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Quantitative Analysis of Acetic Acid Traces in Alcohol Aqueous Solution during Fermentation

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Declaration

I certify that the thesis submitted for the degree of master is the result of my research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed :.....

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Date: 21/12/2024

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Abstract

This study introduces a validated a novel, user-friendly, cost-effective method to quantify acetic acid traces in alcohol-water solutions during fermentation using Fourier Transform Infrared (FTIR) spectroscopy. Calibration curves were developed by correlating acetic acid concentrations (ranging from 0% to 30% w/w) with their absorption peaks at specific wavenumbers (1703 cm^{-1}, 1233 cm^{-1}, and 1287 cm^{-1}). For ethanol, absorption was analyzed at key wavenumbers such as 1087 cm^{-1} and 1635 cm^{-1}. The study found that the optimal ratio for determining acetic acid concentration was at 1703 cm^{-1} relative to pure water, while the best ratio for ethanol quantification was 1087 cm^{-1} divided by 1635 cm^{-1}. Validation demonstrated 1. Repeatability: A laboratory-prepared sample (4% acetic acid, 5.1% ethanol) was measured 12 times with minimal deviation. 2.Real-world applicability: Market vinegar samples were analyzed 13 times, with results closely matching traditional acid-base titration. The findings confirm the suitability of IR spectroscopy for precise and real-time analysis of acetic acid and ethanol in fermentation products. This innovative approach offers significant advantages for food and beverage industries, providing a robust alternative to traditional methods.

Keywords: Acetic Acid, infrared spectroscopy, calibration curve, fermentation, quantitative analysis, IR absorption Outline

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CHAPTER ONE

Introduction

1.1 Background

Fermentation is an ancient biotechnological technique used to produce desirable food products with long shelf life and good sensory properties. Fermented foods can often be stored at ambient temperatures without spoiling. Some fermentation processes enhance the nutritional value or digestibility of food by breaking it down into simpler, easily digestible compounds (Ramez et al., 2024).

Bioreactors of various types have been widely employed in biological sciences and industrial biotechnology, increasing the demand for new engineering designs and the development of bioreactor technology. The introduction of computers to control fermentation processes represents a significant leap in their development, resulting in systems with high reliability and efficiency (Terefe, 2022).

This process has significant historical and cultural importance, leading to the production of a variety of fermented foods and beverages, such as yogurt, bread, beer, an`8d vinegar. The main types of fermentation include :1. Alcoholic fermentation, where yeast converts sugars into ethanol and carbon dioxide. 2.Lactic acid fermentation, utilized in dairy products and pickled vegetables, 3. Acetic acid fermentation, which transforms ethanol into acetic acid, the primary component of vinegar (Maicas , 2022).

Globally, food spoilage is a significant issue, with approximately one-third of all food produced for human consumption equivalent to about 1.3 billion tons wasted annually (Bélanger and Pilling 2019).

This waste results not only in economic losses estimated at $940 billion but also contributes to environmental concerns, as decomposing food generates greenhouse gases. Locally, in Palestine, approximately 25% of fresh fruits and vegetables are lost due to spoilage, with specific crops like dates experiencing losses of around 30% during the harvesting and post-harvesting stages (Palestinian Ministry of Agriculture, 2022). Similarly, apples can suffe spoilage rates of up to 20%, emphasizing the need for effective management strategies to minimize waste (Bélanger and Pilling 2019).

By reviewing previous studies, it was agreed that fermentation process can be used to transform

spoiled and damaged foods into valuable products, reducing waste and generating income, which in turn helps to increase income. Specific strains of bacteria, such as acetobacter for acetic acid production and Saccharomyces for ethanol `production, play a crucial role in this process. In the Palestinian market, the loss of fruits such as dates and apples not only represents an economic waste, but also highlights the potential of using fermentation techniques to produce products such as vinegar and alcoholic beverages, thus enhancing local food security and promoting `sustainability. By using these innovative methods, we can reduce food waste while creating added value for agricultural products, which ultimately benefits the economy and the environment (Siddiqui et al., 2023).

Through our study, we will aim to achieve the objective of conducting a quantitative analysis of acetic acid traces in alcohol aqueous solution during fermentation. This will involve using advanced techniques such as (IR) and other analytical methods to determine the precise quantities of acetic acid.

1.1.2 Introduction to Fermentation

Fermentation is a metabolic pro2cess that occurs anaerobically, where microorganisms utilize organic molecules as energy sources, resulting in by-products such as organic acids, alcohol, and gases (Liszkowska & Berlowska, 2021). This process supports organisms that thrive in oxygen-free environments and can be categorized into two types based on the presence of biocatalysts: processes involving living microorganisms and those that do not. Common types include homolactic, alcoholic, and mixed fermentations. Fermenting organisms are primarily classified as fermenting bacteria (usually Gram-positive), yeasts (e.g., *Saccharomyces spp.*) (Garofalo et al., 2022).

As one of the oldest food processing and preservation methods, fermentation transforms inedible raw materials into consumable products, significantly contributing to diets and health worldwide while supporting millions of jobs in their production and distribution. However, the complexity of fermentation raises food safety concerns, necessitating a comprehensive understanding of microbial community structures to ensure product safety and quality (Garofalo et al., 2022).

1.1.3 Definition and Importance

Fermentation used to produce beverages (e.g., beer and wine), and various food products like bread, cheese, yogurt, and kimchi, along with biotechnological and pharmaceutical products. Biochemically, fermentation involves the breakdown of organic compounds (mainly carbohydrates, proteins, and lipids) bymicroorganisms (yeasts, lactic acid bacteria, fungi) under anaerobic conditions, generating energy in the form of ATP and other metabolites (Siddiqui et al., 2023).

The Fermentation process is vital for producing significant global products, particularly ethanol on an industrial scale, pharmaceutical products (e.g., antibiotics, amino acids), and energy-efficient food processing. It is also applied in wastewater treatment and biofuel production. Given its importance, research on large-scale fermentation systems is crucial in microbiology and biotechnology. Fermented foods, characterized by microbial activity, include products like yogurt and cheese, while alcoholic beverages are a primary focus of this field. Fermentation has historically influenced world cultures, and controlling fermentation processes is essential for producing safe food products, as fermented foods contain beneficial microorganisms,

particularly lactic acid bacteria (LAB) recognized for their health benefits (Garofalo et al., 2022).

1.2 Classification of Fermentation

Fermentation can be classified in various ways, primarily based on the energy supply for fermentative organisms. It can be categorized into two main types: (1) lithofermentation, where electron donors such as H_2 , $S_2O_3^-$, or Fe^{2+} are utilized for energy, with CO_2 acting as an electron acceptor; and (2) organ fermentation, which involves the fermentation of organic nutrients like carbohydrates, proteins, amino acids, purines, or fatty acids (Garofalo et al, 2022).There may be another, more productive way to classify fermentation based on the sources (substrates) from which they originate. There are five main categories of fermented beverages depending on the raw materials: (a) from grains (such as barley, corn, rye, millet, etc.), resulting in products like Irish whiskey, beer, bourbon whiskey, boza, kvass, and vodka; (b) from fruit juice (such as apples, bananas, cherries, grapes, ginger, etc.), producing cider, wine, cherry wine, ginger beer, among other products; (c) from vegetables (such as potatoes, sugar cane, sauerkraut, etc.), resulting in vodka (in Poland), rum, and sauerkraut juice, etc.; (d) from milk, producing various beverages like ayran, kumis, kefir, and buttermilk; and (e) other raw materials, such as tea leaves, honey, sugar, and palm sap, to produce different beverages (such

as kombucha and mead, etc.) (Kaur et al 2019). Additionally, fermentation can be classified based on the type of metabolic substrate used (e.g., carbohydrates, fats) or the end products (e.g, organic acids, alcohols, gases) (Garofalo et al., 2022).

The most common alcohol fermentations include two main types: **alcoholic fermentation** (ethanol fermentation) and **lactic fermentation**. Ethanol is the primary component of products from alcoholic fermentation, while lactic fermentation produces lactic acid, ethanol, and gases such as carbon dioxide. Fermentation can also be categorized by the growth conditions of microorganisms involved, distinguishing between aerobic and anaerobic fermentation. Furthermore, classification can be based on fermentation vessels, including static, intermittent, continuous, column, and surface fermentation, as well as traditional versus industrial fermentation (Stanbury et al., 2013).

1.2.1 Based on Microorganisms

`8 Fermentation can be divided according to the microorganisms involved, such as bacteria, yeast, and molds. These microorganisms play crucial roles in complex bioconversion systems that produce targeted products. Fermented foods often contain nourishing substances like polysaccharides, proteins, and phenolic compounds (Garofalo et al., 2022). Bacterial fermentation is dominant in terms of the variety and market presence of fermented foods. For instance, (LAB) are responsible for the fermentation of dairy products like yogurt and kefir, while Bacillus subtilis is used in producing natto from soybeans. LAB, yeast, and molds also contribute to fermenting fish sauces and vegetables like kimchi (Babich et al., 2023).

Yeast fermentation is especially important in agriculture, alcoholic fermentation, a type of anaerobic fermentation, traditionally refers to the conversion of sugar solutions from fruits by yeast species such as *Saccharomyces cerevisiae*, resulting in alcoholic beverages. This process produces significant amounts of ethanol and carbon dioxide while releasing energy stored in glucose. Although many fermented foods produced by fungi exist (Babich et al., 2023).

1.2.2 Aerobic Fermentation

Aerobic fermentation occurs in the presence of oxygen. Some prokaryotes, fungi, and yeasts can use aerobic fermentation to generate energy. In some cells, glucose is absorbed by the cell membrane, and that is acted upon by the glycolytic pathway (net 2 ATP), which breaks glucose in a series of enzymatic reactions into pyruvate. For those cells that use aerobic fermentation, pyruvate is further metabolized in the Krebs cycle (net 36 ATP). In this fermentation, glucose is metabolized into carbon dioxide and water, this is the reason that aerobic fermentation is stopped when the oxygen in the medium is depleted. This fermentation is also used in the food & drinks industry for the production of bread, beer, and wine (Siddiqui et al, 2023).

1.2.3 Anaerobic Fermentation

Anaerobic fermentation is the conversion of sugars into cellular energy in the absence of oxygen. A by-product of this process is acids, gases or alcohol. Anaerobic fermentation is a type of fermentation process widely used in food and beverage production (Siddiqui et al., 2023). Yeast and some bacteria perform anaerobic fermentation. Yeast converts sugars into alcohol and carbon dioxide. The process is called Ethanol fermentation or Alcoholic fermentation. Anaerobic fermentation by yeast is exploited in the production of wines, beers and breads (Siddiqui et al., 2023).

In such a process, an energy-poor compound, alcohol, is formed. However, the reactions generating ATP are also producing by-products, such as CO_2 and ethanol, forming what is commonly called “fermentation” with respect to industrial fermentation processes. Bacteria which carry out lactic acid fermentation convert sugars into lactic acid. The process is exploited in the production of yogurt and fermented milk. The lactic acid fermentation is the basis of the sourness of fermented foods (Siddiqui et al., 2023).

1.2.3.1 Alcoholic Fermentation

Alcoholic fermentation is a type of anaerobic fermentation in which microorganisms, including yeast or bacteria, convert sugars (such as glucose, fructose, or sucrose) into alcohol (ethanol) and carbon dioxide gas. Alcoholic fermentation has been used in the brewing and baking industries for centuries. Ethanol and carbon dioxide are the main products of alcoholic fermentation, and alcoholic beverages are produced as a result of this process (Siddiqui et al., 2023).

Since ancient times, fermented alcoholic beverage has been produced, and a myriad of alcoholic drinks has been produced in this way. Fruit-based drinks such as wine and cider, sap-based drinks such as palm wine, honey-based drinks such as mead, and milk-based fermented drinks are included in this group. All of these raw materials contain sugar, so naturally existing yeast turns it into ethyl alcohol, thus making an alcoholic drink Alcoholic beverages produced in a multi-step fermentation process comprise starch in grains and tubers. Starch is saccharified first using enzymes in saliva, mold, and germinated seeds, and thereafter, alcohol fermentation by yeast is conducted. Beer and traditional sorghum liquor of West Africa are included in this group. When distilled, the beverage becomes spirits. (McGovern, 2019). Alcoholic fermentation was discovered by accident at first. However, people recognized this phenomenon

at an early stage and conducted brewing even before scientific explanation was given. Alcoholic fermentation by yeast can be represented by the following equation. In the presence of glucose and absence of oxygen, yeast produces carbon dioxide and ethanol.

