhllch-Wyder and Bachmann, 2004). Propionic acid fermentation begins about 30 days after the start of manufacturing and lasts for about 4 – 7 weeks. In the most common manufacturing procedure, fermentation starts when the cheese is moved from precooling (about 10°C) to a warm room of about 21°C. After keeping in the warm room, Cheese is then stored in a cold room at 1 - 2°C for ripening (Fröhllch-Wyder and Bachmann, 2004). Swiss cheese is cut and packaged 60 days after manufactured to meet the federal regulation (FDA, 21CFR133.182).

2.2 Split and/or Secondary fermentation Defects of Swiss-type Cheese

Split defects manifest as undesirable openings and appear as slits and cracks that are visible in the cut cheese loaf. They lead to downgrading of the cheese. Daly, et al. (2009) state in their review, that there is no consensus as to the definitive causes of the problem. The defects generally do not occur until the cheese is placed in cold storage (1 – 2°C) for further ripening. Daly et al. (2009) stated “Development of secondary
fermentation is a multi-faceted issue where many of the causes of the defect and factors influencing the quality of the cheese are interconnected.”

Two main types of contributing factors to secondary fermentation are changes in rheological properties such as loss of cohesiveness and elasticity and overproduction of gas in the ripening stage during cold room storage. In turn, each of these areas has multiple interconnected factors. As for the changes in rheological properties, contributors include high acidity which causes loss of calcium from the casein micelle during acid production, fat crystallization when placed in the cold room which reduces elasticity, and proteolysis of the casein which reduces the cohesiveness and elasticity of the curd. In terms of overproduction of gas, the contributing factors include microbial growth of propionic acid bacteria (PAB), heterofermentative lactic bacteria and nonstarter culture lactic acid bacteria (NSLAB) involved in secondary fermentation, and also bacterial cell lyses which can release cell contents that provide a source of energy for heterolactic bacteria.

There are some widely accepted methods which control secondary fermentation. These methods include (Harper, 2010):

1) Washing curd to remove leftover lactose which is a major source of energy in secondary fermentation;

2) Increasing citrate fermentation in the warm room to reduce secondary fermentation in the cold room;

3) Eliminating PAB that grow in the cold room;
4) Using NSLAB to inhibit PAB;

5) Using heterolactic acid bacteria that do not form lactate, ethanol and CO₂;

6) Using ultrafiltration or bactofugation to remove undesirable organisms capable of producing secondary fermentation in the warm room.

In order to monitor the cheese ripening and prevent split and/or secondary defects, several indicating parameters are recognized. The testing methods include measurements of following parameters (Harper, 2010):

- pH of cheese curd when out of the press
- residual sugars out of the press and residual sugars out of the warm room
- residue of citric acid in cheese out of the warm room
- amino acids and peptides, carbon dioxide (CO₂)
- aspartase activity and amino peptidase activity (cheese and heterolactic bacteria used as adjuncts)
- free amino acid content
- cold temperature growth characteristics of Propionibacterium freudenreiclii strains and hetero lactic bacteria.

For all the parameters above, varied testing methods are available. High Performance Liquid Chromatography (HPLC) is considered to be a standard method to determine organic acids, amino acids and peptides and sugars in dairy products (Bevilacqua and Califano, 1989; Akalin et al., 2002). Gas Chromatography (GC) and GC-MS (Mass Spectroscopy) is commonly used as the standard method in analysis of
free fatty acids and amino acids (Yang and Choong, 2001; Partidario et al., 1998). Also, enzymatic and colorimetric techniques can also be used to investigate the analysis for cheese components like organic acids and amino acids. However, those methods are limited in detecting certain categories of compounds. Most of the methods outlined are time consuming and not easy to conduct, and might require extensive chemical use for pretreatment and derivation. Therefore, a rapid yet reliable instrumental method is needed. It’s difficult to develop a simultaneous testing procedure, but according to previous studies, a model addressing the need to test a majority of the chemical parameters is possible by using Fourier Transform infrared (FTIR) spectroscopy, which will be discussed in the later part of this chapter (see 2.4.4 and 2.4.5).

2.3 High Performance Liquid Chromatography (HPLC)

2.3.1 The Development of HPLC

High Performance Liquid Chromatography (HPLC) is a chromatographic technique of great versatility and analytical power (Reuhs and Rounds, 2010). The technique shares a similar mechanism with other liquid chromatography techniques where different components of the sample move through the column with moving solvent at different speeds and become separated due to difference in molecular size or weight, solubility, and affinity to the column packing material. HPLC represents the modern culmination of classic liquid chromatography (Snyder et al., 2009). A basic HPLC system consists of a pump, injector, column, detector, and data processing system. In a HPLC
system, the solvent is continuously pumped through the column, and the separated sample components are continuously sensed by a detector when they leave the column (Reuhs and Rounds, 2010). This technique was developed in the mid-1970s and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation between very similar compounds.

By the 1980's HPLC was commonly used for the separation of chemical compounds. Computers and automation added to the convenience of HPLC. Improvements in type of columns greatly enhanced the reproducibility of tests (Reuhs and Rounds, 2010).

2.3.2 Applications of HPLC in Cheese Analysis

High Performance Liquid Chromatography has been widely used in food analysis for decades. For chemical analysis in cheese products, numerous workers have reported the application of HPLC for sugars, organic acids and other cheese related compounds.

Among the compounds which are commonly evaluated by HPLC methods, organic acids have been studied in depth due to their importance in cheese chemistry. These are produced as a result of the hydrolysis of butterfat, normal bovine metabolism, or direct addition of acidulants during manufacture, or bacterial growth and activity in certain products (Marsili et al., 1981). Information about organic acids is critical in
understanding metabolism and quality traits of milk and dairy products (Bevilacqua and Califano, 1989). Marsili et al. (1981) and Bevilaqua & Califano (1989) reported HPLC procedures for profiling and quantitating organic acids in dairy products. Bouzas et al. (1991) investigated the HPLC method to simultaneously determine sugar and organic acids in cheddar cheese. Zeppa et al. (2001) expanded the application of HPLC on organic acids and sugars further to the determination of diacetyl and acetoin. Moreover, many studies have been conducted to monitor chemical changes in cheese manufacturing and ripening (Kaminarides, 2007; Ong and Shah, 2009), and universally take organic acid profile and content as important quality indicators, using HPLC as the reference method for organic acids determination.
2.4 Fourier Transform Infrared (FTIR) Spectroscopy

2.4.1 A Brief History of FTIR

Fourier transform infrared (FTIR) spectroscopy is a simple and rapid technique that monitors the molecular vibrations exhibited by various compounds under infrared light (Subramanian, 2009a). FTIR spectroscopy has been established for several decades and several publications and articles are available (Subramanian, 2009b). The root of all types of IR spectroscopy is the discovery of IR radiation in 1800 by Sir William Herschel, who then developed prism based techniques for measuring IR spectra (Subramanian, 2009b). Later in 1880, Dr. Albert A. Michelson invented the Michelson interferometer - an optical device designed to measure wavelengths of light, which made the measurement of infrared light possible. However, the interferograms obtained by the Michelson interferometer are very difficult to interpret, and require time-consuming calculations to be converted into spectra. To obtain an infrared spectrum, interferograms have to undergo a complex mathematical calculation, called a Fourier transform (Smith, 1996). The establishment of the Fast Fourier Transform (FFT) algorithm, which conducts quick Fourier transforms, had turned FTIR into a practical laboratory instrument (Cooley and Tukey, 1965).

The development of FTIR spectroscopy hastened after World War II when several major breakthroughs in the field of Fourier-transformation computation took place. Since then various instrumental and computational improvements including digital signal
processing, FTIR software and diagnostic software development have improved the resolution, signal-to-noise ratio (SNR), sensitivity and speed of detection.

