BIOCHEMICAL MARKERS AS A TOOL FOR RAPID DETECTION OF MICROBIAL SPOILAGE IN MINIMALLY PROCESSED FRUITS

By

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Dedicated to my Family

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1	A_f	accuracy factor
2	a_w	water activity
3	ATR	attenuated total reflectance
4	ANN	artificial neural network
5	APS	ammonium per sulphate
6	B_f	bias factor
7	BP	back propagation
8	CFU	colony forming unit
9	e-nose	electronic nose
10	Ea	activation energy
11	FTIR	fourier transform infrared spectroscopy
12	FSSAI	Food safety & standards authority of India
13	GCMS	gas chromatography mass spectrometry
14	HS/SPME	headspace/solid phase micro-extraction
15	LAB	lactic acid bacteria
16	LV	latent variable
17	PC	principal component
18	MLP	multilayer perceptron
19	MPF	minimally processed fruit
20	Na_2CO_3	sodium carbonate
21	PCA	principal component analysis
22	PLS-R	partial least square regression
23	R^2	coefficient of determination
24	SD	standard deviation
25	SEP	standard error of deviation
26	TVC	total viable counts
27	VOC	volatile organic compound
28	Y&M	yeast & mould count
29	μ_{max}	maximum specific growth rate

Chapter 1

Introduction

1.1 Overview

Fruits and vegetables comprise an essential part of the human diet as they are the major source of dietary nutrients of great importance. Consumption of fruits has been found to counteract many of the chronic diseases, including cancers and cardiovascular diseases. Therefore, recommendations for a balanced diet must include the consumption of fresh fruits and vegetables. Consumers have become more concerned about the nutritional and sensory aspects as well as the safety of the food they eat due to growing health awareness. At the same time, consumer demand for convenience products is increasing and so is the demand for minimally processed fruits and vegetables. The minimally processed fruit market has expanded considerably in recent years. However, the quality and safety of such products are an issue of concern as these products can act as vehicles for transmitting infectious diseases. Furthermore, owing to the available nutritive surface, fresh-cut produce is more susceptible to spoilage and can facilitate the rapid growth of spoilage micro-organisms as well as the micro-organisms of public health significance. Monitoring microbial quality is a critical factor in assuring the safety and quality of food products in modern supply chains [1]. Conventional plate count techniques require 48-72 h to evaluate the microbial quality, which is not suitable for fast deteriorating products with a short shelf life. Moreover, this method is cumbersome, time-consuming and destructive which makes rapid detection of microbial quality of food extremely difficult [2]. Therefore, it is of interest to develop rapid methods to detect microbial spoilage of minimally processed fruits.

1.2 Minimally processed fresh produce

International Fresh-Cut Produce Association (IFPA), defines fresh-cut produce as ‘any fresh fruit or vegetable or any combination thereof that has been physically altered from its

original form, but remains in a fresh state'. The necessity for minimal processing of fresh produce has gained its relevance after the catering industry considered purchasing vegetables and fruit that are already peeled and possibly also sliced, grated or shredded for reasons of expense, labour and hygiene. However, the fresh-cut industry was first developed to supply hotels, restaurants, catering services and other institutions. Today, with changing market trends, consumers looking for healthier options seek to replace unhealthy snack foods with fresh-cut fruit and vegetable products. The availability of fresh-cut fruits in vending machines in schools and at workplaces would constitute an excellent strategy to improve the nutritional quality of snacks and convenience food at a time when obesity and nutrition-related illnesses affect large percentages of the population [3]. Besides, minimal processing also offers several advantages such as ease in serving portion of large and difficult to peel fruits, reduce cost in packaging and transportation, extend the shelf life of the product and offer choice of selection for the consumer. The industry has expanded with more recent additions of minimally processed fruits at quick-service restaurants and in retail stores. These trends have led the fresh-cut industry to increase investment in research and development to address issues regarding raw product supply, packaging technology, processing equipment, refrigeration and safety and hygiene aspects about such commodities. Minimally processed tropical fruits available in the market today (Figure 1) include melons, cantaloupe, watermelon, mangoes, mangosteen, rambutan, jackfruit, pummelo, papaya, durian, grapefruit, pineapples and fruit mixes [4].

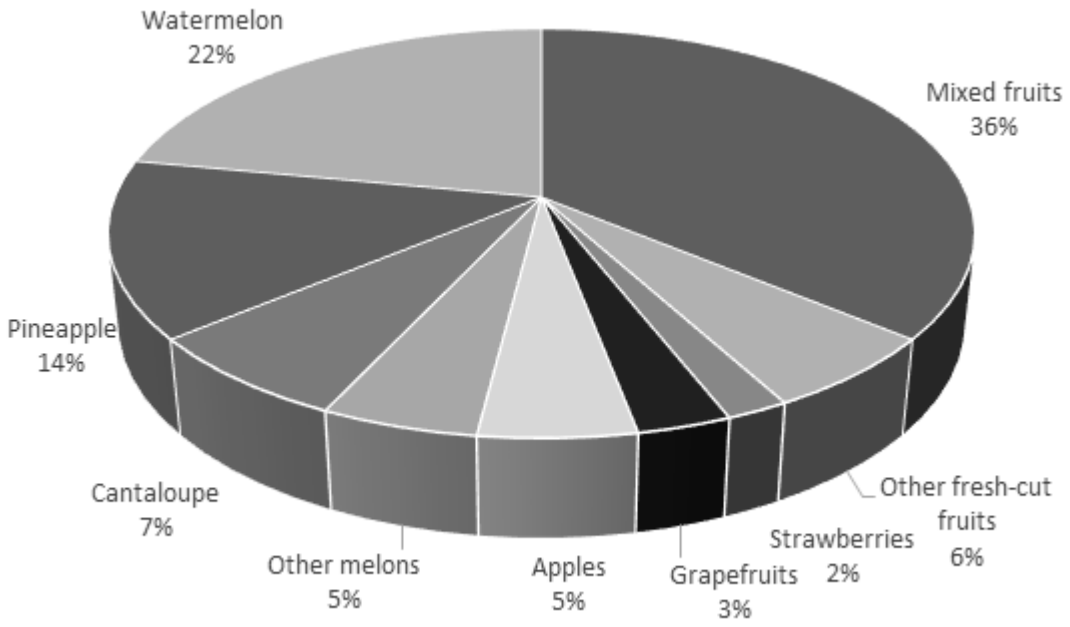


Figure 1: Fresh cut market share (Source Adapted from Perishables Group. 2008. U.S. fresh cut produce trends)

1.3 Quality changes in minimally processed produce

Minimally processed fruits are much more susceptible to deterioration than their corresponding whole fruits due to wounding during preparation [5, 6]. Quality of minimally processed produce can be affected both by internal factors including morphological, physiological and biochemical defense mechanisms, type of fruit, genotype, stress-induced senescence programs and external factors representing environmental situations such as storage temperature, humidity, sharpness of cutting-knife, and chemical treatments [7]. Minimally processed produce deteriorates because of physiological ageing, biochemical changes and microbial spoilage, which may result in degradation of the colour, texture and flavour of the produce [8,9]. During peeling and grating operations, many cells are ruptured and intracellular products such as oxidizing enzymes are liberated which leads to browning or lipid oxidation. Ethylene production also increases during processing that leads to tissue softening (over

ripening). The respiration activity increases by several folds which leads to further enhanced product deterioration. The surface of the produce is also exposed to air which leads to microbial contamination of bacteria, yeast and moulds. Microbial activities bring about several biochemical changes that affects the product quality.

1.4 Microbial Spoilage in minimally processed fruit

Microbial spoilage has been used by quality assurance departments in the fresh-cut industry as the objective indicator for quality for more than 50% of fresh-cut vegetable commodities and almost 100% of fresh-cut fruit products [1]. Contamination sources of fresh-cut fruits and vegetables include raw materials and contact with processing equipment. The microorganisms that exist on the surfaces of raw, whole produce appear to be the major source of microbial contamination and consequent spoilage of fresh-cut fruits and vegetables. During the preparatory steps of minimal processing, the natural protection of fruit is generally removed and hence, they become highly susceptible to microbial spoilage. In addition, cross-contamination may occur during cutting and shredding operations because sanitation in whole fruit may not have been carried out properly. Leakage of juices and sugars from damaged tissues allows the growth and fermentation of some species of yeasts such as *Saccharomyces cerevisiae* and *Saccharomyces exiguous* [10]. Many types of microorganisms can be found on a cut fruit or vegetable, including Gram-negative bacteria, Gram-positive bacteria, and fungi (yeasts and moulds). At present, many parameters have been used as useful and effective indicators for the microbial quality evaluation of food products such as total viable counts (TVC), lactic acid bacteria (LAB), *Pseudomonas, Enterobacteriaceae, Brochothrixthermosphacta* (*B. thermosphacta*), etc.

1.4.1 Total Viable bacterial count (TVC)

TVC indicates the total counts of viable individual microorganism and is always recorded as colony forming units (CFU) per g (or per ml) of target sample. TVC may include bacteria, yeasts and mould species. The mesophilic aerobic bacterial counts ranges from 2 to 5 Log_{10} CFU/g fresh weight on finished cut fruits, depending on the commodities, seasons of the year, and growing regions [11].

1.4.2 *Pseudomonas*

The family *Pseudomonadaceae* consists of the four genera: *Pseudomonas, Xanthomonas, Zoogloea* and *Frauteuria* [12]. The type genus is *Pseudomonas,* and members are often referred to as pseudomonads. They are Gram-negative rods, occurring singly or in pairs, motile with one or more polar flagella, strictly aerobic, catalase positive and oxidase positive or negative. Pseudomonads are also characterized by their ability to easily grow in food products under simple nutritive conditions. They have been reported to be the predominant group of micro-organisms on fresh produce. They are capable of synthesizing enzymes, even under refrigeration conditions that facilitate the breakdown of food components and cause spoilage [13,14]. Some antagonistic strains of pseudomonads have also been identified and isolated from fresh-cut produce which restrict the growth of pathogenic species of *E. Coli* [15].

1.4.3 Lactic Acid Bacteria (LAB)

LAB is a family of Gram-positive, non-motile and non-spore-forming bacteria and easily causes the quality degradation of food products [16]. The term lactic acid bacteria describe a number of genera of Gram-positive bacteria (rods and cocci) that are traditionally known as fermentative organisms associated with fermented food products and food spoilage. These genera commonly associated with spoilage of foods include *Lactobacillus*, *Leuconostoc*

and *Pediococcus*. Based on chemotaxonomic and phylogenetic studies, these three genera are closely related, with overlap between them. There are three main groups: *Lactobacillus delbrueckii* group, which includes mainly homofermentative lactobacilli; the *Lactobacillus casei/Pediococcus* group; and the *Leuconostoc* group, including some obligate heterofermentative lactobacilli [17]. Lactic acid bacteria form the primary spoilers under modified atmosphere packaging during cold storage.

1.4.4 *Enterobacteriaceae*

Enterobacteriaceae is a large type of Gram-negative and rod-shaped bacteria and includes hazardous *Salmonella* and *Escherichia coli* (*E. coli*). This class of organisms are present on fresh produce when contaminated water is used for processing.

1.4.5 Yeast & Moulds (Y&M)

Yeasts and moulds can be found in a wide variety of environments, such as in plants, animal products, soil, water and insects. This broad occurrence can be explained by the fact that yeasts and moulds can utilize a variety of substrates such as pectin and other carbohydrates, organic acids, proteins and lipids. Moreover, yeasts and moulds are relatively tolerant to low pH, low water activity, low temperature and the presence of preservatives. Yeasts are single-celled eukaryotic organisms of which many genera are associated with the fermentation and spoilage of foods. Fermentative species of yeasts such as *Kloeckera* and *Hanseniaspora* occur naturally on the surfaces of fruits and are capable of causing fermentative spoilage [18]. Other fermentative species such as *S. cerevisiae* and *S. exiguus* may contaminate fruits during processing and cause explosive fermentative spoilage. Yeast populations of 10^3–10^4 have been reported for processed fruits [19]. Tournas *et al*. [20] found yeast levels ranging from less than 2 to 9.72 log CFU/g on a majority of 38 fruit salad samples (cantaloupe, citrus fruits, honeydew, pineapple, cut strawberries and mixed fruit salads). The most common yeasts were *Pichia* sp.,

Rhodotorula sp., *Candida pulcherrima, C. lambica, C. sake,* and *Debaryomycespolymorphus.* Yeasts have a slightly higher growth rate than moulds. Moulds are fungi that cover surfaces as fluffy mycelia and usually produce masses of asexual, or sometimes sexual, spores. A wide range of mold species can be present on fruits. Moulds reported on berries are *Botrytis cinerea, Rhizopusstolonifer*, *Mucorpiriformis*, *Rhizoctoniasolani*, and *Phytophtoracactorum*. Table 1 provides the TVC and Y&M counts of some fresh cut fruits studied previously.

Table 1. TVC and Y&M counts of fresh cut fruits

Fresh cut Produce	**Microbial Population (Log_{10} CFU/g)**		**References**
	Total Viable Count	**Yeast & Mould count**	
Fresh fruit salad	3.0		21
Kiwi fruit	3.15	2.46	22
Cantaloupe	1.05	-	23
Pineapple	4.05	2.90	24
Honey dew	2.32		24
Papaya	4.34	2.84	24
Cantaloupe	4.14	1.87	24

1.5 Factors determining microbial spoilage of minimally processed fruit

Microbial spoilage of food quality is largely influenced by intrinsic, extrinsic and implicit factors. Intrinsic factors include water activity, acidity, redox potential, available nutrients and antimicrobial substances. Extrinsic factors are the environmental factors notably temperature, humidity and atmospheric composition where the food is stored. Physical or chemical treatments also have impact on the microflora associated with the produce. Implicit factors are the result of association of contaminated organisms on the food surface that may have synergistic and antagonistic effects.

1.5.1 pH

The optimum pH for growth of most microorganisms is near neutrality (pH 7.0). However, yeasts and moulds are usually acid tolerant and are therefore associated with the spoilage of acidic foods. Yeasts can grow in a pH range of 3–10. Moulds can grow from pH 2 to 11, but favour an acidic pH. Due to the low pH values of most fruit, the main typical flora consists of moulds and yeasts. *Botrytis cinerea* and *Aspergillus niger* have been found to be important moulds while yeasts such as *Candida*, *Cryptococcus*, *Fabospora*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, and *Zygosaccharomyces* are the most prominent [25]. However, some commonly occurring bacteria, such as the lactic acid or acetic acid bacteria, can grow at pH 4.0 or less. Fungi are much more tolerant to acidic pH than are bacteria and can grow at pH values as low as 1.5 [26].

1.5.2 Refrigeration

Storage temperature is one of the most important factors that influences spoilage as well as safety of fresh produce. Cold storage can change both the nature of spoilage and the rate at which it occurs. There may be qualitative changes in spoilage characteristics as low temperature exerts a selective effect, preventing the growth of mesophiles and leading to the

microbiota being dominated by pychrotrophs. With respect to safety of minimally processed fruit, they are safer because conditions used with fresh produce are usually unfavourable for the growth of most pathogens (refrigeration temperatures, low pH of fruits, short shelf life). The spoilage microorganisms in refrigerated produce are usually psychrotrophic and therefore have a competitive advantage over most pathogens. Sometimes this competition prevents the growth of pathogens [27, 28, 29, 30]. Nevertheless, foodborne disease can and does occur with consumption of fruits and vegetables, especially when fresh-cut produce is packaged under modified atmosphere, as it increases the shelf life of the products, and pathogens have more time to reach infectious numbers before the product is notably spoiled. Pseudomonads are heat sensitive, however, they are able to grow at refrigeration temperature (the minimal temperature for growth is at 4° C) and have been found in a variety of frozen and refrigerated foods, including fresh-cut produce and are the primary spoilers in aerobic storage conditions [1].

1.5.3 Water activity

Following temperature, water activity is the second important factor for maintaining quality. According to US Food Drug Administration (FDA) the water activity (a_w) of a food is defined as "the ratio between the vapour pressure of the food itself when in a completely undisturbed balance with the surrounding air media, and the vapour pressure of distilled water under identical conditions". Shelf life and value of fruits and vegetables decreases with water loss because it causes appearance deterioration, tissue softening, wilting, shrivelling, and weight loss. Such changes also affect product suitability for the fresh market and the fresh-cut industry. Fresh produce is usually packed with porous materials filled with their own internal atmosphere, which has a high relative humidity. They lose water through the skin or abscission cuts, because of relative humidity differences between the internal atmosphere and that surrounding the product. Therefore, fresh produce should be stored under high relative humidity environments, as a complement to optimum storage temperature. The high humidity

conditions within a package and the presence of a large area of cut surfaces, which provide a rich source of nutrients, create an environment conducive to growth of microorganisms. In terms of water requirements for growth, yeasts are intermediate between bacteria and moulds [31]. Microbes vary in the minimum water activity necessary for growth and survival. Most bacteria require an a_w of at least 0.90 to grow and many cannot grow below an a_w of 0.95. Most yeasts can grow at a minimum a_w of 0.87 and most moulds can grow down to a_w of only 0.80. Some specialized bacteria and fungi can even grow at a_w of 0.65. No microbial growth will occur below a_w of 0.60 [32]. Except for a few specialized species, all microbes will grow better at higher water activities. Virtually all fresh fruits and vegetables have a_w of 0.95 or greater [1].

1.5.4 Atmospheric Composition

The use of modified or controlled atmospheres for storage of fruits and vegetables has become very popular in recent years. The most common way to modify the atmosphere is to reduce the O_2 while increasing the CO_2 concentration [33]. However, there are many changes that can be made to the atmosphere that can rightly be considered modified. Modified atmosphere includes any process that causes the gaseous environment of the produce to differ from that of ambient atmospheric conditions. Microorganisms differ in their sensitivity to gases normally used in modified atmospheres. Nitrogen is often used in modified atmospheres but is primarily used to displace O_2 and has little other direct effects on microorganisms. Gaseous composition of the atmosphere surrounding the fresh-cut produce has a profound effect on the microbial quality and shelf life. Low oxygen-modified atmospheres may inhibit the growth of spoilage micro-organisms and increase the shelf life of packaged produce [34].

1.5.5 Packaging

Packaging is an increasingly important parameter that has several features that make it desirable for use with fresh produce. First, it is quite useful from a marketing standpoint because it allows processors to provide labelling information but still allows consumers to see the product. More importantly, it minimizes dehydration of the product, a major cause of deterioration of minimally processed refrigerated produce [35]. In addition, some packaging is specifically designed to exploit the use of modified atmospheres. Others can unintentionally become modified atmosphere systems as a result of metabolic activity of the product [36]. The use of packaging can have substantial impact on the microflora of fresh fruits and vegetables. Many of the microbial changes that occur within packaged fresh fruits and vegetables result from changes in humidity within the package [36]. Respiration by the plant tissues increases relative humidity and thereby also increases the likelihood for mould growth [36]. The increased humidity also increases the likelihood for condensation to occur within the package. This can allow water droplets to form both on the product and on the inner surface of the package, particularly during refrigerated storage. This accumulation of water droplets can itself affect the microflora. First, droplets can serve as a transport medium and allow microorganisms to be distributed more easily to other parts of the product. In addition, these droplets can dissolve usable carbohydrates leaking from plant tissues and serve as a growth medium. Packaging is one of the important factors influencing the microbial quality of fresh-cut products. Fresh-cut products are mostly packaged under modified atmospheric conditions or they develop passive MAPs on storage under refrigeration which gives rise to a favourable environment and time for proliferation of spoilage micro-organisms and micro-organisms of public health significance [30]. The economic value of fresh-cut products is impaired by micro-organism proliferation because it may lead to decrease in product shelf life, through spoilage, and also pose a risk to public health by causing food-borne illnesses [19, 37].

1.6 Shelf life of minimally processed produce under refrigeration conditions

The product should have a shelf life sufficient to make its distribution feasible to its intended consumers. Each food processor is responsible for setting the shelf-life date on products put on the market. Regarding fresh-cut fruits and vegetables, this shelf-life date will be relatively short, so a "best before" date should be mentioned on each package. The microbiological, sensory and nutritional shelf life of minimally processed vegetables or fruit should be at least 4-7 days, but preferably even longer, up to 21 d depending on the market. Attention has to be given to the selection of the correct parameters that need be followed and evaluated during the shelf-life studies. Microbial spoilage is a limiting factor for shelf life of fruit pieces stored under controlled atmosphere conditions [24]. In Europe, microbial specifications have been established for quality of fresh-cut produce. The Spanish legal limit (RD 3484/2000, 2001) for microbial populations on minimally fresh processed fruit for safe consumption are 7, 5, and 3 Log_{10}CFU/g for aerobic bacteria, yeasts and moulds, respectively given in Table 2.

Table 2: Permissible microbial limits for fresh cut fruits

Parameter	Fresh samples	At best before date[b]
Total aerobic psychrotrophic count	10^5	10^7
Lactic acid bacteria[a]	10^3	10^7
Yeast	10^3	10^5
Moulds	10^2	10^3

a When the number of lactic acid bacteria on the best before date is greater than 10^7 CFU/g and the food product can be rejected only if found sensorially unacceptable.

b Best before date is the end of the shelf life, above these guidelines when notable spoilage will occur

In India, the acceptable regulatory limits of microbial counts on minimally processed fruits for safe consumption are 7 and 4 Log CFU/g for Total viable aerobic bacterial count and yeast and mould counts, respectively as set by FSSAI [38].

1.7 Physiological & biochemical changes associated with microbial spoilage

Microbial spoilage leads to formation of off-flavor (e.g., fermented aroma with cut lettuce odour, sour taste with cantaloupe and bell pepper odour) slimy surface (e.g., "baby" carrots), wetness and soft rot (e.g., cut bell pepper), discoloration (e.g., apple wedges), and visual microbial growth/colonies (such as apple wedges, cantaloupe chunks, and cored pineapple). The following have been used as a main or exclusive objective criterion to determine shelf life of fresh-cut products [24, 39].

1. Soft rots—maceration of the tissue caused by enzymatic degradation of the plant cell wall by pectinolytic enzymes [40]

2. Formation of off-odors and off-flavors—activity of lipolytic and proteolytic enzymes and fermentation reactions [24, 41]

3. Wilting—brought on by vascular infections [42]

4. Brown discoloration—polyphenol oxidase activity of the microflora may contribute to browning [43]

5. Fermentative spoilage—fermentation of carbohydrates to produce acid, gas or alcohol.

Yeasts have the ability to ferment simple carbohydrates to produce alcohol, gas and flavour components such as esters, acids and higher alcohols, and the ability of some species to grow at relatively low temperatures (10–15°C). Less fermentative species, such as *P.*

membranifaciens, *Candida krusei* and *Kluyveromyces*, may also spoil fresh-cut products through the formation of films or off-odours [44, 45]. Pseudomonads may also contribute to the yellowing of vegetable products during storage, through the production of the ripening hormone ethylene [46, 47]. LAB leads to fermentation of sugars to produce acid and gas, which is undesirable in fresh-cut products. Their fermentative metabolism and ability to grow in anaerobic conditions enables lactic acid bacteria to cause spoilage, such as souring of the product, gas production and slime formation [48, 49].

1.8 Need for rapid assessment of microbial spoilage

Quality of minimally processed produce is measured in terms of colour, texture and microbial quality. Loss of colour and texture can be monitored with analytical instruments or sensory analysis. Moreover, microbial quality assessment is an objective indicator of quality of fresh produce that also provides a measure of safety of the produce. Conventional methods such as microscopy [50], plate count method, biological techniques (e.g. PCR & real time PCR) [51, 52] and immunological methods (e.g. ELISA) [53, 54] have been widely used for quantitative microbial measurement. They are objective and reliable but time-consuming, labour-intensive and destructive, thereby not very suitable for rapid and online measurement. Moreover, trained or skilled personnel are needed to operate the equipment. Food safety is one of the main objectives of food industry and application of Hazard Analysis and Critical Control Point (HACCP) is a prerequisite during handling, processing and distribution. Microbial contamination always has a negative impact on the food quality and human health [55, 56, 57]. Foods containing none or minimal microbial contamination will reduce food hazard thereby building consumer confidence. Therefore, microbial evaluation is an essential step during the food processing, in order to guarantee food quality and safety. Novel techniques are therefore warranted for improving the detection efficiency and reducing cost.

1.9 Detection of microbial spoilage based on biochemical markers

Quality of fruits during storage is influenced by two major processes. On the one hand, fruits are living materials, as they keep respiring after harvesting, resulting in different physiological processes [58]. The produce is also prone to microbial contamination with different types of micro-organisms during pre-harvest/post-harvest. The composition of the microbiological population can vary depending on the handling and processing protocols followed. Once fruits are minimally processed, micro-organisms can further influence different quality attributes due to metabolite production as a result of microbiological activity [59]. The correlation between biochemical changes associated with microbial growth during storage has been demonstrated to be a useful quantitative measure to monitor quality of fresh cut produce [16].

Sugars, organic acids, and amino acids significantly contribute to sweetness and aroma of fruits. Sweetness and aroma are the two most important quality indicators in fruits. [60, 61]. In addition, organic acids are important flavor precursors and energy sources in plant cells [62]. Metabolite production or sugar consumption in fruits and vegetables has been reported [56, 63]. A range of volatile organic compounds produced by yeast on strawberry agar such as acetone, ethyl acetate, ethanol, isopropyl acetate, ethyl butyrate, 1-propanol, 2-methyl-1-propanol, 1-butanol, 2-methyl-1-propanol, 1-hexanol and hexyl acetate were noted [64]. A simultaneous decrease in sugar concentration was also observed. Ethyl esters were formed when ethanol reached to a high concentration toxic to microbes, thus detoxifying the effects of ethanol. Several studies have also been carried out on off-odour production resulting from changes in volatile composition as well as the impact on changes in organic acid. [65, 66, 67, 68]. Lactic acid production was shown to increase with subsequent reduction of malic acid in minimally processed cantaloupe under airtight chambers [23]. Total amino acid content also decreased rapidly at higher storage temperature. The type of microbial species present at

different storage conditions and post processing operation finally influences the qualitative and quantitative composition of biochemical indicators. Comprehensive profiling of the metabolic changes due to microbial activity is therefore currently being targeted for correlation with microbial counts.

1.10 Instrumental technique for online monitoring of microbial quality

Recently some interesting analytical approaches based on the biochemical changes occurring in the food have been proposed for rapid and quantitative assessment of microbial quality in fresh produce. These include imaging [69], spectroscopic [70, 71], hyperspectral imaging [72, 73], & e-nose [74]. Imaging technique gives the morphological information in the form of pixels that registers external deformities or structural defects. Spectroscopic techniques evaluate the biochemical changes in the form of spectra. E nose provides the volatile changes associated with product spoilage in the form of signals. This comprehensive information obtained in the form of pixels, spectra or signal is multi- dimensional and requires advanced statistical tools to develop correlation with microbial growth. Thus, development of a "robust system" that can automatically classify the input into a "diagnostic tool" based on extracted variables is necessary before widespread adoption of such an analytical tool. The application of multivariate statistical methods and predictive tools (Partial least square regression, artificial neural network etc.) can be used for qualitative and quantitative estimation of microbial quality.

1.10.1 Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) is a fast, easy to use, reagent less and non-destructive technique for obtaining biochemical information of food samples. Owing to its benefits it has attracted considerable interest and several potential applications has been explored in food and related sector [75]. Molecular vibrations that are excited by infrared radiation are monitored by FTIR. Eventually infrared spectrum represents a "fingerprint"

which is the characteristic of the biochemical composition. This technique has been widely explored in muscle food for microbial spoilage [70, 76, 77, 78, 79].

1.10.2 Headspace Solid Phase Micro-Extraction Gas Chromatography & Mass-spectrometry (HS-SPME-GC/MS)

HS-SPME-GC/MS is a rapid solvent free technique that employs SPME for extraction of volatiles from the headspace of the foodstuff. Several volatile changes associated during the process of food spoilage provide the biochemical information about the status of food. GC-MS, a hyphenated analytical technique separates the volatiles which are subsequently fragmented and further identified based on their mass. Volatilomics, is the field of detection, characterization and quantification of volatile metabolites from the biological system. This field has recently gained importance in several areas of food science such as food safety, quality and food authenticity. Different aspects such as sensory, microbial, biochemical and nutritional attributes of several foodstuffs based on food volatiles are exploited using GC-MS because of rapid advancement of this analytical technique in recent years, easy sample preparation and rapid and on-line techniques for extraction. The processes of washing, peeling and chopping cause damage to the fruits. The accompanying reactions allow the formation of volatiles such as alcohols, aldehydes, terpenes, esters and acids in fresh cut fruits. Compounds that are frequently detected in many fruits are terpenoids such as linalool, geranyl acetate, limonene, etc. A common pattern in most fruits as far as maturity is concerned, is presence of C-6 aldehydes and alcohols in high concentration at the beginning of maturation process. As the desired level of maturity is approached, a decreasing trend in the content of aldehydes and an increase in esters is observed [80]. Several studies conducted on food matrix have documented marker volatile compounds such as 2-methyl butanol, 3-methyl butanol and 2,3-butanediol, alcohol, ethyl esters that are linked with vegetables, fruits & meat spoilage. They are the common metabolites of many bacteria and yeast under various storage conditions [65, 66, 68].