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + 1875\ kJ$

Equ.1: Alcoholic fermentation by yeast

This equation shows that supposedly yeast consumes glucose to produce ethanol and carbon dioxide (Solomons et al., 2016).

1.2.3.2 Lactic Acid Fermentation

Lactic acid fermentation is a specific type of fermentation where sugar (usually glucose) is converted into cellular energy and the metabolite lactate, which takes place in almost all animal tissues, is one of the three major metabolic pathways to regulate redox homeostasis Dairy products such as curd, cheese, and yogurt are made by the lactic acid fermentation of milk by certain (LAB). Other foods such as pickles, olives, and sauerkraut are made by the fermentation of vegetables (Vijayakumar et al., 2008).

The sour taste in yogurt and sour milk products is caused by the lactic acid produced by *Lactobacillus* Bacteria Lactic acid fermentation is used in the preparation of various foods (Joshua Ashaolu & Reale, 2020).

1.2.3.3 Yeast-Based Fermentation

Without yeast fermentation, many foods would not exist today, like yogurt, cheese, and oil. Nowadays, fermentation is used in the production of insulin, antibiotics, and ethanol, a crucial biofuel. Fermentation works as a metabolic process in which sugars or other organic compounds are converted into alcohol and/or acids and gases. Much of the flavor, aromas, and texture of fermented foods and beverages come from the metabolism of carbohydrates, phenols, alcohols, and other compounds by the fermenting microorganisms (Liszkowska & Berlowska, 2021).

The basic metabolic process of all fermentations is the same, yeast and bacteria convert specific organic substances, like glucose into ethanol, lactic acid, carbon dioxide, and other by-products. Yeast or microorganisms grow during and after this process, which gives fermented foods or beverages unique flavors, aromas, textures, and nutritional effects. The large diversity of food and beverages fermented can be attributed to the diversity in "substrates" fed to the fermenting microorganisms, the huge variety of microorganisms used for fermentation, and the different environmental conditions under which fermentation takes place (temperature, pH, duration, etc.). Fermentation has, in general, the potential of improving or generating a variety of sensory properties in food. For some different food and beverage (Sharma et al 2020).

1.2.3.4 Butyric Acid Fermentation

Butyric acid fermentation is a type of anaerobic fermentation in which certain organisms convert sugars into butyric acid, carbon dioxide, and hydrogen gas. It is a process of decarboxylation of pentoses and hexoses, in which several microorganisms, including some

Clostridium species, participate. Heterofermentative species such as *C. bothrium* oxidize glucose and similar sugars to butyric acid, CO_2, and H_2. These bacteria can be isolated from various sites such as the intestines of humans and animals, soil, and sludge in wastewater treatment plants (Xie and Gänzle, 2023). Butyric acid fermentation is involved in the production of several food products, including cheese, butter, and fermented cream (Szymanowska-Powałowska, 2014). It functions mainly in the gut of herbivores, such as cattle and deer, and in the reticulated chambers of the stomach of these animals. It is also involved in some industrial fermentations, such as the production of chloroform, acetone, butanol, and isopropanol. Some *Clostridia* are industrially important as they convert cellulose to butyric acid, which in turn can be converted to acetic anhydride, an important chemical used in the synthesis of pharmaceuticals (Qureshi et al., 2024).

1.2.3.5 Propionic Acid Fermentation

Propionic acid fermentation is a type of fermentation process, which is carried out by some bacteria as *Propionibacterium*. Propionic acid results from the fermentation of any sugar such as glucose, galactose, or lactate. Propionic acid is a three carbon acid, which is used widely in dairy, food, and agricultural industries. Anaerobic fermentations are generally carried out in a mannitol-glucose culture medium to produce propionic acid and also for the production of vitamin B12, which is a bulkey product of food industry (Ranaeet al., 2020). Fermentation of Swiss cheese is an important application of propionic acid fermentation. To make Swiss cheese, lactic acid is fermented into propionic acid by propionibacteria. This acid is then used to flavor cheese (Cogan, 2002). Similar to the butyric acid fermentation, conversion of lactate to propionic acid is a two-step process. First, lactate is converted to PCR, a five carbon compound, by lactate/pyruvate conversion. Here, one molecule of carbon dioxide is removed from lactate. Subsequently, this five-carbon compound is converted to propionic acid by a series of reactions. Utilization of mannitol or glucose as a carbon source, phosphonate consumption, production of PHB or propanol, amino acid biosynthesis (alanine, aspartate, isoleucine, leucine, methionine), thiamine biosynthesis and so on are the other metabolic pathways of bacteria, nevertheless, cheese making using lactic acid fermentation and propionic acid group of fermentation are the important commercial applications of this fermentation type (Zhu, 2008).

Aceti Acid Fermentation

Acetic acid fermentation is a type of fermentation, where acetic acid (the major component of vinegar) is produced from ethanol. In this process, ethanol is first oxidized to acetaldehyde. The reaction is catalyzed by the enzymes alcohol dehydrogenase. The produced acetaldehyde is oxidized, subsequently, to acetic acid by the action of enzymes aldehyde dehydrogenase. The enzyme complex alcohol dehydrogenase–acetaldehyde dehydrogenase, containing a coenzyme that is specific for NADH. Most bacteria in nature reduce acetaldehyde and produce ethanol (Chiang 2005).

Acetic acid fermentation is used in the manufacture of vinegar. The process uses the pure culture of acetic acid bacteria of the genera *Acetobacter* and *Gluconobacter*. This process is called the Schutzenbach process. The submerged acetic acid fermentation is carried out in large fermenters (acetator) having a working volume ranging from 20 to 200 m^3. Vinegar has been

used for thousands of years as a food preservative, flavoring agent, and as a medicinal drink. Wine vinegar originated with the discovery of fermentation from wine, with the simultaneous formation of acetic acid (Plioni et al., 2021). Subsequently, other types of vinegar derived primarily from cereals, sweet potatoes, palm sap, cane sugar, aspergillus, and molasses, as well as rice and grain vinegar. The production process, flavor, and properties of vinegar vary with the region of origin and raw materials. Most vinegar consumed worldwide (more than 50% at present) is produced from different kinds of wine. Vinegar is a liquid product containing 4% or more acetic acid and is consumed as-is or used in various food products. Acetic acid is produced commercially by catalytic processes or fermentation. Fermentation is a naturally occurring biological process in which an organic compound is converted to an organic compound with the simultaneous production of energy (Plioni et al., 2021).

1.3. Industrial Applications of Fermentation

Fermentation predominantly takes place in nature and has been exploited at the industrial level since ancient times. Fermentation results in an increase in the number of bacteria, yeast, or protozoa in a sample. Fermentation involves the process of converting sugar into carbon dioxide and alcohol in the absence of air or oxygen. There are several types of fermentation processes with different industrial applications. Various fermented products are manufactured by utilizing different raw materials through fermentation technology. The fermented food industry is a well-established industry all over the world. Fermented foods, such as bread, curd, horseradish, green olives, and sauerkraut, are produced regularly by utilizing technology or by traditional culture. These industries play an essential role in the agricultural economy and help to increase food production. Fermented foods are regarded as healthy food all over the world due to their nutritional benefits and health-friendly products (Plioni et al., 2021).

Fermentation technology is also used for the production of commodities like amino acids, organic acids, enzymes, alkaloids, proteins, steroids, nucleotides, vitamins, and antibiotics on an industrial scale. Fermentation produces a large amount of substrates, which are microbial products that are excreted into the environment, such as organic acids, alcohol, gas, enzymes, and antibiotics. Fermentation is also used for the bioremediation of waste, which includes the removal of chemicals, acids, alcohols, metals, and phenolics. Several waste products can be fermented, including starchy waste, molasses, whey, etc (Bhalla et al., 2007).

1.4 Applications of IR Spectroscopy in Quantitative and Qualitative Analysis

Is a very informative and non-destructive analytical technique useful in almost all scientific fields, including but not limited to chemistry, biology, physics, and environmental, material, pharmaceutical, and food sciences. Based on the observation of molecular vibrations and transitions, absorption of IR radiation by the sample enables both qualitative and quantitative analyses of different types of compounds. IR spectra can practically be obtained from all types of substances and in all physical states (solid, liquid, and gas), thus making this powerful analytical technique a very versatile and valuable tool for the study of organic and inorganic molecules, biological tissues, and metabolites of biological systems. The region of the spectrum from about 400 to 300 cm^{-1} is called the far-IR, while a range from 400 to 4000 cm^{-1} is known as the mid-IR region. Within the mid-IR, regions can be differentiated with distinctions established especially for the study of small organic, inorganic, and foreign

species: (a) the functional group (F/G) region, (b) the condensed phase inorganic region, (c) the near-IR, and (d) the fingerprint (FP) region. Generally, the F/G region is especially regarded in qualitative analyses because the established regions are unique for each inorganic element **(**Shriner et al 2004). Infrared absorptions usually occur at low wave numbers without previous confirmative evidence of spectra (regions usually thinner or improved with stronger peaks), and the measurement and data treatment can be fully performed with marginal errors, even with small fiber or single reflection ATR measuring cells and detectors of limited wavelength range. To understand the interaction of IR radiation and matter, studying the mechanism by which an absorption process occurs, especially due to bands associated with (F/G), is also crucial in the realization of qualitative methods and in the choice of the related mathematical analysis applied to such bands (Johnson et al., 2023).

1.4.1 Basic Principles and Instrumentation

Spectroscopy generally studies the interaction between electromagnetic radiation and matter, which results in the absorption of radiation energy by matter. (IR) spectroscopy, also known as vibrational spectroscopy, is based on the principle that molecules absorb IR radiation, producing vibrational transitions between the energy levels of the molecules. The frequencies of the transitions are determined not only by the types of atoms and how they are connected but also by the molecular environment, which leads to characteristic spectral features in the IR spectrum. The IR spectroscopy technique is widely used in qualitative and quantitative analysis in various analytical fields **(**Shriner et al., 2004).IR spectrometry can be divided into different types based on how the interference pattern is measured. The two most commonly used instruments are dispersive (IR) spectrophotometers, which measure the entire emission of the IR light source and isolate discrete bands using a wavelength filter. Diode arrays or Fourier-transform infrared (FTIR) spectrophotometers establish the spectral region of interest using monochromatic light for wavelength scanning to acquire a full IR spectrum. A principle of diode arrays (or arrays of detectors for FTIR) is sufficiently durable to be utilized for drug substance analysis, while more advanced designs are required for the measurement of dosage forms. The basic FTIR instrument consists of an IR light source, a Michelson interferometer with a moving mirror, a fixed mirror, a beam splitter, a sample-holding compartment, and an IR detector, with associated electronics to transduce the detector signal into a standard, observable output. The absorbance spectrum of the sample is produced after transformation of the interferogram data using software for processing. For reliable methods development and instrument validation, a qualification tool and software are necessary to confirm that the instrument hardware is intact and connected appropriately. Standard test parameters are available for an FTIR qualification test. The most common sample presentation form for IR analysis is the KBr pellet, an intimate mixture of solid sample and KBr, pressed under high pressure, or the sample can be diluted with a reflective agent, producing a matrix. Cleaning and possibly drying inactive, powdered KBr for use in the pellets using a vacuum desiccator and filtering through glass wool are necessary actions for the reasonable operation of the IR instrument (Fahelelbom, et al., 2022).

1.4.2 Quantitative Analysis using IR Spectroscop y

In the course of its development over the decades, much attention has also been devoted to the aspects of the intended use of (IR) spectroscopy. The molecular vibrations are related to the

structure, function, and reactivity of compounds, and information about these can be obtained from the 2D frequency map of the absorptive IR spectra. The position, intensity, and polarity of the IR bands are closely related to the atomic composition, bond, and molecular shape of the material. Most importantly, it is the use of IR spectroscopy for quantitative analysis. IR can measure the concentration of an analyte in a mixture. The determination of the concentration of the constituents is a very important part of analytical chemistry. Since the objective of an analytical chemist is to determine 'how much' of the analyte is present. These days, IR spectroscopy is used for quantification in many areas. The analytical technique of IR- based quantification uses various calibration methods to develop an accurate, linear, and precise relationship between the analyte concentration and the IR measurement (Sun, 2009).