With the adaptation of attenuated total reflectance (ATR) and diffuse reflectance (DRIFTS), FTIR provided more advantages for the analysis of liquids and solids with higher speed and reproducibility. In the early 1980s, the introduction of infrared microscopes brought a great advance in the use of samples in the technique. This accessory enables FTIR to collect spectra from sample as small as 10 micrometers in diameter (Smith, 1996). Due to rapid commercial development and extensive research, FTIR spectroscopy is considered one of the most powerful techniques for chemical analysis. Because of its simplicity, sensitivity, versatility and speed of analysis, its application in biological and chemical analysis in the food science field is growing at a rapid pace (Koca, 2007).
2.4.2 FTIR Spectrometer Mechanism

Although there are different types of FTIR instruments, the core component and basic design of FTIR spectrometers is still the Michelson interferometer (Smith, 1996). The core components of a Michelson interferometer are a beamsplitter, a fixed mirror, and a moving mirror (Figure 2.1). The infrared light (the source of light can be internal or external) is split into two by the beamsplitter. After splitting, half of the light is transmitted to the fixed mirror and the other half is reflected to the moving mirror. Following the reflections of light off the two mirrors respectively, the two light beams recombine at the beamsplitter before leaving the interferometer to pass through the sample and finally strike the detector (Smith, 1996). The signal profile produced by the interaction of infrared beam and sample forms the interferogram.

![Optical Diagram of a Michelson Interferometer](source: Smith, 1996)

Figure 2.1. An illustration of the optical diagram of a Michelson interferometer (Source: Smith, 1996)
2.4.3 Principle of Attenuated Total Reflectance (ATR)

ATR plays very important role in expanding FTIR’s application to solid and liquid samples, which greatly simplifies sample preparation and increases instrumental reproducibility (PerkinElmer, 2005). The core of an ATR accessory is an optically dense crystal, which directs a total internal reflectance of the infrared beam from a certain angle several times according to the crystal structure (indicated in Figure 2). The internal reflectance creates an evanescent wave, or more likely a cloud of infrared light, which extends beyond the surface of the crystal into the sample held closely in contact with the crystal. The size of the evanescent cloud is a few microns (0.5 µm - 5 µm) beyond the crystal surface and reaches into the sample. Therefore, a good contact between the sample and the crystal surface is required to ensure the functioning of ATR. Sharing the same light path with the total beam, the evanescent wave, which is attenuated in regions of the infrared spectrum where the sample absorbs energy, will be passed back to the IR beam which is eventually received by the detector (PerkinElmer, 2005). Subsequently, the system generates an infrared spectrum.

![Figure 2.2. An illustration of a multiple reflection ATR system (Source: Anonymous, ©2005 PerkinElmer)](image-url)
2.4.4 Advantages and Limitations of FTIR

FTIR has become a predominant way of obtaining infrared spectra. Compared with other types of infrared instruments, FTIR has significant higher signal-to-noise ratio (SNR), which is considered to be the ultimate performance indicator of any infrared spectrometer (Smith, 1996). Under a given sample and set of conditions, an instrument is more sensitive and applicable to more kinds of samples with higher accuracy in measurements if it has a higher SNR. Moreover, among several analytical techniques widely applied in food science field, FTIR offers many advantages. A brief list below is a summary of advantages and disadvantages of FTIR from various research study sources (Subramanian, 2009; Koca, 2007; Chalmers and Griffiths, 2002; Smith, 1996; Smith, 1999).

There are several compelling advantages that make this technique a great choice for chemical analysis:

1. The need of minimal sample preparation and low sample volumes, especially with the applications of newly developed accessories and instruments.
2. There is possibility of non-destructive analysis depending on the application.
3. High-throughput, which means the ability to analyze several samples in a short time. In the infrared spectrometer system, all the infrared radiation passes through the sample and gets to the detector at once. Therefore in one scan the detector gets the maximum amount of infrared light at all points, renders the best quality of spectrum and achieves high-throughput.
4. It is a universal technique, in which various types of samples can be analyzed, including solids, liquids, semi-solids and powders. It is simple, sensitive, and the speed of spectra collection is fast.

5. There is less or zero use of hazardous solvents that pose environmental and health hazards.

6. Relatively low operational cost. With good maintenance, FTIR instruments can last a long time.

Despite the above advantages, FTIR spectroscopy has some shortcomings that may limit its application to certain types of analyses.

1. FTIR cannot detect atoms and monatomic ions, elements, or inert gases which show no IR radiation.

2. Biological samples including food are complex mixtures and hence FTIR spectra are complicated with overlapping peaks and signal masking.

3. Most biological samples contain water, which has a strong absorption band that can mask certain important signals. Commonly, sample preparation procedures are required to reduce the nuisance effect of water.

4. Since most FTIRs are single beam instruments, change in environments (e.g. carbon dioxide and water vapor occurring during an experiment) can cause uncertainties in the spectra. The major reason is that the background spectrum is collected at a different time point than the sample spectrum collection. Thus even minor changes in instrument or environment, such as the interference of
water vapor and carbon dioxide, can bring certain spectral artifacts in sample spectra.

5. The information from the spectra shows detectable functional groups, but the complete structure of an unknown chemical molecule is not necessarily predictable. Infrared Spectroscopy needs to be combined with other techniques which reflect other molecular structural information to address more specific analysis problems.

Over all, FTIR is a very useful chemical analytical tool. If not targeting on specific analytical use, most of the limitations can be overcome by developing sample preparation procedure and better experiment design. And as quality and flavor of food are correlated to relatively large molecular or even macromolecular compounds in food matrices (detection as specific to the grade of atoms and monatomic ions is not common), application of FTIR spectroscopy has great potential in food chemistry.
2.4.5 Application of FTIR in Cheese analysis

Due to the great potential of applying FTIR spectroscopy in food, and as previous studies have been done on applying FTIR spectroscopy in dairy products, the Association of Analytical Communities (AOAC) International has approved an FTIR method to determine fat, protein, lactose and total solid contents in some dairy products (AOAC, 2005).

Most of the applications of FTIR spectroscopy in cheese have been focused on the determination of chemical parameters and composition of cheese, and the studies on a chemical testing regimen for the split defects are limited. Martin-del-Campo, Bonnaire, Picque, and Corrieu (2009) monitored ripening in Swiss cheese and Mazerrolles et al. (2001) investigated protein structure and interactions during ripening of semi-hard cheeses. The use of FTIR to monitor flavor-related minor compounds was explored by Koca, Rodriguez-Saona, Harper, and Avarez (2007). They analyzed the water-soluble fraction (WSF) of Swiss cheese to determine short-chain fatty acids. A comparison of analysis of WSF with direct analysis of cheese clearly showed a significant increase in predictive capability of regression models, where a correlation coefficient greater than 0.90 was reported for prediction of acetic, propionic, and butyric acid contents using data collected by FTIR spectroscopy. Subramanian, Alvarez, Harper and Rodriguez-Saona (2009a) developed a novel sample preparation method in Cheddar cheese and demonstrated the possibilities of predicting cheese composition and flavor quality by
FTIR analysis of cheese WSF obtained through the sample preparation method. Since then, the same method was applied to Swiss-type cheese to study the correlation between FTIR spectra and descriptive sensory analysis (Kacaoglu-Vurma et. al, 2009) and the effect of adjunct culture during ripening (Chen et al., 2009). Most recently, Subramanian, Harper and Rodriguez-Saona (2009a) presented the capability of a simple sample preparation method and FTIR spectroscopy to simultaneously determine pH, fat, moisture, salt and flavor quality of Cheddar cheese. Later, the same group further applied FTIR spectroscopy in monitoring amino acids, organic acids, and ripening changes in Cheddar cheese and showcased the potential of FTIR spectroscopy to simultaneously monitor 20 amino acids, three organic acids, age, and some biochemical changes during cheese ripening (Subramanian et al., 2011).
2.5 Infrared Spectral Interpretation and Multivariate Analysis

2.5.1 Basics of Infrared Spectral Interpretation

The fundamental measurement obtained in infrared spectroscopy is an infrared spectrum. The spectra show as plots of measured infrared intensity versus wavelength (or wavenumber) of light (Smith, 1999). Spectral information alone is insufficient for analytical purposes. Therefore the interpretation of spectra is critical for the application of Infrared Spectroscopy.