1.11 Chemometric based intelligent system for quality assessment

The enormous volume of information generated by the analytical tools demand application of various multivariate statistical tools for data analysis. Multivariate analysis also coined as chemometrics can be defined as “the simultaneous statistical analysis of a collection of random variables” [81]. The process involves extraction of chemically relevant information out of the enormous analytical chemical data by mathematical and statistical tools [82].

Multivariate analysis is primarily divided into two categories: unsupervised and supervised learning. Unsupervised methods “attempt to disclose naturally occurring groups and structures within the dataset without previous knowledge of any class assignment” [83]. It principally extracts the information such as patterns, trends or clusters and in turn it also facilitates performing outlier analysis. The techniques included in unsupervised learning are principal component analysis (PCA) and cluster analysis. On the other hand, supervised learning algorithms “make use of a priori knowledge of classes to guide the characterisation or classification process” [83]. These algorithms generate prediction models for classification, regression, pattern recognition or machine learning tasks. Characteristic examples of supervised learning involve Partial Least Squares (PLS) and Artificial Neural Networks (ANN), among many others.

1.11.1 Unsupervised learning

1.11.1.1 Principal Component Analysis

Datasets with large number of variables will have large dimensionality in data, redundancies & correlation among variables. Principal Component Analysis (PCA) is the most commonly used technique for dimensionality reduction, data compression and feature extraction [84, 85]. The PCA algorithm reduces the initial number of possibly correlated variables into a new lower number of uncorrelated variables, known as the Principal

Components (PCs). Geometrically, we can imagine the input data as a cloud of points in a high-dimensional space. As illustrated in Figure 3, this cloud of points is probably longer in a certain direction of the pattern space; in this direction the data appear to be most different and PCA draws the first axis (PC_1). The first PC places all points the farthest apart from each other, extracting thus the highest variance. Similarly, a perpendicular to the first PC axis is drawn for the second PC (PC_2), which accounts for the second highest variance. The process is repeated to get multiple orthogonal principal components. Each successive orthogonal axis displays a decreasing amount of the total variance.

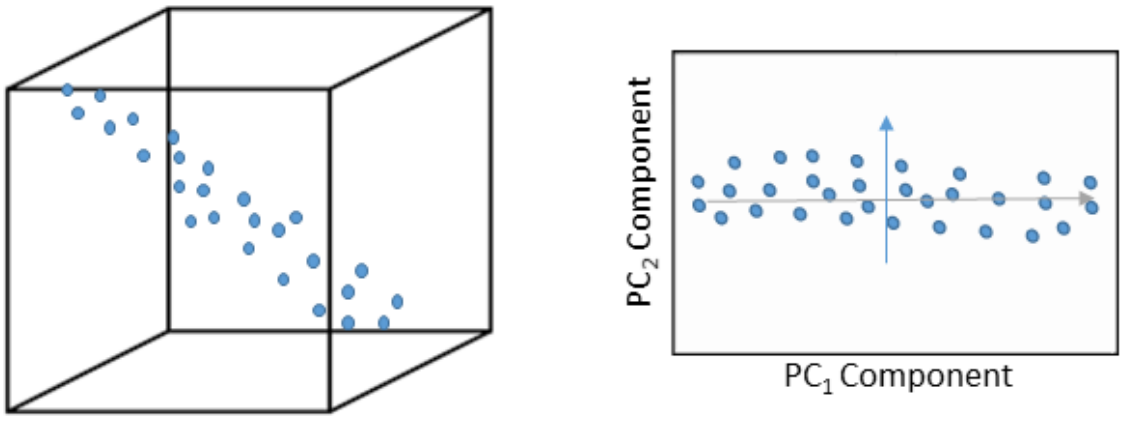


Figure 3. Extracting the principal components

1.11. 2 Supervised learning

1.11.2.1 Partial Least Square Regression

Partial Least square regression (PLS-R) is a multivariate regression tool utilised for prediction of dependent variable (Y) from multivariate independent variables (X). Its goal is to predict or analyse a set of dependent variables from a set of independent variables or predictors. This prediction is achieved by extracting from the predictors a set of orthogonal factors called latent variables (LV) which have the best predictive power. PLS regression searches for a set of latent variables that performs a simultaneous decomposition of X and Y with the constraint

that these components explain as much as possible the covariance between X and Y. The number of LVs selected for model building depends on the data. Building the model on more latent variables improves the model fit to the observed data, but extracting too many factors can cause over-fitting, that is tailoring the model too much to the current data that leads to the detriment of future predictions. The PLS procedure enables you to choose the number of latent variables by cross validation, that is, fitting the model to part of the data and minimizing the prediction error for the unfitted part. Various methods of cross validation are available, including one-at-a-time validation, splitting the data into blocks, and test set validation. PLS-Regression is a preferred choice when the variables are collinear and number of variables are larger than number of observations.

1.11.2.2 Artificial neural networks

Artificial neural networks (ANNs) are biologically inspired computational networks. ANNs works on similar principles as the human brain with two key similarities between biological neural networks and ANNs. First, the building blocks of both networks are simple computational devices that are highly interconnected. Second, the connections between neurons determine the function of the network. A human brain consists of approximately 10^{10}neurons, computing elements, which communicate through a connection network (approximately 10^{4}connections per element). ANNs function as parallel distributed computing networks and are analogous to biological neural systems in some basic characteristics [86, 87].

Multilayer perceptrons (MLPs) with back propagation learning algorithms, also called multilayer feed forward neural networks, are very popular and are used more than other neural network types for a wide variety of problems. MLPs are based on a supervised procedure, i.e., the network builds a model based on examples in the data with known outputs. An MLP has to extract this relation solely from the presented examples, which together are assumed to

implicitly contain the necessary information for this relation. An MLP comprises three layers (input, hidden, and output) with nonlinear computational elements (also called neurons and processing units). The information flows from input layer to output layer through the hidden layer connected to neurons in the adjacent layers. These connections are represented as weights (connection intensity) in the computational process. The weights play an important role in propagation of the signal in the network. They contain the knowledge of the neural network about the problem-solution relation. The number of neurons in the input layer depends on the number of independent variables in the model, whereas the number of neurons in the output layer is equal to the number of dependent variables. The number of output neurons can be single or multiple (Figure 3).

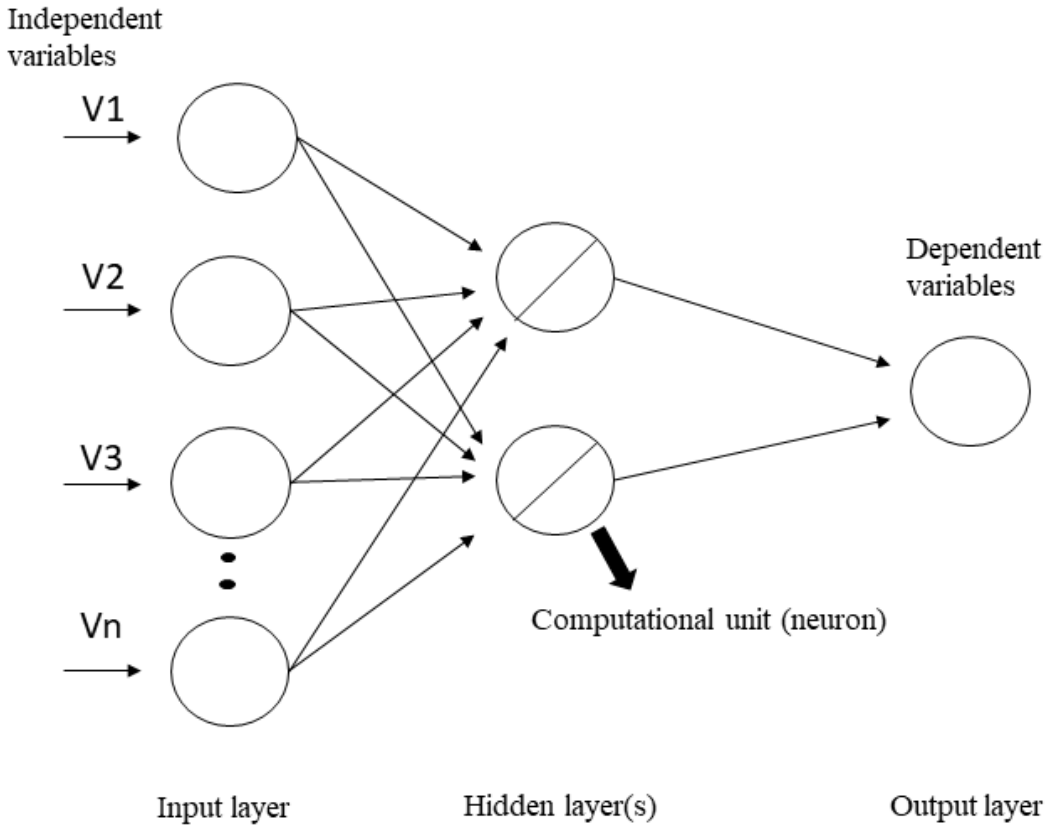


Figure 3: Network architecture of ANN

In the development of data-driven models, the dataset has to be split into two subsets which are respectively used for training and validation/testing. The proportion may be 1:1, 2:1, 3:1, etc. for these two sets. However, the training set still has to be large enough to be representative of the problem and the test set has to be large enough to facilitate correct validation of the network. This procedure of partitioning the data is called k-fold cross validation, sometimes also called the hold-out procedure [88, 89, 90].

1.12 Intelligent packaging system for real time monitoring of spoilage

Modern-day food packaging provides containment and protection for food produce along with being informative, attractive and ease of use. Consumer demands are primarily focussed towards food products that are fresh and minimally processed. Online monitoring of the quality of the refrigerated perishables have been largely studied using several instrumental techniques which offers a rapid method to serve as control points suitable for food processing industry. However, the shelf life of these refrigerated fresh cut produce needs to be critically displayed for consumer's safety and informed choice at retail establishment. The quality loss of ready to eat fruit is dependent on its microenvironment represented in the terms of shelf life. Temperature is deemed to be the most important external factor controlling food spoilage. Storage temperature has a direct influence on the kinetics of the chemical and biological changes that occur in food products. Usually shelf life information given in the form of '**best before'** or '**use by'** dates are based on assumptions of either a most probable average temperature for the food or a worst case temperature exposure. This may lead to unacceptable quality before the stated end of shelf life in temperature abusive condition or may lead to waste of perfectly good product. This problem could be solved if a way could be devised to monitor the temperature history of the product and to estimate the remaining shelf life. Time temperature indicators (TTI) offer a potential solution; these are smart, inexpensive, colour

changing labels that can be attached to the food package most suitable for retail establishments and consumers to monitor the quality of the package.

1.12.1 TTI's for microbial quality assessment

TTIs rely on modelling and predictive behaviour of microbial growth when temperature abuse occurs. Temperature dependence of microbial activity on food is an established fact and is a primary cause of spoilage in fresh cut industry [1]. It also serves as operational check point for quality and safety assurance in such products. Effective application of TTI requires systematic study of parameter governing shelf life by establishing correlation with the primary cause of spoilage. Subsequently, the TTI response in the form of visual change should match the quality loss of food. This requires thorough kinetic studies of colour development of TTI as well as the product spoilage parameter at different storage temperatures. Several TTI's have been previously worked out based on different principles such as polymerization, chemical, microbial and enzymatic reaction which has been reviewed extensively in literature [91]. TTI has been widely applied in the food industry for fresh milk, frozen fish, meat, sea food and frozen vegetables to reflect the time temperature history [92, 93, 94]. However, applications of TTI are not explored for minimally processed refrigerated fruits based on the literature data, although use of several commercial TTI's for application to this food commodity has been suggested [95]. Thus, the current study attempts to examine the real time application of cheap chemical TTI on these commodities never explored before.

1.13. Selection of minimally processed fruits

Minimal processing of fruit is important to supply it in a convenient form for its wider consumer demands, yet the produce should maintain fresh like attributes without losing its nutritional quality and the product should have a shelf life sufficient to make its distribution feasible to its intended consumers.

1.13.1. Minimally processed jackfruit bulbs

Jackfruit is a tropical fruit, native to India which is rich in energy, dietary fiber, minerals, and vitamins moreover; it lacks saturated fat or cholesterol. India is the second largest producer of the fruit in the world and is considered as the motherland of jackfruit (*Artocarpus heterophyllus)*. It is grown in an area of 97,536 ha with annual production of 348 million fruits and productivity of 3,568 fruits per ha. [96]. The fruit is very bulky ranging from 5 – 70 kgs. Their large size makes them cumbersome to process. Recently minimal processing of jackfruit has gained importance due to ease in serving portion for large and difficult to peel fruit and reduction in cost of packaging and transportation. The jackfruit bulbs are commercially sold in supermarkets in India as minimally processed and packed product.

1.13.2 Minimally processed pomegranate arils

Pomegranate (*Punica granatum*) is an exotic fruit bearing deciduous shrub. Aril is the specialized outgrowth from the funiculus (attachment point of the seed) that covers or is attached to the seed. India ranks first in the world with respect to pomegranate cultivation area (0.125 million ha) and production (1.14 million tonnes) [97]. Pomegranate consumption is limited, since extracting the pomegranate arils is very difficult and time consuming therefore consumption is not widespread. Commercial production of pomegranate arils is now available with different technologies available in market [98]. Thus, boosting opportunity in minimal processing industries provides desirable options for consumers [99]. However, minimally processed pomegranate seed have a greatly reduced post-harvest life compared to whole fruit. This commodity is highly prone to microbial contamination because of removal of thick protective cuticle.

1.13.3 Minimally processed pineapple slices

Pineapple (*Ananas comosus*) is one of the tropical fruit which has huge economic importance for its excellent flavor and taste. Minimal processing of pineapple requires skill and provides convenience to consumer and motivation to consume this health boosting fruit with essential minerals, fiber and vitamins. In India, minimally processed pineapple consumption has become increasingly popular in urban population living in metropolitan cities. Microbial quality assessment of minimally processed pineapple available in Indian market has been studied previously [100].

1.14 Research Hypothesis

According to the report submitted by planning commission India, up to 18% of the fruit and vegetables produced in the country gets spoiled during its transport from farm to fork. The production of fruits and vegetables has increased to 170 % in a decade. With increased awareness of health benefits associated with consumption of fruits and vegetables there has been an increased consumer demand for these food commodities. However, uncontrolled post-harvest loss of these perishable foodstuffs during storage and transportation has led to higher market price (approx. by 20%) in the last decade. It is therefore crucial to control spoilage of these products in order to increase availability and affordability.

Minimal processing of fruits is one of the processing approaches that have gained increase in consumer demand in food processing sector. The consumption of minimally processed food products including ready-to-eat and ready-to-cook has increased worldwide in last decade due to their convenience, freshness and improved quality. Total microbial load has been used by quality assurance departments in the fresh-cut industry as an objective indicator for quality of fresh-cut minimally processed fruit products [1]. However, traditional microbial enumeration techniques to determine the microbial load are cumbersome, destructive and time

consuming, thus making timely detection of food spoilage very difficult and challenging [2]. Therefore, it is of interest to develop rapid methods for microbial quality assessment. Rapid evaluation of microbial quality has been previously studied on meat and fish products using techniques such as FTIR [101, 102], hyperspectral imaging [73] and electronic nose [103]. However very few reports on processed fruits exist in current literature even though they have a short shelf life and are highly susceptible to microbial spoilage. The aim of the study is to investigate the possible biochemical changes and explore for possible specific markers pertaining to fruit spoilage process. The final goal of the research is to develop a measurement system which rapidly predicts the microbial spoilage from biochemical changes as measured by instrumental analysis of the minimally processed product. The theory behind the method is that the microbial activity brings about several changes in the biochemical metabolite during the spoilage process. Thus, monitoring the volatile profile & other non-volatile biochemical changes of minimally processed food product may determine the spoilage status. GC-MS and FTIR will be exploited to monitor changes in the product which will then be correlated with the microbial counts. Finally, sensor-based assessment of the microbial quality of the packed minimally processed food will be performed. It is anticipated that the technology produced from this research will give a viable and low-cost solution to help minimize preventable food waste from consumers as well as improving food industry process efficiency, especially in the field of food supply chain management.

1.15 Aims & Objective of the work

The present study aims at evaluating analytical techniques such as GC/MS & FTIR as rapid detection methods for microbial quality status of minimally processed food products. A new sensor such as time temperature sensor based on colour responses will also be developed for rapid monitoring freshness of minimally processed perishables.

Objective

1. To evaluate the microbial quality of minimally processed fruits (jackfruit, pomegranate, pineapple) during the storage.

2. To evaluate the biochemical changes in minimally processed fruits during storage using various techniques such as GC/MS and FTIR.

3. To correlate microbial growth with instrumental data obtained, using various multivariate statistical techniques such as principal component analysis, partial least square regression analysis, artificial neural networks, etc.

4. To identify various biochemical changes in minimally processed fruits responsible for observed correlations between microbial quality and instrumental data.

5. Possible development of sensor based on identified biochemical changes for detection

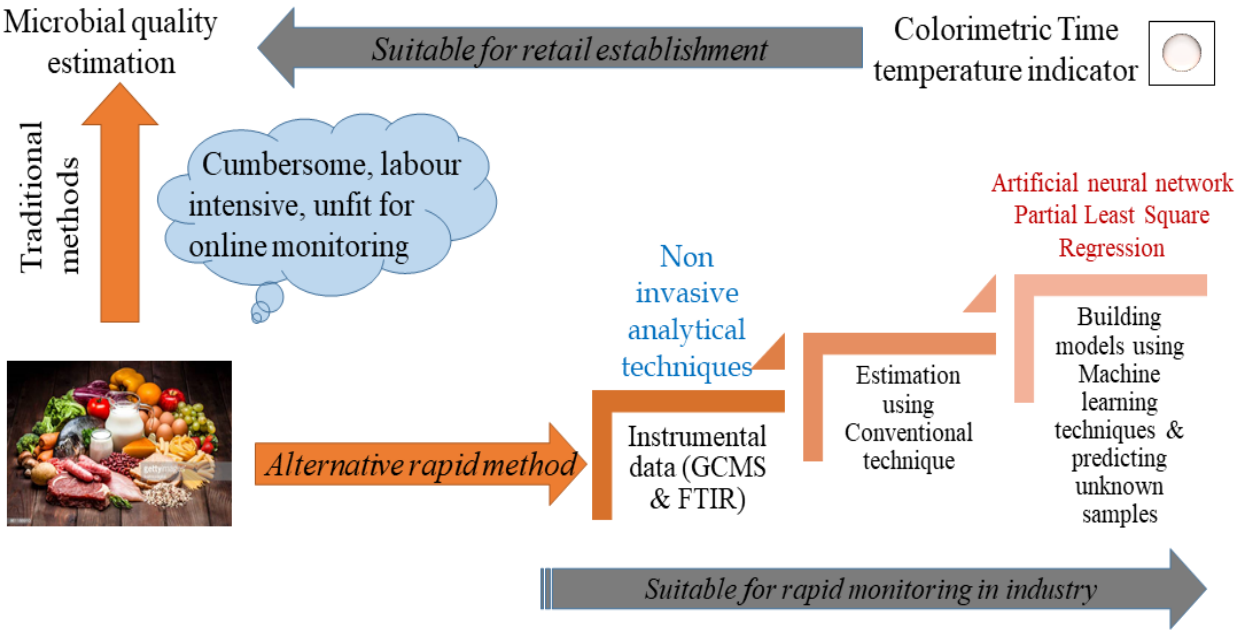


Figure 4. Schematics of the work carried out in the thesis

Chapter 2

Materials and Methods

2.1 Materials

2.1.1. Minimal processing and storage of fruit samples

2.1.1.1 Minimal processing of Jackfruit

Jackfruit (*Artocarpus heterophyllus)* of brownish yellow skin colour at optimum ripening stage was procured from local fruit markets of Mumbai, India (Weight – 7 to 15 Kg). The fruits were opened by manually cutting along the axis using sharp-edged stainless steel knives and the bulbs were removed from rind. The bulbs were light yellow in colour, fibrous and slightly juicy. The processed fruits were packed and immediately stored at 4 °C. Segregation of samples for storage at different conditions was carried out after final processing and packaging.

2.1.1.2 Minimal processing of Pomegranate

Pomegranate (*Punica granatum*) at fully ripened stage was procured from local market in Mumbai. The healthy fruits uniform in size and appearance were chosen for extraction of pomegranate arils. Fruits were washed to remove all adhered dust with running tap water before cutting. Husks were cut in a cone shape from the top and carefully sliced till bottom using sharp knife and the arils were manually extracted. All the extracted arils were collected in trays and maintained at 4 °C until completion of the processing steps. The collected pomegranate arils were mixed to ensure random and homogenous packaging.

2.1.1.3 Minimal processing of Pineapple

Ripe pineapples (*Ananas comosus*) were procured from local market in Mumbai. Pineapples were peeled and cut into thin slices (thickness, 0.2 cm) and mixed to ensure random and homogenous packaging. The slices were shifted to cooled incubators (4 °C) until complete processing.

2.1.1.4 Packaging and storage

75 g of sample (jackfruit bulbs, pomegranate arils and pineapple slices) were placed in polystyrene trays (9 cm x 9 cm x 2.5 cm), packaged by wrapping with cling film (Flexo film wraps ltd. Aurangabad, Maharashtra, India) and then sealed tightly to avoid any leakage. The packed samples were stored at 4 °C and 10 °C to replicate the market conditions of storage. The number of packages stored and the final storage days of all the three minimally processed fruits is given in Table 4 below. Samples were withdrawn until total viable aerobic bacterial counts (TVC) reached > 7 log CFU g^{-1} and no further increase in counts was observed after achieving stationary phase.

Table 4. Total number of samples and the final storage period for all the three fruit samples

Fruit samples	**4 °C**		**10 °C**	
	No. of samples	Final storage period	No. of samples	Final storage period
Jackfruit	23	19 d	32	6 d
Pomegranate	21	28 d	72	6 d
Pineapple	32	22 d	42	6 d

d- days

2.1.2 Withdrawal of samples for analysis

Withdrawal of stored packages kept at 10 °C was carried out every day from day 0 till the product spoiled. Samples stored at 4 °C were withdrawn every 3rd day of storage. The packed products were stored in high precision (± 0.5 °C) incubation chambers (MIR-153, Sanyo Electric Co., Osaka, Japan). For every storage day, samples were withdrawn in triplicate and microbial, HS-SPME-GCMS and FTIR analysis was carried out. Samples were initially

subjected to microbial quality assessment using standard plate count method followed by instrumental analysis.

2.1.3 Microbial analysis

The enumeration of microorganisms was made using standard plate count techniques. Each packet was opened in a laminar flow cabinet and a 25 g sample was then aseptically transferred to stomacher bag (Seward, UK) containing 225 ml of sterile saline. The sample was homogenized (230 rpm, 1 min) using a stomacher (Model: 400 circulator, Seward, UK). Serial dilutions of these samples were prepared in sterile saline (0.9 %) solution. 1 ml of appropriate dilution was then pour plated in plate count agar (PCA) for total viable aerobic bacterial counts (TVC) and potato dextrose agar (PDA) for total yeast and mould count (Y&M). PCA plates were incubated at 37 ˚C for 48 h, while PDA plates were incubated at 28 ˚C for 4 days. The microbial load was determined in the form of colony forming units (CFU). Results were expressed as Log_{10} CFU/g.

2.1.4 Headspace gas chromatography and mass spectrometric analysis (HS-GCMS)

Packages of minimally processed jackfruit were incubated at 37 °C for 45 min. Headspace volatiles were then extracted by inserting a pre-conditioned SPME (Solid Phase Micro Extraction) -PDMS/DVB/CAR (50/30 µm polydimethylsiloxane (PDMS)/ carboxen (CAR)/ divinyl benzene (DVB), Supelco, Bellefonte, PA) fibre through a septum pasted on polystyrene tray under extraction conditions of 37 °C for 20 min. After extraction, the fibre was exposed from SPME assembly injected in GC-MS injection port. In case of pomegranate arils (25 g in 2.5 ml distilled water) and pineapple slices (30 g in 7 ml distilled water) the samples were homogenized in omni mixer (Sorvall, Waterbury, CT) for 3 min at a speed corresponding to the mark 2.5 on the instrument. Resultant slurry was strained through muslin cloth and then centrifuged at 12,850 g for 10 min at 4 ^{o} C. 15 mL of the juice was added in SPME vial

containing 4.5 g of NaCl. 3-hexen-1-ol at final concentration of 28 ug/L was used as internal standard. Headspace volatile compounds were isolated using a pre-conditioned (250 °C, 5 min) SPME fiber as above. Conditions of extraction were: sample equilibration; 40 °C for 10 min with magnetic stirring, fibre exposure for absorption of volatiles; 10 min at same conditions, desorption; on the injection port kept at 250°C for 2 min. The length of the fibre in the headspace was always kept constant. Before each analysis, the fibre was preconditioned to remove any volatile contaminant by exposing on the injector port for 10 min. Analysis was carried out on GCMS (QP2020, Shimadzu Corporation, Japan) equipped with a Rxi-5ms capillary column (length = 10 m, inner diameter = 0.1 mm, film thickness =0.1 µm, Restek Corporation, USA). Helium was used as a carrier gas at a constant flow of 0.4 ml/min. The injector port was equipped with a liner (0.75 mm ID, supelco) suitable for SPME analysis. Injections were conducted in split mode with a split ratio of 5. GC temperature settings were: Initial oven temperature was 40 °C with a hold time of 5 min. The oven temperature was then increased to 200 ºC with change in rate of 13 ºC per minute and finally to 280 °C at the rate of 33 ºC per minute. Oven was maintained at final temperature for 3 min. The interface temperature was set at 280 ºC. MS parameters were: ionization voltage 70 eV, electron multiplier voltage, 1 kV and scan mode from m/z 35 to 500. The peaks were identified by comparing the Kovat indices based on a homologous series of n-alkanes (C5-C24, Aldrich chemical company, WI, USA) with that of standard compounds as well as from MS data available in the Wiley and NIST library (NIST/EPA/NIH, 2014 compilation). Automated mass detection and identification (AMDIS) software (v 2.62) was used for identification and quantification of target compounds with match factor 90. The peak areas of the targeted volatile compounds were evaluated and quantified based on internal standard to generate a data matrix of the identified volatile compounds.

2.1.5 Fourier transform infrared (FTIR) spectroscopy analysis

Thin slices of jackfruit and the juice extracted for pomegranate aril and pineapple slices (juice preparation mentioned in section 2.2.3) were used for FTIR analysis. FTIR spectra was obtained by placing the sample on a ZnSe 45° ATR (Attenuated Total Reflectance) crystal of the FTIR spectrometer (Jasco, 4100) equipped with a DLaTGS (deuterated l-alanine doped triglycene sulphate) detector with KBr beam splitter. The spectrometer was controlled by Jasco spectra manager version 2 software to collect spectra in the range of wave number 4000–650 cm^{-1}, accumulating 40 scans with a resolution of 4 cm^{-1}. Sample analysis was done in duplicate and mean values of measurements was later used. Background scans were obtained from the cleaned blank surface of the crystal before each sample analysis to avoid any contaminating peaks. After each sampling, the crystal surface was thoroughly cleaned with distilled water and dried with lint free tissue.

2.1.5.1 Data pre-processing of FTIR data

FTIR spectral data in range of 2000 to 800 cm^{-1} was used for data analysis of jackfruit samples and FTIR data in range of 2000 to 1000 cm^{-1} was utilized for data analysis of pomegranate and pineapple samples. This spectral range was utilized to obtain metabolic fingerprint of pomegranate juice during storage. There were hardly any peaks detected in the remaining spectral region. Spectra obtained were baseline corrected and smoothed using the Savitzky-Golay algorithm of Spectra Manager software (Jasco, Japan). FTIR data was then pre-processed in two different ways before correlating with the microbial quality. A) FTIR spectral data was exported in ASCII format to Microsoft excel. This processed data is henceforth termed as FTIR spectral data. B) First derivative of FTIR spectral data was calculated by Savitsky-Golay (SG) procedure using a fourth order polynomial with five points. This processed data was then exported in ASCII format to Microsoft excel and henceforth

termed as FTIR first derivative spectral data. Data obtained was subsequently mean centered and standardized before further statistical analysis.

2.1.6 Principal component analysis (PCA)

PCA is an exploratory qualitative method that accomplishes two major tasks, data reduction i.e. compression of data without affecting original data structure and thereby also allows graphical representation of the data. It displays relationship amongst samples and variables and highlights the most informative variable from the large data set. It generates set of few uncorrelated variable designated as principal components (PCs) that contains valuable information of similarities and differences amongst variables and sample. PCA was applied on GCMS and FTIR data (FTIR spectral data & FTIR first derivative data; Section 2.1.5.1) of all the three fruits as variables for stored fruit samples to study the effect of storage on samples stored at 4 & 10 °C. The PCA type used was Pearson in XLSTAT_v 2013.4.05 (Addinsoft Co., Paris, France), that standardises the data before PCA operations.