Qualitative analysis involves the process of determining 'what' is in a sample when we have some knowledge of what analytes might be present but not how much. However, in the case that the other analytes are already known and the method is not new, this can be classified as semi-quantitative or multicomponent analysis rather than traditional qualitative analysis. To implement the quantitative analysis, an IR method must follow a systematic approach to method validation in relation to complete procedures from sampling, sample preparation, and analysis to the final reporting, especially in the amount of polyherbal and polypharmacy in a preparation. For a complex analysis, such as the one for in vitro chromatographic dissolution profile in a biowaiver test, the full subject of qualitative analysis, such as an alternative to the performance of UV-Visible spectroscopy, could be infrared spectroscopy (Eserian and Lombardo, 2015).

1.4.2.1 Calibration Methods

Quantitative analysis using (IR) spectroscopy is thoroughly descriptive and highly overlapping for structural discrimination. With ever-decreasing labor costs, it seems that quantification is less demanded, especially for complicated mixtures. On the other hand, the field of process analysis provides a very promising market for rapid, simultaneous, real-time, and quantification screening applications. Calibration is usually used to prepare for quantitative analysis by correlating the absorbance of a tested sample from a given instrumental method

with the analyte concentration of the mixture through a straight-line equation. In the area of infrared spectroscopy, reference spectra with concentration for individual quantitative analysis purposes are also obtainable, and the commonly used calibration methods are the following (Johnson et al., 2023).

Calibration Curves: Absorbance versus analyte concentration, which is drawn in a straight line equation. Range and standards are the two crucial steps implied in the construction of a suitable calibration curve. Linearity and instrument precision are also other crucial considerations. The most common pitfall in probe and qualitative analysis is the sample matrix effect, poor measurement, and the low resolution of the fitting spectrum. In the area of IR, a newly developing multivariate calibration technique enhances the capability of the method but tends to be more complicated than the method itself. While the software package available commercially improves data acquisition, handling, and interpretation, a suitability study addressed according to official guidelines or other appropriate approaches is suggested for verifying the analytical and documentary methods (Johnson et al., 2023).

1.5 Validation

Validation is an applied approach to verify that a method is suitable to function as a quality control tool. The objective of any analytical measurement is to obtain consistent, reliable and accurate data. Validated analytical methods play a major role in achieving this goal. An analytical method consists of the techniques, method, procedure and protocol. Analytical method validation includes the determination of accuracy, precision, LOD, LOQ, linearity and range. The results from method validation can be used to moderator the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice (Yang et al., 2022). Validation of analytical methods is also required by most regulations and quality standards that impact laboratories. According to FDA (food and drug administration) validation is a procedure for production and process control designed to assure that the drug products have their identity, strength, quality and purity. According to FDA guidelines in may 1987, the validation package must provide the necessary information and test procedures required to prove that the system and the process meet the specified requirements. The importance of validation is 1. Assurance of quality time bound process optimization reduction of quality cost. 2.Nominal mix-ups, and bottle necks minimal batch failures .3. Improved efficiently and productivity reduction in rejections. 4. Increased output Avoidance of capital expenditures .5. Fewer complaints about process related failures. 6. Reduced testing in process and in finished goods.7. More rapid and reliable start-up of new equipment's Easier scale-up form development work .8. Easier maintenance of equipment. 9 . Improved employee awareness of processes ideally validation starts in the very beginning, in the laboratory, scientists discover exactly how the product reacts, as well as the parameters that are required to produce such a product. They learn under what conditions the product fails or become. unstable, unusable and when its quality begins to suffer.

Once the laboratory has established the boundary processing criteria, this information can then be used for establishing requirements for validation. Validation of a system never truly ends. Once a new system and process have been validated the system still requires maintenance, periodic calibrations and adjustment. Therefore, the process is always under scrutiny and constant evaluation (Chavan and Desai, 2022). It is important for to understand the parameters or characteristics involved in the validation process. The various performance parameters, which are grouped as follows: Accuracy Precision, Repeatability, Intermediate precision, Reproducibility Specificity/Selectivity, Limit of Detection (LOD), Limit of Quantitation (LOQ), Linearity Range, Robustness, Ruggedness and System suitability testing (Chavan and Desai, 2022).

1.5.1 Linearity

Linearity is the ability of the method to obtained test results that are directly proportional to the analyte concentration within a given range. A linear relationship should be evaluated across the range of the analytical procedure. It may be established directly on the drug substance by dilution of a standard stock solution. Linearity should be evaluated by visual inspection of a plot a graph of concentration (on x – axis) vs mean response (on Y – axis). Calculate the regression equation, Y- intercept and correlation coefficient. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. For the determination of linearity, a minimum of 5 concentrations is recommended. The method proved that the calibration curve is linear when taking more than one sample with varying concentrations as will be shown (Chavan and Desai, 2022).

1.6 Research Focus

This study is centered on the quantitative analysis of acetic acid traces in alcoholic aqueous solutions during fermentation using IR Spectroscopy. The focus is on evaluating different analytical methods and their effectiveness in addressing the challenges associated with fermentation process monitoring and control.

1.7 Aim and objective

- to quantitatively analyze the acetic acid traces content in fermented products using techniques such as (IR).
- To develop an innovative, user-friendly, and cost-effective quantitative analysis method for determining the composition of fermentation broths using IR Spectroscopy, while maintaining affordability.
- To validate the proposed method across a range of fermentation broth samples to ensure its applicability, robustness, and accuracy.

The objective is to use accurately calculate the acetic acid traces content in fermented products. This curve will establish a mathematical relationship between known concentrations of acetic acid and the readings obtained from the analysis, facilitating precise calculations of the content in various samples. findings of the study, offer conclusions based on the results, and provide recommendations for future research and practical applications in the field of fermentation science.

Chapter two:

Literature Rreview

1. Literature review

2.1 Chromatographic Techniques for Acetic Acid/Ethanol Quantification

2.1.1 High-Performance Liquid Chromatography (HPLC)

HPLC involves the use of a liquid mobile phase to transport the sample through a column packed with a stationary phase. The separation is based on the interactions between the analyses and the stationary phase. Detection is typically performed using a UV detector or a refractive index detector (Skoog et al.,1998). It includes a wide range of chromatographic principles and property modes, alongside the possibility for hyphenation with different methods. This technique is likely the best solution for the quantification of acetic acid traces. The advantages of applying HPLC lie in the effectiveness of acetic acid analysis in the presence of complex mixtures, especially in fermentation samples, as well as in precision and accuracy (Sanarico et al., 2003).The analyte is separated from different compounds, so there should be no effect on the co-elution of peaks. Preparation of the sample, evaluation of particular properties, and separation conditions that influence the above conclusions are, therefore, significant. The most popular type of chromatography is reversed phase mode Reversed Phase (RP). Many changes of HPLC, and Ultra High-Performance Liquid Chromatography (UHPLC) versions, can be practiced in the analytical assessment of acetic acid. Concerning the determination of acetic acid, UHPLC methods are mainly conducted for the study of wines with reduced concentration. Standard valid values and unusual problems in quantifying acetic acid using HPLC and UHPLC are outlined. Analysis validation and the most important cause of error in HPLC and UHPLC have been highlighted. Application areas in the alcohol fermentation process have been indicated. HPLC is a necessary technique because it provides accurate and valid data on the qualitative and quantitative content of acetic acid (Castellari et al., 2000). HPLC has been extensively used to monitor acetic acid levels in wine, beer, and other fermented beverages, providing accurate and reliable results (Skoog et al., 1998). HPLC is used to separate and analyze quantitatively components, that may occur in fresh or fermented vegetables and fruits as glucose, fructose, glycerol, ethanol, 1-propanol, malic acid, succinic acid, lactic acid, acetic acid, propionic acid, and butyric acid (McFeeters, 1993).

Many difficulties have been reported, such as interference sources, sampling strategies, calibration curve strategies, and sample preparation for the final analysis. In the selection of an appropriate and practical analytical method, trade-offs among minimizing uncertainty measurement, time consumption, and lower detection limits must be considered. Interfering agents in analysis have been frequently reported as one of the main causes to be considered (Castellari et al., 2000).

2.1.2 Gas Chromatography

This method is used to analyse volatile compounds (Skoog et al 1998). GC fitted with a flame ionization detector was able to analyze quantitatively the concentrations of ethanol and volatile organic acids, up to six carbon atoms, including acetic acid in the fermentation liquids (Playne, 1985 and Adorno et al, 2014). GC coupled to mass spectrometry (GC-MS) was used as an accurate method for quantification of major volatile metabolites found in different food and beverages and fermented solutions, including ethanol, acetic acid and other aroma compounds (Pinu and Villas-Boas, 2017).

2.2 Spectroscopic Techniques for Acetic Acid/Ethanol Quantification

Techniques like visible and IR can analyse the sample, providing information about the concentrations of the compounds in the mixture. These methods are essential tools in modern analytical chemistry and represent a significant advancement from older methods that depended on manual experiments and visual estimates.

2.2.1 Near-infrared spectroscopy (NIR)

Near-infrared spectroscopy (NIR) ranging from 4000-25000 cm^{-1}, is a nondestructive analytical method,was used for the simultaneous quantitative determination of the concentrations of ethanol and acetic acid in the fermentation of a rice vinegar. Quantitative analysis usually carried out with the aid of statistical and mathematical and software programs (Yano et al, 1997). Quantitative determination of acetic acid and ethanol in human blood serum was determined by NIR in the aid of Partial least-squares (PLS) analysis (Paprocki et al., 2023).

2.2.2 UV-visible spectroscopy

Aqueous solution containing ethanol potentially can be analyzed quantitatively using visible spectrophotometer. Since ethanol and other alcohols make red complex with ammonium cerium nitrate solution, according to the following reaction **(**Shriner et al., 2004).

$$(NH_4)_2Ce(NO_3)_6 + ROH \rightarrow (NH_4)_2Ce(OR)(NO_3)_5 + HNO_3$$

where:
-Ammonium cerium nitrate(yellow)

Alcohol (RoH)reacts to form a red complex(Equ.2)

2.3 Problem Statement

Despite the numerous advantages of fermentation, the industry encounters several challenges that impede its efficiency and effectiveness. These challenges include:

- Complex and Expensive Analytical Methods: Traditional methods for analyzing fermentation broth composition, such as (HPLC) and various spectroscopic techniques, are often complex, costly, and require specialized equipment and technical expertise.
- Time-Consuming Procedures: Many conventional analytical methods involve lengthy sample preparation and analysis, which can be a significant hindrance in a fast-paced production environment.
- Limited Accessibility: Advanced analytical techniques are typically restricted to well-equipped laboratories, which limits their accessibility for smaller-scale or less-resourced operations.
- Difficulties in Real-Time Monitoring: There is a notable challenge in monitoring component concentrations, such as acetic acid, in real-time during fermentation. This lack of real-time data can lead to inconsistencies in product quality.
- Quality Control Issues: Ensuring consistent product quality is challenging when there are difficulties in maintaining precise control over component concentrations during fermentation.
- Slow Development of New Products: The challenges in analyzing fermentation broths and monitoring processes can slow down the development and introduction of new fermen
- tation products.

Chapter three

Methodology

3. Methodology

3.1 Chemicals:

- Glacial acetic acid (Sigma Aldrich, ≥99% purity).
- Vinegar samples from AL-Zahraa company.
- Ethanol (analytical grade) (Sigma Aldrich, ≥99% purity).
- Distilled water

3.2 Instruments

- Analytical balance (Hitachi).

Figure 1: Analytical Balance (Hitachi)

- Bruker TENSOR II Infrared Spectrophotometer equipped with an ATR (Attenuated Total Reflectance).

Figure 2: Bruker Tensor II device.

3.3 Experimental part:

3.3.1 Samples Preparation

Different samples of a ternary system consisting of ethanol, acetic acid, and water were prepared as weight percentages using an analytical balances listed table1:

Table 1: Composition of solutions which are used in this study to to construct the calibration curve.