There are two significant uses of infrared spectra, to identify unknown components or compounds and to determine identities (classification with known samples). Based on the mechanism of infrared spectroscopy that it is sensitive to the presence of chemical functional groups, thus with given wavenumber ranges of the bands of a functional group, the functional group can be identified in different samples. Combined information of different functional groups can be used to identify the existence of a kind of molecule or compound. The second use of identity confirmation requires comparison of the spectra of two samples to determine if they have the same composition (same pattern of spectra) (Smith, 1999).
2.5.2 Soft Independence Modeling of Class Analogy (SMICA)

Infrared spectroscopy obtains a simultaneous detection of a large range of wavenumbers of light, which creates rich information including peak positions (corresponding to wavenumbers), intensities and shapes (Smith, 1999). In many research and technical fields, when dealing with a large amount of data, classification is commonly performed to distinguish one or several specific groups (Infometrix, 2001). The Soft Independent Modeling of Class Analogy (SIMCA) is one of the classification model based on similarity, with the assumption that the closer samples lie in measurement space, the more likely they belong to the same category (CAMO, 2012). SIMCA is a supervised model-establishing technique for sample classification, which builds a distinct confidence region around each assigned classes; and a principal component model is created for each class based on the confidence regions (Maesschalck et al, 1999).

In this approach, at the first stage it runs a global Principal Component Analysis (PCA) or Partial Least Squares (PLS) regression (depending on the data structure) on the whole dataset in order to identify principle component spaces. The models are constructed by SIMCA using samples pre-assigned to different classes. Finally, new sample observations are classified to one of the established class models on the basis of their best fit to the respective model (Infometrix, 2001). Therefore, this approach will compute a reliable classification according to the proximity (the similarity of a set of measurements for an unknown sample to that of one specific group) of data within one
group. In addition, a rich set of diagnostics that address other interesting aspects of classification is provided. For example, interclass distances show how well the classes are distinguished and discriminating power points out the most important variables in the data set. Moreover, the variance structure of each class indicates information about category complexity and may even reveal the phenomena which cause one category to differ from another (Infometrix, 2001).

3.1 Abstract

In order to study the organic acid profile in Swiss cheese and to investigate the potential changes in the profile related to the occurrence of the split defects, four pairs of Swiss cheeses were measured using a standard HPLC method. The chromatographs of Swiss cheese extract were compared with organic acid standards. Three major organic acids were identified (oxalic acid, pyruvic acid and lactic acid), and the concentrations of pyruvic acid and lactic acid showed differences. Standard curves of these two organic acids were made to quantify the concentration of each acid in all sample extracts. The results showed a trend of lower level of overall lactic acid concentration and a higher ratio of pyruvic acid’s concentration to lactic acid’s concentration in the defective Swiss cheese samples.

3.2 Introduction

High performance liquid chromatography is the reference method for determining various components in food analysis (Nollet, 2000). This technique is widely applied in both qualitative and quantitative analysis (Harris, 2006). In dairy products, the
information about organic acids is extremely valuable in understanding the quality traits (Bevilacqua and Califano, 1989). Moreover, it has been reported that lower levels of lactic acid and higher contents of other organic acid salts (e.g. acetate, succinate, and glutamate) were observed in Swiss cheeses with secondary fermentation, which is the possible cause of the split defects (Daly et al., 2009). In addition, the organic acid profile in Swiss-type cheese has not been well studied before.

To better understand the organic acid profile in Swiss cheese and confirm the trend of the changes, HPLC analysis were performed on eight Swiss cheese samples from the four pairs, each containing one defective sample and one non-defective sample.

3.3 Material and Methods

3.3.1 Swiss Cheese Samples

For the study with HPLC, four pairs of Swiss cheese samples were evaluated. The samples were provided by one factory of a commercial cheese manufacturer in Ohio (Table 3.1), which is designated as factory A. The two members of the cheese pair were from the same vat. Within the pair one cheese was identified with split defects and the other one has good quality.
Table 3.1. Information of Swiss cheese samples for study on evaluation of organic acids in Swiss cheese on HPLC

<table>
<thead>
<tr>
<th>HPLC study and initial FTIR evaluation</th>
<th>Swiss Cheese samples</th>
<th>Date Manufactured</th>
<th>Date arrived</th>
<th>Date Processed</th>
</tr>
</thead>
</table>

* Same samples were applied in FTIR evaluation (See Chapter 4, 4.3.1 Swiss cheese samples.)

According to the manufacturing procedure of Swiss-type cheese, after manufacturing, cheeses are placed in warm room and held for 20 – 30 days to get eye-formation (Kosikowski, 1977). Then the cheese undergoes a ripening process for about one to three months. All the cheese samples used in this study were cheeses which had undergone ripening in manufacturing plants and which were transported in cold foam boxes and stored in a 4°C walk-in cooler (Bally Refrigerated Boxes; Thermolinear; Morehead City, NC) immediately after being received. Sampling was finished within one week after receiving each cheese.
Sampling was conducted by selecting a pair of cheeses and cutting each pair member, into pieces weighing between 35 – 50 grams. From each pair member, about 20 cheese sample pieces resulted, then three pieces from each pair member were randomly selected and labeled to indicate which pair member they had come from. All samples were then vacuum-packaged and stored at -20°C (Continental Freezer, Bensalem, PA) until sample preparation and analysis.

### 3.3.2 HPLC Sample Preparation

Several methods of cheese extraction for HPLC, from Zeppa et al. (2001), Ong & Shah (2009), Kaminarides (2007) and Bouzas et al. (1991), were compared in a series of preliminary trial tests. By comparing the instrument system (the chromatograph shape and the peak heights), a HPLC sample preparation method was chosen and modified from the combination of the methods used by Zeppa et al. (2001) and Ong & Shah (2009).

The randomized 35 – 50 grams cheese pieces were shredded and weighed to 5 grams. Then shredded cheese samples (5g) were added to 15 mL of 0.009 N H₂SO₄ (mobile phase) in 50 mL centrifuge tubes where after they were homogenized for 1 minute using a homogenizer (OMNI Tissue Homogenizer, TH-01, Omni International, GA). The homogenized mixture was incubated for 45min in a 50°C water bath. After the water bath, three separated layers had emerged. The top layer was an oil phase, the middle layer was an aqueous phase and the bottom layer was solid, containing mostly insoluble proteins. The aqueous phase subsequently underwent two steps of filtration,
firstly through filter paper (Cat No. 1001-110, Whatman, GE Healthcare UK Limited, Buckinghamshire, UK) then through a 0.20 µm disposable syringe membrane filter (Fisher brand, Fisher Scientific, USA). The filtrate was then ready for HPLC measurements.

3.3.3 HPLC System and Operation Conditions

The HPLC system applied in this study was equipped with a degasser (DGU-20As) and two Shimadzu LD-6AD pumps (Shimadzu, Shimadzu Manufacturing Inc, IL), a multiple autosampler (SIL-20A HT) and a UV detector (SPD-M20A) set to 210 nm for organic acids detection. Data were collected on the LCSolution system (Shimadzu, Shimadzu Manufacturing Inc, IL).

The analyses were performed at 0.6 mL/min and 35 °C with a 300 mm × 7.8 mm cation exchange column (Aminex HPX-87H, Bio-Rad Laboratories, Hercules, CA) equipped with a cation H⁺ Micro-Guard cartridge (Bio-Rad Laboratories, Hercules, CA). Mobile phase was 0.009 N H₂SO₄, prepared by diluting reagent grade sulfuric acid with HPLC grade water and filtered through a 0.45µm membrane filter (Millipore Cooperation, Billerica, MA). Filtered mobile phase was then ready for experimental use, which is recommended to be used within one week.

The organic acid analysis standard (125-0586, Bio-Rad Laboratories, Hercules, CA), provided by the same manufacturer of the column, is a lyophilized mixture of sodium salts of six organic acids. When dissolved in the acidic mobile phase, the anions
from the salts returned as oxalic acid, citric acid, malic, succinic acid, formic acid and acetic acid. This standard is used to evaluate the performance of the HPLC system and column condition. It is also used to identify certain organic acids in the sample (Swiss cheese extract) chromatograph. In addition, based the organic acid profiles (Kaminarides et al., 2001; Ong and Shah, 2009; Mullins and Emmons, 1997; Marsili et al., 1981) of different cheese varieties, several organic acids were chosen to make a Swiss cheese organic acid analysis standard, including: acetic acid (Fisher, A38-500), citric acid (Fisher, A940-500), lactic acid (Fisher, A 162-500), propionic acid (Sigma, P-1386) and pyruvic acid (Aldrich, 107360-25G). Both organic acid standards were used in this study to determine the major organic acids showed in the chromatographs of Swiss cheese extract.