2.1.7 Partial least square regression modelling

The PLS algorithm built a linear (or polynomial) relationship between X and Y matrices. PLS works on the basis of extracting a smaller number of orthogonal latent variables (LVs) that are linear combinations of the original (X). For building PLS-R model, dataset was randomly partitioned into training and testing subset (80:20). Test data were not employed in any step of training the PLS model but they were used exclusively to determine its performance. A series of PLS models were created using a number of latent variables ranging from 1 to 8, hence 8 models were developed in total for each analysis. The performance of PLS models generated were evaluated based on standard error of prediction (SEP). Final regression models were prepared using that number of latent variables which on further increase resulted in either constant or increased SEP. The performance parameters of the resulting model were then

evaluated for the test set. XLSTAT v 2013.4.05 (Addinsoft Co., Paris, France), available as an add-in software to MS-Excel, was used to perform PLS analysis. The performance of PLS models generated were evaluated based on standard error of prediction (SEP), accuracy factor (A_f) and Bias factor (B_f). PLS-R models were built for GCMS and FTIR data (FTIR spectral data & FTIR first derivative data, Section 2.1.5.1) of all the fruit samples at both the storage temperature.

2.1.8 Artificial neural network modelling

Nonlinear neural multiple layer perceptron (MLP) network was applied for prediction of TVC and Y&M. In the present study, back propagation (BP), which is the most commonly used training algorithm for neural networks, was employed. Original GCMS and FTIR data due to large number of input variables (volatiles or wave numbers) is not suitable for ANN training and secondly, the strong correlation among volatiles and FTIR variables (i.e. wave-numbers), would seriously deteriorate the modeling procedure. Therefore, uncorrelated principal components (PCs) were utilized as input variables. Volatile profile of GCMS data and processed FTIR data (FTIR spectral and first derivative data) was first subjected to PCA. Principal components (PC) were then used as input variables for ANN modelling. The number of PCs extracted as input variables accounted for 95% of cumulative variance observed in the experiment. The database was randomly partitioned into a training, validation and test subset (70:15:15). Training data was employed for building and training the model while test data was not used for training but for determining performance of the model.

MLP network chosen had one hidden layer with varying number of neurons from 1 to 14. The output layer contained two nodes one for prediction of TVC and other for Y&M counts. Numbers of input neurons were kept same as number of PC's chosen in training dataset. The training algorithm utilized for ANN was Levenberg-Marquardt. Training for all architectures

was carried out for six times after re-initialization of weights for maximum of 1000 generations. However, training was stopped if the validation mean square error (MSE) showed no improvement for 6 epochs. Transfer functions for hidden and output layer were chosen as sigmoidal and hyperbolic tangent, respectively. Best neural network architecture was selected network with highest R^2 values of training, validation and test data and lowest training, test and validation performance errors was selected. The MLP network was developed in MATLAB version 7.0 code (Mathworks, Inc., Massachusetts, USA). ANN networks were developed in this work with GCMS and FTIR data (FTIR spectral data & FTIR first derivative data, Section 2.1.5.1) of all the fruit samples at both the storage temperature.

2.1.9 Performance parameters of the generated model

To evaluate the performance of the models generated by PLS-R and ANN, various statistical parameters such as Standard error of prediction (SEP), accuracy factor, bias factor and correlation coefficient (R^2) between the observed and predicted counts were calculated. The bias (B_f), accuracy (A_f) factors and SEP [102] were expressed as follows:

$$B_f = 10^{((\sum \log(y_i/y)/n)} \quad (1)$$

$$A_f = 10^{(\sum |log(y_i/y)|/n)} \quad (2)$$

$$SEP = \left(\sqrt{\frac{\sum (y_i - y)\text{^}2}{n}}\right) \quad (3)$$

where y_i is the predicted value of the ith observation, y is the measured value of the ith observation, and n is the number of observations.

A_f provides a measure of how close predictions are to observations while B_f gives a measure of systematic under or over prediction by the model.

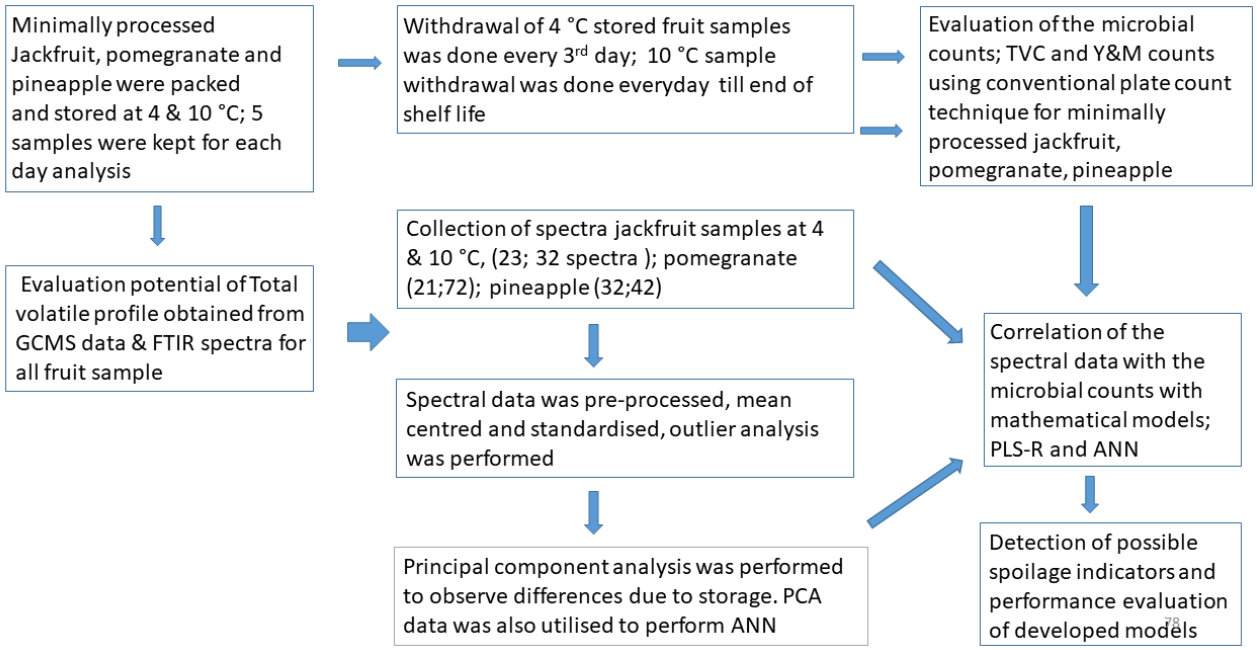


Figure 5. Flow diagram for instrumental based rapid assessment of all the three fruit samples using GCMS & FTIR.

2.2 Preparation of Time Temperature Indicator and its evaluation for real time application

2.2.1 Chemicals

Phenol and agarose were procured from Sigma (Steinheim, Germany). Ammonium per sulphate (free radical initiator) was purchased from HiMedia (Mumbai, India). Sodium carbonate, were purchased from CHEMCO chemicals, Mumbai.

2.2.2 Preparation of minimally processed fruits samples

Pineapple thin slices (0.5cm) were obtained after removing the peel and cutting it into slices, pomegranates were manually processed to obtain arils and jackfruit was processed to obtain ripe bulbs. Minimally processed fruits (75 g each) were enclosed in polystyrene trays (75g each) using cling film (Flexo film wraps ltd. Maharashtra, India). The packaged samples were stored at constant temperature (4, 10, 20 and 37 °C) in high precision (± 0.2 °C) temperature incubator (Panasonic Incubator, MIR-154-PE).

Withdrawal of minimally processed fruit packages stored at different temperature conditions was done on regular time interval depending on the shelf life of the product. Triplicate packages of all the minimally processed fruit from each storage temperature were sampled at appropriate time interval to allow for effective kinetic analysis of microbial growth during microbial spoilage either under isothermal or dynamic storage conditions. All the experiments were conducted twice. Table 5 provides the final storage period for all the fruit samples.

Table 5: Final days of storage for minimally processed fruit samples

Fruit samples	4 °C	10 °C	20 °C	37 °C
Pineapple	528h (22 d)	168h (7 d)	77h (3.2 d)	23h
Pomegranate	668h (27 d)	240h (10 d)	96h (4 d)	28h
Jackfruit	456h (19 d)	264h (11 d)	69h (3d)	28h

h- hours; d - days

2.2.3 Microbial analysis of minimally processed fruit samples

TVC and Y&M counts were evaluated during storage for all the three minimally processed fruit samples as detailed in Section 2.1.3.

2.2.4 Microbial growth rate kinetics in minimally processed fruits

The growth data of both TVC and Y&M in the stored fruit samples at different constant temperatures were modelled as a function of time using Baranyi and Roberts model (1994) [104]. For curve fitting, DMFit program of IFR (Institute of Food Research, Reading, UK) available in Combase (2020) was applied. Curve fitting allowed the estimation of the kinetic parameters like the maximum growth specific rate μ_{max} (h^{-1}).

For determination of activation energy (Eα) of microbial spoilage, the temperature dependence of the maximum growth rate μ_{max} (h^{-1}) of TVC and Y&M for all the fruit samples at different storage temperature was modelled using Arrhenius equation. The logarithmic function of

maximum growth rate was plotted against the reciprocal of temperature to obtain the activation energy (Eα) of the microbial reaction.

$$ln\ \mu_{max} = ln\ A_{ref} - \frac{E\alpha}{R}\left(\frac{1}{T} - \frac{1}{Tref}\right) \quad (4)$$

The logarithmic function of μ_{max} was plotted against the reciprocal of storage temperature (T) in Kelvin. Data was fitted in linear regression and the activation energy (Eα; kJ/mol) was calculated from the slope of the curve.

2.2.5 Preparation of TTI prototypes

TTI hydrogel were prepared containing sodium carbonate (Na_2CO_3), Ammonium per sulfate (APS) and phenol using 2 % agarose. Phenol undergoes oxidative browning in presence of free radical generator APS under alkaline conditions aided by sodium carbonate with colour changing from transparent to dark brown. Aqueous solution of Na_2CO_3 of different concentrations in the range of 23-59 mM and freshly prepared APS in distilled water (21-43 mM) were added in the microtiter 96-well plate. Freshly prepared 2 % (wt/vol) phenol in distilled water (50µl) was added to the mixture. Finally, 100 µl of 2 % agarose solution was added to the reaction mixture, mixed thoroughly and allowed to solidify at 4 °C for 10 mins. Solidified hydrogels of different combinations of TTI (Table 6) were incubated at different temperatures (10, 20, 30, 37 and 45°C) to observe the colour change at regular intervals for different time period to attain the final chronochromic change. These experiments were repeated twice and duplicate samples were kept in microtitre plate. The absorbance of the developed TTI prototypes was recorded at regular intervals at 440 nm using microplate spectrophotometer (Thermo Scientific™, Multiskan Go, Finland).

Table 6: Different combination of TTI prototype with varying concentration of sodium carbonate and APS. Different concentrations of APS are depicted as A (21 mM); B (32 mM); C (43 mM). Concentrations of Na_2CO_3 are shown as 1 (23 mM), 2 (35.4 mM), 3(47 mM), 4 (59 mM).

	Na_2CO_3 ⟶	1	2	3	4
		23 mM	35.4 mM	47mM	59mM
	APS ⇩				
A	21 mM	A1	A2	A3	A4
B	32 mM	B1	B2	B3	B4
C	43 mM	C1	C2	C3	C4

2.2.6 Determination of rate constant and Arrhenius parameter of time temperature indicator

According to the indicator kinetics described by Taoukis and Labuza [105], the color change of the TTI can be represented as follows

$$X = \frac{-d[A]}{dt} = k\,[A]^n = k_A \exp(-Ea/RT)\,[A]^n \quad (5)$$

Where X is rate of change in colour of TTI, A is the absorbance measured at 440 nm, n is the reaction order, and k is the reaction rate constant. The rate constant is an exponential function of inverse absolute temperature, T (°K) given by the shown Arrhenius expression, where k_A is a constant and Ea is the activation energy of the reaction that controls quality loss and R is the universal gas constant (8.314 kJ/mol).

The equation (1) can be transformed as the following one

$$\log X = \log k + n \log [A] \quad (6)$$

Log of rate of change in absorbance (Log X) was plotted versus log of absorbance (log [A]). Value of k was calculated from intercept of this plot. For every TTI prototype, value of k was calculated at each storage temperature.

The temperature dependence of a chemical reaction is given by Arrhenius expression. Therefore, the estimation of activation energy of the chemical reaction of TTI can be described by the Arrhenius equation expressed in logarithmic terms

$$lnk = ln\, A_{pef} - \frac{E\alpha}{R}\left(\frac{1}{T} - \frac{1}{Tref}\right) \quad (7)$$

Where k is rate constant (h^{-1}), Ea is activation energy (kJ/mol), T is temperature (in K), A_{pef} is pre-exponential factor (h^{-1}) and R is universal gas constant.

The logarithmic function of k was plotted against the 1/T where T is storage temperature in Kelvin. Data was fitted in linear regression and the activation energy (Ea; kJ/mol) was calculated from the slope of the curve.

Statistical analysis: A full factorial screening experimental design (Design Expert 8.0, Stat-Ease Inc., USA) was performed for investigating the effect of individual variable (Sodium carbonate and APS) on the color development reaction of TTI as determined in terms of Ea. The range of the variable selected was: Na_2CO_3(24-59mM) and APS (21-43 mM) consisting of 12 experiments in triplicate. Experimental data were fitted in third order polynomial (quadratic) equations. Equations obtained after fitting were utilized to generated 3D plot to elucidate the relationship between response and experimental levels of variable.

2.3.7 Camera based rapid read out system to determine the microbial counts on a real time basis

In order to check the feasibility of developed TTI's for their use in market conditions, the selected candidate TTI hydrogels were prepared in petri plates and allowed to solidify at 4 °C for 10 mins. Later, TTI hydrogels in petriplates were kept on ice pack and cut in circles (diameter-1cm), packed in low density polyethene (LDPE) films and placed along with the minimally processed pineapple, pomegranate & jackfruit packages. Performance of TTI was

evaluated during storage both isothermally at 10 °C for 7 days and under non-isothermal conditions (to mimic possible market conditions) with periodic 24 h cycle of 16 h at 10 °C and 4 h at 15 °C then finally for 4 h at 20 °C in high precision (± 0.5 °C) incubation chambers (MIR-153, Sanyo Electric Co., Osaka, Japan) for 5 days. Different colour intensity of TTI was captured in DSLR camera at different period of storage and microbial counts were estimated simultaneously as described in microbial analysis section above. The RGB scores were estimated by employing image processing packages in R. ΔRGB scores were computed and were plotted against log CFU/g counts and their correlation established.

$$\Delta RGB = \sqrt{(R_{ref} - R_i)^2 + (G_{ref} - G_i)^2 + (B_{ref} - B_i)^2} \quad (8)$$

Where, reference value of RGB are at 0 hr just after TTI preparation whereas, i^{th} values of RGB are at different time points.

The performance of the TTI based on the ΔRGB for estimating TVC and Y&M were evaluated by estimating accuracy factor (A_f) between observed and predicted counts expressed as equation (2).

Chapter 3

Results & Discussion

3.1 Rapid assessment of microbial quality of minimally processed fruits using instrumental techniques

3.1.1 Microbial analysis of minimally processed fruits

The tropical Indian fruits selected for the study such as jackfruit (*Artocarpus heterophyllus*), pomegranate (*Punica granatum*) and pineapple (*Ananas comosus*) are rich in nutritive value. They have wide acceptance in Indian market as minimally processed products due to cumbersome peeling and cutting techniques involved. Minimally processed packed jackfruit, pomegranate and pineapple were processed as described in materials and methods (section 2.1.1) and stored at 4 and 10 °C for different time intervals to monitor the growth of total viable counts (TVC) and yeast &mould counts (Y&M) during storage.

3.1.1.1 Microbial analysis of jackfruit

The results for TVC and Y&M on packaged jackfruit samples stored at 4 ºC and 10 ºC are shown in Figure 6A and Figure 6B, respectively. Freshly packed samples at 0 day had TVC of <4 Log_{10}CFU/g. The TVC on freshly packaged samples indicates level of surface contamination resulting from various processing steps such as cutting, washing and packaging. A significant ($p<0.05$) increase in TVC was observed during storage at both the temperatures studied. Samples stored at 4 ºC demonstrated an increase in the TVC to 6 Log_{10}CFU/g in 19 days, while for those stored at 10 ºC the TVC reached 9Log_{10}CFU/g after 6 days. Y&M counts were < 2.5 Log_{10} CFU/g for freshly packed samples. Y&M increased to 4and 6.6 Log_{10} CFU/g for samples kept at 4 and 10 °C, respectively, at the end of storage period. Significantly ($p<0.05$) higher microbial growth was observed for samples stored at 10°C as compared to samples kept at 4°C.

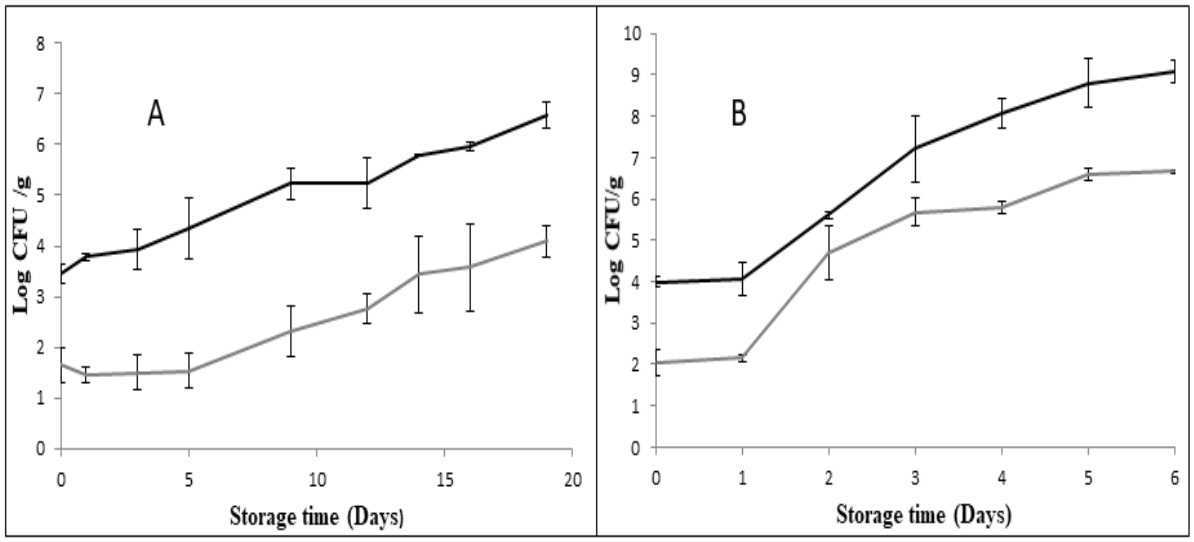


Figure 6. Total viable count (—) and Yeast & Mould count (—) during different storage period at 4 °C(A)and 10 °C (B) for minimally processed Jackfruit. Values represented here are mean ± SD.

3.1.1.2 Microbial analysis of pomegranate

The results for TVC and Y&M on packaged pomegranate samples stored at 4 ºC and 10ºC are shown in Figure7A and Figure7B, respectively. The initial microbial count (day 0, freshly packed samples) was ≤3 LogCFU/g for both TVC and Y&M. A very slow growth for both TVC and Y&M was recorded for samples stored at 4 °C. Microbial counts (TVC and Y&M) reached to 5 Log_{10}CFU/g on 28th day of storage at 4°C. However, a rapid increase in both TVC and Y&M was observed during storage at 10 °C (Figure7B). After storage period of 3 and 7 days at 10 °C the TVC increased to 5.8 ± 0.168 and 7.0 ± 0.44 Log_{10}CFU/g, respectively. Y&M counts increased to 6 ± 0.38 and 7.1 ± 0.365Log_{10}CFU/g on day 4 and 7, respectively at 10 °C. These results indicate that the packaged pomegranate sample have undergone a rapid deterioration in quality during storage at 10 °C. Interestingly, higher Y&M counts as compared to TVC counts were recorded in minimally processed pomegranate sample at all storage conditions (Figure 7). Similar observations were also noted by Caleb *et al*. [69].

This could be attributed to the fact that the lower pH (3.2) in pomegranate favours the growth of Y&M in comparison with aerobic mesophilic bacteria [106, 107].

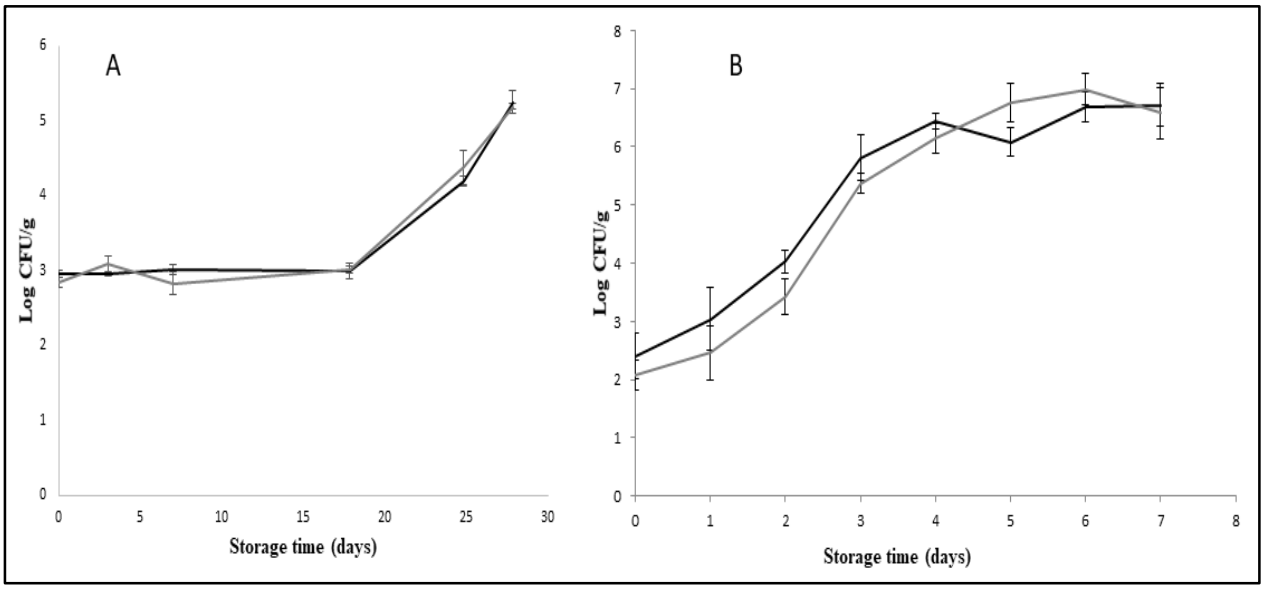


Figure 7. Total viable count (——) and Yeast & Mould count (—) during different storage period at 4 °C(A)and 10 °C (B) for minimally processed pomegranate. Values represented here are mean ± SD.

3.1.1.3 Microbial analysis of pineapple

The initial microbial counts of freshly packed minimally processed pineapple on day 0 were found to be 3.49 ± 0.31 and 3.56 ± 0.42 Log_{10} CFU/ g for TVC and Y&M, respectively as shown in Figure 8. Samples stored at 4 °C demonstrated a marginal but significant ($p<0.05$) increase in the microbial counts to 4.77 ± 0.21 and 4.89 ± 0.24 Log_{10} CFU/ g for TVC and Y&M, respectively by the end of the storage period at 22 days (Figure 8A). Although, microbial counts demonstrated an increase of only 1 Log_{10}CFU/g even after the storage period of 22 days, physiological deterioration with browning and water loss was observed in pineapple slices stored at 4°C after10 days of storage. On the contrary, samples stored at 10 ^{0}C demonstrated a rapid increase in TVC and Y&M with counts reaching to 7.92 ± 0.32 and 7.66± 0.15 Log_{10} CFU/ g, respectively by the end of the storage period of 7 days (Figure8B).

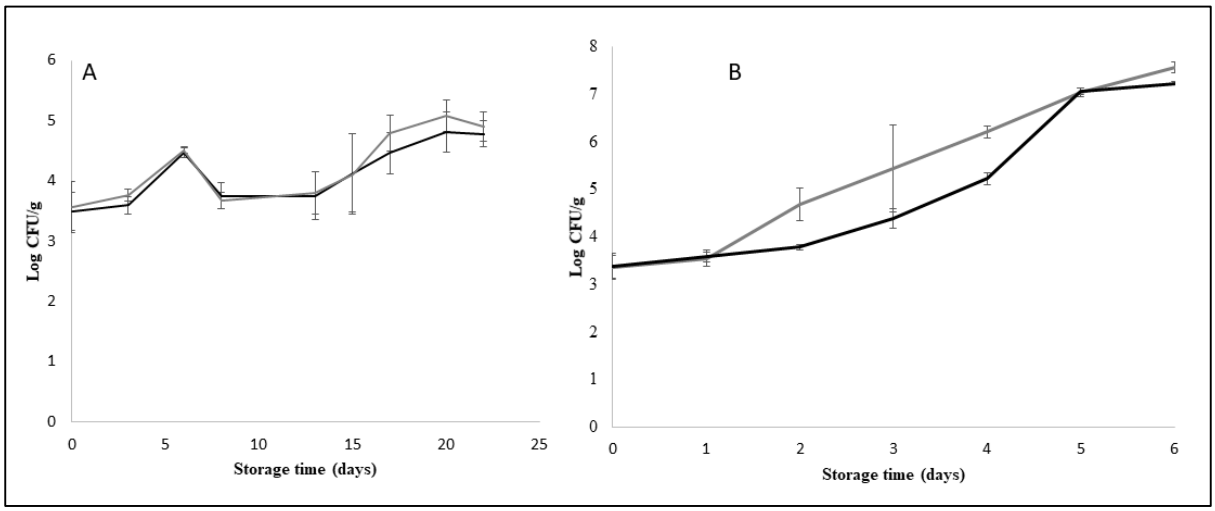


Figure 8. Total viable count (——) and Yeast & Mould count (—) during different storage period at 4 °C (A)and 10 °C (B) for minimally processed pineapple. Values represented here are mean ± SD.

3.1.1.4 Inference from microbial analysis

The microbial load (TVC and Y&M) on fresh minimally processed fruit samples were obtained in the range of 10^2 to 10^4 CFU/g. Total counts on any fresh cut products just after processing are reported in the range of 10^3 to 10^6 CFU/g [19]. Microbial analysis revealed that temperature had significant effect on microbial growth ($p<0.05$). Sample stored at 4 °C did not show significant increase in microbial growth however the counts increased by 1 Log_{10} CFU/g even after a prolonged storage period of 19, 28 and 22 days for minimally processed jackfruit, pomegranate and pineapple, respectively. Similar observations were demonstrated in the previous studies wherein it was observed that microbial counts did not increased significantly at 4 °C for minimally processed pineapple [24, 108]. Samples stored at 10 °C showed an increase in microbial growth. All the three fruit samples exceeded permissible microbial limits beyond 3 or 4 days suggesting a short microbiological shelf life. The European standards limits maximum TVC not to exceed 10^7 CFU/g and Y&M counts not greater than 10^5 CFU/g during the entire storage period for minimally processed produce [109]. Thus, it can be concluded that

minimally processed products have short shelf life of few days primarily due to microbial growth beyond a storage temperature of 4 °C. Moreover, market samples are stored at temperature between 8 to 15 °C, that are additionally subjected to temperature abusive conditions during handling, transport and storage which may lead to rapid microbial growth and quality deterioration [110]. While minimally processed products are prone to rapid microbial spoilage, traditional plate count methods require long time (48-72 h) to assess microbial quality and thus may not be applicable for such products. Therefore, there is a need for development of rapid methods of assessment for microbial quality of minimally processed products.

3.1.2 Biochemical changes in packed minimally processed fruit samples during storage

Microbial growth during storage could lead to several biochemical changes in the product. These biochemical changes if monitored could possibly provide an indication of extent of microbial spoilage in the product. Microbial spoilage leads to generation of volatile organic compound (VOC), several of them contributes to off-odours specific to spoilage. In this study, VOCs were targeted for monitoring product microbial quality by correlating them with microbial counts. Changes in volatile constituents during the product deterioration can be rapidly monitored by HS-SPME in combination with GCMS [68]. On the other hand, changes in the non-volatile constituents can be monitored by FTIR. It can be used directly on the surface of food to produce biochemical interpretable “fingerprints” (metabolic snapshots), thus enabling early detection of microbial spoilage [71]. Therefore, in the present work, changes in biochemical constituents were monitored both by HS-SPME-GCMS and FTIR.

3.1.2.1 Analysing biochemical changes using GCMS

The total volatile profile of minimally processed jackfruit, pomegranate and pineapple during storage was studied using HS-SPME-GCMS. Qualitative and quantitative distribution of different volatiles at the beginning and end of the storage period at 4 and 10 °C is listed in Table 6, 7, 8, respectively. Changes in the volatile profile during storage at both the temperatures studied are also depicted in Figure 9, 10 and11 for the respective minimally processed products.

3.1.2.1.1 Jackfruit

A total of 45 volatile compounds were detected in minimally processed jackfruit at day zero which is in accordance with that reported in previous studies [111,112]. The profile demonstrated predominance of esters followed by alcohol, aldehyde and ketone. However, there was complete absence of terpenoids, which was unique to jackfruit [113]. The major compounds isolated were methyl isovalerate, butyl acetate, 1-butanol 3-methyl acetate, 1-butanol 2-methyl acetate, isoamyl isovalerate. The predominance of sweet and fruity aroma is reported to be due to dominance of esters imparting jackfruit aroma [114].