Solution Number	Contents	% (W/ W) Ethanol	% (W/ W) Glacial acetic acid
1	Water 100%	0%	0%
2	Ethanol pure	100%	0%
3	glacial acetic acid	0%	100%
4	3g ethanol and 7g water	30%	0%
5	2.8g ethanol,7g water and 0.2g **acetic acid**	28%	2%
6	2.6g ethanol,7g water and 0.4g **acetic acid**	26%	4%
7	2.4g ethanol, 7g water and 0. 6g **acetic acid**	24%	6%
8	2.2g ethanol,7g water and 0.8g **acetic acid**	22%	8%
9	2 g ethanol,7g water and 1g **acetic acid**	20%	10%
10	1.8g ethanol,7g water and 1.2g **acetic acid**	18%	12%
11	1.6g ethanol,7g water and 1.4g **acetic acid**	16%	14%
12	1.4g ethanol,7g water and 1.6g **acetic**	14%	16%

	acid		
13	1.2g ethanol,7g water and 1.8g **acetic acid**	12%	18%
14	1gethanol,7gwater and 2g **acetic acid**	10%	20%
15	0.8gethanol,7gwater and 2.2g **acetic acid**	8%	22%
16	0.6gethanol,7gwater and 2.4g **acetic acid**	6%	24%
17	0.4gethanol,7gwater and 2.6g **acetic acid**	4%	26%
18	0.2gethanol,7gwater and 2.8g **acetic acid**	2%	28%
19	0.0g ethanol ,7g water and 3g **acetic acid**	0%	30%

Different samples of system consisting of acetic acid and water were prepared as weight percentages using an analytical balances listed in table(2):

Table 2: Composition of solutions which are used in this study to construct the calibration curve.

Acetic Acid (Mass %)	Mass of Acetic Acid (gm)	Mass of Water (gm)
0	0	100
0.5	0.5	99.5
1	1	99
2	2	98
3	3	97
4	4	96
5	5	95
6	6	94
7	7	93
8	8	92
9	9	91
10	10	90
15	15	85
20	20	80
30	30	70

Different samples of system consisting of ethanol and water were prepared as weight percentages using an analytical balances listed in this table 3:

Table 3: Composition of soluions which are used in this study to construct the calibration curve.

Ethanol (Mass %)	Mass of Ethanol (gm)	Water Mass (gm)
0	0	100
0.5	0.5	99.5
1	1	99
2	2	98
3	3	97
4	4	96
5	5	95
6	6	94
7	7	93
8	8	92
9	9	91
10	10	90
15	15	85
20	20	80
30	30	70

IR Spectroscopy Measurement:

the ATR (Attenuated Total Reflectance) crystal was cleaned thoroughly using a solvent like ethanol to remove any residues or contaminants. Allow it to dry completely before placing each sample. Background correction was carried out at the beginning of each measurement. Each sample solution was scanned 120 times in the range 400–4000 cm^{-1}. The scanning results were stored as data points and then transferred to Excel sheet. The obtained spectrum was compared with reference libraries or the peaks were interpreted manually to identify functional groups and determine the composition of the sample. Peaks corresponding to ethanol, water, and acetic acid appeared at characteristic wavelengths, allowing for identification and quantification. Data points on each Excel sheet was analyzed to establish the mathematical equations for determining the concentration of acetic acid and ethanol in each aqueous sample. A ratio was found between the absorption of acetic acid and ethanol and was determined through more than

one calibration curve.

3.3.3 Validation

3.3.3.1 Preparation of Laboratory Sample:

A sample containing 4.00% acetic acid and 5.1% ethanol was prepared to simulate fermentation conditions. This sample was measured 12 times using the Bruker TENSOR II (IR) Spectrophotometer to evaluate the repeatability of the method.

3.3.3.2 Analysis of Market Samples:

Samples of white vinegar collected from the market were measured 13 times using the Bruker TENSOR II (IR) Spectrophotometer to ensure consistency and assess the method's applicability to real-world products.

3.3.3.3 Acid-Base Titration:

The acetic acid concentration in the vinegar samples from the market was also determined using the traditional acid-base titration method. The results were compared to those obtained from the FTIR measurements to validate the accuracy of the FTIR method.

3.4 FTIR Spectral Measurements:

All samples were analyzed using FTIR spectroscopy in the range of 400–4000 cm^{-1}.

3.4.1 Key wavenumbers used were:

3.4.1.1 1703 cm^{-1} for the carbonyl group (C=O) of acetic acid.
3.4.1.2 1087 cm^{-1} for the C-O bond of ethanol.

Chapter four

Result and Discussion

4.1 Result and discussion

Regarding the practical procedure to obtain the results, we used the Bruker Tensor II spectrometer, an advanced Fourier Transform Infrared (FTIR) device known for its high sensitivity and resolution. Figures 3and 4 shows the IR spectrum for acetic acid and ethanol, respectively from 50-4000 cm^{-1}. Figure 5 shows IR spectrum of a real sample of acetic acid (15% W/W) and ethanol (15% W/W) in water. All solutions were measured using the Tensor II Bruker IR spectrophotometer with the ATR accessory. The data was collected as data points, and the significant wavelengths were recorded according to Tables 4 and 5.

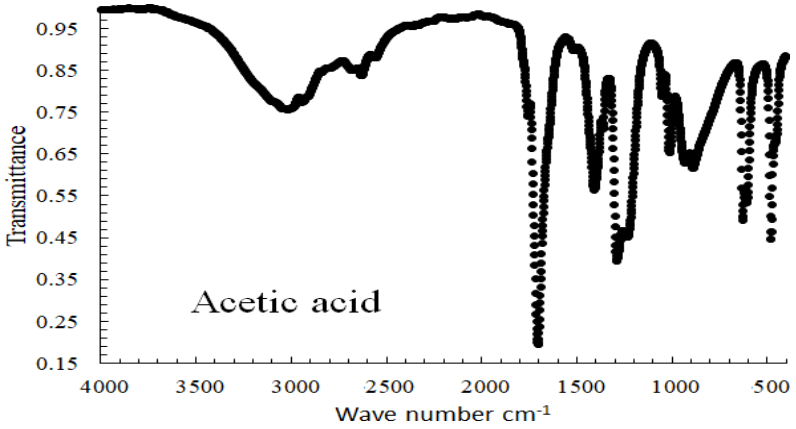


Figure 3: Spectrum of acetic acid.

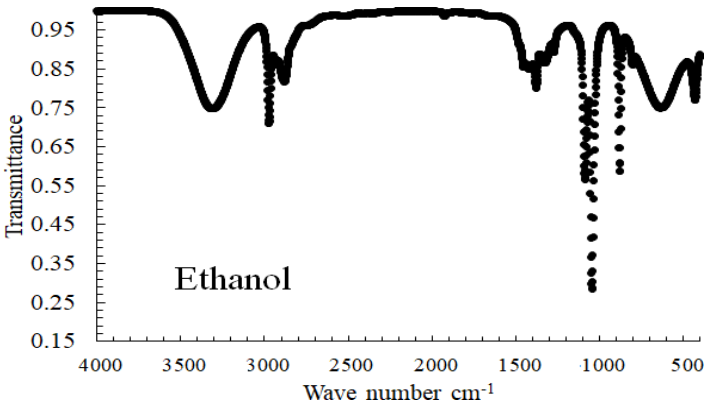


Figure 4: Spectrum of ethanol.

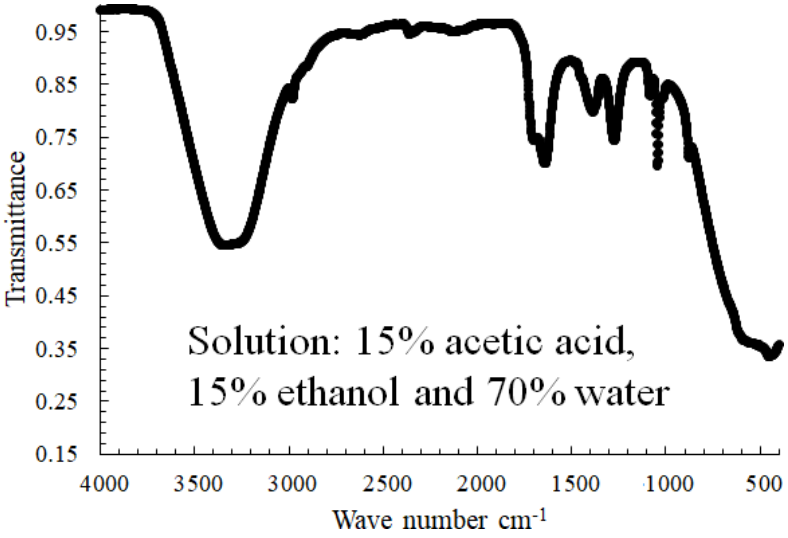


Figure 5: Spectrum of real sample.

Table 4: Absorbance of fermented solutions was measured at the most important peaks of acetic acid.

Acetic Acid(mass%)	2629.5 cm^{-1}	2553.8 cm^{-1}	1704.4 cm^{-1}	1407.5 cm^{-1}	1287.6 cm^{-1}	1233.4 cm^{-1}	626.7 cm^{-1}
0	0.01601	0.01442	0.09784	0.13323	0.10746	0.09309	0.60487
2	0.89365	0.98078	0.87753	0.85909	0.87172	0.89882	0.39496
4	0.11033	0.02299	0.14165	0.14813	0.14506	0.10793	0.60276
6	0.11159	0.0268	0.16284	0.15459	0.16243	0.11556	0.60348
8	0.0382	0.03073	0.18191	0.16145	0.17838	0.12339	0.60223
10	0.04377	0.03462	0.20266	0.1684	0.19514	0.13183	0.60207
12	0.4858	0.3851	0.22069	0.17437	0.20954	0.13972	0.60274
14	0.05343	0.04217	0.23895	0.18091	0.22407	0.14801	0.6024
16	0.05851	0.04557	0.25682	0.18747	0.23818	0.15677	0.60226
18	0.06424	0.05012	0.27709	0.19509	0.25404	0.16733	0.60173
20	0.06832	0.05327	0.29157	0.20004	0.2655	0.17532	0.60299
22	0.07537	0.05845	0.31483	0.20971	0.28346	0.18913	0.60189
24	0.07894	0.06106	0.3262	0.21419	0.2925	0.19644	0.60241
26	0.08432	0.0651	0.34337	0.22139	0.30539	0.20748	0.60219
28	0.08765	0.06762	0.35331	0.22556	0.31332	0.21464	0.60222
30	0.09225	0.07107	0.36686	0.23127	0.32342	0.22353	0.60401

Table 5: Absorbance of fermented solutions was measured at the most important peaks of ethanol.

Solution #	Ethanol (mass%)	Absorbance at 3317.5cm^{-1}	Absorbance at 2972.1cm^{-1}	Absorbance at 1046.4cm^{-1}	Absorbance at 879.3cm^{-1}	Absorbance at 803.7cm^{-1}
1	30	0.47353	0.16024	0.39131	0.30666	0.36613
2	28	0.47042	0.15858	0.37516	0.30195	0.36506
3	26	0.46372	0.15975	0.37083	0.3012	0.3624
4	24	0.46219	0.15862	0.35593	0.29702	0.36198
5	22	0.4567	0.15895	0.34665	0.29425	0.36068
6	20	0.45236	0.15911	0.33437	0.35915	0.29133
7	18	0.45171	0.15874	0.32075	0.28791	0.35884
8	16	0.44809	0.15877	0.30668	0.28468	0.35796
9	14	0.44484	0.15951	0.29565	0.28232	0.35714
10	12	0.44094	0.16014	0.27834	0.27848	0.35633
11	10	0.43956	0.15978	0.25878	0.27398	0.35595
12	8	0.43348	0.16542	0.23939	0.27082	0.35442
13	6	0.43165	0.16102	0.22422	0.26713	0.35428
14	4	0.42735	0.16248	0.20573	0.26488	0.35357
15	2	0.42457	0.16296	0.18997	0.26162	0.35293
16	0	0.42527	0.16301	0.15885	0.25642	0.35312

Tables 4 and 5 show the absorbance of acetic acid (A.A) and ethanol in different solutions with different concentrations at specific wavenumbers. The concentration of acetic acid range from 0% to 30% by mass, where the absorption was measured at specific wavenumbers such as (2523.5 cm^{-1}, 1704.5 cm^{-1}, 1407.5 cm^{-1}, 1233. 4 cm^{-1} and (626.7 cm^{-1}). The absorbance at specific wavenumbers for ethanol as (3317.5 cm^{-1}, 2972.1 cm^{-1}, 1046.3 cm^{-1}, 879.3 cm^{-1}, 803.7 cm^{-1}) is also presented in Table 5. The absorption increases as expected with increasing acid concentration, indicating a direct relationship between the concentration of acetic acid and its absorption behavior at different wavenumbers. Additionally, ethanol's absorption pattern changes in parallel with acetic acid concentration, which may suggest an overlap in the absorption between the two substances at the studied wavenumbers. These results are part of our analytical study using (IR Spectroscopy) to measure acetic acid concentrations in aqueous solutions containing ethanol and water aiming to provide an accurate analysis of the interaction between the acid and alcohol at multiple wavelengths.Through the experiment and study, focused on three specific wavenumbers for acetic acid: (1704 cm^{-1}, 1233 cm^{-1}), and (1287 cm^{-1}), as well as two wavenumbers for ethanol: (3317 $cm^{-1,}$ and 2972 cm^{-1}), a relationship was established based on the ratio of acetic acid absorption to ethanol absorption at these wavenumbers as defined in the relevant definitions and illustrated in Table 6.