The method reproducibility was confirmed by comparing the chromatographs of the commercial organic acid analysis standard. Those chromatographs were obtained each time before the sample measurements were ran. The method linearity was determined by making two calibration curves for pyruvic acid and lactic acid using two series of standard solutions of each organic acid. The series of solutions cover a range of known concentrations within the detection limit of the system. The same calibration curves were used for standard curves for concentration determination.
3.4 Results and Discussion

3.4.1 Chromatograph Interpretation and Standard Curves

Chromatographs were obtained from each cheese sample extract, in which five organic acids were identified, including oxalic acid, citric acid, pyruvic acid, lactic acid and acetic acid. The determination was based on the comparison between retention times of notable peaks on sample chromatographs and those of organic acid analysis standards.

Figure 3.1. a. Swiss cheese extract chromatograph; b. Chromatograph of organic acid analysis standard made in laboratory; c. Chromatograph of commercial organic acid analysis standard. HPLC chromatographs of organic acids (1. oxalic acid; 2. citric acid; 3. pyruvic acid; 4. lactic acid; 5. acetic acid; 6. propionic acid; 7. malic acid; 8. succinic acid; 9. formic acid) in Swiss cheese extract and organic acid analysis standards with UV detector set at 210nm.
In all the chromatographs collected from every Swiss cheese extract, three peaks are most predominant. They are peaks of oxalic acid, pyruvic acid and lactic acid. The peak height of oxalic acid kept consistent, while the peak heights of pyruvic acid and lactic acid showed differences between the groups of non-defective cheeses and defective cheeses. These indicated that the concentrations of pyruvic acid and lactic acid might vary in Swiss cheeses with different quality grades. In order to determine the concentrations of pyruvic acid and lactic acid in the Swiss cheese extract, two series of standard solutions covering a range of known concentrations of each cheese were made separately. All the solutions were measured by HPLC under the same operating conditions. The standard curves (Figure 3.2) were constructed by plotting the known concentrations (x axis) versus their peak heights, corresponding to the maximum absorbance at UV 210nm(y axis). The standard curves demonstrated very good linear relationship, with the $R^2$ as 0.9992 and 0.9983 respectively, indicating their reliability for the determination of the concentrations of the two organic acids.
Given the purities according to the chemical assay and the densities of both organic acids, the following equations for unit conversion were calculated and used for organic acid concentration determination.

Pyruvic acid concentration (mg/100mL): $1.242 \times a$ (a = the value on x axis)

Lactic acid concentration (mg/100mL): $1.0656 \times b$ (b = the value on x axis)
3.4.2 Statistical Analysis on the Concentrations of Organic Acids

By the equations of the trend lines and the ones for the unit conversion, the concentrations of pyruvic acid and lactic acid (Appendix A) were determined in every Swiss cheese extract from all eight pair members, and then analyzed by statistical tests using the software Minitab 16 (Minitab, Inc., USA).

One-way ANOVA tests showed the effect of the quality attributes (split defective or non-defective) is significant to the concentration of lactic acid (C [Lactic Acid]). However there’s no significant effect of quality attributes on the concentration of pyruvic acid (C [Pyruvic Acid]) according to the test. The result of T-test with 95% confidence interval showed that the concentration of lactic acid in pair members with splits is significantly lower than that in non-defective pair members. This confirms previous research in which overall lactic acid content is observed to be lower in Swiss cheese with split defects, potentially serving as a chemical indicator of the defects (Daly et al., 2009). Figure 3.3 shows the boxplot of the concentration of lactic acid versus the quality levels of samples. The boxplot is created with the T-test. (See Appendix B)
Although the concentration of pyruvic acid was not shown to be significantly affected by the quality attributes of split or non-split, pyruvic acid serves as a very important metabolic intermediate in a great number of pathways; hence the change of pyruvic acid content is always interconnected with the change of other metabolites (Thierry and Maillard, 2002; Adda, 1982). To investigate the potential correlation between split defects and the pyruvic acid level, the ratio of the concentration of pyruvic acid (C [PA]) to that of lactic acid (C [LA]) was calculated and applied to the same statistical analysis. The one-way ANOVA test showed significant effect of quality attributes to the ratio of C [PA] to C [LA]. And the T-test proved that the ratio of C [PA] to C [LA] is significantly higher in the samples with splits. The results are illustrated in Figure 3.3. Boxplot of the concentration of lactic acid versus quality attribute in four pairs of Swiss cheeses (NS – non-defective cheeses; S – Split defective cheeses) (P = 0.019 < 0.05)
Figure 3.4 and data in detail are listed in Appendix B. This finding can be correlated to the higher microorganism activity in defective cheese, causing potential overproduction of CO$_2$.

![Boxplot of ratio of C[PA] to C[LA]](image)

Figure 3.4. Boxplot of the ratio of the concentration of pyruvic acid to the concentration of lactic acid versus quality attribute in four pairs of Swiss cheeses (NS – non-defective cheeses; S – Split defective cheeses) (P = 0.001 < 0.05)

### 3.5 Conclusion

HPLC is a very accurate way to determine organic acid profile both qualitatively and quantitatively. However the method is rather time consuming and limited in the ranges analytes it can determine simultaneously. In this study, five organic acids were identified in the Swiss cheese organic acid profile and three of them have notable peaks.
In the profile acids, pyruvic acid and lactic acid content showed trends of differentiation between pair members. Statistical analysis of the concentrations of both organic acids confirmed the differences. The concentration of lactic acid is significantly correlated with split defects, where the split Swiss cheeses were observed to have lower concentrations of lactic acid. In addition, the ratio of the concentration of pyruvic to that of lactic acid is significantly higher in defective Swiss cheese samples.
4. Chapter 4: Evaluation of Swiss Cheese Extract using FTIR

4.1 Abstract

In order to evaluate the capacity of Fourier Transform Infrared (FTIR) Spectroscopy in monitoring the chemical changes related to the split defects, the four cheese pairs investigated using HPLC were also evaluated with FTIR. Relatively good differentiation of defective and non-defective samples was obtained from two out of the four cheese pairs with statistical significance. The important variables fall into the wavenumber ranges representing organic acids, which supports the results of previous HPLC studies.

To better investigate the possible chemical changes in defective cheese, a new sampling method was applied. Nine new cheeses from three different factories were evaluated. Sampling was done in each single block, from the areas of the split, eye and blind. The modified sampling resulted in better differentiation of the samples containing splits from the other samples. However, the significant variable partly shifted to the wavenumber range representing amino acids and peptides. The differentiation among factories outweighed the differentiation between defective areas and non-defective areas of the cheeses from single blocks and single factories. There is a need to determine the predominant bands to identify specific compounds related to the changes in cheese with split defects.
4.2 Introduction

The capacity for the applying Fourier Transform Infrared (FTIR) Spectroscopy with multivariate analysis in various chemical testing procedures has been reported by numerous researchers (Pillonel et al., 2002; Martin-del-Campo et al., 2009; Koca, 2007; Subramanian, 2009a; Kacaoglu-Vurma et al., 2009; Chen et al., 2009; Subramanian et al., 2011), yet there’s a need for a rapid, reliable testing method addressing the problem of split defects. It is possible to expand the application of FTIR for this need, to overview the chemical changes occurred with the split defects.

Initially FTIR evaluation was performed using the same samples with the HPLC study. Only two out of four pairs of cheeses showed significant differentiation between the defective and non-defective samples within the pair. In order to better confirm the trend of chemical changes related with split defects, a sampling method more specific to the defective area was approached and resulted in better differentiation.