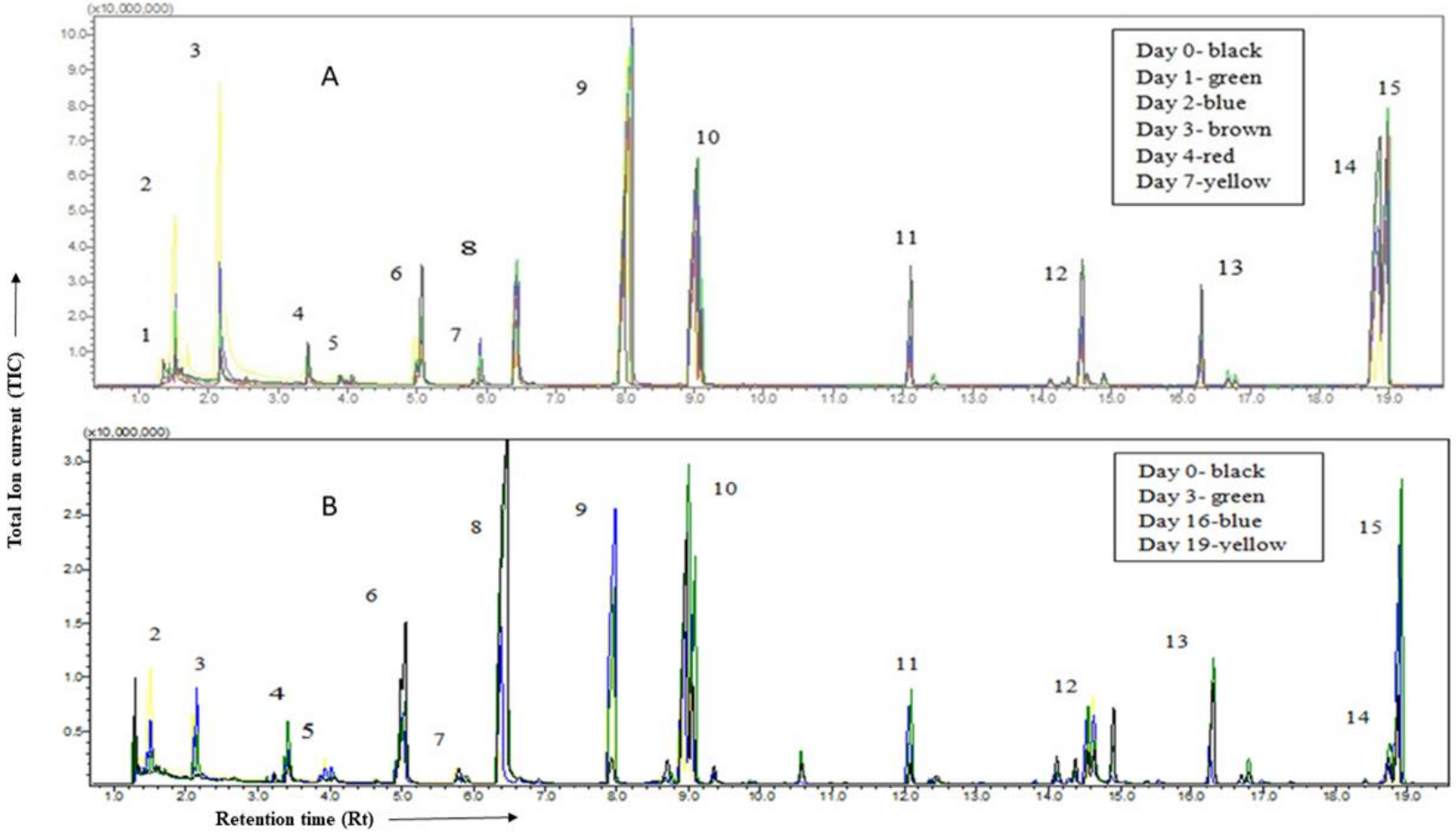


Figure 9: Volatile profile of minimally processed Jackfruit during storage at A) 10 °C and B) 4 °C.

Significant changes ($p<0.05$) in the volatiles profile of jackfruit samples was observed during storage at 4 and 10 °C (Figure 9). With respect to alcohols, varying trends were observed at different storage temperature. Ethanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 1-butanol and 2-ethyl hexanol showed an increase during storage. Their concentrations were significantly ($p<0.05$) higher at 10 °C when compared to 4 °C (Table 6).1-hexanol and 1-nonanol showed a decreasing trend at both the storage temperatures, with a greater decrease at 4 °C due to longer storage duration up to 19 days. Aldehydes such as 2-butenal, octanal, decanal, 2-decenal, dodecanal were either unaffected or showed a slightly decreasing trend during storage. However, acetaldehyde and hexanal exhibited a significant increase at both the temperatures with greater increase at the higher storage temperature (10 °C). Esters such as methyl acetate and ethyl acetate showed an increasing trend at both the storage temperatures**.** Isoamyl acetate, methyl isovalerate, ethyl butyrate, butyl acetate, butanoic acid 3-methyl ethyl ester, 1-butanol 3-methyl acetate, 2-butanol 3-methyl acetate, propyl isovalerate, ethyl hexanoate, isobutyl isovalerate, hexyl acetate, butyl isovalerate, amyl butyrate, iso amyl valerate, iso amyl isovalerate, n-amyl isovalerate showed an increased content at both the temperatures studied. However, at both the storage temperatures, volatiles such as hexyl acetate, hexyl isovalerate showed a decreasing trend.

Table 6: Headspace volatile concentrations of minimally processed jackfruit during storage at 4 and 10 °C

Sr. No	Compound Name	KI	Day 0	Day 19 (at 4 °C)	Day 7 (at 10 °C)
1	acetaldehyde	412.24	0.285±0.09	110.92±19.1	966.3408± 76.5
2	ethanol	512.83	1.77±0.12	539.8976±71.3	4693.7±517.3
3	methyl acetate	550.56	17.07±3.40	330±18.65	515.17±67.34
4	ethyl acetate	616.93	17.07±3.40	703±108.65	5502.17±627.34
5	2-butenal	652.21	4.01±0.65	7.01±1.55	8.8±3.37
6	1-butanol	663.21	3.24±1.5	6.81±3.11	7.2±2.50
7	propyl acetate	714.84	54.09±41.00	135.63±83.48	1032.63±478.15
8	1-butanol, 3-methyl-	732.54	4.24±1.73	3.42±0.56	294.07±53.68
9	1-butanol, 2-methyl-	735.75	2.03±0.78	59.79±20.27	270.23±110.26
10	2-butenal, 2-methyl-	739.0	8.17±2.58	44.91±26.05	4.25±2.68
11	isoamyl acetate	775.06	95.56±24.26	188.31±20.65	2691.84±732.18
12	methyl isovalerate	777.96	175.61±20.42	110.94±14.04	1478.22±304.65
13	hexanal	804.25	12.42±1.30	13.68±3.05	26.01±10.84
14	ethyl butyrate	806.73	14.15±8.41	24.01±4.76	2.26±0.92
15	butyl Acetate	820.54	982.35±58.32	812.32±371.82	3326.6±781.53
16	butanoic acid, 3-methyl-, ethyl ester	858.91	22.81±5.81	646.30±52.08	7713.18±593.26
17	1-hexanol	876.85	29.03±18.23	8.52±4.17	13.61±6.68
18	1-butanol, 3-methyl-, acetate	883.24	659.59±138.11	860.92±356.31	5453.79±1028.45
19	1-butanol, 2-methyl-, acetate	885.71	194.93±111.63	860.92±314.81	3469.46±1023.24
20	n-amyl acetate	920.38	21.49±4.10	2.09±0.17	3.10±0.585
21	propyl isovalerate	954.44	16.03±10.38	60.47±0.42	184.01±76.78

Sr. No	Compound Name	KI	Day 0	Day 19 (at 4 °C)	Day 7 (at 10 °C)
22	benzaldehyde	962.51	5.88±1.52	16.92±4.24	39.80±5.89
23	ethyl hexanoate	1004.21	0.29±0.16	6.90±1.92	30.32±12.85
24	octanal	1006.31	15.59±3.11	12.51±4.64	14.86±2.14
25	isobutyl isovalerate	1010.18	30.62±11.62	85.68±22.60	794.31±236.11
26	p-dichlorobenzene	1012.5	35.05±7.48	158.11±21.53	0
27	hexyl acetate	1017.89	118.76±47.08	8.49±3.19	36.18±11.23
28	2-ethyl hexanol	1033.72	1.25±0.37	9.10±1.54	5.68±1.23
29	butyl Isovalerate	1050.11	57.73±6.49	24.33±11.52	129.14±36.24
30	isoamyl isobutyrate	1059.37	9.58±3.15	2.64±1.16	26.70±14.122
31	amyl butyrate	1061.69	10.73±4.07	13.38±6.16	41.72±12.23
32	1-nonanol	1075.51	10.17±6.48	1.05±1.24	4.03±2.33
33	isoamyl valerate	1107.53	31.47±23.96	22.27±9.13	1862.79±927.17
34	iso amyl isovalerate	1111.89	79.14±64.85	406.38±133.30	522.75±287.63
35	n-amyl isovalerate	1145.89	0.16±0.14	0.36±0.08	7.26±3.19
36	benzenepropanal	1165.17	1.18±0.72	11.44±7.68	5.06±1.35
37	dodecane	1200	2.61±0.22	16.00±6.62	12.05±2.34
38	decanal	1207.19	20.19±3.11	39.85±18.57	7.85±4.74
39	hexyl isovalerate	1243.80	6.3±3.13	2.87±1.36	1.68±0.86
40	2-decenal	1264.69	0.44±0.30	2.39±0.87	1.68±0.86
41	tridecane	1300	1.26±0.11	7.95±2.52	8.07±1.05
42	trans-dodec-5-enal	1383.10	3.07±0.37	3.6±0.75	0.29±0.02
43	dodecanal	1406	1.29±0.159	23.97±14.66	4.38±2.97
44	Propanoic acid, 2,2-dimethyl-, 2-phenylethyl ester	1492.12	2.56±0.07	12.44±5.12	21.24±4.82
45	butanoic acid, 3-methyl-, 3-phenylpropyl ester	1609.96	0.82±0.33	6.14±2.36	19.19±4.35

KI-kovat index; concentrations of volatiles expressed in µg/kg

3.1.2.1.2 Pomegranate

In total 10 volatiles were identified in GC profile of pomegranate at both the storage temperature (Figure 10). They can be classified as aldehydes: hexenal, 2-hexenal; ketone: 2-octanone, ester: ethyl acetate, alcohols: ethanol, 1-heptanol, 2-ethyl, 1-hexanol, mono terpene alcohol such as terpineol, 4-terpineol, p-cymene-2-ol. Very few changes were observed in the volatile profile of pomegranate (Table 7) at both the storage temperature. Hexanal and 1-hexanol however, showed a significant increase at 10 °C ($p<0.05$) but remain unchanged at 4 °C. Moreover, ethyl acetate showed a low but significant decrease in concentration during storage at both the storage temperatures. No other marked trend could be observed in the volatile profile for minimally processed pomegranate during storage.

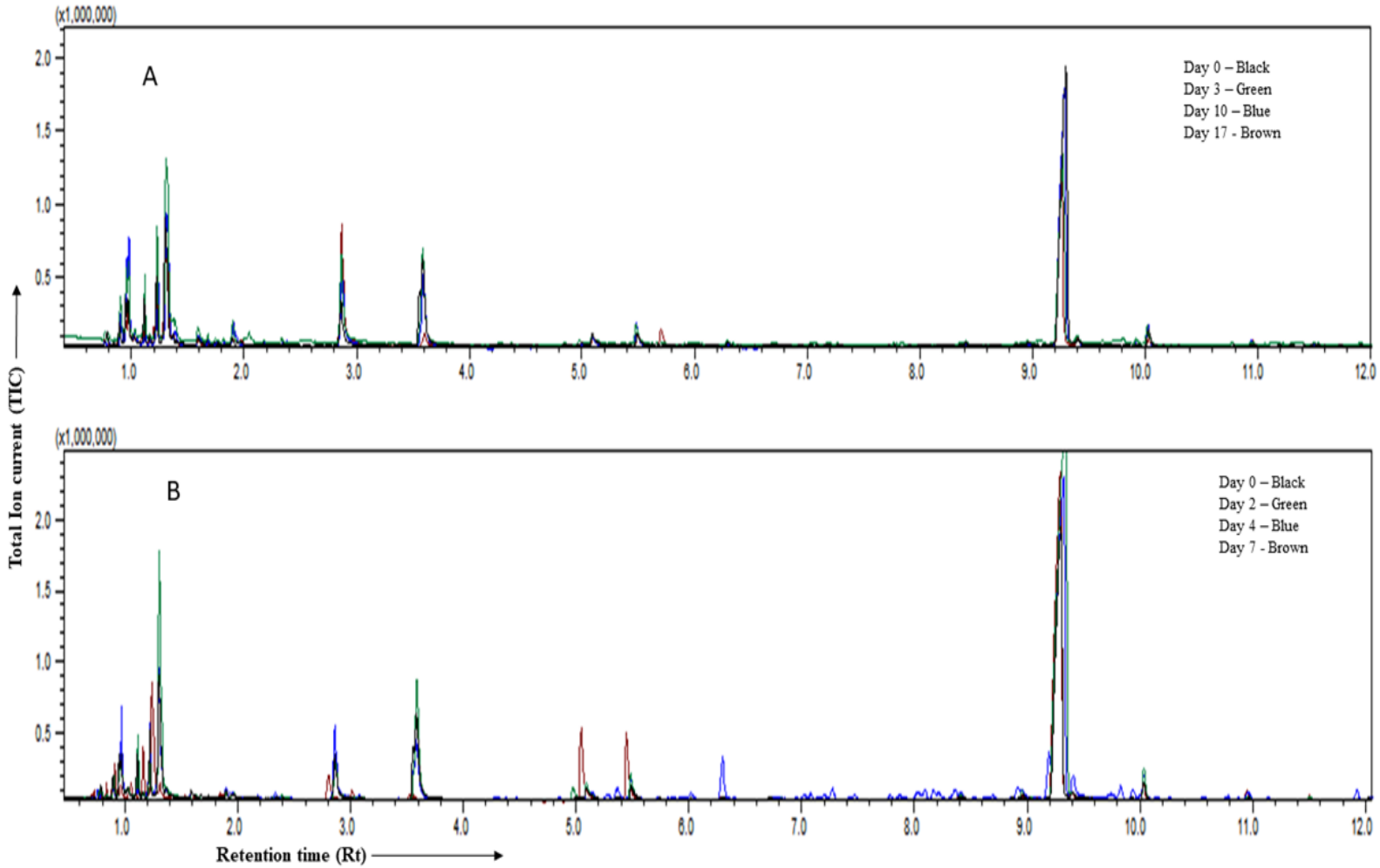


Figure 10: Volatile profile of minimally processed Pomegranate during storage at A) 4 °C and B) 10 °C.

Table 7: Headspace volatile concentrations of minimally processed pomegranate during storage at 4 and 10 °C.

Sr. No.	Compound name	KI	Day 0	Day 22 (at 4 °C)	Day 7 (at 10 °C)
1	ethanol	515.13	0.37±0.37	1.28±0.73	0.84±0.23
2	ethyl acetate	616.93	7.97±1.78	4.79±0.78	5.41±1.02
3	hexanal	801.42	8.29±2.05	1.42±0.66	5.26±0.12
4	1-hexanol	876.85	1.08±0.45	0.825±0.13	4.10±0.98
5	2-heptanol	901.59	0.11±0.04	0.23±0.12	0.48±0.11
6	2-octanone	989.28	0.23±0.06	0.19±0.04	0.20±0.06
7	1-hexanol, 2-ethyl-	1033.72	1.32±0.34	0.37±0.12	0.98±0.27
8	4-terpineol	1169.77	0.018±0.003	0.099±0.018	0.067±0.013
9	α-terpineol	1182.55	0.79±0.18	0.75±0.11	0.81±0.24
10	p-cymene-2-ol	1295	0.81±0.32	0.28±0.11	0.77±0.34

KI-kovat index; concentrations of volatiles expressed in µg/kg

3.1.2.1.3 Pineapple

GCMS profile showed presence of 62 volatile compounds in the stored pineapple samples with a variation in volatile constituents under different temperature conditions (Figure 11). Table 8 shows the quantitative distribution of the 40 identified compounds that were present at the beginning and end of storage period. The majority of identified compounds were alcohol, esters, ketones, terpenes and aldehydes reported previously [115, 116].

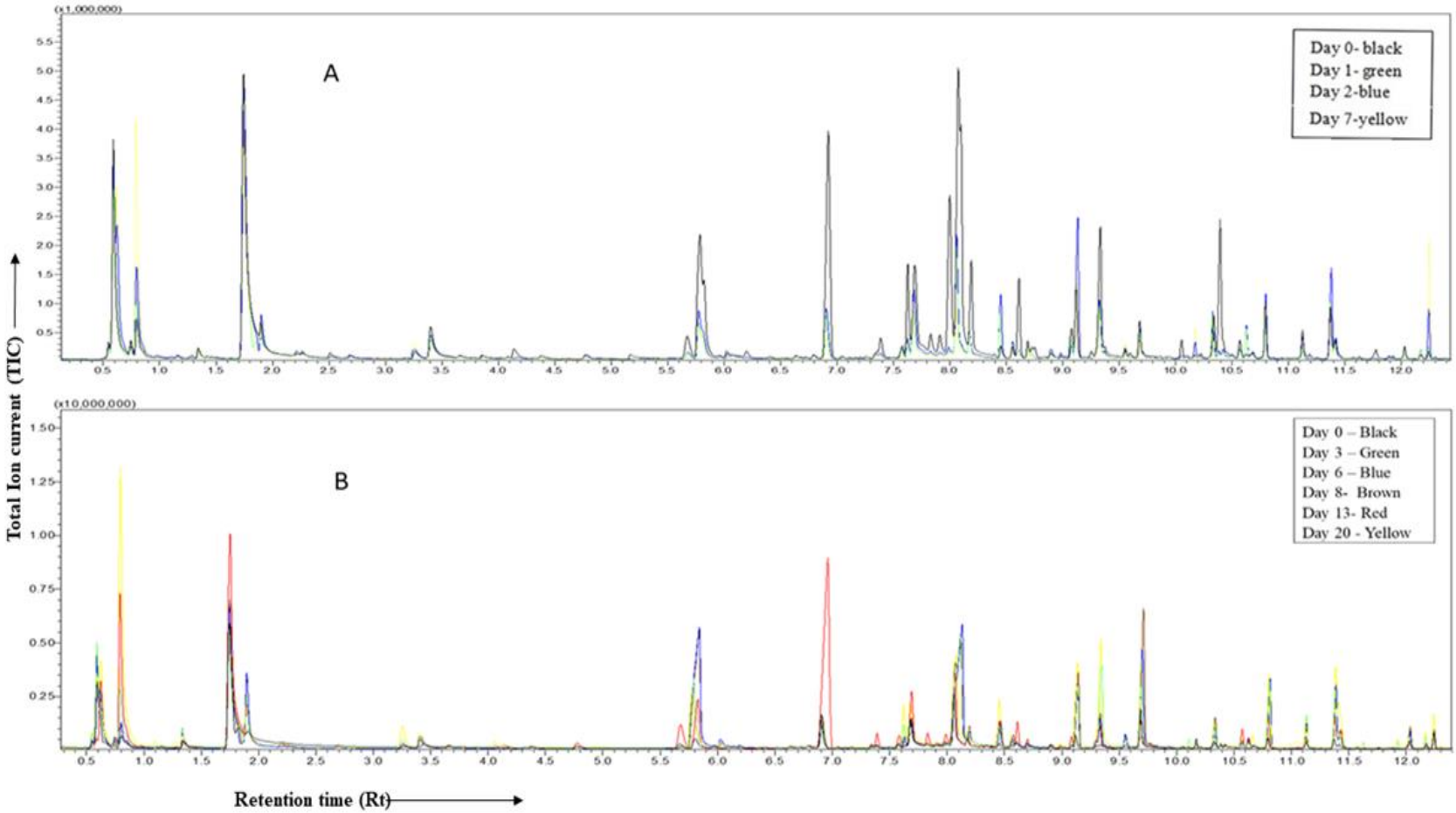


Figure 11: Volatile profile of minimally processed pineapple during storage at A) 10 °C and B) 4 °C.

Volatile profile demonstrated changes occurring during storage at 4 and 10 °C in the stored pineapple samples (Figure 11). Alcohols were found to be stable with no significant change ($p>0.05$) at 4 °C with the exception of 2-ethyl-1-hexanol that showed a decrease. However, a significant increase ($p<0.05$) in concentration of alcohols was observed at 10 °C (Table 8). Ethanol, 1-hexanol, 1-heptanol, 2-heptanol, 3-methyl-1-butanol, phenyl ethanol and 2-ethyl-1-hexanol showed no significant change ($p>0.05$) at 4 °C and an increase at 10 °C. Among the ketones, 2-heptanone and acetophenone showed a decreasing trend at 4 °C and an increase at 10 °C. Esters identified can be grouped into methyl esters and their corresponding ethyl esters. Methyl esters detected include methyl butanoate, methyl 2-methyl butanoate, methyl hexanoate, methyl-3-hexenoate, methyl 3-(methyl thio) propanoate, methyl octanoate. The corresponding ethyl esters were ethyl butanoate, ethyl 2-methyl butanoate, ethyl hexanoate, ethyl 3-hexenoate, ethyl 3-(methyl thio) propanoate, ethyl octanoate. It was observed that methyl esters showed a decrease at both the temperatures with the exceptions of methyl 2-methyl butanoate that showed an increase at 10 °C. Contrasting results were observed for ethyl esters that remain unchanged at 4 °C while a significant increase ($p<0.05$) was observed at 10 °C. Methyl, ethyl and propyl acetates showed an increase at both the temperatures. However, 1-butanol, 3-methyl-, acetate and 1-butanol, 2-methyl-, acetate showed no change at 4 °C while it increased at 10 °C.

Table8: Headspace volatile concentrations of minimally processed Pineapple during storage

Sr. No	Compound	KI cal	Day 0	Day 22 (For 4 °C)	Day 7 (For 10 °C)
1	ethanol	512.13	6.96 ± 0.79	6.80 ± 0.81	130 ± 12.57
2	methyl acetate	545.96	0.00	48.48 ± 2.85	125 ± 11.38
3	ethyl acetate	616.63	12.59 ± 1.54	106.06 ± 7.01	1636 ± 123.12
4	n propyl acetate	704.44	0.05 ± 0.01	0.74 ± 0.08	15.45 ± 1.94
5	methyl butanoate	710.89	6.18 ± 1.07	1.02 ± 0.64	4.61 ± 0.45
6	3-methyl, 1-butanol	723.70	0.57 ± 0.03	0.64 ± 0.08	9.34 ± 1.75
7	methyl, 2-methyl butanoate	770.26	11.87 ± 0.82	3.30 ± 0.88	50.51 ± 6.28
8	hexanal	801.42	1.06 ± 0.02	3.86 ± 0.92	0.50 ± 0.07
9	ethyl butanoate	803.34	0.00	1.28 ± 0.07	82.39 ± 3.42
10	isobutyl acetate	828.14	2.16 ± 0.25	0.42 ± 0.06	0.00
11	ethyl 2-methylbutanoate	841.75	2.30 ± 0.36	3.60 ± 1.23	82.39 ± 3.44
12	1-hexanol	865.61	1.90 ± 0.04	3.56 ± 0.12	3.06 ± 1.60
13	1-butanol, 3-methyl acetate	871.64	0.17 ± 0.04	0.24 ± 0.03	114.82 ± 24.90
14	1-butanol, 2-methyl acetate	874.75	3.13 ± 0.77	0.11 ± 0.05	30.59 ± 4.68
15	2- heptanone	888.29	0.108 ± 0.01	0.207 ± 0.05	18.32 ± 2.04
16	2-heptanol	901.59	0.00	0.239 ± 0.02	9.59 ± 2.24
17	α thujene	929.89	11.59 ± 1.49	3.14 ± 0.42	4.94 ± 0.58
18	methyl hexanoate	934.82	107.96 ± 2.58	3.04 ± 0.58	63.88 ± 4.67
19	methyl 3-hexenoate	943.53	0.31 ± 0.08	0.58 ± 0.15	2.21 ± 1.08
20	α sabinene	975.25	97.91 ± 2.04	20.36 ± 0.61	44.29 ± 6.43
21	1-heptanol	979.95	0.00	2.15 ± 0.67	2.26 ± 0.36
22	ethyl-3-hexenoate	1009.02	0.19 ± 0.07	0.63 ± 0.05	7.39 ± 2.99

Sr. No	Compound	KI cal	Day 0	Day 22 (For 4 °C)	Day 7 (For 10 °C)
23	(+)-4-carene	1013.53	6.22 ± 0.67	1.23 ± 0.22	2.16 ± 0.96
24	o-cymene	1022.55	4.84 ± 1.18	25.20 ± 1.25	7.04 ± 2.20
25	D- limonene	1026.89	65.41 ± 3.20	25.20 ± 0.66	84.20 ± 8.58
26	3-(methylthio) methyl propanoate	1029.78	182.98 ± 17.13	26.52 ± 3.70	69.45 ± 5.29
27	2-ethyl-1-hexanol	1033.74	14.17 ± 4.73	15.45 ± 2.61	21.98 ± 3.05
28	γ terpinene	1057.01	9.26 ± 2.57	2.47 ± 0.86	5.75 ± 1.51
29	acetophenone	1063.97	0.45 ± 0.07	1.45 ± 0.08	2.57 ± 1.01
30	dimethyl malonate	1074.83	0.40 ± 0.27	0.79 ± 0.05	6.86 ± 2.88
31	2,3-butanediol,diacetate	1077.70	0.72 ± 0.029	1.81 ± 0.71	16.65 ± 4.9
32	3-(methylthio) ethyl propanoate	1097.38	57.11 ± 1.01	26.52 ± 1.70	428.04 ± 169.39
33	phenyl ethanol	1109.17	0.00	0.07 ± 0.01	41.95 ± 4.44
34	methyl octanoate	1121.89	107.51 ± 2.32	97.61 ± 0.45	74.37 ± 16.41
35	Menthol	1166.01	18.51 ± 3.09	10.21 ± 2.43	62.76 ± 30.65
36	L-4-terpineol	1169.77	55.16 ± 8.02	1.36 ± 0.54	3.65 ± 1.43
37	L-α-terpineol	1182.55	1.45 ± 0.27	5.47 ± 1.08	20.81 ± 4.89
38	benzene methanol, alpha.-methyl-, acetate	1186.51	1.21 ± 0.07	5.16 ± 0.01	20.79 ± 7.82
39	ethyl octanoate	1189.43	0.26 ± 0.12	0.55 ±0.06	55.56 ± 12.82
40	2-phenylethyl acetate	1252.11	0.46 ± 0.60	0.43 ±	3.72 ± 0.85

KI-kovat index; concentrations of volatiles expressed in µg/kg

3.1.2.1.4 Inference from headspace volatile analysis of fruit samples

Concentration of volatiles remained more or less unaffected with no significant changes for samples stored at 4 °C in case of all the three fruit samples. However, major changes in volatile constituents could be noted in all the three fruits for storage temperature of 10 °C. Major changes in the volatile profile were observed for ethanol and ethyl acetate peak for jackfruit and pineapple samples, however**,** pomegranate did not demonstrate similar trend for these volatiles. Moreover, several other compounds also demonstrated significant changes at 10 °C. The packaging film used in the present study, cling film, does not result in anaerobic conditions. Several previous studies on minimally processed products using cling films report package headspace atmosphere values close to atmospheric values [117].

It was observed that concentration of ethanol increased during storage at 10 °C in case of jackfruit and pineapple. Ethanol is produced in fresh cut pineapple by several specific spoilage yeast such as C. *sakae* and C. *argentea* under oxygen limiting condition [118] Additionally, Crab free positive S. cerevisiae is also known to produce alcohol under glucose rich condition wherein glucose is converted to pyruvate. This increases the activity of pyruvate decarboxylase and alcohol dehydrogenase that in turn produces alcohol to recycle depleted NAD^+ [67]. Branched chain alcohol such as 2-methyl-1-butanol and 3-methyl-1-butanol showed an increase at 10 °C in case of jackfruit and pineapple. It has been reported that production of these alcohols can be due to amino acid metabolism of leucine and iso-leucine by the microorganisms present, primarily by *Pseudomonads* that can bring about these metabolic changes under aerobic condition [119, 120]. Significant increase in 2-ethyl hexanol was observed in case of jackfruit and pineapple while 1-hexanol showed increase in pomegranate. Long chain aliphatic alcohols such as 1-hexanol, 1-heptanol and 2-heptanol are formed from the corresponding reduction of long chain fatty acid by *Enterobacteriaceae* family [121]. These detected alcohols have also been reported as possible products of lipid oxidation [122, 123].