Table 6: Ratios of IR absorbance at certain wavenumber maximum peak of acetic acid to maximum absorption peak of ethanol. These ratios are determined from table 4 and 5.

Acetic Acid (Mass%)	1704cm^{-1}/3317cm^{-1}	1704cm^{-1}/2972cm^{-1}	1287cm^{-1}/3317cm^{-1}	(1287cm^{-1}/2972cm^{-1}	(1233cm^{-1}/3317cm^{-1}	(1233cm^{-1}/2972cm^{-1}
0.05	0.220627	0.679459	0.247668	0.762734	0.16043	0.486264
0	0.206618	0.610584	0.226934	0.670619	0.19659	0.580941
4	0.305465	0.886698	0.312818	0.908044	0.23275	0.675618
6	0.352323	1.026604	0.351436	1.02402	0.25003	0.728534
8	0.398314	1.144448	0.390585	1.12224	0.27018	0.776282
10	0.448006	1.27371	0.431382	1.226447	0.29143	0.828546
12	0.488566	1.390261	0.463882	1.32002	0.30931	0.880181
14	0.533263	1.505007	0.500056	1.411287	0.33031	0.932229
16	0.577331	1.610056	0.535428	1.493198	0.35242	0.982822
18	0.628407	1.730298	0.576133	1.586362	0.37949	1.044898

20	0.663322	1.824822	0.604013	1.66166	0.39885	1.097259
22	0.726285	1.903216	0.653917	1.713578	0.43631	1.143332
24	0.755705	2.025835	0.677632	1.816545	0.45509	1.219973
26	0.803487	2.113306	0.714613	1.879554	0.4855	1.276957
28	0.83216	2.168078	0.73797	1.92268	0.50555	1.317133
30	0.862652	2.250537	0.760505	1.98405	0.52562	1.371266

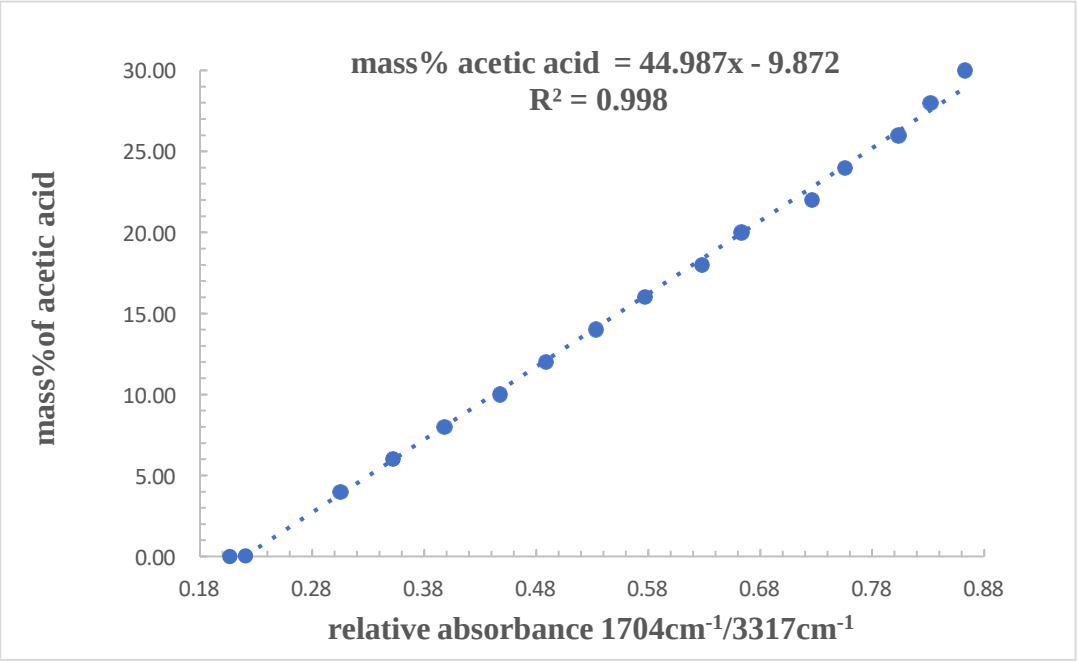


Figure 6: calibration curve of mass % of acetic acid versus absorbance ratio at 1704 $^{-1}$ absorbance at 3317 cm $^{-1}$

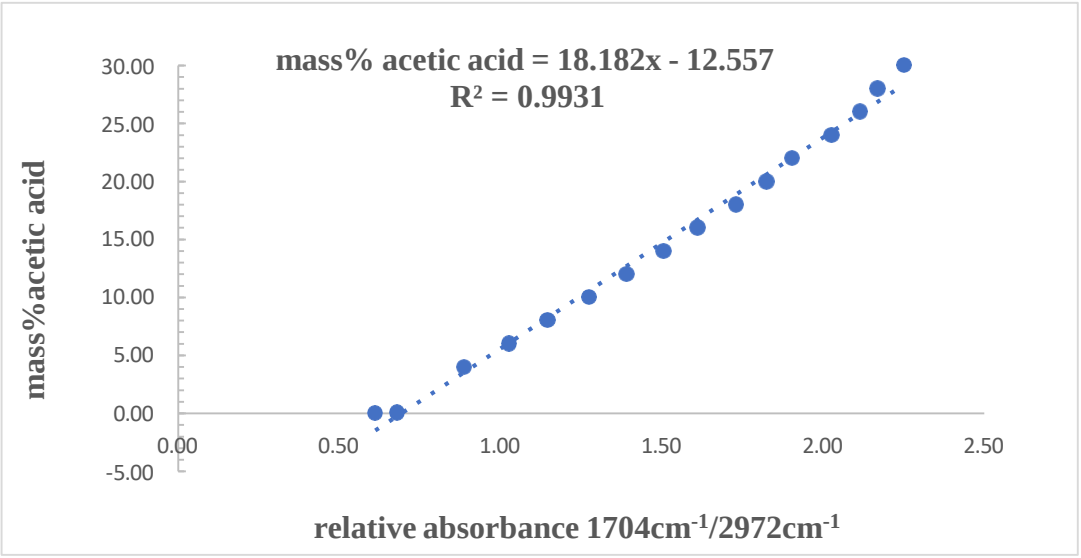


Figure 7: calibration curve of mass % of acetic acid versus absorbance ratio at 1704 $^{-1}$ absorbance at 2972 cm $^{-1}$

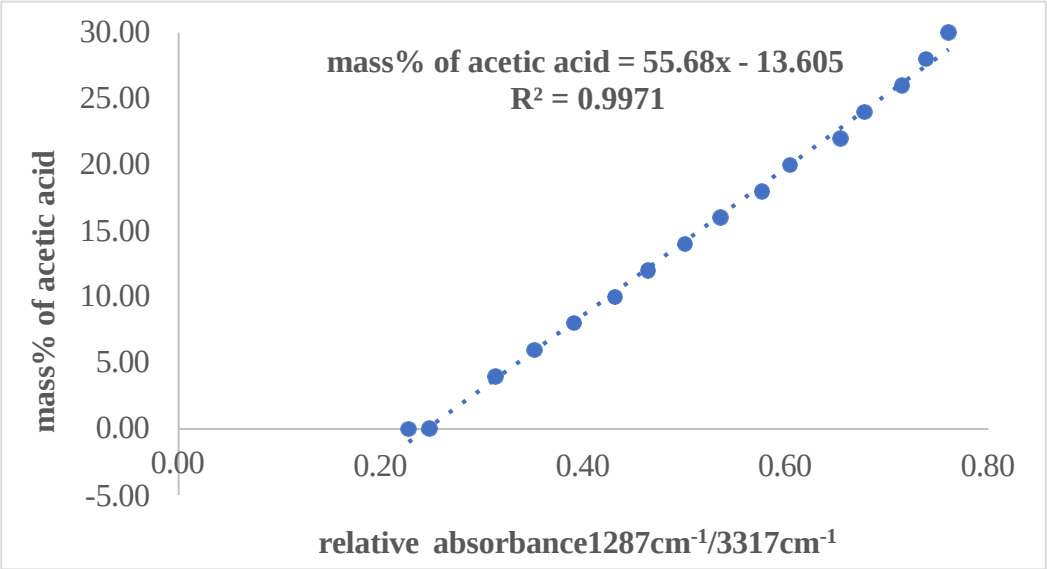


Figure 8: calibration curve of mass % of acetic acid versus absorbance ratio at 1287 cm $^{-1}$ absorbance at 3317 cm^{-1}

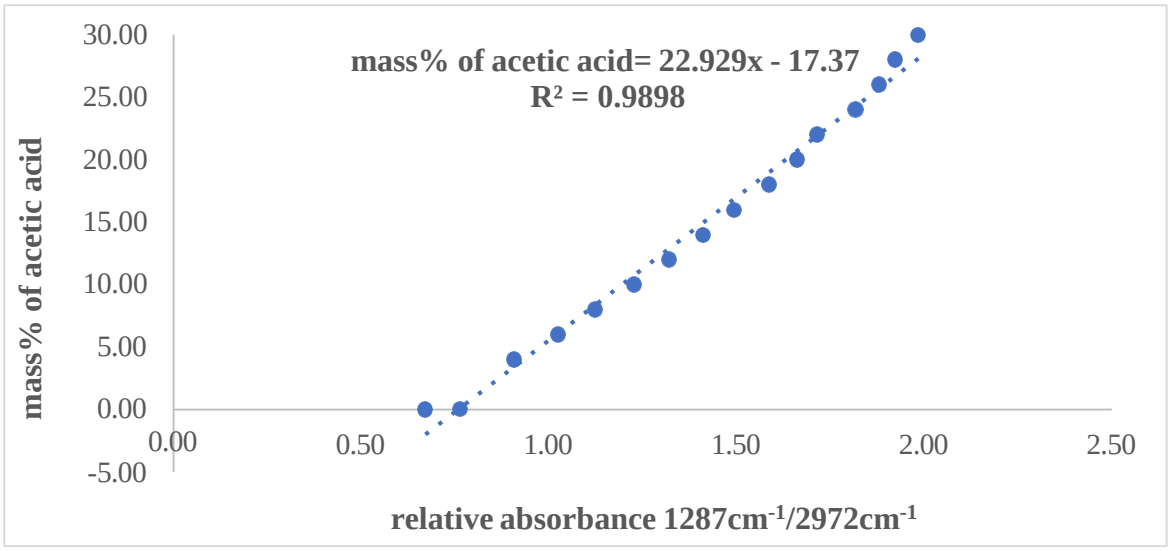


Figure 9: calibration curve of mass % of acetic acid versus absorbance ratio at 1287 cm $^{-1}$, absorbance at 2972 cm $^{-1}$