4.3 Material and Methods

4.3.1 Swiss cheese samples

The four pairs of Swiss cheese samples used in the HPLC study (Table 3.1) were also evaluated with FTIR spectrometry. The same sampling method was used for the FTIR, by selecting a pair of cheeses and cutting each pair member into pieces weighing
between 35 – 50 grams. Three pieces were randomly selected and labeled from the 20 cheese sample pieces resultant from cutting each pair member. All samples were then vacuum-packaged and kept frozen until sample preparation and analysis.

For the later part of the study on FTIR evaluation, the sampling method was modified and other nine cheeses were evaluated from different blocks that all had splits, eyes and blind areas in the single block (Table 4.1). These cheeses were provided by commercial cheese factories belonging to three different manufacturers: two in Ohio including factory A and B, and one in Iowa (designated as C). Three cheese samples were provided from each factory, for FTIR analysis (Figure 4.1).

Figure 4.1. Sample supply from three different factories
Table 4.1. Information of Swiss cheese samples for FTIR evaluation using modified sampling method

<table>
<thead>
<tr>
<th>Sample Origin</th>
<th>Sample Codes</th>
<th>Date Manufactured</th>
<th>Date Arrived</th>
<th>Date Processed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ohio (OH)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Iowa (IA)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For FTIR evaluation, the blocks of the 9 individual cheeses were cut into slices about 2cm thick. On one slice, the areas of splits, eye and blind were identified and accordingly cut into different sections. Split and eye sections were cut along the splits or eyes with edges about 0.5 cm around the targeted areas. Blind sections were selected in blank areas at least 1 inch (2.54 cm) away from the splits and eyes, and they were cut into $1 \times 2 \times 2$ cm cuboidal pieces. From each type of area on one cheese, three pieces were
randomly selected, then separately vacuumed packed and kept frozen till sample preparation, immediately followed by measurements.

Figure 4.2. Illustration of sampling from a single block of cheese

4.3.2 Swiss Cheese Extraction for FTIR measurements

In this study, the Swiss cheese extracts were prepared as described by Subramanian et al. (2009a) with slight modification. Frozen cheese samples were fine shredded, and then approximately 0.100g of shredded cheese sample was weighed and placed in a 1.5mL centrifuge tube. 0.5mL of distilled water was added to each centrifuge tube and the mixtures were sonicated (Sonic Dismembrator Model 100, Fisher, Pittsburgh, PA) for 10s with an output of 5 watts. Subsequently, 0.5mL of chloroform (Fisher) was added to the mixture, vortexed and centrifuged at 13000 × g for 3.0 min by
using a micro centrifuge (Model 5415R, Eppendorf, Westbury, NY). The supernatant (200 μL) was collected and transferred into another 1.5mL centrifuge tube, and then mixed with an equal volume of ethanol (Reagent Alcohol, A995-4, Fisher). The ethanol – supernatant mixture was again centrifuged at 13,000 × g for 3.0 min to precipitate the remaining macromolecular peptides and proteins.

Aliquots (10 μL) of the supernatant were dispensed onto the surface of a triple-reflection ZnSe crystal mounted on an attenuated total reflectance (ATR) accessory (Pike Technologies, Madison, WI) and vacuum dried in a cap which tightly sealed the ATR platform and was connected to the vacuum pump. The vacuum drying procedure takes approximately 3 min at room temperature, after that the thin film formed on the crystal is ready for spectrum collection. Between every measurement, the crystal was carefully cleaned using lint-free delicate test wipers (Kimwipes, Kimberly-Clarks professional, Roswell, GA) with 70% ethanol and then distilled water.

4.3.3 FTIR Spectroscopy and Operation Conditions

Measurements of Swiss cheese extract were performed in an infrared spectrometer (Varian FTS-3500GX, Varian Inc., Randolph, MA) equipped with a Zinc selenide (ZnSe) ATR accessory (Pike Technologies, Madison, WI). The optical bench included a dynamically aligned Michelson interferometer, potassium bromide beam splitter, and a deuterated tri-glycine sulfate (DTGS) detector. Spectra were observed and
collected using commercial software Varian Resolutions Pro (Version 4.0.5.009, Varian Inc., Randolph, MA).

The infrared spectra were obtained from 700 to 4000 cm\(^{-1}\) (mid-infrared) with a resolution of 8 cm\(^{-1}\) and 64 scans were co-added per spectrum to enhance signal-to-noise ratio. Measurements were performed on a zinc selenide (ZnSe) crystal, with refractive index of 2.5 and incidence angle of 45° yielding 3 internal reflections.

The instrument was calibrated before measurements using the ZnSe crystal alone as background, with one background collected before each cheese sample section. Two spectra were collected for each cheese extract; therefore 96 spectra (4 pairs × 2 pair members × 3 random pieces out of the block × 2 spectra) in total were collected for the samples of the four cheese pairs, and 162 spectra (9 cheeses × 3 sections × 3 pieces × 2 spectra) in total were collected for nine individual cheese blocks.

### 4.3.4 Multivariate Analysis

In this study, the multivariate analysis was conducted using soft independent modeling of class analogy (SIMCA) by the software Pirouette ® (Version 4.0, Infometrix Inc., Woodville, WA.) Spectra were collected via software Varian Resolutions Pro and were imported as GRAMS (*.spc) files to Pirouette. The IR spectral data were mean-centered, and transformed to their 2\(^{nd}\) derivative to reduce the noises. After SIMCA was run, a set of plots and tables were created to show the results. Projections of the data onto
three principal component axes were used to visualize sample clustering (classification plots).

Among the set of results demonstrated in SIMCA, the interclass distance (ICD) tables shows how well the reassigned classes are distinguished from each other based on the average residual variances. Classes with close average residual variance will lie close to each other whereas those with a large difference in their average residual variance will lie further away in the class projection plot. If the average residual variances are significant enough to make interclass distance between two classes greater than 3, the classes are considered to be statistically different (Kvalheim and Karstang, 1992). The significance of variables, also known as discriminating powers, can be used to define the variables (wavenumbers) that have a predominant effect on sample classification.

4.4 Results and Discussion

4.4.1 Spectral Analysis

A representative spectrum and its 2nd derivative for the water soluble fraction of Swiss cheese are shown in Figure 4.3. The 2nd derivative algorithm was conducted and applied in the analysis to remove the baseline variance, to improve band resolution and to reduce noise and variability between replicates (Schmitt and Flemming, 1998; Kansiz and others, 2001). Prominent spectral features in the 2nd derivative spectral are shown in the range between 900 and 1800 cm\(^{-1}\). The extraction method removed most of the lipid and protein components, leaving most water soluble compounds from cheese in the extract,
such as carboxylic acid (fatty acids), amino acids, sulfur compounds, lactones, aldehydes, ketones, and esters among others (Subramanian et al., 2011).

Figure 4.3. Representative spectrum (top, blue line) and 2nd derivative spectral transformation (bottom, red line) of Swiss cheese extract collected by FTIR-ATR. AU = arbitrary unit. (Source of typical spectral range information: Subramanian et al., 2011)

The region from 4000 to 3100 cm\(^{-1}\) of the spectrum consists of absorbance from O–H and N–H stretching vibrations of hydroxyl groups and primary amide of polypeptides and amino acids, respectively (Smith, 1999). The absorbance caused by C–H stretching vibrations of –CH\(_3\) and –CH\(_2\) functional groups of fatty acids appears between 3100 and 2800 cm\(^{-1}\). The spectral range of 1800 to 900 cm\(^{-1}\) contains signals from polypeptides, amino acids, carbonyl groups of fatty acids, hydroxyl groups, carboxylic acid groups, and fatty acid esters (typically short chain). In addition, the typical spectral ranges are shown in Figure 4.3.
4.4.2 Initial Study on Cheese Pair Member Differentiation

The infrared spectral region of 900 – 1800 cm\(^{-1}\) was applied to build the Soft Independent Modeling of Class Analogy (SIMCA) models to classify Swiss cheese samples based on their quality grades. In four pairs of cheese samples, good differentiation was obtained in two pairs, where the interclass distances between the pair members were greater than 3. Figure 4.4 separately showed the SIMCA class projections of the two well differentiated pairs.