Ketones probably originate from several fatty acid oxidation reactions chemical auto-oxidation and enzymatic α- or β-oxidation [123, 124]. 2-Heptanone showed an increase at 10 °C in case of pineapple samples during storage. Some methyl ketones can be derived from a lipolytic process as well as from several other pathways, such as alkane degradation by *Pseudomonas* through a unique alpha-oxidation, with no change in the carbon skeleton [125]. Methyl ketones are also formed by bacterial dehydrogenation of secondary alcohols, a reaction that appears to be part of the alkane oxidation sequence [125]. Hexanal showed an increasing trend in case of jackfruit and pomegranate at 10 °C. Saturated aldehydes with more than five carbon atoms can be produced from oxidative degradation of fat. It has been reported to be derived from oxidation of unsaturated omega-6 fatty acid [126]. Significant increase in ethyl acetate concentration was observed in jackfruit and pineapple and several other ethyl ester derivatives also showed increase at 10 °C (Table 6&8). Ethyl esters are also known to be formed by pseudomonads at increasing temperature under aerobic conditions [120]. Yeasts are reported to produce acetate esters and medium chain ethyl esters during fermentation [127]. In conclusion, several biochemical changes in volatile constituents could be clearly observed due to microbial growth during storage in minimally processed fruit sample.

3.1.2.2 Analysis of biochemical changes using FTIR

The biochemical changes associated with minimally processed fruits during storage at 4 and 10 °C were studied using FTIR. FTIR spectra in the range of 4000 to 650 $cm^{-1}$was obtained for all the three minimally processed fruit samples as depicted in Figure12, 13, and 14, respectively for jackfruit, pomegranate and pineapple. Since the FTIR spectra in the fingerprint region carried maximum information, it was utilised for further analysis. FTIR spectra was obtained in the range of 2000 to 800 cm^{-1} for minimally processed jackfruit samples (Figure 15), while for pomegranate and pineapple a range between 2000 to 1000 $cm^{-1}$was found

to be appropriate as shown in Figure 16&17, respectively.

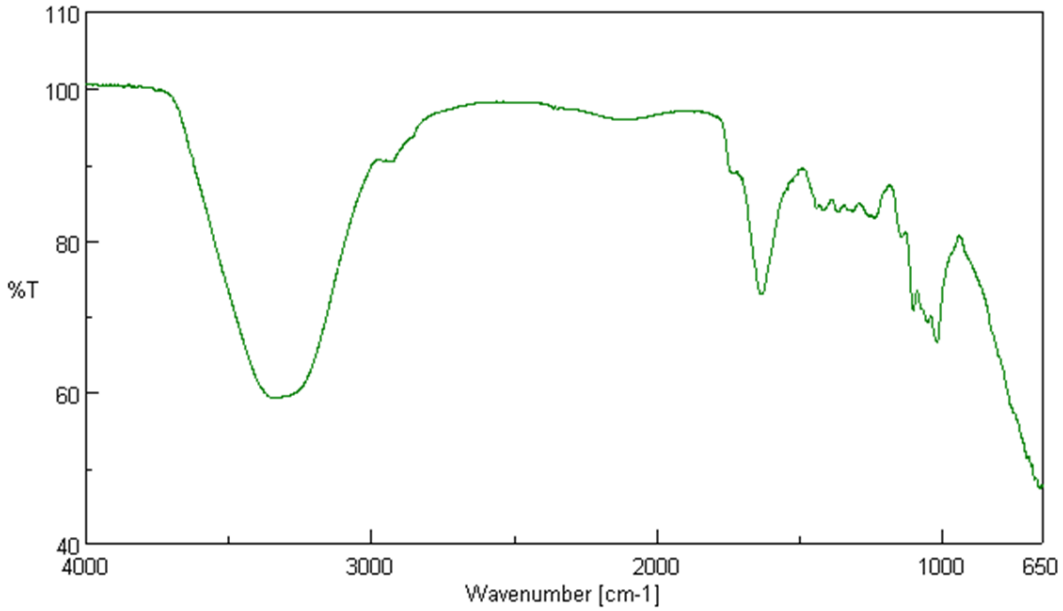


Figure 12: FTIR spectra of jackfruit bulbs

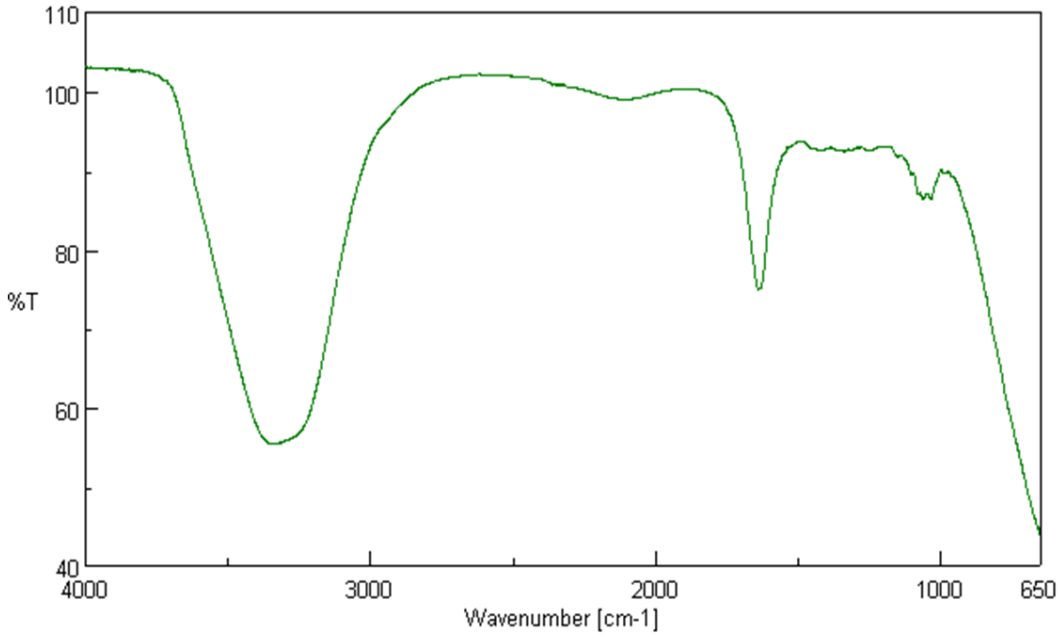


Figure 13: FTIR spectra of pomegranate arils

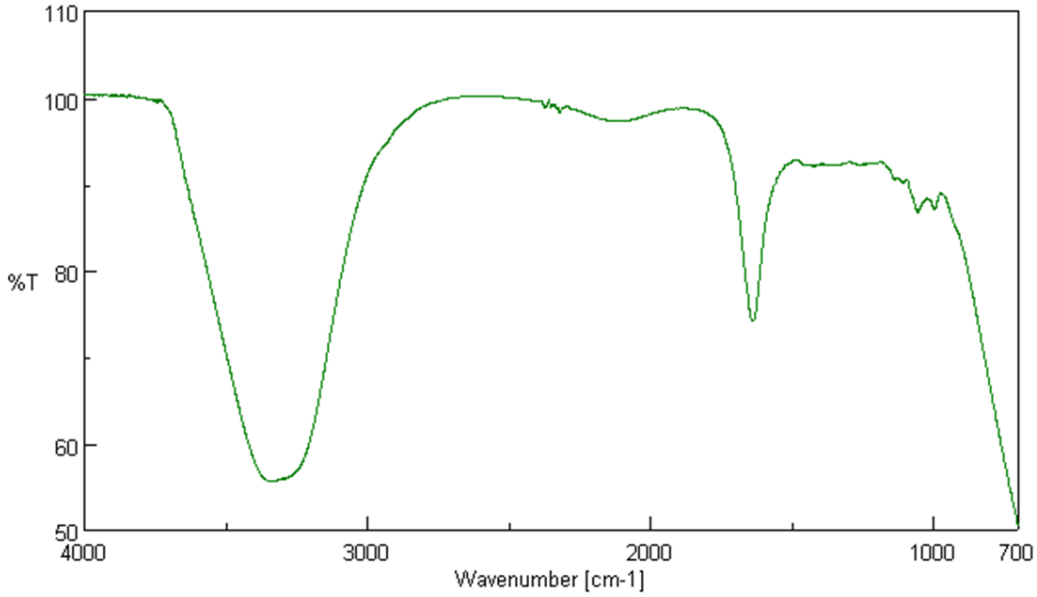


Figure 14: FTIR spectra of pineapple slice

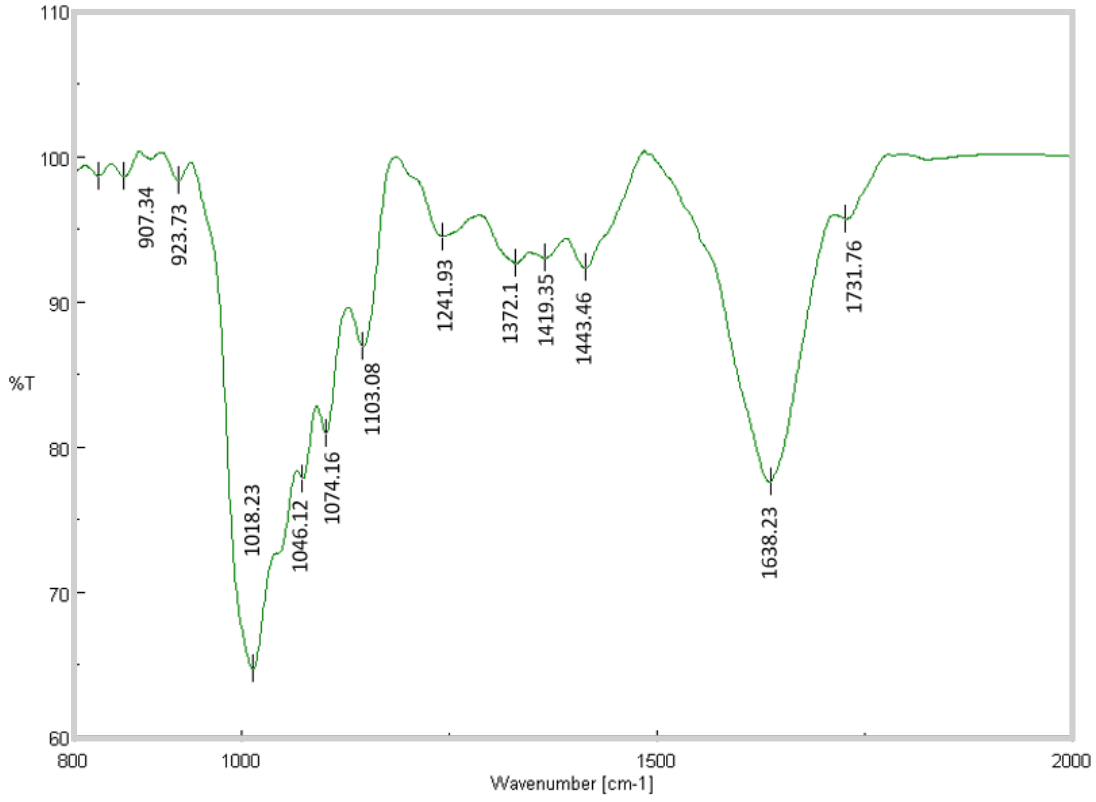


Figure 15: FTIR spectra of minimally processed jackfruit bulbs from 800 to 2000 cm^{-1}

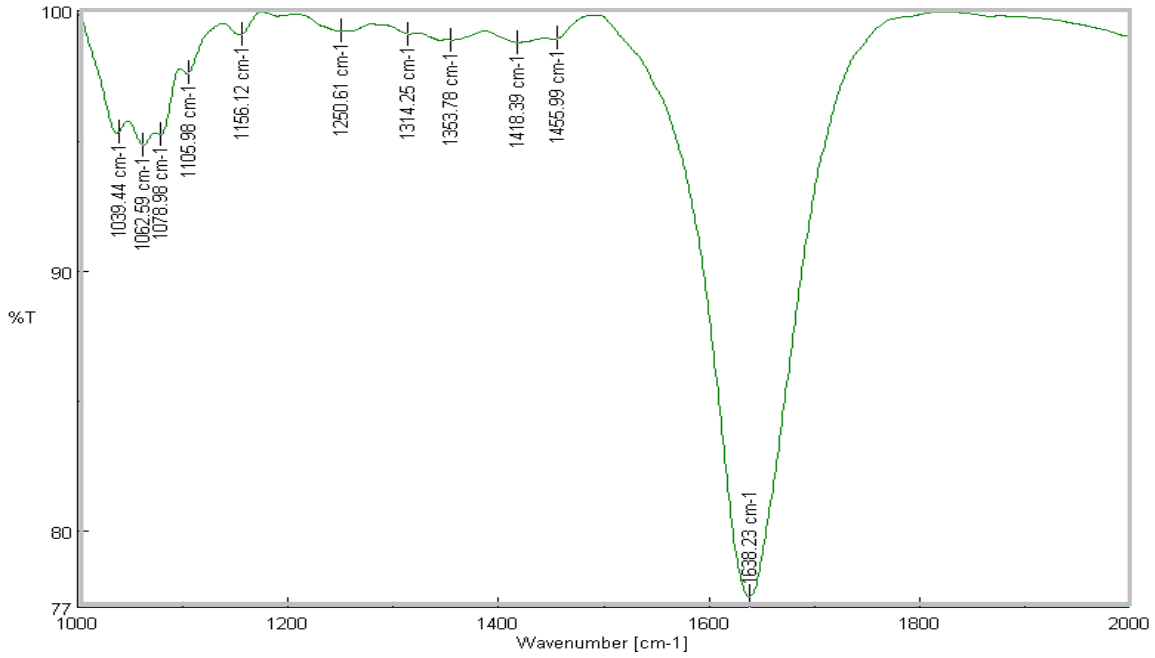


Figure 16: FTIR spectra of minimally processed pomegranate arils from 1000 to 2000 cm^{-1}.

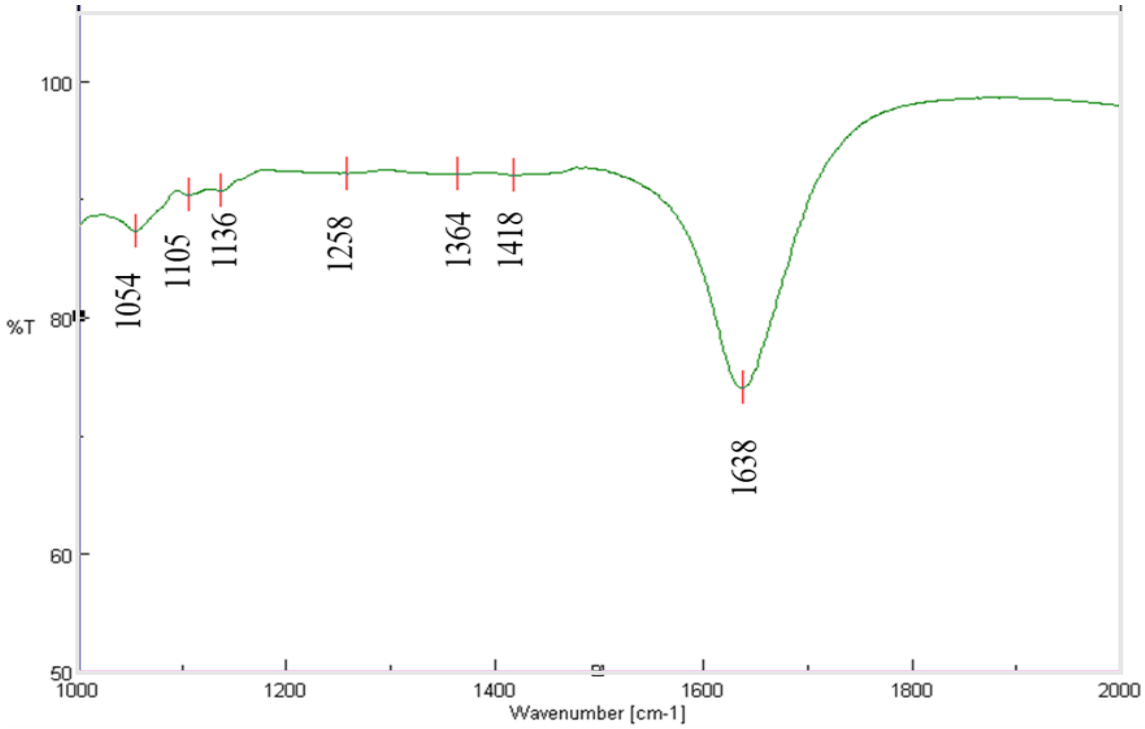


Figure 17: FTIR spectra of minimally processed pineapple slices from 1000 to 2000 cm^{-1}.

The detailed assignment of each peak in the FTIR spectra is provided in Table 9. A major peak that appeared at 1638 cm^{-1} in all the fruit samples could be attributed to moisture (O-H stretch) and C=O acid stretch. Peaks at 1353 could be assigned to CH_2 rocking and O-H bending of organic acids while 1418 cm^{-1} could be assigned to O-C-H, C-O-H and C-C-H bending in the carbohydrate molecules. Absorption band at 1250.6 cm^{-1} was attributed to C-O acid stretching. O-H deformation of secondary and tertiary alcohols resulted in peaks at 1156 cm^{-1}. Spectral peaks at 1105, 1078, 1039and 1062cm^{-1} are due to presence of C-O and C-H stretching of sugars such as glucose and sucrose. Spectral peak at 923 cm^{-1}can be attributed to C-C stretching of sugars such as fructose.

Table 9: Observed FTIR frequencies and possible assignments of the vibration modes

Frequency (cm^{-1})	**Assignment**	**References**
1720-1580 (1638)	C=O stretching and H-O-H def	[128]
1433-1460(1443)	-	
1400-1421 (1418)	O-C-H, C-C-H, C-O-H bending of carbohydrates	[129]
1350-1382 (1353,1364,1372)	CH_2 rocking, O-H bending of organic acids	[128]
1330-1294 (1314)	O-C-H, C-C-H, C-O-H deformation of carbohydrates	[129]
1239-1270 (1250)	-C-O acid stretching	[130]
1142-1187 (1156)	C-O stretching of secondary & tertiary alcohol	
1078 and 1105	C-O & C-H stretch of sugars, C-O-H bending	[131, 132]
1062	C-O & C-H stretch of sugars	[133]
1039	C-O & C-H stretch of sugars	[133]
1018	-	
923	C-C stretch of sugar (fructose)	[131, 132]

IR absorption spectra depicting the changes occurring during storage of minimally processed jackfruit, pomegranate and pineapple at the two temperatures studied are shown in Figure 13,14 and 15. It was observed that the absorption spectra of all the three fruit samples showed either no change or slight enhancement in the intensity of peaks during storage at both the temperatures (Fig 13A & C; Fig 14 A & C; Fig 15 A & C). Therefore, the FTIR spectra was processed to obtain its first derivative to differentiate overlapping peaks. Enhanced peak intensity in the first derivative spectra could also be clearly observed for all samples as depicted in Figures 13, 14 and 15 (Fig 13 B&D; 14 B&D; 15 B&D) for jackfruit, pomegranate and pineapple respectively. The first derivative shows a maxima were the signal has a maximum slope and crosses zero were the signal has a peak [133]. Thus, several biochemical changes were clearly observed in the FTIR spectra due to microbial growth during storage.

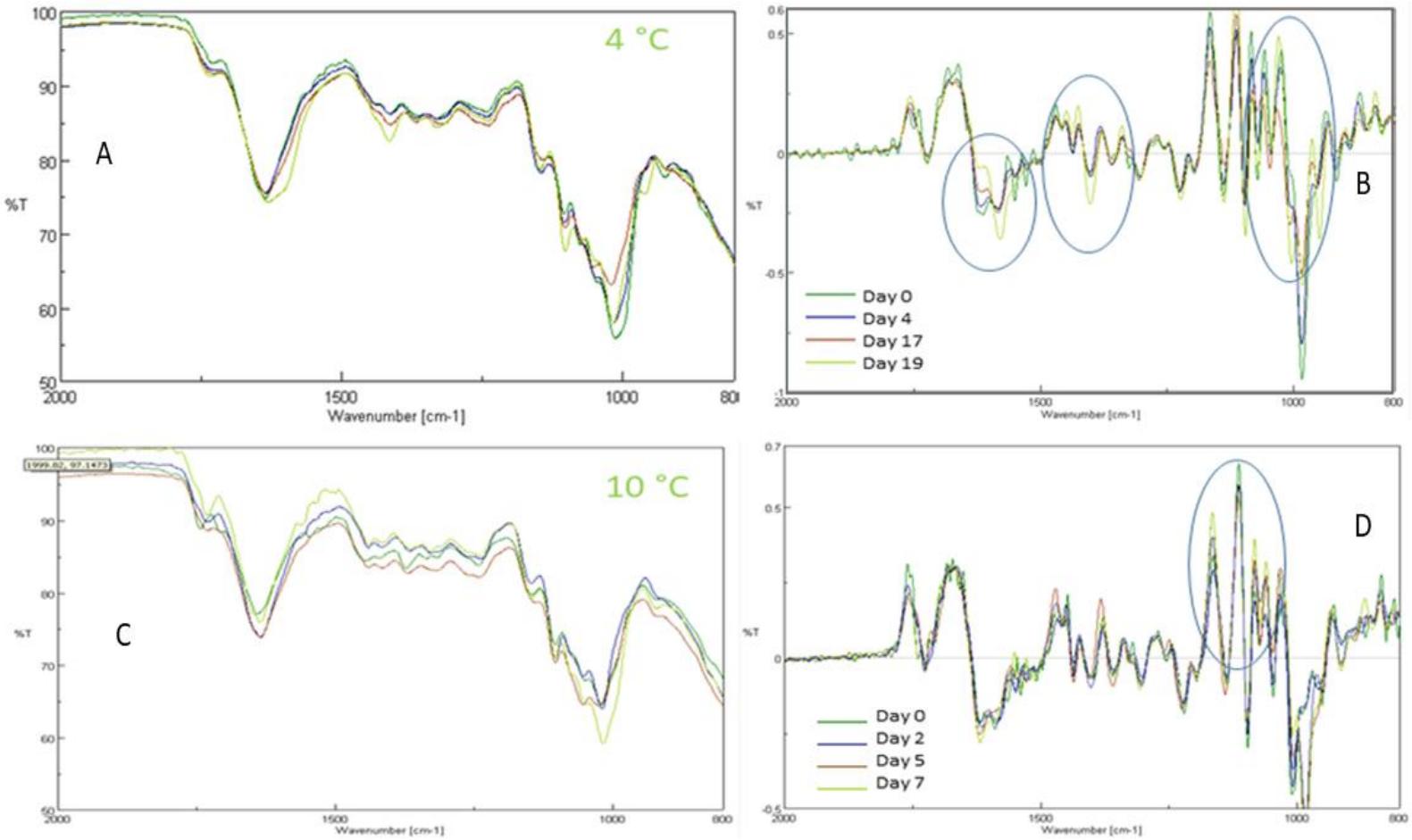


Figure 18: FTIR spectra (A & C) and FTIR first derivative spectra (B&D) for different days of storage for minimally processed jackfruit at 4 °C (A & B) and 10 °C (C&D).

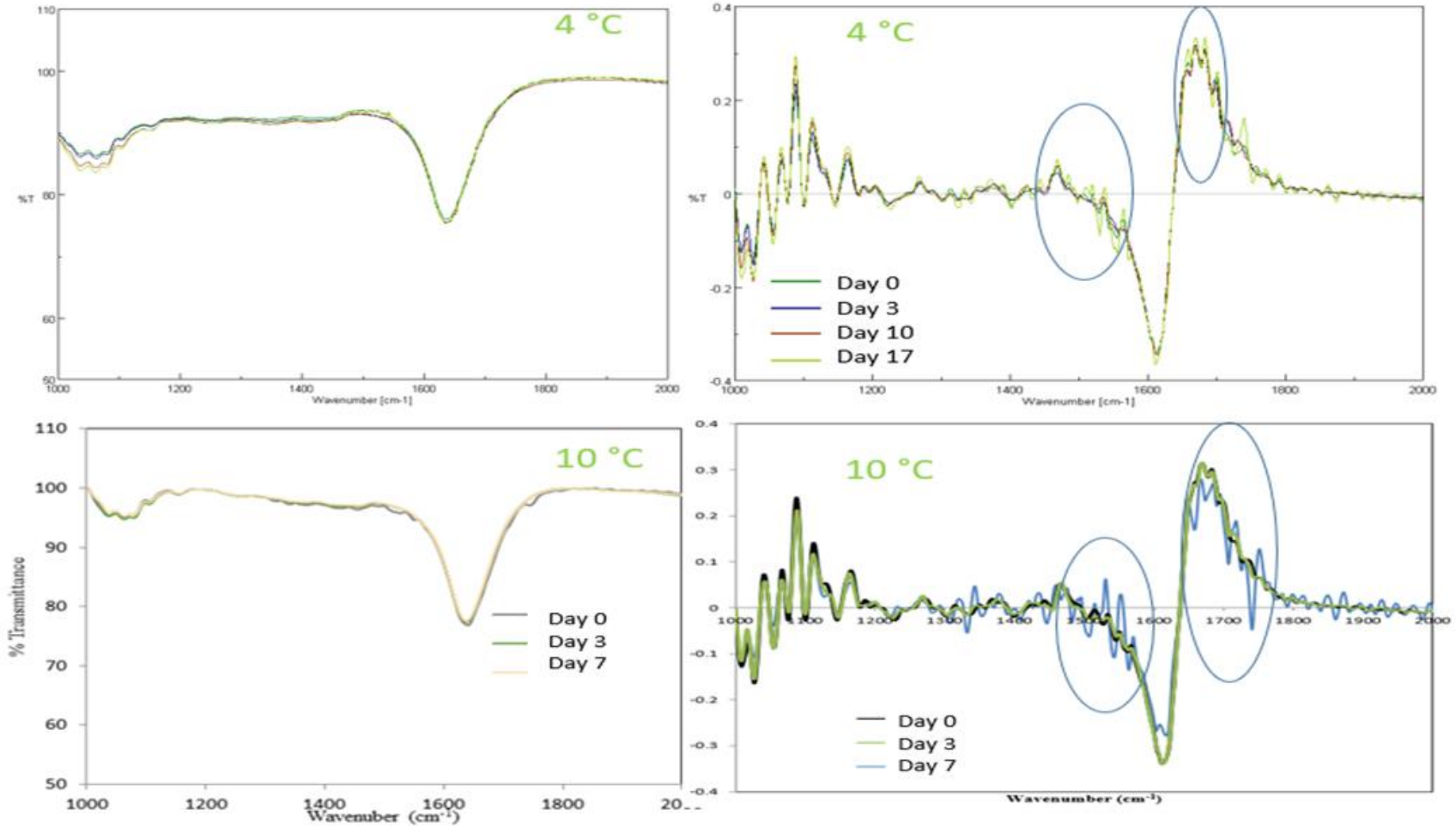


Figure 19: FTIR spectra (A & C) and FTIR first derivative spectra (B &D) at different days of storage for minimally processed pomegranate at 4 °C (A & B) and 10 °C (C&D).

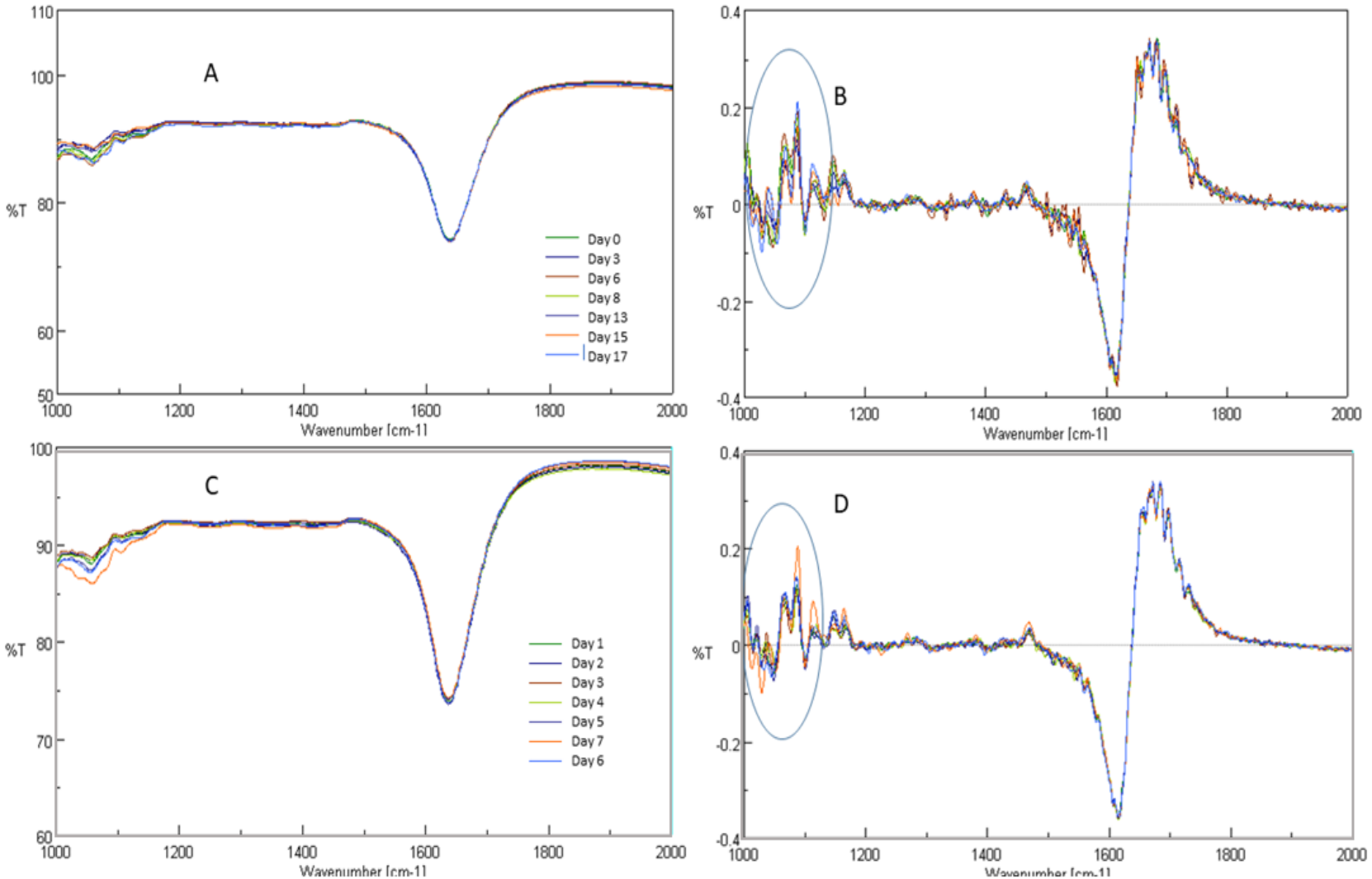


Figure 20: FTIR spectra (A & C) and FTIR first derivative spectra (B &D) at different days of storage for minimally processed pineapple at 4 °C (A & B) and 10 °C (C&D).