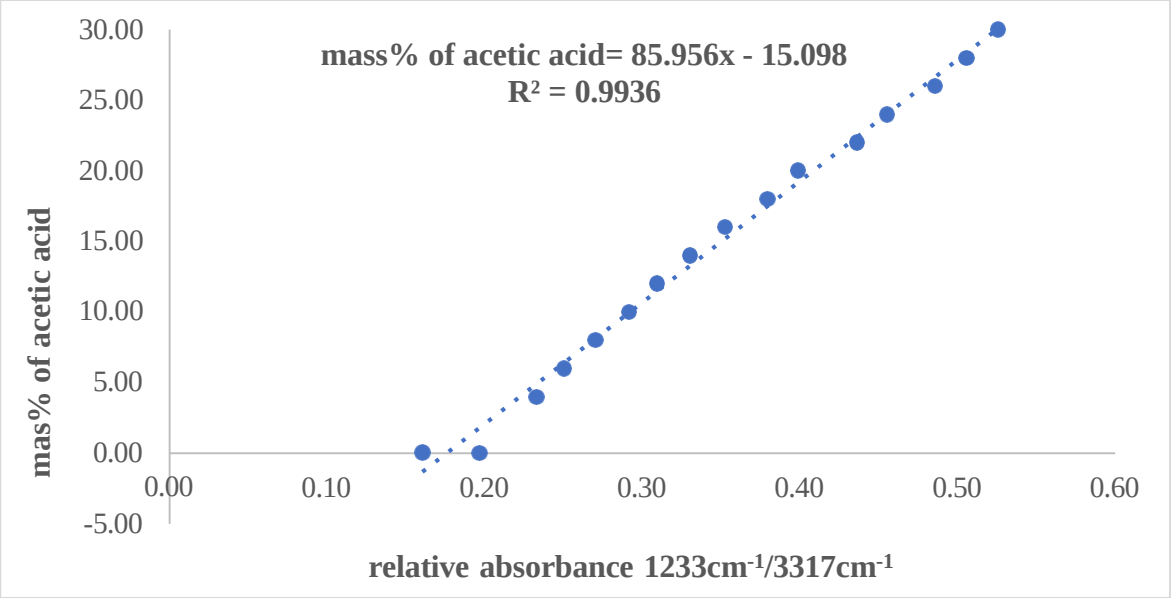


Figure 10: celibration curve of mass % of acetic acid versus absorbance ratio at 1233 cm $^{-1}$ absorbance at 3317 cm $^{-1}$

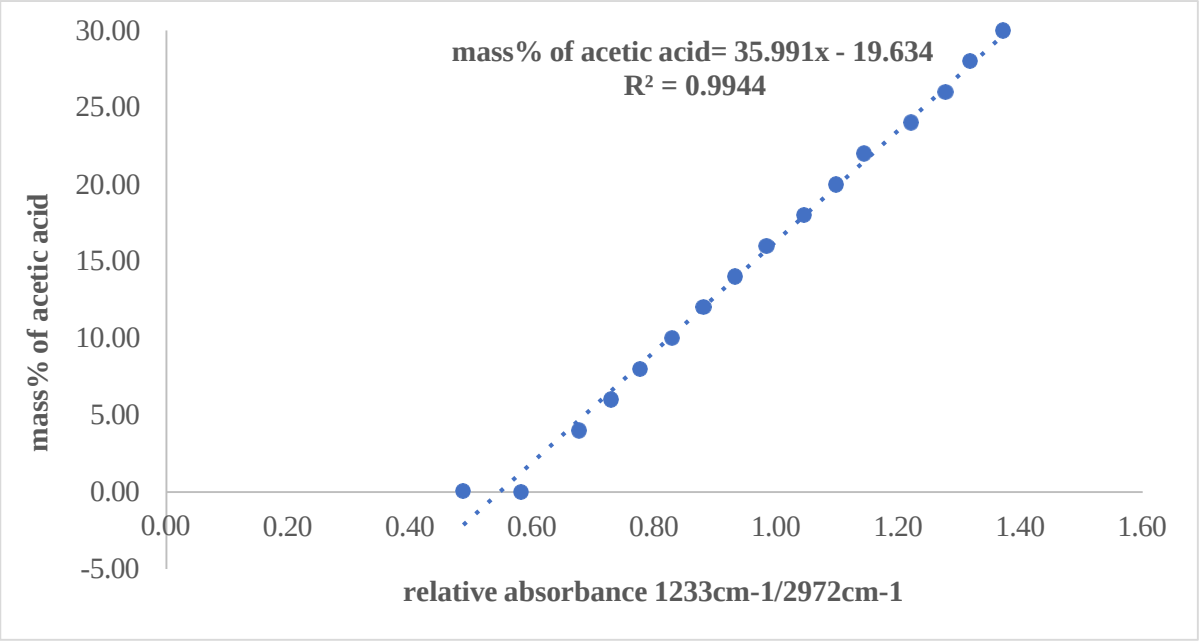


Figure 11: calibration curve of mass % of acetic acid versus absorbance ratio at 1233 cm $^{-1}$ absorbance at 2972 cm $^{-1}$

Mass percent of acetic acid in ternary system consisting of ethanol, acetic acid, and water was plotted versus the ratios of IR absorption at one certain maximum peak of acetic acid relative ethanol, six straight line correlations are obtained (Figures 6A-F). From Fig 6 a, when plotting mass % of acetic acid versus absorbance ratio at 1704cm^{-1}/absorbance at 3317 cm^{-1}, the following straight-line correlation is obtained in equation 3.

mass% of acetic acid = 44.987x - 9.8718 $R^2 = 0.998$, ***Equ 3***

From Fig 6b, when plotting mass% of acetic acid versus absorbance ratio at 1704cm^{-1}/absorbance at 2972 cm^{-1}. The following straight-line correlation is obtained in equation 4.

mass% of acetic acid = 18.182x - 12.557 $R^2 = 0.9931$, ***Equ 4***

From Fig 6c, when plotting mass % of acetic acid versus absorbance ratio at 1287cm^{-1}/absorbance at 3317 cm^{-1}. The following straight-line correlation is obtained in equation 5.

mass% of acetic acid = 55.68x - 13.605 $R^2 = 0.9971$, ***Equ 5***

From Fig 6d, when plotting mass % of acetic acid versus absorbance ratio at 1287cm^{-1}/absorbance at 2972 cm^{-1}. The following straight-line correlation is obtained in equation 6.

mass% of acetic acid = 22.929x - 17.37 $R^2 = 0.9898$, ***Equ 6***

From Fig 6e, when plotting mass % of acetic acid versus absorbance ratio at 1233cm^{-1}/absorbance at 3317 cm^{-1}. The following straight-line correlation is obtained in equation 7.

mass% of acetic acid = 87.746x - 15.83 $R^2 = 0.9944$, ***Equ 7***

From Fig 6F, when plotting mass % of acetic acid versus absorbance ratio at 1233cm^{-1}/absorbance at 2972 cm^{-1}. The following straight-line correlation is obtained in equation 8.

mass% of acetic acid = 37.29x - 21.065 $R^2 = 0.9988$, ***Equ 8***

Based on the calibration curves obtained and the R-squared (R^2) value, it was determined that the first calibration curve is the most suitable. The (R^2) value indicates how well the data points fit the calibration curve. It measures the proportion of the variance in the dependent variable (e.g., absorbance or signal) that can be explained by the independent variable (e.g.,

concentration) in the calibration model. An R^2 value close to 1 suggests a strong linear relationship and a good fit between the measured data and the calibration curve. This means the calibration curve can accurately predict unknown concentrations. Therefore, the calibration curve with the highest R^2 value is considered the best because it provides the most reliable and precise results for quantitative analysis. After applying the previous calibration curves to some vinegar solutions found in the market, it was found that these linear curves obtained were only valid for the same types of solutions prepared in the laboratory, and not for all fermented solutions containing acetic acid and ethanol, even in small proportions. Therefore, other ratios were used to draw calibration curves that are more valid, where neither ethanol nor acetic acid depends on each other.

4.2 **Calibration Curve for Measuring Acetic Acid**

Table 7: Absorbance of fermented solutions was measured at at the most important peaks of ethanol and acetic acid .

Acetic acid (mass%)	Absorbance 1703 cm^{-1}	Absorbance a t 1636 cm^{-1}	Absorbance at 1288 cm^{-1}	Absorbance at 1233 cm^{-1}	Absorbance at 1088 cm^{-1}	Absorbance at 1046 cm^{-1}
0	0.12725	0.30932	0.10203	0.10099	0.10145	0.10556
0.5	0.13195	0.30961	0.10691	0.10328	0.10466	0.11056
1	0.13635	0.30921	0.11133	0.10315	0.10152	0.10644
2	0.14599	0.31221	0.12171	0.10707	0.10427	0.1109
3	0.15501	0.31336	0.13199	0.11121	0.10766	0.11617
4	0.16403	0.31442	0.14167	0.11477	0.10907	0.11873
5	0.17278	0.31617	0.15117	0.11782	0.11008	0.12131
6	0.18353	0.31903	0.16028	0.11934	0.10604	0.11515
7	0.19276	0.32014	0.16941	0.1226	0.10676	0.11652
8	0.20184	0.3219	0.17842	0.12611	0.10753	0.11868
9	0.21062	0.32385	0.18641	0.12927	0.10807	0.11968
10	0.22081	0.32517	0.19524	0.1329	0.10877	0.12168
15	0.26448	0.33366	0.23516	0.15192	0.11281	0.13048
20	0.30586	0.34239	0.27068	0.17308	0.11728	0.14015
30	0.37939	0.35261	0.33015	0.22445	0.12457	0.15951

Table 8: Ratios of IR absorbance at certain wave number maximum peak of acetic/ that of pure water. These ratios are determined from table 7

acetic acid (mass %)	Absorption Ratio ($1703cm^{-1}$/that of Pure Water)	Absorption Ratio ($1287.8cm^{-1}$/that of Pure Water)	Absorption Ratio ($1233.3cm^{-1}$/that of Pure Water)
0	1	1	1
0.5	1.036935	1.047829	1.022676
1	1.071513	1.09115	1.021388
2	1.147269	1.192884	1.060204
3	1.218153	1.293639	1.101198
4	1.289037	1.388513	1.136449
5	1.3578	1.481623	1.16665
6	1.442279	1.570911	1.181701
7	1.514813	1.660394	1.213982
8	1.586169	1.748701	1.248737
9	1.655167	1.827012	1.280028
10	1.735246	1.913555	1.315972
15	2.078428	2.304812	1.504307
20	2.403615	2.652945	1.713833
30	2.981454	3.235813	2.222497

When plotting the calibration curve between the concentration of acetic acid and the absorbance at each wavenumber, the result was linear. However, there was an issue with interference. To address this, a ratio was calculated between the absorbance at each wavenumber for acetic acid and the absorbance of pure water alone, rather than taking the ratio at each point for water. Using the absorbance of pure water as a reference proved to be more effective, as determined experimentally. The calibration curve was then plotted, yielding an excellent linear result.

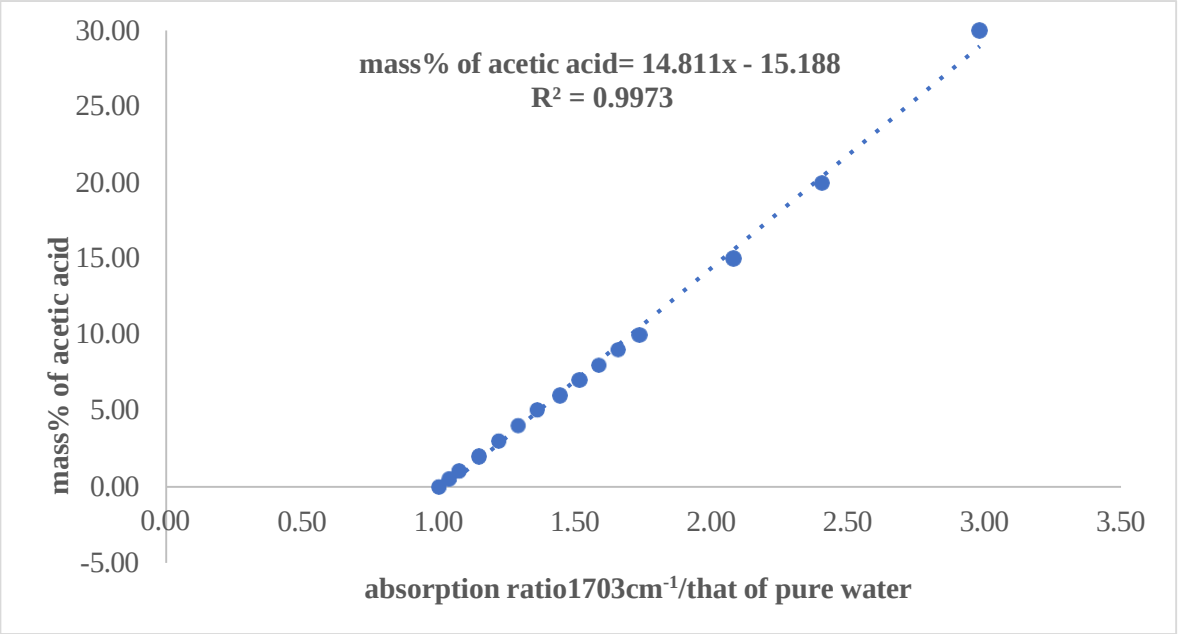


Figure 12: Calibration curve of mass % of acetic acid versus absorbance ratio at 1703 cm $^{-1}$ that of pure water.