Figure 4.4. Soft independent modeling of class analogy (SIMCA) classification plot showing the difference in pair members in two pairs of Swiss cheese samples. a. OH A – Pair 1; b OH A – Pair 2. (Brown – cheese without split defects; Red – cheese with split defects)

<table>
<thead>
<tr>
<th>Pair number (See Table 3.1)</th>
<th>OH A – Pair 1</th>
<th>OH A – Pair 2</th>
<th>OH A – Pair 3</th>
<th>OH A – Pair 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interclass Distances</td>
<td>6.78</td>
<td>9.47</td>
<td>*1.63</td>
<td>*1.25</td>
</tr>
</tbody>
</table>

* Samples in two pairs didn’t get significant differentiation
The interclass distances (ICD) in Table 4.2 show that OH A – Pair 1 and 2 are well differentiated by the SIMCA model, while the pair members of Pair 3 and 4 were not distinguished from each other. Therefore Pair 1 and Pair 2 were selected for further analysis. The spectral information of the two pairs were combined together and applied to SIMCA.

Figure 4.5a shows the SIMCA classification plot of pair members of both Pair 1 and 2 based on their grades. Samples with splits formed fairly distinctive clusters from samples of good grade, implying that cheeses with the defects and the ones without are chemically distinct.

Figure 4.5. (a) Soft independent modeling of class analogy classification plot showing the overall compositional difference between pair members in both OH A–Pair 1 and Pair 2. (Brown – cheese without split defects; Red – cheese with split defects) (b) Discriminating power plot shows the regions of the FTIR spectra that contributed to the discrimination.
As it shows in Figure 4.5b, the majority of discrimination between pair members was due to the absorbance in wavenumber ranges of 1100 – 1230 and 1720 – 1750 cm\(^{-1}\). The bands in the range of 900 – 1200 cm\(^{-1}\) contain significant absorbance of C–O and C–C stretching in organic acids, and the bands between 1700 and 1730 cm\(^{-1}\) contain absorbance of saturated C=O stretching (Smith, 1999), these findings indicate that the chemical changes of certain organic acids are correlated to the difference between cheese with split defects and cheese with good quality grade. In addition, the absorbance in the range of 1380 – 1450 cm\(^{-1}\) is relatively high, which correlates to the vibration of O–H in-plane bend (Smith, 1999), confirming that the significant wavenumbers represent some organic acids. This finding supports the HPLC results, where the content of pyruvic acid and lactic acid showed changes.

Overall, the high discriminating powers obtained in this model are in the range of 300 – 600 AU, which is relatively low, compared with discriminating powers obtained in other research on cheese evaluation using FTIR. The discriminating power reaches thousands or even higher in the said studies (Subramanian et al., 2011; Koca, 2007; Rodriguez-Saona, 2006).

### 4.4.3 FTIR Evaluation on Individual Cheeses with Modified Sampling Method

As discussed previously, a modification in the sampling method was applied to improve the specificity to the defective area of the Swiss cheese samples. In nine individual Swiss cheese samples, good differentiation of samples from three different
areas of the cheese was obtained from six of them, showing interclass distances (ICD) greater than 3 between all three types of samples. Table 4.3 shows the ICD of split samples from the other two classes in all nine cheeses.

Table 4.3. Interclass distances of split samples from blind and eye samples in nine individual cheeses

<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ICD from Blank</td>
<td>4.92</td>
<td>15.21</td>
<td>*2.08</td>
<td>5.37</td>
<td>10.81</td>
<td>11.05</td>
<td>13.84</td>
<td>*1.02</td>
<td>*1.26</td>
</tr>
<tr>
<td>ICD from Eye</td>
<td>4.80</td>
<td>6.09</td>
<td>*1.47</td>
<td>3.39</td>
<td>26.74</td>
<td>7.45</td>
<td>5.59</td>
<td>*1.44</td>
<td>*1.17</td>
</tr>
</tbody>
</table>

*Samples in three cheeses didn’t get significant differentiation*

In the above six cheeses in which split areas showed significant differences, clear clustering in classification plots were obtained and the discriminating power plots showed very similar predominant bands. Figure 4.6a shows the representative SIMCA classification plot of the three different types of samples from one individual cheese. The three types of different sampling areas formed very distinctive clusters from each other, indicating that cheeses sampled from different areas are chemically distinct.

The discriminating power plot of the same samples is shown in Figure 4.6b. The classification mostly resulted from absorbance in the ranges of 1000 – 1200 and 1350 – 1680 cm\(^{-1}\). The wavenumber range of 1250 – 1530 generally contains absorbance of side chains in various amino acids and aliphatic chains in fatty acids (Barth, 2000;
Subramanian et al., 2011). In addition, the range of 1515 – 1680 represents the absorbance of characteristic functional groups in secondary amides which forms the amino bonds in peptides and proteins (Smith, 1999). Given that most proteins were removed from the water extract fraction of the cheese sample, the findings on the interpretation of prominent wavenumber ranges emphasize the importance of certain amino acids and peptides in the differentiation of split sections.

Although with lower discriminating power (Figure 4.6b), organic acids which are represented in the wavenumber range 1000 – 1200 cm$^{-1}$ also play an important role in SIMCA classification based on cheese sampling areas.

Figure 4.6. (a) Soft independent modeling of class analogy classification plot showing the compositional difference between three different sampling areas in OH A–1. (Brown – Blind; Red – Eye; Green – Split)

(b) Discriminating power plot shows the regions of the FTIR spectra that contributed to the discrimination.
The discriminating power tables were collected from the SIMCA results of the six samples, and the top 15 wavenumbers with highest discriminating powers in each table were compared (See Appendix C). Overall the discriminating power increased significantly compared with the initial study using cheese pair members, since in six groups the DP all reached over one thousand. The most important bands in individual cheeses vary, but they all fall into the range of 1400 – 1700 cm\(^{-1}\), where the absorbance from amino acids and peptides dominates, implying that the chemical changes in amino acids and peptides in Swiss cheese contribute the most to split defects.

**4.4.4 FTIR Evaluation on Individual Cheeses by Factories**

As discussed previously, spectra information collected from two Ohio A cheeses and all three Ohio B cheeses showed great differentiation individually, they were combined as two groups according to their factories and applied to SIMCA analysis. Within the factories, good differentiations were also obtained between the three different sampling areas (see Table 4.4 for interclass distances). The discriminating powers reached the similar values with those in the results of the individual cheeses. However, the dominant bands in the two cheese groups fall into the two wavenumber ranges of 950 – 1150 cm\(^{-1}\) and 1300 – 1450 cm\(^{-1}\), which mostly consists of absorbance of both organic acids and amino acids.
Table 4.4. Interclass distances of split samples from blind and eye samples in two factory groups

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>OH A (including A – 1 &amp; 2)</th>
<th>OH B (including B – 1, 2 &amp; 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICD from Blank</td>
<td>6.69</td>
<td>25.71</td>
</tr>
<tr>
<td>ICD from Eye</td>
<td>4.97</td>
<td>16.09</td>
</tr>
</tbody>
</table>

Figure 4.7. (a) Soft independent modeling of class analogy classification plot showing the overall compositional difference between three different sampling areas in OH B three cheeses. (Brown – Blind; Red – Eye; Green – Split)

(b) Discriminating power plot shows the regions of the FTIR spectra that contributed to the discrimination.

The spectra information collected from all six cheeses showing great differentiation were combined together and applied to SIMCA analysis. The classes were initially assigned according to the sampling areas. However, instead of getting differentiation based on the sampling areas, the classification was clustered into the pattern corresponding to the three factories. The classes were re-assigned according to
factories for analysis. Table 4.5 shows significant differentiation based on sample production sites, presenting as high interclass distances between each factory.

Table 4.5. Interclass distances between classes in SIMCA classification of cheeses based on factories

<table>
<thead>
<tr>
<th>Factories</th>
<th>OH A</th>
<th>OH B</th>
<th>IA C</th>
</tr>
</thead>
<tbody>
<tr>
<td>OH A</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OH B</td>
<td>3.69</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>IA C</td>
<td>72.60</td>
<td>58.46</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Sample clustering in Figure 4.8a illustrates the differentiation of the classes. The projection of samples manufactured in factory ‘Iowa C’ lies far away from the other two sample groups originating in Ohio. Although being plotted closely, with interclass greater than 3, samples from the two factories in Ohio are also statistically significantly distinguished from each other.