3.1.2.3 Inference from biochemical analysis using GCMS and FTIR

Several biochemical changes during storage were observed by employing both the techniques. These changes can be correlated to microbial quality of products and subsequently used for obtaining information regarding microbial quality of products by instrumental analysis. However, since the data generated by both the techniques had large number of variables (volatile constituents in GCMS and wave number in FTIR) multivariate statistical tools such as PCA, PLS-R and ANN was used to generate prediction models.

3.1.3 Analysis of biochemical changes using principal component analysis

PCA is a bilinear modelling method that reduces the number of variables by using orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of uncorrelated variables called principal components (PCs). The number of principal components are less than or equal to the number of original variables. The first principal component covers as much of the variation in the data as possible. The second principal component is orthogonal to the first and covers as much of the remaining variation as possible, and so on. In this study, since the biochemical changes as monitored both by volatile analysis using GCMS and FTIR spectra generated huge amount of multivariate variability, it becomes cumbersome to study the effect of storage on changes in fruit samples with simple statistical tool. PCA was therefore applied to reduce the size of the data set and to investigate differences between samples during storage as a result of product deterioration. PCA thus aided in visualizing complicated data in an easy interpretable manner.

3.1.3.1 Principal component analysis of GCMS data

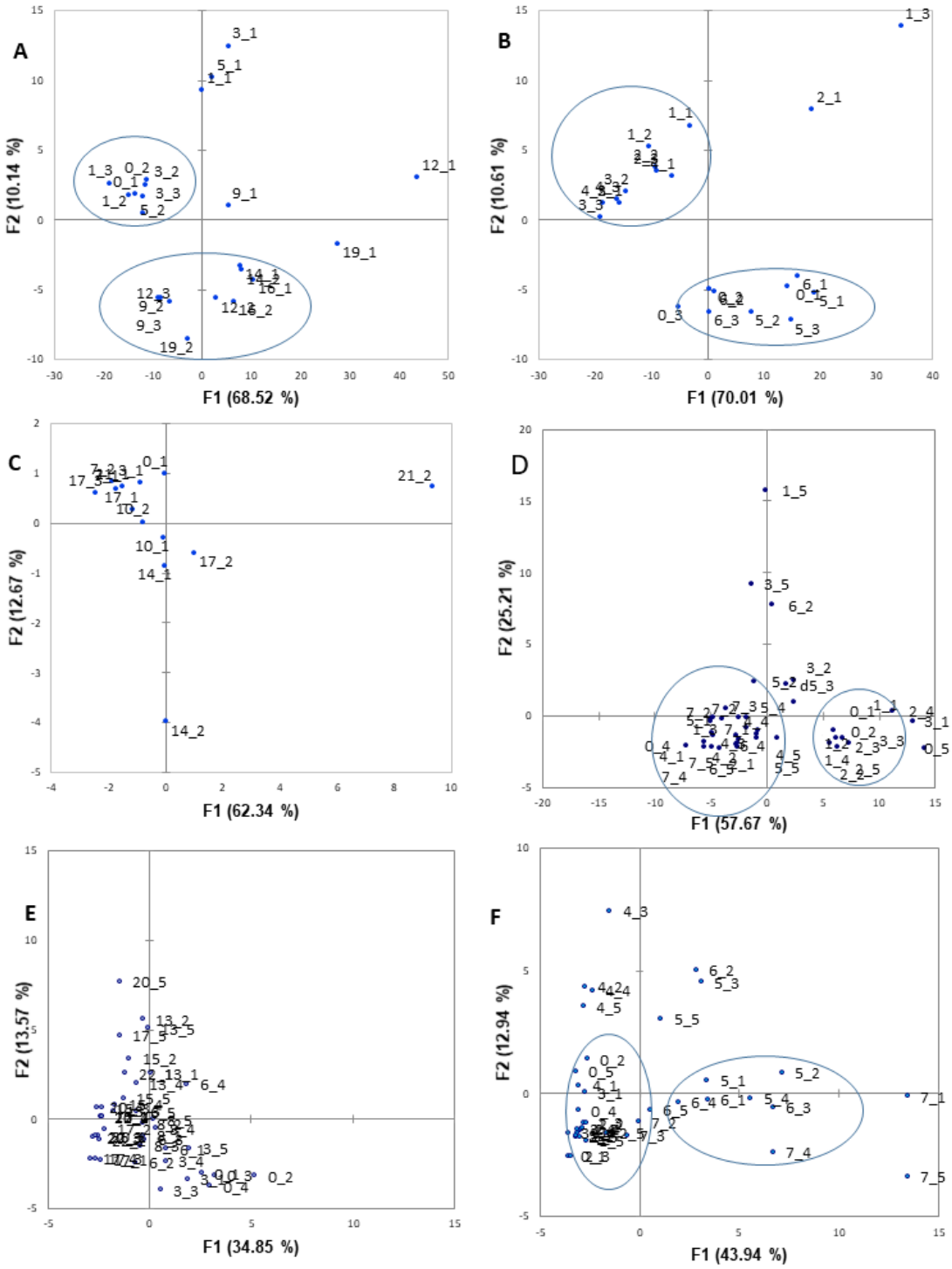


Figure 21: Principal component analysis of volatile profile of jackfruit (A, B), pomegranate (C, D) and pineapple (E, F) at 4 ºC (A, C, E) and 10 ºC (B, D, F).

GC/MS profile of all the three minimally processed fruit samples when individually analysed by PCA (Figure 21) demonstrated segregation according to the period of storage**.** For jackfruit samples kept at 4 °C, 79% of the total variation was accounted for by the first two principal components. Segregation in three groups was observed for samples stored at 4 °C (Figure 21A). First group had samples stored up to day 5 and was located on negative side of PC1 and on the positive side of PC2**.** Samples stored beyond a storage period of 5 days and up to 19 days constituted second group located on negative side of PC2 (Figure 21A). For samples stored at 10 °C first two principal components cumulatively explained 81 % of total variation. Score plot for 10 °C stored samples is demonstrated in Figure21B. The score distribution from first two PCs demonstrated two separate groups in the samples analysed. Although two distinct groups were observed there were a few outliers in every group. Samples stored from day 1 till day 4 constituted the first group and was located on the negative side of PC1 and positive side of PC2. Samples stored for day 5 and day 6 constituted second group located on positive side of PC1 but negative side on PC2 (Figure 21B).

In case of pomegranate, the cumulative variance explained by two PCs was 75% for samples stored at 4 °C (Figure 21C). It could be observed that samples did not show distinct day-wise segregation, however spread in the data due to different days of storage could be observed during the storage period of 21 days. For samples stored at 10 °C, the samples showed clear segregation in two groups. The first group consisted of samples stored up to 2 days and were located on positive side of PC1 and negative side of PC2. The second group comprised of samples stored beyond storage period of day 3 up to storage period of 7 days (Figure 21D).

In case of pineapple, no segregation was observed in principal component score plots (Figure 21E) for samples stored at 4 °C suggesting no significant changes in volatile constituents during storage. Score plot for 10 °C stored samples however, showed segregation in three different groups. The cumulative variance explained by the first two PCs was 56.88%.

First group had samples stored up to 3 days located on negative side of PC1, while second group comprised of samples stored from day 5 to day 7 located on the positive side of PC1 (Figure 21F).

3.1.3.2 Principal component analysis of FTIR data

Principal component analysis of the FTIR data obtained in the form of FTIR spectral data and its first derivative termed as FTIR first derivative data (detailed in methodology section) revealed difference in the stored samples for all the three fruits (Figure 22, 23 & 24).

Segregation based on duration of storage at 4 and 10 °C was observed for jackfruit samples in the PCA of FTIR data. For jackfruit samples stored at 4 °C, it can be observed from PCA plots that there was no distinct day-wise segregation in case of FTIR spectral data (Figure22A). FTIR first derivative data could, however, distinguish various stored samples with day 2 to day 8 stored samples forming one group located on positive side of PC1 and PC2. The second group was located on positive side of PC1 and negative side of PC2 (day 10 to 19). For 10 °C stored jackfruit samples, it was observed that both FTIR spectral data and FTIR first derivative data showed day-wise spread with samples stored in the initial period (day 0) segregating from the stored samples from day 3 to 6 (Figure 22 C&D). Thus, it can be seen that FTIR data of jackfruit samples could give distinct day wise segregation for both storage temperatures.

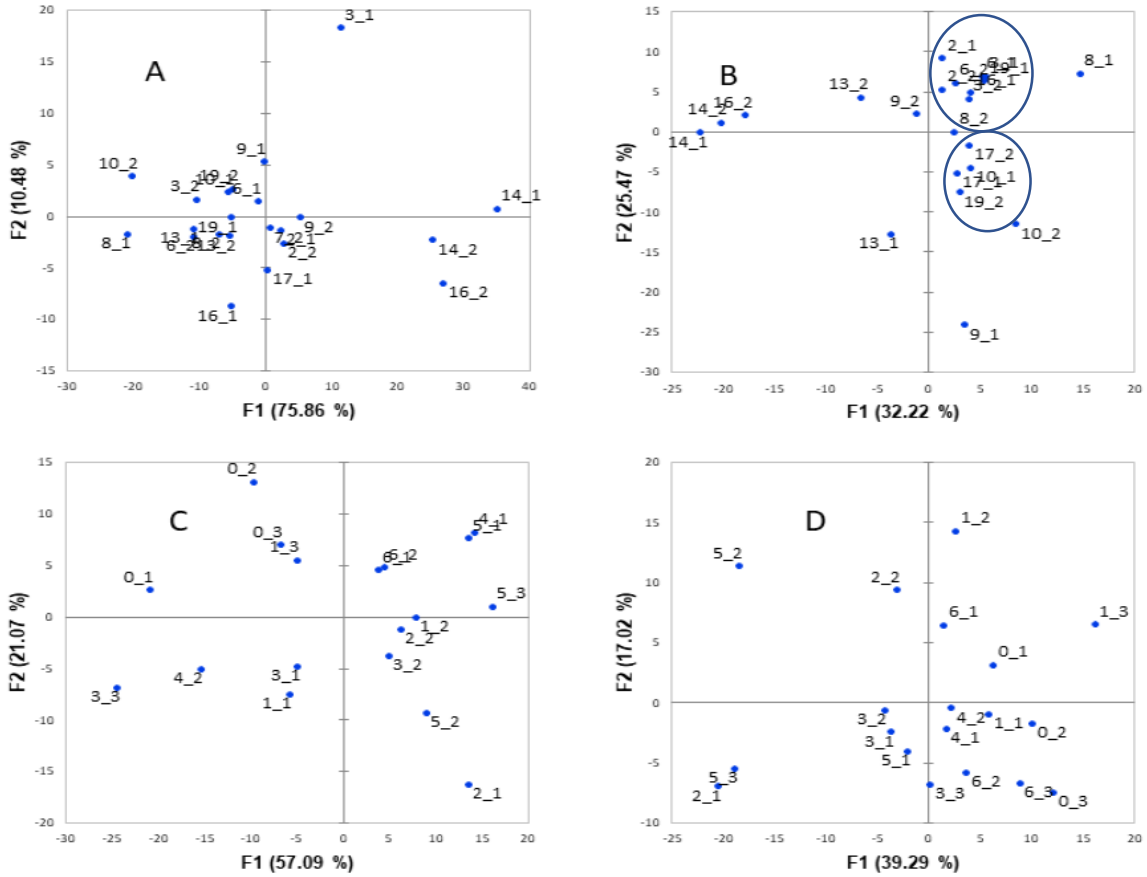


Figure 22: Principal component analysis of FTIR spectral data of jackfruit (A, C), FTIR first derivative data of jackfruit (B, D) at 4 °C (A, B) and 10 °C (C,D).

Segregation of pomegranate sample based on storage time was observed in the PCA of FTIR data. Day wise segregation was observed among the 4 °C stored sample for both FTIR spectral data and FTIR first derivative data (Figure 23 A & B). In case of 10 °C stored pomegranate samples, segregation of FTIR spectral data into two groups was observed as shown in Figure 23C. The first group comprised of sample stored up to 2 days (0 to 2 day) and was located on the positive side of PC1 & negative side of PC2. The second group comprised of sample stored beyond 3 days and until 7 days and was located on the positive side of PC2. Similarly, segregation based on storage period was also observed when PCA was applied to the FTIR first derivative spectral data. PC1 & PC2 accounted for 56.32 % of the total variance and samples were segregated into four groups (Figure 23D). The first group located on positive

side of both PC1 and PC2 consisted of freshly packed samples (Day 0). The second group consisted of samples stored from day 1 till day 3 and was located on positive side of PC1 & negative side of PC2. Samples stored from day 4 to day 6 constituted the third group located on the negative side of both PC1 and day 7 constituted to fourth group located on the negative side of both PC1 and PC2.

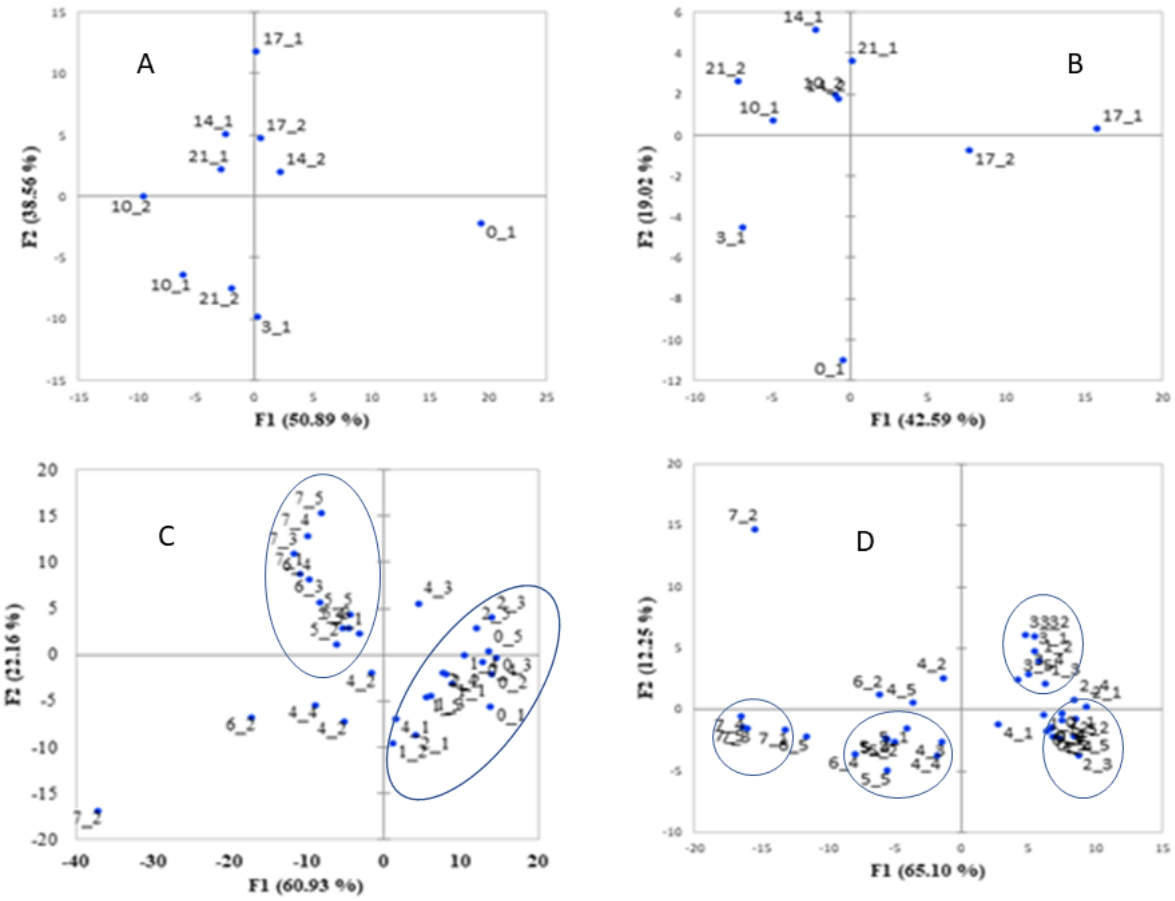


Figure 23: Principal component analysis of FTIR spectral data of pomegranate (A, C), FTIR first derivative data of jackfruit (B, D) at 4 °C (A, B) and 10 °C (C,D).

In case of pineapple, day-wise segregation was not observed among samples stored at 4 °C for both FTIR spectral data and FTIR first derivative data. (Figure 24A and B). In case of samples stored at 10 °C, PCA analysis of FTIR spectral data revealed no segregation of samples based on storage time (Figure 24C). However, application of first derivative function to FTIR

spectra resulted in segregation of samples based on storage period. Samples stored up to 4 days constituted one group and was located on negative side of PC2 whereas samples from 5 to 7 days formed another group located on positive side of PC1 and PC2 (Figure 24D). Use of first derivative function thus revealed the difference in the stored samples.

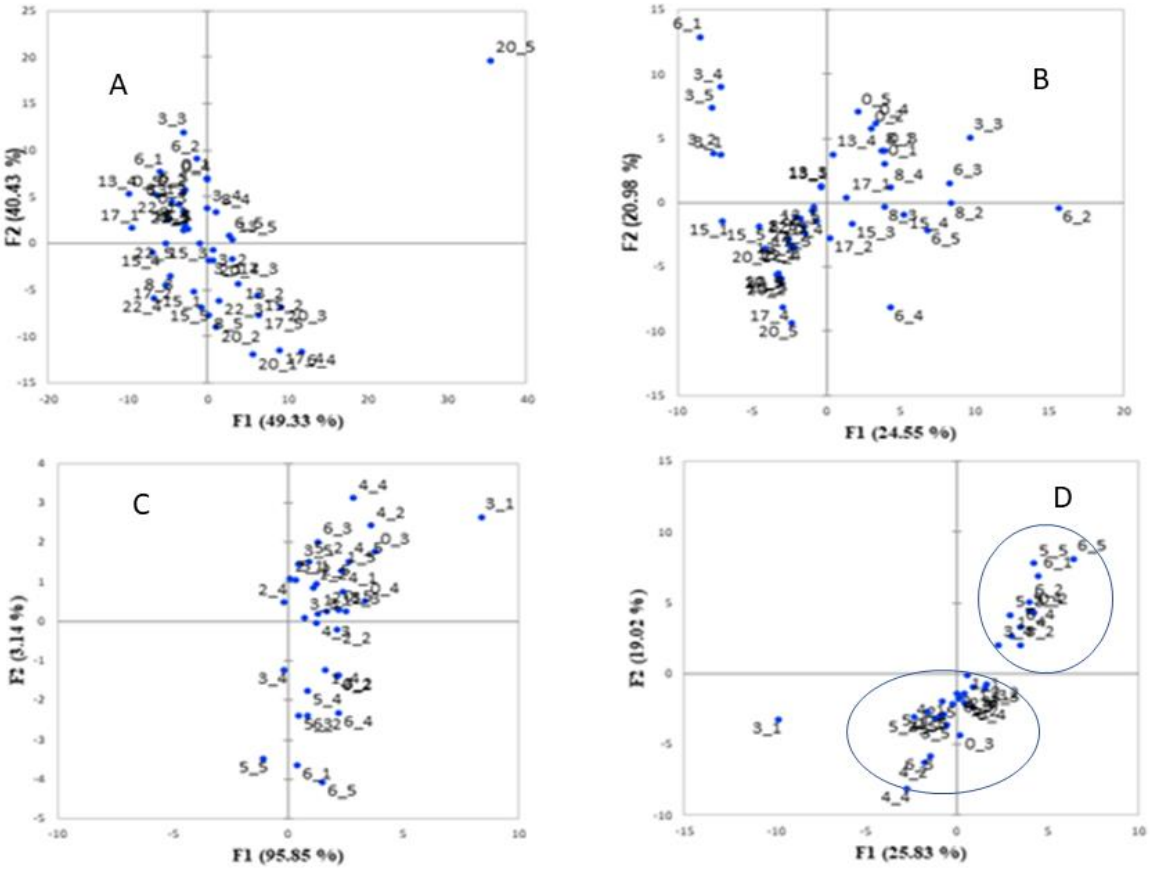


Figure 24: Principal component analysis of FTIR spectral data of pineapple (A, C), FTIR first derivative data of jackfruit (B, D) at 4 °C (A, B) and 10 °C (C,D).

3.1.3.3 Conclusions from PCA analysis of GCMS and FTIR data

PCA analysis of volatile profile obtained from GCMS and biochemical changes as monitored by FTIR revealed difference amongst stored fruit samples suggesting chemical changes occurring during storage. It was also evident that the biochemical changes in the stored samples were significantly affected by temperature. PCA analysis (both GCMS and FTIR) showed segregation for all the fruit samples according to storage period at 10 °C. However, this was not always the case for samples stored at 4 °C. Jackfruit samples showed segregation in PCA score plot with GCMS and FTIR data, but pomegranate and pineapple samples did not follow this trend at 4 °C. FTIR first derivative data demonstrated better segregation when compared to FTIR spectral data. In conclusion, this difference in PCA plots also suggests that microbial activity does induce chemical changes in the stored fruit samples. Thus, quantitative prediction of microbial counts was performed from the data obtained from GCMS and FTIR using machine learning tools such as PLS-R and ANN.

3.1.4 Quantitative prediction of microbial counts in minimally processed fruits

Principal component analysis of data obtained from GCMS as well as FTIR revealed biochemical differences in all the fruit samples stored for different time periods. Observed changes in headspace volatile composition might be due to microbial growth or metabolic changes in the product [135, 136, 137]. These results suggested the possibility of using supervised chemometric techniques such as partial least square regression (PLS-R) and artificial neural network (ANN) for generating regression models to predict microbial quality of minimally processed fruits based on biochemical changes. Further, as only a marginal increase in microbial counts was observed in pomegranate and pineapple samples stored at 4 °C, quality assessment using chemometrics tools was not further carried out for these samples.

3.1.4.1 Quantitative estimation for predicting microbial quality in minimally processed fruit using GCMS data

The supervised tools, ANN and PLS-R were utilised to built models for TVC and Y&M counts using GC volatile data as independent variables and Log_{10} CFU/ g as dependent variable.

3.1.4.1.1. Supervised ANN for predicting microbial quality in minimally processed fruit using GC/MS

ANN models were built for TVC and Y&M counts using GCMS volatile data as independent variables and Log_{10} CFU/g as dependent variable. A multilayer perceptron (MLP) neural network based on back propagation was used to estimate TVC and Y&M. The number of uncorrelated PCs that could explain 95% of total variance of data was utilized as input variables, while TVC and Y&M were two output neurons in ANN architecture. Among fourteen architectures (fourteen networks with hidden neurons varying from 1 to 14) tested, network with highest R^2 values of training, validation and test data and lowest training, test and validation performance errors was selected.

3.1.4.1.1.1 Jackfruit

The performance of the MLP networks to predict TVC and Y&M in minimally processed jackfruit samples in terms of statistical indices is presented in Table 10. The R^2 obtained for ANN models built for both TVC and Y&M was 0.85 at 4 °C. The SEP (standard error of prediction), A_f (accuracy factor) and B_f (bias factor) were 0.73, 13% and 0.89. TVC counts for test samples (described in Section 2.1.7 & 2.1.8) stored at 4 °C could be predicted within 13% average deviation suggesting good accuracy. The SEP, A_f and B_f for Y&M were 1.01, 27% and 0.76, respectively. Prediction of Y&M counts in test samples (4 °C) demonstrated significantly higher ($p<0.05$) deviation of 27% in comparison to TVC counts.

Models built for samples stored at 10 °C had a R^2 value of 0.89 for both TVC and Y&M. The SEP, A_f and B_f were 0.89, 17% and 0.99 and 0.85, 15% and 0.97 for TVC and Y&M, respectively (Table 10). In test samples, average deviation between actual and predicted counts was less than 20% for both TVC and Y&M.

Thus, ANN could be successfully applied for prediction of TVC and Y&M in test samples using GCMS data. Microbial counts could be predicted with average deviation of < 20% for jackfruit samples stored at both the temperatures (4 and 10 °C) with the exception of Y&M counts at 4 °C that had a large error (27%) in predicted values.

Table 10. Performance indices of models build using ANN for GCMS data for minimally processed Jackfruit

		Observed (Log_{10} CFU/g)	Predicted (Log_{10} CFU/ g)	PC	Hidden neuron	A*f*	B*f*	*SEP*	R^2
4 °C	TVC	3.6	3.78	5	1	13%	0.89	0.73	0.85
		5.01	3.88						
		6.86	6.55						
	Y&M	2.00	1.35	5	1	27%	0.76	1.01	0.85
		2.77	1.35						
		3.30	4.11						
10 °C	TVC	4.8	3.82	8	3	17%	0.99	0.89	0.89
		8.63	7.58						
		5.51	6.96						
	Y&M	4.67	5.61	8	3	15%	0.97	0.85	0.89
		5.83	4.81						
		6.43	5.88						

PC-principal components; SEP- Standard error of prediction; A_f-Accuracy factor; B*f*- Bias factor

3.1.4.1.1.2 Pomegranate

Table 11 demonstrated the performance parameters of ANN models built using GCMS data for pomegranate samples stored at 10 °C. Models built had R^2 of 0.85 for both TVC and Y&M. TVC prediction for the test samples was achieved with SEP of 0.71 and 14 %. Similarly, Y&M also showed a good prediction with SEP of 0.72 and only 9 % average deviation between actual and predicted counts. Thus, these results suggest suitability of ANN in combination with GCMS for prediction of microbial counts in minimally processed pomegranate.

Table 11: Performance indices of models build using ANN for GCMS data for minimally processed pomegranate and pineapple stored at 10 °C.

Statistical	Pomegranate		Pineapple	
	TVC	Y&M	TVC	Y&M
R^2	0.86		0.98	
PC	5		8	
SEP	0.71	0.72	0.134	0.423
A_f	14%	9%	2%	6%
B_f	0.97	0.96	1.01	0.98
Hidden neuron	4	4	3	3

PC-principal components; SEP- Standard error of prediction; A_f-Accuracy factor; B*f*- Bias factor

3.1.4.1.1.3 Pineapple

Results of ANN models for pineapple samples stored at 10 °C are shown in Table 11. It can be observed from the table that models built using GCMS had R^2 of 0.98 for both TVC and Y&M. In test samples, TVC counts could be predicted with very low SEP of 0.13. Average deviation between actual and predicted values was observed to be only 2%. Y&M counts in test samples could be predicted with average deviation of only 6% between actual and predicted counts.

3.1.4.1.1.4 Discussion & conclusion of ANN models using GCMS data

Rapid quantitative estimation of microbial quality could be successfully demonstrated using ANN on GCMS data for all the three fruit samples. $R^2 \geq 0.85$ was obtained for all the developed models for TVC and Y&M in case of all minimally processed fruit samples. The accuracy factor was lower than 20% for all the models except for Y&M counts of jackfruit stored at 4 °C. Similar performance was observed for TVC and Y&M in individual fruit samples. ANN is a less explored tool for microbial quality estimation in food samples [72]. Moreover, ANN has not been applied for microbial quality estimation using GCMS data for food samples so comparisons of our results with those published in literature was not possible. The current study demonstrates that nonlinear relationship could exist between some specific microorganisms and volatile profile, which thus led to a successful attempt in applying ANN prediction tool for microbial quality assessment using GCMS data.

3.1.4.1.2 Supervised PLS-R for predicting microbial quality in minimally processed fruit using GCMS

PLS-R models were built to correlate TVC and Y&M with GCMS data. Selection of number of latent variables for model building is a critical step in PLS. Very few variables could lead to insufficient model while too many variables results in over fitting of data [102]. In the present study, before the final model preparation, number of latent variables were finalized based on standard error of prediction (SEP) for test data as detailed in materials & methods (section 2.1.7).

3.1.4.1.2.1 Jackfruit

The performance of the PLS-R models to predict TVC and Y&M in minimally processed jackfruit samples in terms of statistical indices is presented in Table 12. R^2 of above 0.9 was observed in models built for TVC and Y&M counts for samples stored at both temperatures (4

and 10 °C). A close agreement of actual and predicted values was observed. The accuracy factor (A_f) is a measure of the average deviation between predictions and observations. Based on results for A_f the lowest average deviation between actual and predicted counts was for TVC at 4°C (4.6%) while highest was for Y&M at 4 °C (16.8%). Thus, the models developed for jackfruit samples had good performance indices and could be successfully utilised for predicting microbial quality.