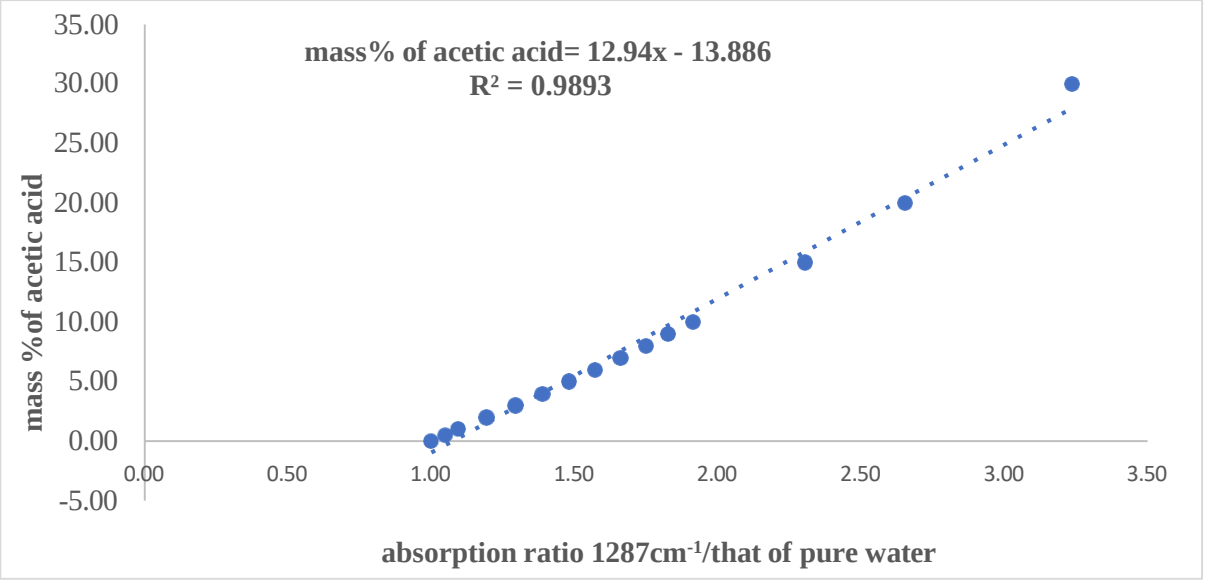


Figure 13: Calibration curve of mass % of acetic acid versus absorbance ratio at 1287 cm $^{-1}$, that of pure water

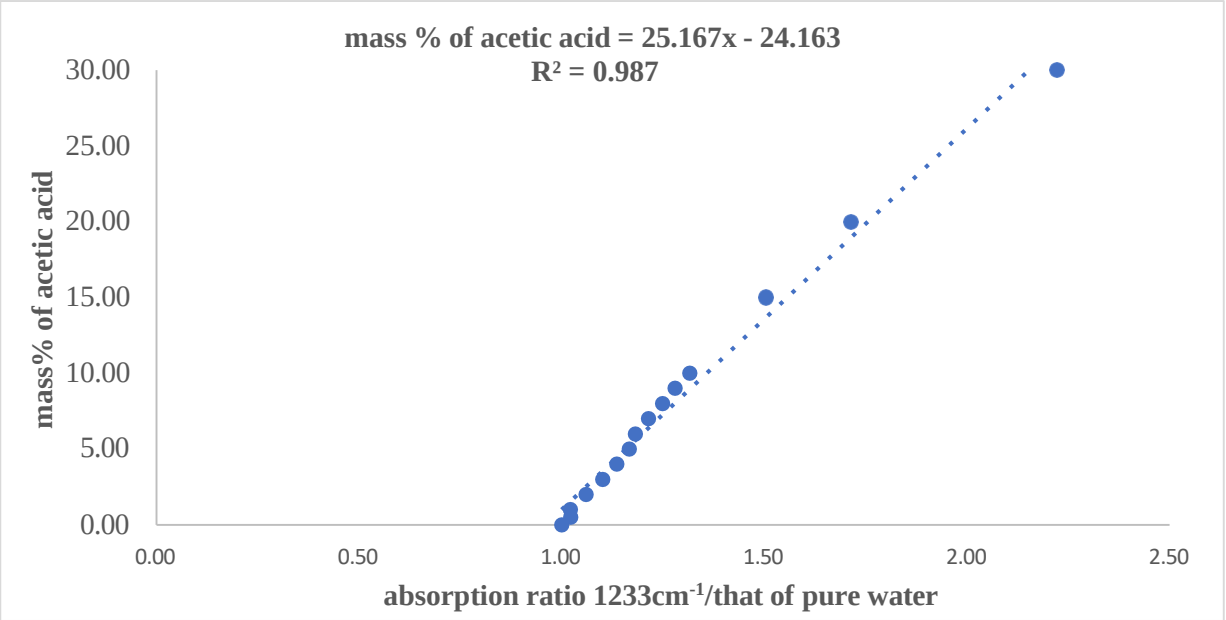


Figure 14: Calibration curve of mass % of acetic acid versus absorbance raio at 1233.3 cm $^{-1}$ that of pure water.

4.3 Calibration Curve for Ethanol Measurements

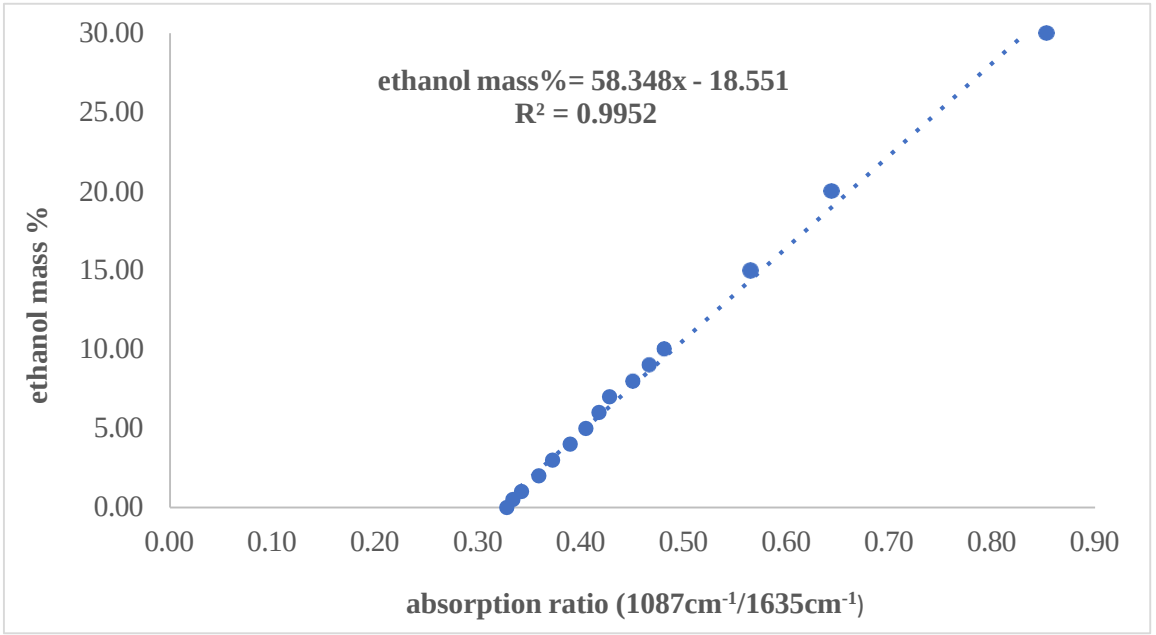
Table 9: Absorbance of fermented solutions was measured at the most important peaks of ethanol and acetic acid.

EtOH(mass %)	Absorbance at 1703 cm^{-1}	Absorbance at 1636 cm^{-1}	Absorbance at 1288 cm^{-1}	Absorbance at 1233 cm^{-1}	Absorbance at 1088 cm^{-1}	Absorbance at 1046 cm^{-1}
0	0.12725	0.30932	0.10203	0.10099	0.10145	0.10556
0.5	0.12646	0.3076	0.10182	0.10055	0.10266	0.11196
1	0.1267	0.30646	0.10222	0.10066	0.10488	0.11847
2	0.12565	0.30336	0.10257	0.10059	0.10889	0.13167
3	0.12428	0.30031	0.10278	0.10041	0.11181	0.14369
4	0.12352	0.2968	0.10304	0.10014	0.11561	0.15545
5	0.12324	0.29461	0.10342	0.09983	0.11928	0.16801
6	0.12671	0.29578	0.10413	0.09997	0.12347	0.18107
7	0.12757	0.29494	0.10418	0.09972	0.12618	0.18957
8	0.12495	0.28994	0.1046	0.09954	0.1307	0.20406
9	0.12523	0.2884	0.10485	0.09945	0.13453	0.21474
10	0.12493	0.28621	0.1052	0.09903	0.13759	0.22483
15	0.12267	0.27474	0.10639	0.09776	0.15517	0.27478
20	0.11968	0.2649	0.10714	0.09644	0.17051	0.31387
30	0.1109	0.24182	0.10785	0.09236	0.20627	0.3937

Table 10: Ratios of IR absorbance at certain wave number maximum peake of ethanol to acetic acid. These ratios are determinted from table 9

Ethanol (Mass%)	Absorption Ratio ($1087.8cm^{-1}/1635.5cm^{-1}$)	Absorption Ratio ($1046.8cm^{-1}/1635.5cm^{-1}$)
0	0.327977	0.341265
0.5	0.333745	0.363979
1	0.342231	0.386576
2	0.358946	0.434039
3	0.372315	0.478472
4	0.389522	0.523753
5	0.404874	0.570279
6	0.417439	0.612178
7	0.427816	0.642741
8	0.450783	0.703801
9	0.46647	0.744591
10	0.480731	0.785542
15	0.564789	1.000146
20	0.643677	1.184862
30	0.85299	1.62807

Figure 15: calibration curve of mass % of ethanol versus absorbance ratio at 1087.8 cm $^{-1}$ absorbance at 1635.5 cm $^{-1}$



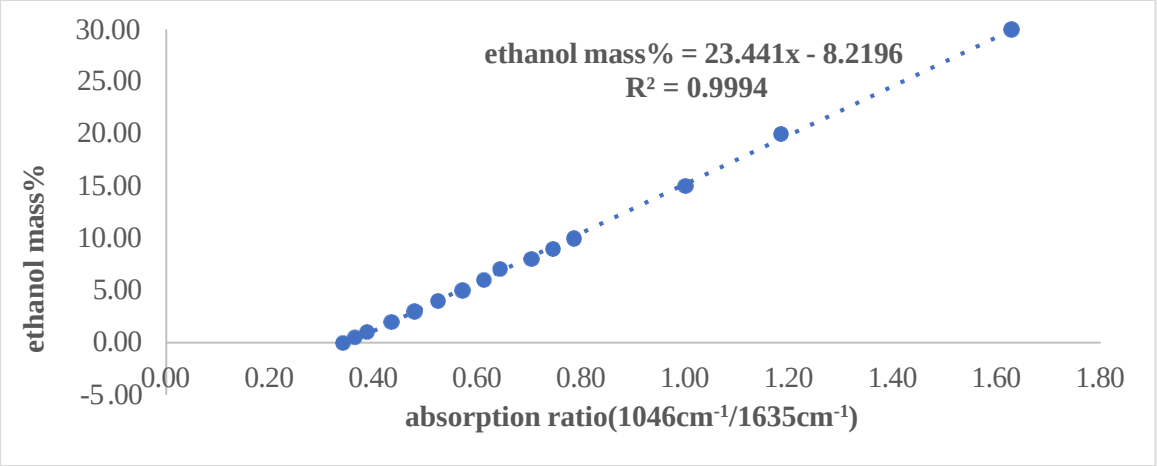


Figure 16: Calibration curve of ethanol versus absorbance ratio at 1046 cm^{-1}, absorbance at 1635.5 cm^{-1}.