As it shows in Figure 4.8b, the discriminating power (DP) in this SIMCA model is markedly high (over $1 \times 10^5$). The plot of DP highlights the dominant bands which mostly fall into organic acid band ranges. The organic acid composition in Swiss cheese greatly distinguishes samples with different manufacturing sites.
Figure 4.8. (a) Soft independent modeling of class analogy classification plot showing the overall compositional difference between cheeses manufactured in three different factories. (Brown – Factory Ohio A; Red – Factory Ohio B; Green – Factory Iowa C)

(b) Discriminating power plot shows the regions of the FTIR spectra that contributed to the discrimination.

Furthermore, the spectral information of cheeses from factory Iowa C were excluded in the model to determine if differentiation can be obtained within manufacturing sites in the same state. This follow-up SIMCA analysis showed similar results comparing with the previous differentiation acquired from three factories, which is demonstrated in Figure 4.9. The interclass distance between these two factories is 47.42, supporting the significance of this classification model. Figure 4.9b shows that the dominant wavenumbers are related to both organic acids and amino acids or peptides absorbance. In addition, the discriminating power value reaches a very high value, which is over $1 \times 10^4$. Both the organic acid and amino acid or peptide compositions in Swiss cheese distinguish sample groups by factories within one state.
Figure 4.9. (a) Soft independent modeling of class analogy classification plot showing the overall compositional difference between cheeses manufactured in two factories in Ohio. (Brown – Factory Ohio A; Red – Factory Ohio B)

(b) Discriminating power plot shows the regions of the FTIR spectra that contributed to the discrimination.

The discriminations shown in both Figure 4.8 and 4.9 can be attributed to the differences in cheese-making procedures at different manufacturing sites, such as the use of starter cultures and the size and shape of the vat, which affects the setting and cooking of milk, and the use of technological procedures to heat the milk. The samples’ geographic origin may correlate to various factors contributing to the chemical changes in Swiss cheese. The climate, geology, forage and breed of cows influence the milk quality, which has great effect on cheese making (Pillonel et al., 2002).

As discussed previously, various contributors, both physically and chemically, can cause split defects in Swiss-type cheese. In this regard, a broader research was conducted in the same laboratory at the same time with this research presented, using a
sample supply from five Swiss cheese factories including the three in this study. The broader research includes different approaches to compare cheeses of split defects with regular cheese areas. Combining the results obtained from other approaches, the potential causes of split defects are different from factory to factory. In addition, factory Iowa C is the only one out of five cheese factories showed statistical significant difference in the texture of cheese samples obtained around split areas from the regular cheese areas (Personal communication, 2012). With this finding, combined with the poor chemical differentiations of defective cheeses from factory Iowa C, the possible cause of split defects in this specific factory might be related to the change of rheological properties of the cheese curds.

4.5 Conclusion

The FTIR spectral information of cheese samples showed clear difference between the pair members in 50% of cheeses from the same vat. In addition, chemical differences of the split areas from the regular cheese areas were obtained with the modification of sampling method. With multivariate analysis, the spectra gave rich information about the chemical difference between split Swiss-type cheese and the non-defective cheese. The classification analysis determined how well the sample groups are differentiated, and also evaluated all wavenumbers’ effects on the differentiation. The dominant wavenumber ranges were easily identified from each set of analysis.
In the initial study where cheeses were evaluated as pair blocks from the same vat, the chemical difference in organic acids resulted in the differentiation of pair members, demonstrated as relatively high discriminating power (DP) in the organic acid ranges. However, much higher DPs were achieved in later analysis with a modified sampling procedure. In nine individual cheeses, 67% (six cheeses) showed significant discrimination of samples from split areas, compared to blind and eye areas. The wavenumber ranges which render the differentiation fall into the ranges mostly containing absorbance from functional groups in amino acids and peptides. The bands of organic acids also contribute to the classification.

In the SIMCA model where the spectral information collected from the six cheeses was combined, the discrimination based on factories greatly outweighed the discrimination caused by the differences of sampling areas, i.e. the differences between factories are much greater than the differences of samples from split areas. The majority of discrimination by factories was based on the wavenumber ranges associated with organic acids.

In conclusion, this study obtained trends from a modest number of cheese samples. Fourier Transform Infrared Spectroscopy (FTIR) combined with multivariate analysis demonstrated great capability in determining the difference of cheese with defects and without. According to the classification models built on FTIR results, chemical changes related to the occurrence of split defects, affect organic acids, amino acids and peptides. While previous work has proven the capability of FTIR in
simultaneously determining amino acids and organic acids in Swiss cheese, the potential of developing a specific testing method targeting the split defects using FTIR is highly possible.

Further research to confirm characteristic bands in the spectra to identify specific compounds, would help to better apply FTIR to detect the split defects in Swiss cheese.
REFERENCES


Personal communication. 2012. Research on the causes and control of split defects in Swiss-type cheese. Unpublished research report. Parker chair laboratory, Department of Food Science and Technology. The Ohio State University, Columbus, OH.


Weinrichter, B., Luginbühl, W., Rohm, H., Jimeno, J. 2001. Differentiation of Facultatively Heterofermentative Lactobacilli from Plants, Milk, and Hard Type Cheeses by SDS-PAGE, RAPD, FTIR, Energy Source Utilization and Autolysis Type. LWT – Food Science and Technology. 34(8):556-566