Table 12: Predicting microbial loads and performance parameter in packaged jackfruit using PLS-R of GCMS data for minimally processed jackfruit at both storage temperatures.

		Observed (Log_{10} CFU/ g)	Predicted (Log_{10} CFU/ g)	LV	A*f*	B*f*	*SEP*	R^2
4 °C	TVC	3.6	3.3	4	4.6%	0.96	0.26	0.98
		5.01	4.7					
		5.86	5.99					
		3.75	3.71					
	Y&M	2	1.6	4	16%	1.05	0.61	0.9
		2.77	2.4					
		4	4					
		1.6	2.1					
10 ºC	TVC	5.52	6	6	7.6%	0.98	0.67	0.95
		7.81	6.63					
		8.04	7.72					
		8.94	9.29					
	Y&M	4.67	4.2	3	11%	0.9	0.9	0.95
		6.43	5.9					
		6.6	6.1					
		8.18	7.6					

LV- latent variable; SEP- Standard error of prediction; A_f-Accuracy factor; B*f*- Bias factor

3.1.4.1.2.2 Pomegranate

In case of pomegranate samples stored at 10 °C, the R^2 values for the generated prediction models for TVC and Y&M were 0.75 and 0.84, respectively (Table13). The SEP values obtained for TVC and Y&M were 0.35 and 0.51 with corresponding high accuracy of 6 and 10 %, respectively. PLSR models could successfully predict microbial counts in minimally processed pomegranate using GCMS.

3.1.4.1.2.3 Pineapple

For pineapple samples stored at 10 °C, it can be observed from Table 13 that models built using GCMS gave a high R^2 of 0.93 and 0.98, respectively for TVC and Y&M. The SEP values obtained for TVC (0.39) was lower when compared for Y&M (0.58) with corresponding high accuracy of 9 and 13 %, respectively.

Table 13: Performance indices of PLS-R models generated for TVC and Y&M of minimally processed pomegranate and pineapple samples

PLS Prediction parameters	Pomegranate		Pineapple	
	TVC	Y&M	TVC	Y&M
R^2	0.759	0.84	0.93	0.98
LV	4	5	7	7
SEP	0.35	0.51	0.397	0.581
A_f	6%	10%	9 %	13%
B_f	0.97	1.008	1.009	0.948

LV- latent variable; SEP- Standard error of prediction; A_f-Accuracy factor; B*f*- Bias factor

3.1.4.1.2.4 Discussion & conclusions of PLS-R models using GCMS data

Argyri *et al*. [138], reported an overall 9 % and 12 % of A_f of TVC and Y&M of minced beef sample using GCMS, similar results were obtained in the current study for all the samples. GCMS volatile data could be successfully utilised for estimation of TVC and Y&M counts for all the three fruit samples with $R^2 \geq 0.73$ with all the developed models. GCMS as an instrumental technique has been shown suitable for microbial quality assessment in the current study for minimally processed fruits such as jackfruit, pomegranate and pineapple. It was also observed that the models developed for jackfruit and pineapple had $R^2 \geq 0.90$ for both TVC and Y&M. When the models for TVC and Y&M counts were compared, it was evident that models developed for TVC had better performance indices for jackfruit compared to pomegranate and pineapple. On the other hand, models build for Y&M had better performance indices in case of pomegranate and pineapple. This was in accordance with the microbial growth profiles where TVC counts are higher than Y&M at the end of storage period for jackfruit samples, while Y&M counts superseded TVC in case of low acidic fruits such as pomegranate and pineapple.

In PLS-R models it is desirable to know the variables that correlate best with the attributes to be predicted. Compound showing positive correlation with microbial counts (TVC and Y&M) are depicted in Table14. Since, in the present study GC/MS was used for volatile analysis, it offered possibility of identifying volatiles that had highest correlation with increasing microbial counts. From PLS-R correlation matrices of jackfruit, pomegranate and pineapple, it was observed that ethanol demonstrated high correlation (>0.4) with microbial counts (TVC and Y&M) as shown in Table 14. Other alcohols that demonstrated high positive correlations were 3-methyl-1-butanol and 1-hexanol. Ethanol is a well-known compound associated with microbial spoilage. Gram-negative bacteria (e.g. *Pseudomonas, Shewanela, Moraxella*) have been found to specifically produce ethanol, methanol, 2-methyl propanol (precursor valine)

and 2-methylbutanol (precursor isoleucine) [139]. *Br. thermosphacta*, produces different types of alcohols according to the storage conditions due to the changes in its metabolism. Anaerobically, it produces mainly ethanol (precursor glucose), whereas aerobically, it produces ethanol, 3-methylbutanol (precursor leucine) and 2-methylbutanol (precursor isoleucine), from Strecker degradation of amino acids during the proteolysis [140, 141]. Correlation matrices of GCMS data of jackfruit and pineapple showed that ethyl acetate had the highest correlation with TVC and Y&M (>0.6) (Table14). Several other ethyl esters of organic acid such as 2-methyl, butanoic acid, hexanoic acid & 3-hexenoic acid showed positive correlation greater than 0.5 for TVC and Y&M in case of pineapple. Caleb *et al*. [68] demonstrated positive correlation of ethyl acetate with microbial growth. Ethyl esters such as ethyl acetate, ethyl butanoate and ethyl octanoate are reported to be produced as Y&M counts increases to 6-7 Log_{10}CFU/g and the headspace O_2 is rapidly converted to CO_2 resulting in fermentative conditions [142]. This results in esterification of various alcohols and carboxylic acids. Other esters that showed strong positive correlation were n-propyl acetate, 1-butanol, 3-methyl-, acetate, 1-butanol, 2-methyl-, acetate and 2-phenyl ethyl acetate. Our results also showed a large increase in total esters during storage. Increase in ester concentration in inoculated samples was also demonstrated by Vikram *et al.*, [143]. Thus, biochemical changes reflected in the headspace volatile profile of the fruit samples as demonstrated by GCMS were utilised for quantitative prediction of TVC and Y&M.

Table14: Correlation of volatile compounds with microbial counts for minimally processed fruits

Correlation coefficient (R)						
Compound name	**Jackfruit**		**Pomegranate**		**Pineapple**	
	(TVC)	**Y&M**	**TVC**	**Y&M**	**TVC**	**Y&M**
Ethanol	0.461	0.488	0.78	0.80	0.452	0.579
3-methyl 1-butanol	0.423	0.447	ND	ND	0.520	0.647
1-hexanol	0.403	0.348	0.342	0.389	0.44	0.525
phenyl ethyl alcohol	ND	ND	ND	ND	0.628	0.764
Methyl acetate	0.582	0.486	ND	ND	0.586	0.703
Ethyl acetate	0.718	0.637	-0.423	-0.415	0.707	0.881
1-butanol, 3-methyl-, acetate	0.338	0.223	ND	ND	0.657	0.823
1-butanol, 2-methyl-, acetate	0.354	0.241	ND	ND	0.638	0.793
butanoic acid, 2-methyl, ethyl ester	0.490	0.304	ND	ND	0.539	0.694
hexanoic acid, ethyl ester	ND	ND	ND	ND	0.572	0.780
3-hexenoic acid, ethyl ester	ND	ND	ND	ND	0.542	0.730
hexanal	0.505	0.272	0.272	0.393	0.104	-0.014
2-heptanone	ND	ND	ND	ND	0.651	0.741
2-heptanol	ND	ND	ND	ND	0.454	0.546

3.1.4.2. Quantitative estimation for predicting microbial quality in minimally processed fruits using FTIR data

The supervised tools, ANN and PLS-R were utilised to build models for TVC and Y&M counts using FTIR data as independent variables and Log_{10} CFU/g as dependent variable.

3.1.4.2.1. Supervised ANN for predicting microbial quality in minimally processed fruits using FTIR data

ANN models were also built for TVC and Y&M with FTIR spectral data and FTIR first derivative spectral data as independent variable and microbial counts as dependent variable.

3.1.4.2.1.1 Jackfruit

Performance indices for models built using both the forms of FTIR data are shown in Table 9 for minimally processed jackfruit. Models built for samples stored at 4 °C using FTIR spectral data had R^2 value of 0.84 & 0.94 for TVC and Y&M, respectively. The SEP, A_f and B_f were 0.63, 17% and 0.93 and 0.90, 12% and 1.07 for TVC and Y&M, respectively. Comparable performance for both TVC and Y&M was observed for samples stored at 4 °C using FTIR spectral data with average deviation between actual and predicted counts of < 20%. ANN models were built for FTIR first derivative data as well. In case of first derivative data, the R^2 obtained was 0.85 for both TVC and Y&M in samples stored at 4 °C. The SEP, A_f and B_f for TVC and Y&M counts were 0.47, 13% and 1.13 and 0.71, 35% and 1.35, respectively. Although, prediction for TVC counts had low average deviation of 13% between actual and predicted counts but Y&M could be predicted with large average deviation of 35%. These results suggest that FTIR spectral data provided better performance than FTIR first derivative data for prediction of microbial counts in samples stored at 4 °C.

Models built for samples stored at 10 °C using FTIR spectral data had a good R^2 value of 0.83 and 0.85 for TVC and Y&M, respectively. The SEP, A_f and B_f were 0.63, 10 % and 1.04

and 0.90, 20 % and 1.01 for TVC and Y&M, respectively (Table 15). These results suggest good accuracy for predictions of microbial counts with average deviations of ≤ 20% employing FTIR spectral data for samples stored at 10 °C.

Building of prediction models using FTIR first derivative data was also attempted for samples stored at 10 °C. The R^2 obtained for TVC and Y&M were 0.95 and 0.87, respectively. In case of FTIR first derivative data as well, acceptable performance for both TVC and Y&M was observed with average deviation between actual and predicted counts of ≤ 10%. Thus, ANN could be successfully applied for prediction of TVC and Y&M counts for stored jackfruit samples at 4 and 10 °C.

Table 15: Performance indices of models build using ANN for FTIR data for minimally processed jackfruit stored at 4 and10 °C.

		Hidden neurons	PC		SEP	A_f	B_f	R^2
				TVC	0.637	17 %	0.93	0.84
	FTIR spectral data	2	4	Y&M	0.909	12 %	1.07	0.94
4 °C	FTIR first derivative data	1	9	TVC	0.470	13 %	1.13	0.85
				Y&M	0.711	35%	1.35	0.85
	FTIR spectral data			TVC	0.637	10%	1.045	0.93
		3	3	Y&M	0.909	20%	1.014	0.85
10 °C	FTIR first derivative data			TVC	0.470	6%	1.004	0.95
		9	9	Y&M	0.711	10%	0.989	0.87

PC-principal components; SEP- Standard error of prediction; A_f-Accuracy factor; B*f*- Bias factor

3.1.4.2.1.2 Pomegranate

The performance indices of the MLP networks to predict TVC and Y&M in minimally processed pomegranate samples stored at 10 °C is presented in Table 16. The network selected for FTIR spectral data demonstrated $R^2 = 0.909$ while model built using FTIR first derivative gave R^2 of 0.619. It is clearly evident that lower deviation with higher A_f between actual and predicted counts for both TVC and Y&M was obtained for models built with FTIR spectral data as compared to models built using FTIR first derivative data (Table 16).

Table 16: Performance indices of models build using ANN for FTIR data for minimally processed pomegranate stored at 10 °C.

Statistical Measurement	**FTIR Spectral Data**		**FTIR First Derivative Data**	
	TVC	**Y&M**	**TVC**	**Y&M**
R^2	0.909		0.619	
PC	8		17	
SEP	0.914	0.784	1.003	1.058
A_f	20%	14%	22%	36%
B_f	0.944	0.917	0.991	1.197
Hidden neuron	8		8	

PC-principal components; SEP- Standard error of prediction; A_f-Accuracy factor; B*f*- Bias factor

3.1.4.2.1.3 Pineapple

The performance indices of the MLP networks to predict TVC and Y&M in minimally processed pineapple samples are presented in Table 17. For pineapple samples stored at 10 °C, the R^2 obtained for the model generated using FTIR spectral data was 0.95. The average deviation between observed and predicted counts was 8 and 7% with corresponding low SEP

values of 0.54 and 0.45 for TVC and Y&M, respectively. ANN models could not be built for FTIR first derivative data because 23 PCs could explain 95% total variance. Current sample size was insufficient to built the model with 23 PCs as input variable. However, FTIR spectral data could be successfully utilised for building ANN model for prediction of TVC and Y&M counts.

Table 17: Performance indices of models build using ANN for FTIR data for minimally processed pineapple stored at 10 °C.

Statistical	FTIR spectral data (10 °C)	
	TVC	Y&M
R^2	0.95	
PC	3	
SEP	0.54	0.45
A_f	8%	7%
B_f	1.02	1.04
Hidden neuron	10	10

PC-principal components; SEP- Standard error of prediction; A_f-Accuracy factor; B*f*- Bias factor

3.1.4.2.1.4 Discussion and conclusions of ANN models using FTIR data

Quantitative estimation of microbial spoilage could be successfully demonstrated using FTIR for all the three fruit samples. It was also observed that the models developed using FTIR data in the form of FTIR spectral data showed better performance indices with $R^2 \geq 0.84$ for both TVC and Y&M in case of all the fruit samples. Despite the fact that FTIR first derivative data carried more information than FTIR spectral data, it was observed that FTIR spectral data gave better performance with ANN models. This could be due to the fact that higher PCs were generated in case of FTIR first derivative data to explain 95% variance. Larger number of input variables also requires large sample size. Thus, increasing the number of sample size may also

increase the performance of ANN models using FTIR derivative data. When the models for TVC and Y&M counts were compared, it was evident that models developed for TVC had better performance indices for jackfruit. Models build for Y&M however, had better performance indices in case of pomegranate and pineapple. This is due to the fact that Y&M growth were higher in this acidic fruits thus better correlations could be established.

A study conducted on TVC prediction for meat samples gave R^2 of 0.94 [144]. The application of ANN to correlate FTIR data with microbial counts has been previously reported in meat products [101, 144]. Similar results are also observed in the present study. This is the first study showing use of FTIR for estimation of microbial counts in minimally processed fruits.

3.1.4.2.2. Supervised PLS-R for predicting microbial quality in minimally processed fruits using FTIR data

Partial least square regression models were built to correlate TVC and Y&M with FTIR spectral data and FTIR first derivative spectral data as independent variable and microbial counts as dependent variable.

3.1.4.2.2.1 Jackfruit

The performance of the PLS-R models to predict TVC and Y&M in minimally processed jackfruit samples in terms of statistical indices is presented in Table 18. In models built employing FTIR spectral data for jackfruit samples stored at 4 °C, the R^2 were low for both TVC (0.37) and Y&M (0.32) counts. This suggests FTIR spectral data might not be suitable for prediction of microbial counts in jackfruit samples. However, for models built with FTIR first derivative data, better R^2 values were obtained for both TVC (0.68) and Y&M (0.66). The A_f for TVC and Y&M were 12 and 24%, respectively with corresponding SEP value of 0.76.

Table 18: Performance indices and predicted microbial counts of models generated for TVC and Y&M using PLS-R for FTIR data in minimally processed Jackfruit at 4 °C.

4 °C		Observed (Log_{10} CFU/g)	Predicted (Log_{10} CFU/g)	LV	A*f*	B*f*	*SEP*	R^2
	TVC	4.24	4.15	2	11%	0.89	0.26	0.37
		5.85	4.76					
		6.01	5.36					
FTIR spectral data	Y&M	4.0	2.89	2	27%	0.78	0.96	0.32
		4.8	3.59					
		4.24	4.30					
	TVC	4.24	4.30	2	12%	0.89	0.76	0.68
		5.85	4.68					
		6.01	5.39					
	Y&M	2.3	3.09	3	24%	0.98	0.76	0.66
FTIR first derivative data		4.0	2.97					
		4.83	4.57					

LV- latent variable; SEP- Standard error of prediction; A_f-Accuracy factor; B*f*- Bias factor

For jackfruit samples stored at 10 °C, the R^2 obtained for TVC and Y&M using FTIR spectral data was 0.85 and 0.68, respectively as depicted in Table 19. The models built for TVC had better performance indices with a low SEP (0.615) and higher A_f (9%) when compared to Y&M with SEP of 0.88 and A_f of 16 %. FTIR first derivative data had higher R^2 of 0.90 and 0.85 for TVC and Y&M models when compared to FTIR spectral data having corresponding R^2 values of 0.85 and 0.68. Average deviation between predicted and actual counts was found to be 11 and 23% for TVC and Y&M, respectively.

In general, it was observed TVC prediction performance had better (higher A_f, Table 19) values for jackfruit samples using both the FTIR data when compared with Y&M prediction.

Table 19: Performance indices and predicted microbial counts of models generated from FTIR data for TVC and Y&M using PLS-R in minimally processed Jackfruit at 10 °C.

10°C		Observed (Log_{10}CFU/ g)	Predicted (Log_{10}CFU/ g)	LV	A_f	B_f	SEP	R^2
FTIR spectral data	TVC	5.25	5.74	4	9 %	1.09	0.615	0.851
		6.03	6.21					
		7.08	7.87					
		4.96	5.73					
	Y&M	3.23	4.74	4	16 %	1.13	0.881	0.686
		5.69	5.55					
		6.83	6.70					
		4.36	5.23					
First derivative	TVC	5.25	4.87	4	11 %	1.00	0.78	0.905
		4.96	6.20					
		6.03	5.84					
		7.08	6.39					
	Y&M	3.23	2.42	4	23 %	0.91	0.97	0.857
		4.36	5.58					
		5.69	4.79					
		6.83	5.91					

LV- latent variable; SEP- Standard error of prediction; A_f-Accuracy factor; B*f*- Bias factor

3.1.4.2.2.2 Pomegranate

For pomegranate samples stored at 10 °C, the R^2 obtained for TVC and Y&M using FTIR spectral data was 0.83 and 0.64, respectively as shown in Table 20. The performance indices of models built for TVC had SEP and A_f of 0.79 and 22 % respectively, while for Y&M, the corresponding values were 1.21 and 37 % respectively. Prediction models built using FTIR spectral data demonstrated large deviation (>20%) in the predicted counts for both TVC and Y&M. In case of FTIR first derivative data R^2 of 0.85 and 0.91 for TVC and Y&M models was obtained. TVC prediction had SEP of 0.78 and A_f of 19 % while corresponding values were 0.77 and 23% for Y&M.

Table 20: Performance indices of models generated for TVC and Y&M using PLS-R for FTIR data in minimally processed pomegranate at 10 °C.

Statistical Measurement	**FTIR spectral data**		**FTIR first derivative data**	
	TVC	Y & M	TVC	Y & M
R^2	0.83	0.643	0.851	0.929
B_f	0.91	0.97	0.9	1
A_f	22%	37%	19%	23%
SEP	0.79	1.21	0.78	0.77
LV	6	5	3	7

LV- latent variable; SEP- Standard error of prediction; A_f-Accuracy factor; B*f*- Bias factor

3.1.4.2.2.3 Pineapple

For pineapple samples the performance indices of PLS-R models are shown in Table 21. It was observed that FTIR spectral data had a R^2 of 0.71 for TVC and 0.69 for Y&M, with a SEP of 1.22 and 0.98, and corresponding A_f values of 29 and 24 %. FTIR first derivative data gave promising results with R^2 of 0.92 and 0.91 for TVC and Y&M, and a lower SEP of 0.76 and 0.71 with an A_f of 11 and 10 %, respectively. Similar results were observed for jackfruit and pomegranate sample with FTIR first derivative data performing better when compared to FTIR spectral data using PLS-R.

Table 21: Performance indices of models generated for TVC and Y&M using PLS-R for FTIR data in minimally processed pineapple at 10 °C.

Statistical Measurements	**FTIR spectral data**		**FTIR first derivative data**	
	TVC	Y&M	TVC	Y&M
R^2	0.715	0.692	0.925	0.938
B_f	1.101	1.06	0.958	0.987
A_f	29 %	24 %	11%	8%
SEP	1.22	0.98	0.76	0.53
LV	4	3	6	7

LV- latent variable; SEP- Standard error of prediction; A_f-Accuracy factor; B*f*- Bias factor

3.1.4.2.2.4 Discussion & conclusions of PLS-R models using FTIR data

Quantitative estimation of microbial spoilage could be successfully demonstrated using FTIR for all the three fruit samples stored at 10 °C. An $R^2 \geq 0.68$ was obtained for all the developed models for both TVC and Y&M counts. Ammor *et al*. [145] showed an R^2 of 0.73 for TVC prediction of minced beef sample, while another study conducted on minced pork demonstrated R^2 of 0.88 and A_f of 7.5 % using FTIR spectral data. Our results are thus similar to that reported in literature. It was also observed that the models developed using first derivative FTIR showed better performance indices with $R^2 \geq 0.85$ for both TVC and Y&M in case of all the fruit samples. This could be due to fact that underlying variations in the overlapped FTIR spectra are clearly identified using first derivative spectra. Similar results were observed by Duarte *et al.* [146] while employing FTIR spectra for quantification of sugars in mango juices. When the models for TVC and Y&M counts were compared, it was evident that models developed for TVC had an overall better performance index for jackfruit while models build for Y&M had better performance indices in case of pomegranate and pineapple. A similar trend was also noted for all the fruit samples studied when PLS-R analysis was applied on GCMS data.

The correlation matrix in PLS-R for the annotated peak showed that few IR absorption bands were highly correlated to microbial counts (positively or negatively) and depended on storage period (Table 22). Absorption bands 1156 and 1252 cm^{-1} were found to be positively correlated with microbial counts while 1062, 1078, 1105, 1419 and 1453 cm^{-1} had negative correlations with microbial counts. Wave number 1156 cm^{-1} corresponds mainly to the absorption of alcohols while 1419& 1453 cm^{-1} to that of sugars and 1252 cm^{-1} corresponding to the presence of acids [146-149]. These results suggest the production of alcohols and acids with utilization of sugars during storage. Carlin *et al.* [150] also observed production of

different acids (lactic, acetic, malic, succinic, and pyruvic acids) during storage of minimally processed carrots. Thus, biochemical changes represented by the metabolic fingerprint in the FTIR profiles of the fruit samples were utilised for quantitative prediction of TVC and Y&M.

Table 22: Correlation analysis of annotated FTIR peaks with TVC and Y&M

Frequency (cm^{-1})	Assignment	Fruit	Correlation analysis
1720-1580 (1638)	C=O stretching and H-O-H def	-all-	+
1433-1460(1443)		Jack/Pine	+
1400-1421 (1419)	O-C-H, C-C-H, C-O-H bending of carbohydrates	-all-	-
1350-1382 (1353,1364,1372)	CH_2 rocking, O-H bending of organic acids	-all-	+
1330-1294 (1314)	O-C-H, C-C-H, C-O-H deformation of carbohydrates	Pom	-
1239-1270 (1250)	-C-O acid stretching	-all-	+
1142-1187 (1156)	C-O stretching of secondary & tertiary alcohol	Pom/pine	+
1078 and 1105	C-O & C-H stretch of sugars, C-O-H bending	-all-	-
1062	C-O & C-H stretch of sugars	-all-	-
1032	C-O & C-H stretch of sugars	-jack/pom	-
1018	-	Jack	-
923	C-C stretch of sugar (fructose)	Jack	-

3.1.4.1.3.1 Comparison of HS-SPME-GCMS and FTIR

Biochemical changes monitored using GCMS and FTIR could be successfully employed for rapid microbial quality assessment in all the three fruits using PLS-R and ANN.

It can be observed from Table 23 that GCMS models for all the fruit samples gave the best performance with low average deviation values between observed and predicted counts well within the acceptable range of within 20% error from the observed values [70]. HS-SPME GCMS allows rapid, solvent less extraction of volatiles from the food packages. Thus, it offers rapid monitoring of headspace volatiles that can monitor biochemical changes associated with microbial spoilage [16, 67, 151]. However, GCMS run time in the current study was 30 minutes for separation of the volatile constituents. Moreover, peak alignment, identification and quantification add up to additional labour and expertise involved.

Models build using FTIR data could also predict test samples within 20% of deviation for all the fruit samples with few exceptions, however the average deviation between observed and predicted counts were higher when compared to GCMS models (Table 23). Fourier transform infrared spectroscopy (FTIR) is a fast, easy to use, reagent less and non-destructive technique for obtaining biochemical information of food samples [110]. Ellis et al. [2] has been a pioneer in demonstrating FTIR as a useful tool for early detection and rapid monitoring of microbial spoilage. Till date several reports have been published on the application of this technique in food products such meat, poultry, milk and fruit juices as detailed in the review by [71]. However, there are only few reports that have demonstrated their use for microbial quality assessment [152, 153]. The current study therefore investigated the use of FTIR as a tool for rapid microbial assessment in minimally processed fruit samples. It takes less than a minute for obtaining FTIR spectra. Further, ease of sample preparation and data handling makes it suitable for online monitoring in industrial application. Thus, both GCMS and FTIR demonstrated their utility for assessment of microbial quality. GCMS offers identification of individual spoilage marker volatiles while FTIR provides overall metabolic fingerprint.

3.1.4.3.2 Comparison of PLS-R and ANN as supervised prediction tools

In conclusion, it was observed that both PLS-R and ANN demonstrated good performance when applied to two different instrumental techniques. Best performance was observed when PLS-R & ANN were applied on GCMS data with very low average deviation between observed and predicted counts (Table 23). However, ANN when applied on FTIR spectral data also demonstrated high accuracy in all the fruit samples.

PLS-R can be applied to a set of collinear variables as observed in GCMS and FTIR data for microbial counts. PLS-R is popular due to its ease of use, fast computation, good predictive performance and easy interpretable representations [71]. This automated tool allows ease in performing operations to food scientist with limited mathematical and statistical expertise to perform the challenging task of data mining and predictive modelling. However, PLS-R cannot be applied on uncorrelated variables thus limiting its use for co-linear dataset.

ANN was applied to determine non-linear relationships between instrumental data and microbial counts. MLP-ANN is adaptive and learns from the data-set by creating the required decision function. Therefore, it allows for application on versatile data set [87]. Models build using ANN in the current study also showed good performance suggesting non-linear relation between spectra and microbial quality. However, ANN suffers from the following disadvantages. Firstly, the data set has to be converted into set of un-correlated input variable, Secondly, defining the number of neurons in the hidden layer requires many trial runs along with several iterations for effective learning. Lastly, new training overwrites the properties of the existing network if existing data are not included in the new training process. Application of ANN is therefore a cumbersome task demonstrating its lower suitability than PLS-R.

Table 23. Overall comparison of different techniques based on average deviation between observed and predicted.

		Average deviation between observed Vs Predicted (A_f)			
		PLS-R		ANN	
		TVC	Y&M	TVC	Y&M
Jackfruit	GCMS	7.6 %	11 %	17%	15%
	FTIR spectra data	9 %	16 %	10%	20%
	FTIR first derivative data	11 %	23 %	6%	10%
Pomegranate	GCMS	6 %	10 %	14%	9%
	FTIR spectra data	22 %	37 %	20%	14%
	FTIR first derivative data	19 %	23 %	22%	36%
Pineapple	GCMS	9 %	13 %	2%	6%
	FTIR spectra data	29 %	24 %	8%	7%
	FTIR first derivative data	11 %	8 %	ND	ND

3.2 Development of Time temperature Indicator for real time quality monitoring of minimally processed fruits

Microbial spoilage is reported to be the primary factor in the deterioration of almost all minimally processed fruits [1]. Thus, microbial load in terms of total viable bacterial count (TVC) and Yeast & Mould count (Y&M) are considered the major parameters determining the quality of minimally processed fruits. Like chemical reactions, growth rate of microbes is strongly dependent on temperature. Just as temperature dependence of chemical reaction is expressed in terms of activation energy (Ea), microbial spoilage as influenced by temperature can also be expressed in terms of Eα. It therefore becomes imperative to tailor the Ea for the chemical reaction with that of microbial growth at different temperatures to obtain a correlation between the two processes thereby facilitating development of a smart tag for sensing food quality status. When rates of microbial growth at various temperatures is kinetically synchronised with chronochromic evolution process of TTI an intelligent tag can be designed and applied to each package during refrigerated storage. The pack will then individually monitor the temperature effects on the product based on colour evolved thereby providing real-time quality status of the product.