4.4 Method Validation:

sample was prepared containing 4.00% acetic acid and 5.1% ethanol, was

	Absorbance 1703 cm^{-1}	Absorbance 1635 cm^{-1}	Absorbance 1287 cm^{-1}	Absorbance 1233 cm^{-1}	Absorbance 1087 cm^{-1}	Absorbance 1046 cm^{-1}
a	0.1644	0.30285	0.14342	0.11248	0.12366	0.1763
b	0.16465	0.30331	0.14369	0.11237	0.12256	0.1744
c	0.16422	0.30314	0.14342	0.11233	0.12345	0.17581
d	0.16432	0.303	0.14336	0.11234	0.12334	0.17545
e	0.16477	0.30378	0.14379	0.11256	0.12297	0.17594
f	0.16427	0.30302	0.14349	0.11243	0.12325	0.17637
g	0.16437	0.30322	0.14347	0.11249	0.12346	0.17691
h	0.16441	0.30282	0.14339	0.11247	0.12374	0.17653
i	0.16427	0.30313	0.14326	0.11232	0.12357	0.17668

j	0.16426	0.3022	0.14305	0.11228	0.12283	0.17624
k	0.16411	0.302	0.14304	0.11231	0.12294	0.17622
l	0.16434	0.30307	0.14331	0.11215	0.12332	0.17658

measured 12 times in table 11

Table 11: Absorbance of fermented solutions was measured at the most important peaks of ethanol and acetic acid of different sample.

Table 12: Ratio of acetic acid % to pure water, acetic acid mass %, avarage, st, std and recovery.

1703cm^{-1}/ that of Pure Water	[Acetic Acid Mass%]	1288cm^{-1}/that of Pure Water	[Acetic Acid Mass%]	1233cm^{-1}/that of Pure Water	[Acetic Acid Mass%]
1.291945	3.946997	1.405665	4.303305	1.113774	3.867341
1.29391	3.976095	1.408311	4.337548	1.112684	3.839929
1.29053	3.926047	1.405665	4.303305	1.112288	3.829961
1.291316	3.937686	1.405077	4.295696	1.112387	3.832453
1.294853	3.990063	1.409291	4.350231	1.114566	3.887277
1.290923	3.931866	1.406351	4.312183	1.113279	3.854881
1.291709	3.943505	1.406155	4.309646	1.113873	3.869833
1.292024	3.948161	1.405371	4.2995	1.113675	3.864849

1.290923	3.931866	1.404097	4.283013	1.112189	3.827469
1.290845	3.930702	1.402039	4.25638	1.111793	3.817501
1.289666	3.913243	1.401941	4.255111	1.11209	3.824977
1.291473	3.940014	1.404587	4.289354	1.110506	3.785104
average	3.94302		4.299606		3.841798
ST	0.021239				
RSD	0.53866				
TRUE	4.00%				

- **Calculations of acetic acid:**

  The average was calculated for each ratio provided in the table (12), and it was found that the optimal ratio, where the difference between the experimentally calculated value and the true ratio is minimal, occurs at the wavenumber 1703cm-1. Therefore, this ratio was selected as the preferred one over the others. This is because the acetic acid ratio at 1703cm-1 is approximately 10 times higher than that of ethanol, while at 1233cm-1, it exceeds by about 80 times.

  Recovery (%) = (Measured Concentration /Known Concentration) ×100. Recovery within 98- 102% is generally acceptable.

  Recovery (%) =98.5755%

Table 13: Ethanol mass %, Average, Standard Deviation, and % Recovery.

Absorption Ratio ($1087.8cm^{-1}/1635.5cm^{-1}$)	[Ethanol Mass %]	Absorption Ratio ($1046.8cm^{-1}/1635cm^{-1}$)	[Ethanol w%]
0.408321	5.273711	0.582136	5.426259
0.404075	5.02597	0.574989	5.258724
0.407238	5.210498	0.579963	5.375314
0.407063	5.200295	0.579043	5.353745
0.4048	5.068243	0.579169	5.356704
0.406739	5.181397	0.582041	5.424018
0.407163	5.206153	0.583438	5.456765
0.408626	5.291486	0.582954	5.445415
0.407647	5.23438	0.582852	5.443039
0.406453	5.164701	0.58319	5.450955
0.407086	5.201659	0.58351	5.458456
0.406903	5.190958	0.582638	5.43801
average	5.187454		5.4072837
ST	0.075418		0.060157
RSD	1.453861		1.112519
TRUE	5.1		

- **Calculations of ethanol**

-

from table 13, absorbance at wavenumber ,1087cm^{-1}and 1046 cm^{-1} was divided by the absorbance at wavenumber 1635cm^{-1}, and the attached results were obtained for ethanol. Absorption ratio,1087.8cm^{-1}/1635.5cm^{-1} were better than Absorption ratio ,1046.8cm^{-1}/1635.5cm^{-1}, because The difference between the average and the true value for ethanol at the first ratio,1087.8cm^{-1}/1635.5cm^{-1} is smaller than that at the second ratio,1046.8cm^{-1}/1635.5cm^{-1} with the difference being 0.08 for the first ratio compared to 0.3 for the second ratio .This is because the interference between acetic acid and ethanol at 1046cm^{-1} is greater than at 1088cm^{-1}, even though the peak at 1046cm^{-1} is more sharp. Therefore, the first ratio was adopted.

Recovery (%) = (Measured Concentration /Known Concentration) ×100. Recovery within 98- 102% is generally acceptable.

Recovery (%) =101.7%

Table 14: Measurements of vinegar from market.

Vinegar sample	Absorbance at 1703 cm^{-1}	Absorbance at1635 cm^{-1}	Absorbance at 1087cm^{-1}	Absorption ratio ($1703cm^{-1}$/that of pure water	Acetic acid mass%	Absorption ratio($1087cm^{-1}$/$1635cm^{-1}$)	Ethanol mass%
A	0.16217	0.31289	0.10654	1.27442	3.687441	0.340503	1.316672
B	0.16254	0.31318	0.10778	1.277328	3.730506	0.344147	1.529297
C	0.16383	0.31467	0.10785	1.287466	3.880653	0.34274	1.447194
D	0.16291	0.3125	0.10802	1.280236	3.773572	0.345664	1.617803
E	0.16276	0.31303	0.10753	1.279057	3.756113	0.343513	1.49232
F	0.16347	0.31301	0.10721	1.284637	3.838752	0.342513	1.43395
G	0.16311	0.3123	0.10762	1.281807	3.79685	0.344605	1.555986
H	0.16124	0.30921	0.10669	1.267112	3.579196	0.345041	1.581428
I	0.16317	0.3111	0.10776	1.282279	3.803834	0.346384	1.659802

J	0.16402	0.31487	0.10675	1.288959	3.902768	0.339029	1.230653
K	0.16337	0.31291	0.10711	1.283851	3.827113	0.342303	1.42169
L	0.16307	0.31262	0.10682	1.281493	3.792195	0.341693	1.386091
M	0.12725	0.31009	0.10008	average	3.780749		1.47274
				ST	0.087292		0.125624
				RSD	2.308864		8.529954

Table 15: Acetic acid measured by acid base titration of vinegar from the market.

Trial Number	Acetic Acid Concentration (%)
1	3.85
2	3.85
3	3.8
4	3.69
5	3.76
Average	3.79

Measurements of Vinegar from Market:

Samples of white vinegar were measured at wavenumber 1703cm^{-1} and 1088cm^{-1}, The ratios for both acetic acid(1703cm^{-1}/that of pure water) and ethanol (1087.8cm^{-1}/1635.5cm^{-1}) were calculated as shown in the table14. The values obtained from the ratios were substituted into the equations derived from Figure 7a and Figure 7d. This allowed the calculation of the mass percentages of both acetic acid and ethanol in the vinegar. Subsequently, the mean, standard deviation, and relative standard deviation were calculated as show in table 15. To validate the method, an acid-base titration was performed, and the process was repeated five times. The average concentration of acetic acid was found to be 3.79 as show in table 15, which is very close to the acetic acid concentration calculated from IR, which was 3.78. This demonstrates that the method is reliable for determining the acetic acid percentage in a fermentation solution.

After reviewing the validation results for linearity, accuracy, and precision, it was concluded that the quantitative analysis method for acetic acid in alcoholic fermentation solutions using IR spectrophotometer is linear, accurate, and reliable. Therefore, the method is deemed valid for its intended purpose.

Chapter five

Conclusion

5. Conclusion:

This study has been successfully developed and validated a novel, user-friendly, and cost- effective method for quantifying acetic acid in fermentation broths using (IR Spectroscopy). This new approach represents a significant advancement over traditional methods, offering improved simplicity and affordability.

5.1 Key Findings:

1. Innovative Method Development: The proposed IR spectroscopy-based method effectively measures acetic acid concentrations in alcoholic aqueous solutions. This innovation simplifies the analytical process while ensuring high accuracy, providing a practical and economical alternative to more complex methods.
2. Successful Validation: The method has been rigorously validated using various fermentation broth samples. It demonstrated robustness and precision, confirming its suitability for widespread application in different fermentation contexts. The results indicate that the method provides reliable measurements of acetic acid concentrations.
3. Comparison with Existing Techniques: The new method was compared to established analytical techniques and was found to be more accessible and cost- effective.
4. Enhanced Process Monitoring: The ability to measure acetic acid concentrations in real-time improves the monitoring and control of fermentation processes. This advancement addresses previous challenges and supports better process management and product quality.
5. Recommendations for Future Use: It is recommended to adopt this method for routine fermentation analysis. Future research could explore additional applications, including integration with automated systems to further streamline analysis and enhance process efficiency.

In conclusion, the development of this IR spectroscopy-based method marks a significant step forward in fermentation analysis. It offers a practical, accurate, and cost-effective

solution for monitoring acetic acid, contributing to improved quality control and process optimization in the fermentation industry.

Chapter six

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6. References

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التحليل الكمي لآثار حمض الخليك في محلول كحولي مائي أثناء عملية التخمر

اعداد : سهى راجح الزعارير

اشراف : د. فؤاد الريماوي و د. محمود الخطيب

الملخص

تقدم هذه الدراسة طريقة موثوقة ومنخفضة التكلفة لقياس آثار حمض الأسيتيك في محلول الماء والكحول أثناء عملية التخمير باستخدام تقنية التحليل الطيفي بالأشعة تحت الحمراء .(FTIR) تم تطوير منحنيات معايرة تربط بين تركي ازت حمض الأسيتيك (من 0% إلى 30% بالوزن) وقمم ا لامتصاص عند الأطوال الموجية المحددة (1703 س-م1، 1233 س-م1، و1287 س-م1). كما تم تحليل الامتصاص للإيثانول عند أطوال موجية رئيسية مثل 1087 س-م1 و1635 س-م1. وأظهرت الدراسة أن أفضل نسبة لقياس تركيز حمض الأسيتيك كانت عند 1703 س-م1 مقارنة بالماء النقي، بينما كانت أفضل نسبة لقياس الإيثانول هي 1087 س-م1 مقسومة على 1635 س-م1.

أظهرت نتائج التحقق:

- قابلية التكرار: تم قياس عينة مخبرية تحتوي على 4% حمض أسيتيك و5.1% إيثانول 12 مرة مع انح ارف طفيف.
- التطبيق الواقعي: تم تحليل عينات خل من السوق 13 مرة بنتائج مماثلة للطريقة التقليدية (المعايرة الحمضية القاعدية).
- تؤكد النتائج أن التحليل الطيفي بالأشعة تحت الحمراء هو أداة دقيقة وفعالة لتحليل حمض الأسيتيك والإيثانول في المنتجات المخمرة. توفر
- لهذه الطريقة المبتكرة مزايا كبيرة لصناعات الأغذية والمشروبات، مما يجعلها بديلًا قوياً للطرق التقليدية