### Appendix A: Pyruvic Acid and Lactic Acid Concentrations

The Concentrations of pyruvic acid and lactic acid in Swiss cheese extracts (mg /100 mL)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NS-a-1</td>
<td>3.330</td>
<td>161.537</td>
<td>0.021</td>
<td>NS-d-1</td>
<td>14.084</td>
<td>229.621</td>
<td>0.061</td>
</tr>
<tr>
<td>NS-a-2</td>
<td>2.931</td>
<td>147.470</td>
<td>0.020</td>
<td>NS-d-2</td>
<td>14.173</td>
<td>233.593</td>
<td>0.061</td>
</tr>
<tr>
<td>NS-b-1</td>
<td>5.422</td>
<td>192.367</td>
<td>0.028</td>
<td>NS-e-1</td>
<td>7.551</td>
<td>240.132</td>
<td>0.031</td>
</tr>
<tr>
<td>NS-b-2</td>
<td>5.439</td>
<td>187.938</td>
<td>0.029</td>
<td>NS-e-2</td>
<td>8.349</td>
<td>262.300</td>
<td>0.031</td>
</tr>
<tr>
<td>NS-c-1</td>
<td>5.174</td>
<td>195.901</td>
<td>0.026</td>
<td>NS-f-1</td>
<td>6.443</td>
<td>166.980</td>
<td>0.039</td>
</tr>
<tr>
<td>NS-c-2</td>
<td>5.544</td>
<td>183.075</td>
<td>0.030</td>
<td>NS-f-2</td>
<td>9.059</td>
<td>237.075</td>
<td>0.038</td>
</tr>
<tr>
<td>S-a-1</td>
<td>17.287</td>
<td>96.060</td>
<td>0.180</td>
<td>S-d-1</td>
<td>10.022</td>
<td>194.199</td>
<td>0.051</td>
</tr>
<tr>
<td>S-a-2</td>
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<td>92.333</td>
<td>0.189</td>
<td>S-d-2</td>
<td>9.093</td>
<td>179.463</td>
<td>0.051</td>
</tr>
<tr>
<td>S-b-1</td>
<td>15.426</td>
<td>72.134</td>
<td>0.214</td>
<td>S-e-1</td>
<td>13.245</td>
<td>154.372</td>
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<tr>
<td>S-b-2</td>
<td>16.123</td>
<td>92.345</td>
<td>0.174</td>
<td>S-e-2</td>
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<td>164.820</td>
<td>0.085</td>
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<td>S-f-1</td>
<td>5.415</td>
<td>184.830</td>
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<tr>
<td>S-c-2</td>
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<td>82.249</td>
<td>0.188</td>
<td>S-f-2</td>
<td>5.444</td>
<td>184.173</td>
<td>0.030</td>
</tr>
<tr>
<td>---------------</td>
<td>-------</td>
<td>-------</td>
<td>-------------------</td>
<td>---------------</td>
<td>-------</td>
<td>-------</td>
<td>-------------------</td>
</tr>
<tr>
<td>NS-g-1</td>
<td>8.850</td>
<td>293.894</td>
<td>0.0301</td>
<td>NS-j-1</td>
<td>17.354</td>
<td>253.841</td>
<td>0.068</td>
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<td>NS-g-2</td>
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<td>0.032</td>
<td>NS-j-2</td>
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<td>213.310</td>
<td>0.057</td>
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<td>NS-h-1</td>
<td>26.844</td>
<td>259.568</td>
<td>0.103</td>
<td>NS-k-1</td>
<td>16.438</td>
<td>253.090</td>
<td>0.065</td>
</tr>
<tr>
<td>NS-h-2</td>
<td>23.010</td>
<td>226.712</td>
<td>0.101</td>
<td>NS-k-2</td>
<td>12.766</td>
<td>203.834</td>
<td>0.063</td>
</tr>
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<td>NS-i-1</td>
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<td>338.342</td>
<td>0.029</td>
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<td>NS-i-2</td>
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<td>0.033</td>
<td>NS-m-2</td>
<td>20.393</td>
<td>208.038</td>
<td>0.098</td>
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<td>S-g-1</td>
<td>32.438</td>
<td>249.361</td>
<td>0.130</td>
<td>S-j-1</td>
<td>19.648</td>
<td>270.313</td>
<td>0.073</td>
</tr>
<tr>
<td>S-g-2</td>
<td>29.852</td>
<td>200.628</td>
<td>0.149</td>
<td>S-j-2</td>
<td>16.304</td>
<td>220.889</td>
<td>0.074</td>
</tr>
<tr>
<td>S-h-1</td>
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<td>225.742</td>
<td>0.097</td>
<td>S-k-1</td>
<td>10.868</td>
<td>268.625</td>
<td>0.040</td>
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<tr>
<td>S-h-2</td>
<td>18.855</td>
<td>212.416</td>
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<td>S-k-2</td>
<td>9.608</td>
<td>223.618</td>
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<tr>
<td>S-i-1</td>
<td>13.646</td>
<td>278.994</td>
<td>0.049</td>
<td>S-m-1</td>
<td>11.448</td>
<td>279.556</td>
<td>0.041</td>
</tr>
<tr>
<td>S-i-2</td>
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<td>227.000</td>
<td>0.041</td>
<td>S-m-2</td>
<td>9.782</td>
<td>227.808</td>
<td>0.0430</td>
</tr>
</tbody>
</table>
Appendix B: Statistical Results in HPLC Study

Statistical Analysis Results of Concentrations of Pyruvic Acid and Lactic Acid versus Swiss cheese quality attributes

One-way ANOVA: C [Lactic Acid] versus quality

<table>
<thead>
<tr>
<th></th>
<th>Sum of</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Quality</td>
<td>19793</td>
<td>1</td>
<td>19793</td>
<td>6.03</td>
<td>0.018</td>
</tr>
<tr>
<td>Groups</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within</td>
<td>150992</td>
<td>46</td>
<td>3282</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>170784</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S = 57.29   R-Sq = 11.59%   R-Sq(adj) = 9.67%

One-way ANOVA: C [Pyruvic Acid] versus quality

<table>
<thead>
<tr>
<th></th>
<th>Sum of</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Quality</td>
<td>154.7</td>
<td>1</td>
<td>154.7</td>
<td>3.60</td>
<td>*0.064</td>
</tr>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within</td>
<td>1975.4</td>
<td>46</td>
<td>42.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2130.1</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S = 6.553   R-Sq = 7.26%   R-Sq(adj) = 5.25%

* p-value > 0.05, quality does not have significant effect on C [Pyruvic Acid]

67
One-way ANOVA: ratio of C[PA] to C[LA] versus quality

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>p</th>
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</thead>
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<tr>
<td>Between Quality</td>
<td>0.02878</td>
<td>1</td>
<td>0.02878</td>
<td>11.96</td>
<td>0.001</td>
</tr>
<tr>
<td>Groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.11065</td>
<td>46</td>
<td>0.00241</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.13943</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$S = 0.04905 \quad R-Sq = 20.64\% \quad R-Sq(adj) = 18.91\%$

Two-Sample T-Test and CI: C [Lactic Acid], quality

Two-sample T for C [Lactic Acid]

<table>
<thead>
<tr>
<th>quality</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>24</td>
<td>226.3</td>
<td>43.6</td>
<td>8.9</td>
</tr>
<tr>
<td>S</td>
<td>24</td>
<td>185.7</td>
<td>68.3</td>
<td>14</td>
</tr>
</tbody>
</table>

Difference = mu (NS) - mu (S)

Estimate for difference: 40.6

95% lower bound for difference: 12.7

T-Test of difference = 0 (vs >): T-Value = 2.46   P-Value = 0.009   DF = 39
Two-Sample T-Test and CI: ratio of C [PA] to C[LA], quality

Two-sample T for ratio of C [PA] to C[LA]

<table>
<thead>
<tr>
<th>quality</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>SE Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>24</td>
<td>0.0495</td>
<td>0.0269</td>
<td>0.0055</td>
</tr>
<tr>
<td>S</td>
<td>24</td>
<td>0.0985</td>
<td>0.0639</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Difference = mu (NS) - mu (S)

Estimate for difference: -0.0490

95% upper bound for difference: -0.0249

T-Test of difference = 0 (vs <): T-Value = -3.46  P-Value = 0.001  DF = 30
Appendix C: Predominant Wavenumbers in FTIR Evaluation

Top 15 wavenumbers with high discriminating powers (DP) in six individual cheeses demonstrating significant differentiation of split samples

<table>
<thead>
<tr>
<th>OH A-1</th>
<th>OH A-2</th>
<th>OH B-1</th>
<th>OH B-2</th>
<th>OH B-3</th>
<th>OH C-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wave #</td>
<td>DP</td>
<td>Wave #</td>
<td>DP</td>
<td>Wave #</td>
<td>DP</td>
</tr>
<tr>
<td>1681.929</td>
<td>1218.523</td>
<td>1577.772</td>
<td>1426.315</td>
<td>1693.502</td>
<td>2106.733</td>
</tr>
<tr>
<td>1685.787</td>
<td>1392.701</td>
<td>1577.772</td>
<td>1426.315</td>
<td>1693.502</td>
<td>2106.733</td>
</tr>
<tr>
<td>1473.615</td>
<td>900.3898</td>
<td>1643.353</td>
<td>1269.385</td>
<td>1689.645</td>
<td>1808.042</td>
</tr>
<tr>
<td>1477.473</td>
<td>1316.719</td>
<td>1316.719</td>
<td>1393.192</td>
<td>1392.701</td>
<td>1697.361</td>
</tr>
<tr>
<td>1550.769</td>
<td>1091.458</td>
<td>1643.353</td>
<td>1269.385</td>
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<td>1693.502</td>
</tr>
<tr>
<td>1546.911</td>
<td>712.433</td>
<td>1643.353</td>
<td>1269.385</td>
<td>1426.315</td>
<td>1693.502</td>
</tr>
<tr>
<td>1712.791</td>
<td>660.8447</td>
<td>1643.353</td>
<td>1269.385</td>
<td>1426.315</td>
<td>1693.502</td>
</tr>
<tr>
<td>1504.777</td>
<td>584.534</td>
<td>1643.353</td>
<td>1269.385</td>
<td>1426.315</td>
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<tr>
<td>1708.933</td>
<td>573.023</td>
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<tr>
<td>1689.645</td>
<td>558.7751</td>
<td>1643.353</td>
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<td>1516.05</td>
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<td>1643.353</td>
<td>1269.385</td>
<td>1426.315</td>
<td>1693.502</td>
</tr>
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</table>

*Wavenumbers repeatedly showed in more than three cheeses*