3.2.1 Growth rate evaluation of TVC and Y&M

Microbial growth during storage under aerobic conditions in minimally processed pineapple, pomegranate and jackfruit stored at 4, 10, 20 & 37 °C, expressed as total viable counts (TVC) and yeast & mould counts (Y&M), is shown in Figure 25. Table 24 provides maximum specific growth rate (μ_{max}) at different storage temperature and Eα values for TVC and Y&M growth for minimally processed fruits. It can be clearly observed from Fig. 16 that rate of microbial growth is more for samples stored at higher temperatures of 20 and 37 °C as compared to samples stored at lower temperatures of 4 and 10 °C. This observation is supported

by significant ($p<0.05$) increase in μ_{max} with increasing storage temperature for all the samples studied (Table 24). μ_{max} increased by 9.8, 12.7 and 25 times for pineapple, pomegranate and jackfruit, respectively when storage temperature was increased from 4 to 37 °C. Thus, increase in microbial load could be stimulated by elevated storage temperatures. The corresponding Eα value for growth of TVC on pineapple, pomegranate & jackfruit were 46.28, 52.41 and 71.66 kJ/mol. Additionally, the Eα value for Y&M growth were 41.50, 61.52 and 69.83 kJ/mol for pineapple, pomegranate and jackfruit, respectively. The values of Eα obtained were in the range of 46.28 to 71.66 kJ/mol, the typical activation energy values (83-251 kJ/mol) reported for microbial spoilage [154]. Similar Eα values (42.58 kJ/mol) were reported by Andreas *et al.* [155] for Y&M counts for cut apple cubes under different treatment and packaging conditions. The Eα values obtained from Arrhenius relation are indicative of product shelf life due to microbial growth. The lower Eα value indicates faster microbial growth leading to rapid product deterioration and effective shorter shelf life. In case of cut pineapple slices, Eα for Y&M growth was lower than TVC because of the low pH (3.2) value of the fruit which favours yeast over bacteria [156, 157]. In contrast, jackfruit has a higher pH of 5.1 which is not favourable for Y&M growth (Eα, 69.83 kJ/mol), allowing for a greater competition with TVC [158]. In case of pomegranate, however, even though the pH of pomegranate juice is acidic (4.2), the intact arils did not contribute to acidic environment thus favouring TVC (Eα, 52.41 kJ/mol) over Y&M (Eα, 61.52 kJ/mol).

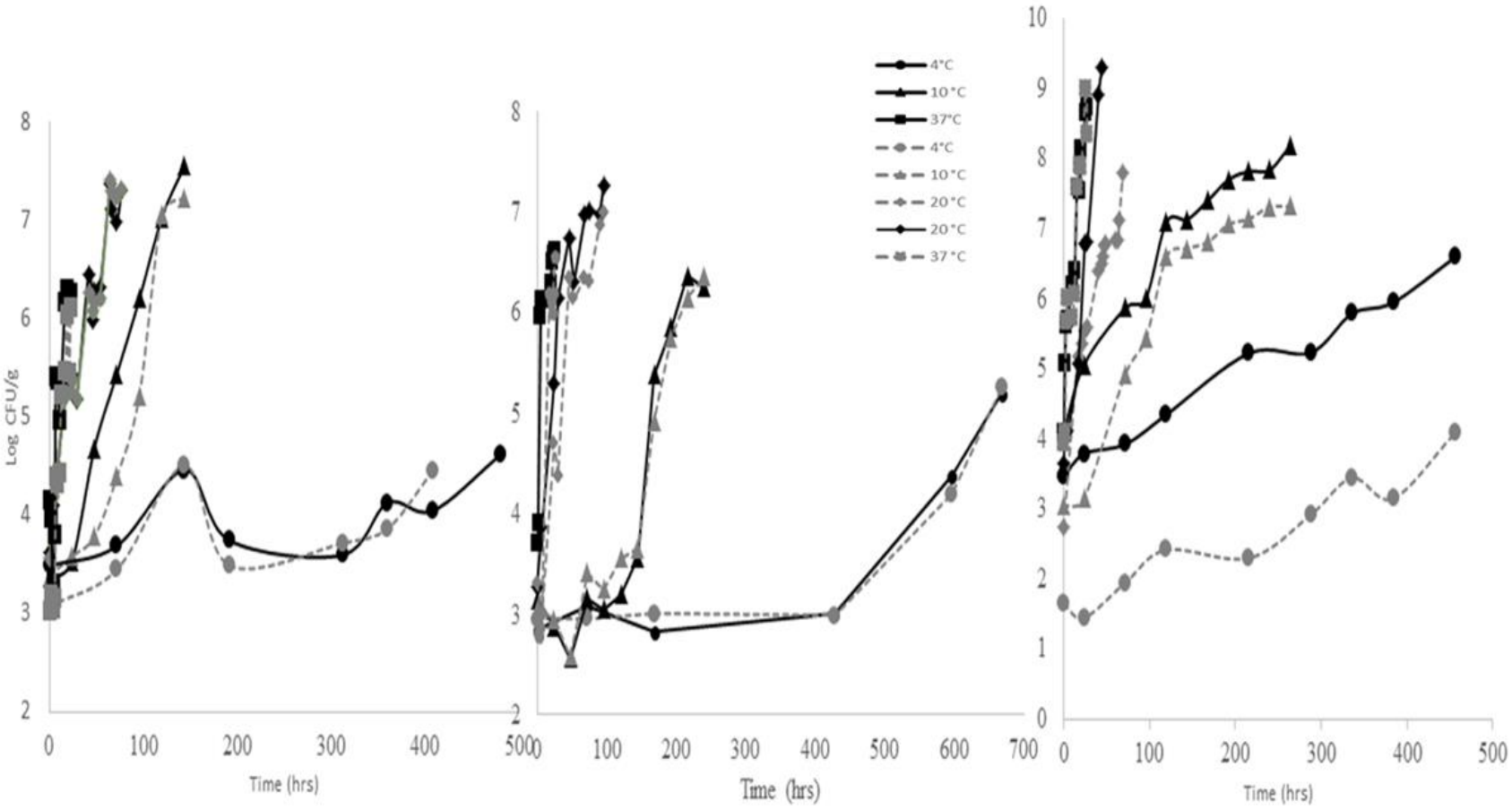


Figure 25: Microbial growth curve of TVC (solid black line) and Y&M (grey dotted line) at 4, 10, 20 and 37 °C of storage

Table 24: Kinetic parameters of microbial spoilage of minimally processed jackfruit, pomegranate and pineapple

		TVC				Y&M			
	Temp-erature (°C)	**μ_{max} (h^{-1})**	**R^2**	**Ea (kJ/mol)**	**R^2**	**μ_{max} (h^{-1})**	**R^2**	**Ea (kJ/mol)**	**R^2**
Pineapple	4	0.020±0.007	0.90	46.28	0.95	0.026±0.004	0.96	41.70	0.78
	10	0.037 ± 0.003	0.98			0.045 ± 0.009	0.84		
	20	0.05 ± 0.005	0.93			0.049 ± 0.004	0.94		
	37	0.196 ± 0.09	0.97			0.224 ± 0.05	0.90		
Pomegranate	4	0.010 ± 0.001	0.98	52.41	0.99	0.014 ± 0.005	0.99	61.52	0.99
	10	0.029 ± 0.004	0.91			0.0266 ± 0.003	0.94		
	20	0.089 ± 0.01	0.96			0.083 ± 0.02	0.92		
	37	0.127 ± 0.005	0.89			0.282 ± 0.06	0.976		
Jackfruit	4	0.0065 ± 0.0003	0.98	71.66	0.80	0.0052 ± 0.001	0.90	69.8376	0.83
	10	0.020 ± 0.001	0.96			0.035 ± 0.004	0.98		
	20	0.146 ± 0.01	0.98			0.085 ± 0.01	0.91		
	37	0.165 ± 0.01	0.95			0.184 ± 0.03	0.87		

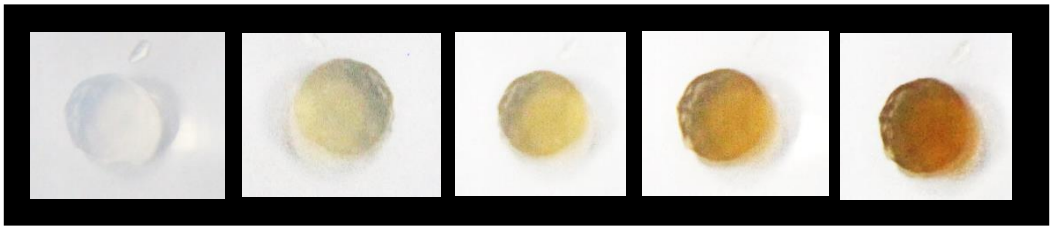
μ_{max}-maximum specific growth rate; R^2-co-efficient of determination; Ea-Activation energy

Further, based on Eα values it can also be concluded that pineapple is more susceptible to microbial deterioration as compared to pomegranate and jackfruit. This observation is also supported by the fact that in stored pineapples microbial counts increased to > 10^7CFU/ g for TVC in 21 h when compared to 24 and 28 h respectively for pomegranate and jackfruit at storage temperature of 37 °C.

3.2.2 Phenol oxidation based TTI

The initial criteria set for TTI development was that the chemical reaction should result in complete colour development between 24 to 36 h at 37 °C. This criterion was set, because it was observed that the microbial counts reached ≥ 7 Log_{10} CFU/g within the period of 21 to 28 h at 37 °C for all the three fruit samples. Phenol showed a slow transition of colour change and could be modulated for its final colour development in the desired period (24-36 h) at 37 °C. The concentration of phenol also played a significant role with a change in colour from less intense light brown to dark brown. Several different concentrations of phenol were tried (data not shown). A final concentration of 0.5 % that gave a distinguishable sharp change in colour from colorless to deep brown was chosen. Figure 26 shows the typical curve for colour development in terms of optical density with respect to time at 5 different temperature of storage for a TTI prototype (0.5% Phenol, 47 mM Na_2CO_3, 21 mM APS).

The colour of TTI hydrogel in all cases changed from colourless to dark brown due to oxidation of phenol to form quinones resulting in absorption of light to 440 nm [159]. Several combinations of TTI prototypes (Table 25) were tried in an array to derive the activation energies comparable to the Eα obtained for microbial growth which was found to be in the range of 52.96–84.05 kJ/mol.



Change in colour of candidate TTI for 24h at 37 °C

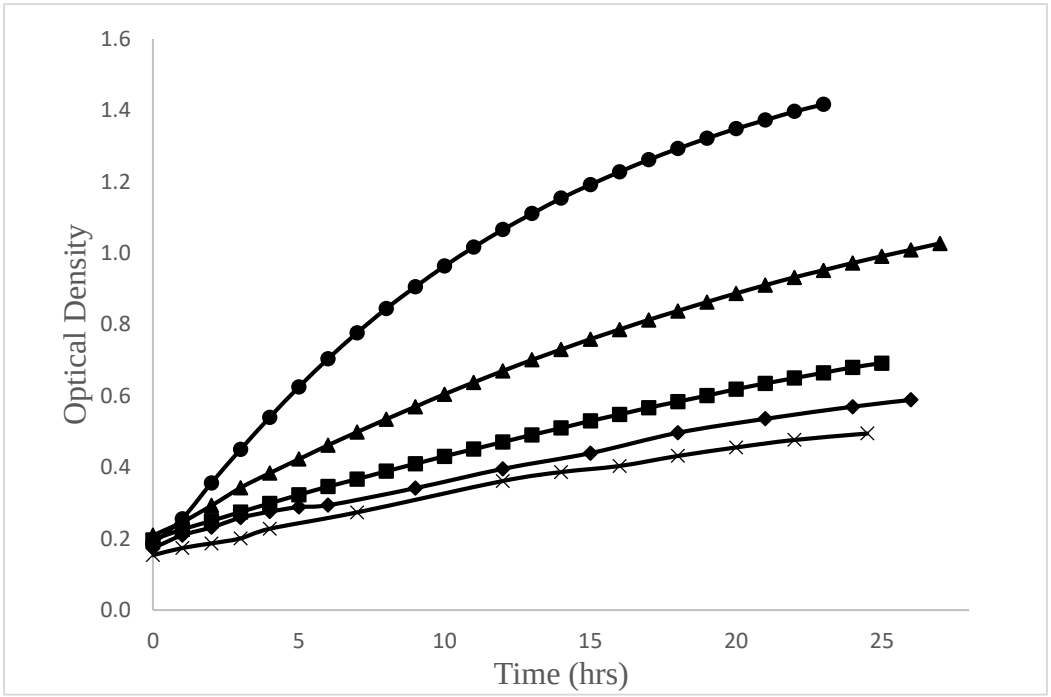


Figure 26: Colour development of Typical TTI in terms of O.D vs time at different temperature of storage; (—×—) 10, (—◆—) 20, (—■—) 30, (—▲—) 37 and (—●—) 45 °C.

Table 25: The activation energy (Ea) values in kJ/mol for different TTI prototype recipe mix.

Na_2CO_3	23 mM	35.4 mM	47mM	59mM
APS ⬇				
21 mM	84.05±4.87	76.77±2.54	68.83±1.89	66.67±1.01
32 mM	76.57±3.75	59.77±1.89	60.44±3.37	52.96±2.19
43 mM	64.68±3.82	60.31±2.06	56.64±1.43	53.59±1.09

Data of concentration of various ingredients and corresponding Ea obtained were analysed by fitting them in full factorial design followed by ANOVA. Results of ANOVA suggest that both sodium carbonate and APS demonstrated significant ($p<0.05$) effect on Ea. The 3D plot shows that as the concentration of both the factors increase there was a corresponding decrease in activation energy of TTI. (Figure 27). It was also observed that when the concentration of one factor is kept fixed at a central value, changing the concentration of other factor decreases activation energy. The effect was more pronounced with sodium carbonate. (Figure 28). Thus, it can be concluded that contribution of sodium carbonate was higher when compared to APS in decreasing the activation energy.

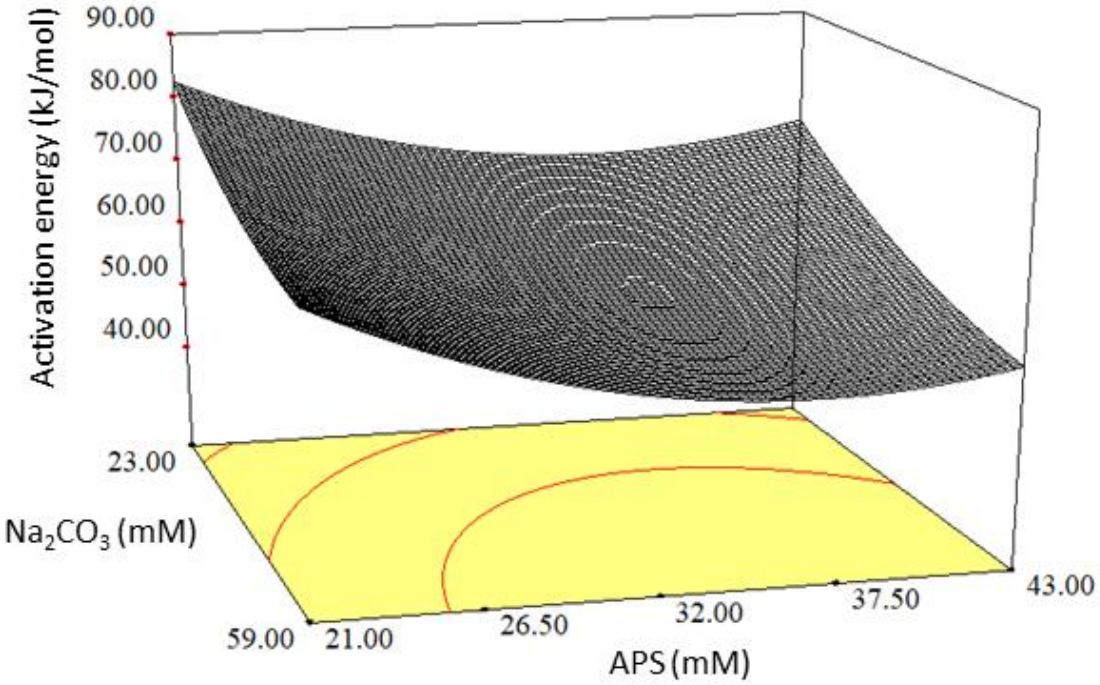


Figure 27: 3D plot showing effect of sodium carbonate (Na_2CO_3) and APS concentration on activation energy

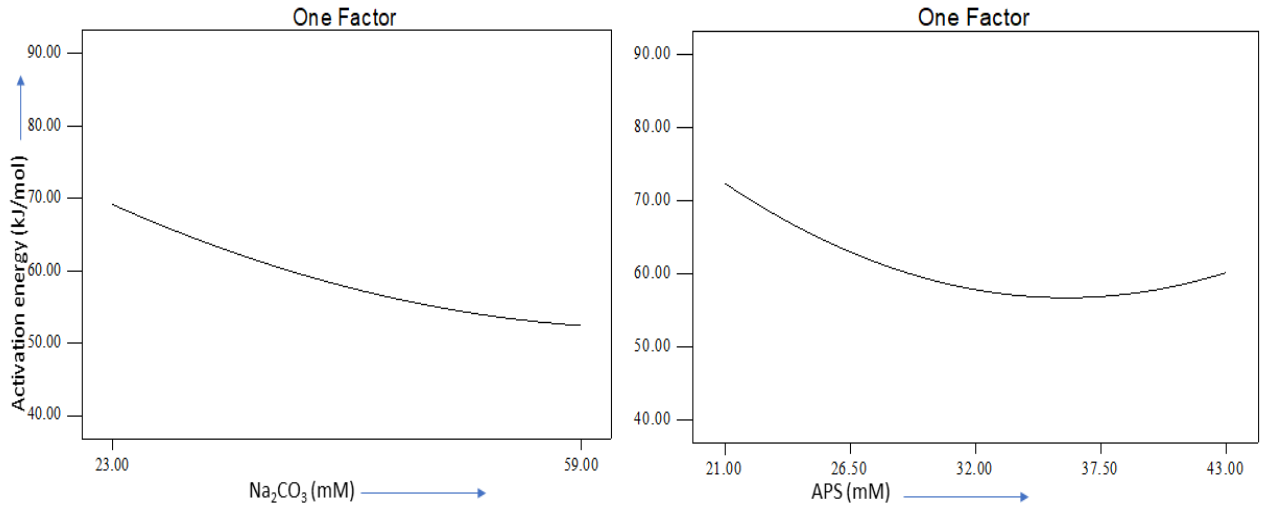


Figure 28. One factor plot when other factor is kept constant at its middle value of concentration

Kim *et al.* [160] utilized a laccase enzyme based oxidation reaction of phenolic compound guaiacol for development of TTI to obtained Ea in the range of 43.9 to 46.9 kJ/mol. Park *et al.* [161] further tuned the activation energy of laccase based TTI for a broader range (48-110 kJ/mol) using sodium azide that falls in the same range as evaluated in the current study. However, enzyme based systems has drawbacks such as instability and substrate specificity, thus limiting the system for wider applicability. Uddin *et al*. [162] developed a TTI based on non-enzymatic browning of fructose and glycine with activation energy range between 58.05 and 95.54 kJ/mol and suggested their usefulness in monitoring food quality. However, the study did not demonstrate its real time application with model food system as conducted in the present study.

3.2.2.1 Studies on Kinetic parameters

Four candidate TTI with Ea values of 59.77 (TTI I: 35.4 mM Na_2CO_3, 32 mM APS), 66.67 (TTI II: 59 mMNa_2CO_3, 21 mMAPS), 76.73 (TTI III 23 mM Na_2CO_3; 32 mM APS) & 84.05 kJ/mol (TTI IV 23 mM Na_2CO_3; 21 mM APS) were selected to evaluate their suitability on minimally processed fruits (Table 26). These candidate TTIs have their Ea values in the range of Eα of microbial spoilage of minimally processed fruit selected (46.28 to 71.66 KJ/mol). Figure 29 shows the Arrhenius plot of the reaction rate constants of these TTIs plotted against temperature. Table 26 provides the value of reaction rate constants of the four candidate TTIs and the Ea value obtained from the Arrhenius plot. The reaction rates of TTI increased as the concentration of APS and Na_2CO_3 increases, implying that the activation energy decreases following the Arrhenius relationship.

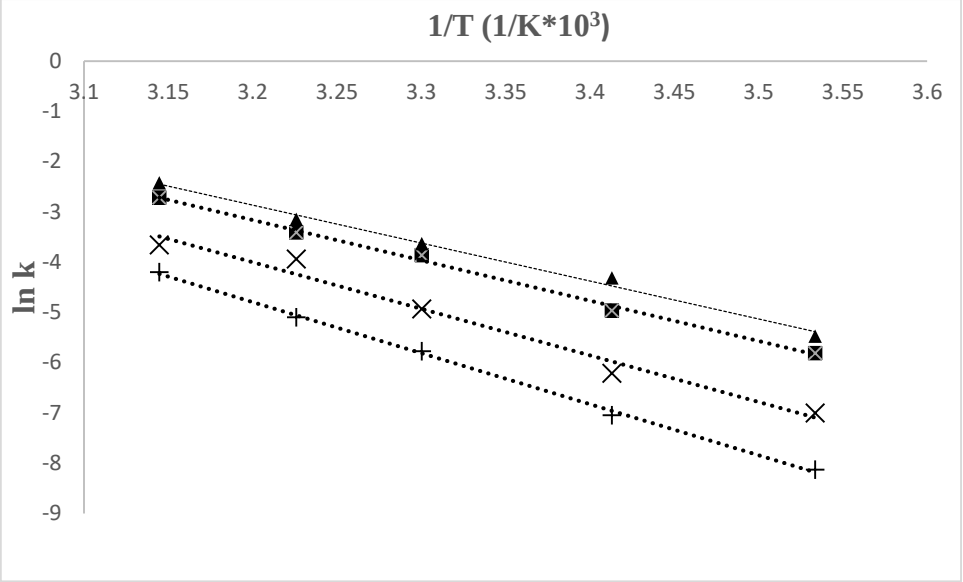


Figure 29: Arrhenius plot of 4 TTI prototypes with varying range of activation energies

Table 26. The reaction rate constants (k) of different TTI prototypes with varying concentrations

Temperature (ºC)	Reaction rate constant (h^{-1}) of TTI prototypes			
	35.4 mM Na_2CO_3; 32 mM APS	59 mM Na_2CO_3; 21 mM APS	23 mM Na_2CO_3; 32 mM APS	23 mM Na_2CO_3; 21 mM APS
10	0.0042±0.0008	0.003±0.0007	0.0009±0.0003	0.0003±0.0001
20	0.0133±0.002	0.007±0.001	0.002±0.0009	0.00087±0.00005
30	0.0263±0.0041	0.021±0.009	0.0125±0.0067	0.0031±0.0012
37	0.0429±0.0009	0.033±0.012	0.0194±0.0032	0.0061±0.003
45	0.0889±0.004	0.066±0.021	0.0257±0.0014	0.015±0.008
Ea (kJ/mol)	59.77±1.89	66.67±1.01	76.57±3.75	84.054±4.87
R^2	0.9924	0.9964	0.9772	0.9985

Ea-activation energy

3.2.3 Establishing correlations between colour change between TTI and microbial growth

Eα for microbial growth in case of pineapple was 46.28 and 41.70 kJ/mol for TVC and Y&M, respectively. Values of Eα obtained for TVC and Y&M were 52.41 and 61.52 kJ/mol, respectively for pomegranate. Therefore, TTI I having Ea of 59.77 kJ/mol would be considered acceptable based on the established criteria that difference in activation energy between TTI and the food system should be less than 25 kJ/mol [104]. Similarly, jackfruit demonstrated Eα values of 71.66 and 69.83 kJ/mol for TVC and Y&M, respectively. Hence, TTI II (Ea-66.67 kJ/mol) could be suitable for jackfruit sample. Correlations were established between the colour change in TTI and microbial counts to identify the suitable indicator for evaluating the microbial spoilage. Table 27 represents the correlations obtained between optical density of chosen TTI prototype with the TVC and Y&M counts at three storage temperatures (10, 20 and 37 °C) for minimally processed fruit. It can be observed from the table that TTI type I (Ea-59.77 kJ/mol) was found suitable for pineapple with high correlation coefficients ($R^2 > 0.94$) for TVC and Y&M ($R^2 > 0.91$) at all the three storage temperatures thus ensuring synchronicity at varied temperature range [163]. TTI type I was also found to be suitable for minimally processed pomegranate with $R^2 > 0.89$ for TVC and Y&M (Table 27). Similarly, TTI type II (Ea-66.67 kJ/mol) gave high correlations with $R^2 > 0.85$ for both TVC and Y&M in jackfruit at all the three storage temperatures. These high R^2 values demonstrate the feasibility of chronochromic evolution of TTI when synchronised with the spoilage kinetics in terms of microbial counts. Correlation studies conducted by Smolander *et al.* [164] between spoilage microorganisms of modified atmosphere packed broiler cut and commercial TTI demonstrated higher R^2 (≥ 0.85) than metabolic analytes such as volatiles, biogenic amines and organic acid for studying loss of food quality.

Table 27: Regression equation of selected TT1 with TVC and Y&M counts of minimally processed fruits at different storage temperature

TTI	T (°C)	Equations for TVC	R^2	Equations for Y&M	R^2
TTI-I With pineapple	10	y = 6.1892x + 1.9634	0.9485	y = 5.445x + 1.7757	0.91
	20	y = 2.8839x + 3.2802	0.9856	y = 2.943x + 3.1873	0.97
	37	y = 4.5642x + 2.8281	0.7897	y = 5.6966x + 1.7883	0.827
TTI-I With pomegranate	10	y = 4.7344x + 1.2567	0.9622	y = 4.2281x + 1.4588	0.9425
	20	y = 3.0762x + 2.9403	0.9819	y = 2.5729x + 2.9553	0.955
	37	y = 4.0246x + 3.4751	0.8144	y = 7.1156x + 0.6148	0.9828
TTI II with jackfruit	10	y = 4.9136x + 2.0477	0.9638	y = 6.3537x - 0.031	0.9549
	20	y = 6.7345x + 2.2458	0.9182	y = 5.383x + 1.9843	0.9969
	37	y = 3.6627x + 3.5091	0.9339	y = 3.9798x + 3.1143	0.8573

Correlations were not established for 4 °C stored fruit samples because even after extended period of storage, increase of 1 Log_{10} CFU/g in case of pineapple and pomegranate was observed. Jackfruit samples reached to the counts of 6.5 Log_{10} CFU/g and 4 Log_{10} CFU/g for the storage period of 19 days which are below the regulatory limits of 7 Log_{10} CFU/g and 5 Log_{10} CFU/g for TVC and Y&M, respectively [109]. Physiological changes instead of microbial spoilage are the primary reason for deterioration of fruit samples stored below or at 4 °C [24]. Similar trend was observed for the developed TTI. A very gradual colour change was observed at 4 °C extending to 21 days for the prototypes with high Ea (data not shown).

Further, to establish suitability of developed TTI, data from the three storage temperatures was merged to obtain a linear regression equation that provides the information of co-evolution of colour with microbial counts. Independent relationship of colour with microbial quality was not affected by time and temperature as shown in Figure 30. The R^2 observed for TTI I in case of TVC and Y&M counts of minimally processed pineapple were 0.82 and 0.75, respectively. For pomegranate samples better correlations were observed for Y&M (R^2=0.83) than TVC (R^2 = 0.66) using TTI I. For jackfruit samples, R^2 of 0.88 and 0.81

were obtained for TVC and Y&M, respectively with TTI II. Inference can be drawn that good correlation were observed even at broad temperature ranges utilised in the study (10 to 37 °C). This range covers the market conditions of storage for minimally processed fruits. The synchronicity of TTI with microbial grow at one temperature necessarily does not guarantee its performance at other temperatures because there can be slight change in Ea value of the system [162]. Thus, synchronicity was demonstrated by studying broad temperature range.

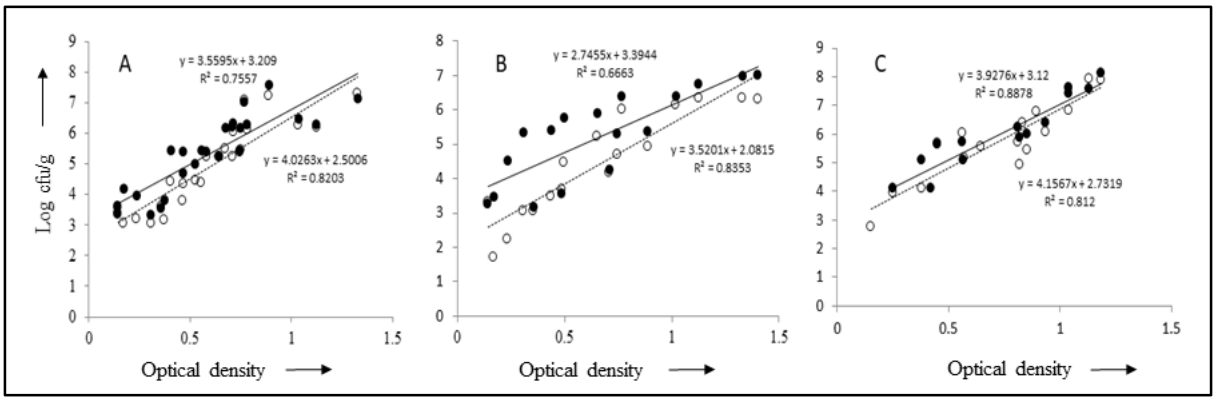


Figure 30. Correlation between O.D vs Log_{10}CFU/g for combined data at different storage temperatures. Solid circles- TVC, hollow circles- Y&M; A-pineapple samples; B-pomegranate samples; C-jackfruit samples.

3.2.4. Camera based rapid read out for quality monitoring

In order to validate the utility of TTI for monitoring the microbial status of minimally processed fruit, the fresh packed minimally processed jackfruit, pomegranate, pineapple and the candidate TTI's (Type I & II) were kept together to demonstrate the synchronicity at constant storage temperature of 10 °C and abusive storage cycle. The quality loss in terms of microbial load for packed produce and colour change of TTI were subsequently monitored simultaneously at different storage intervals. Moreover, for commercial suitability and to ensure rapidity of system the colour changes were monitored using DSLR camera and obtained in terms of ΔRGB scores.

Table 28 gives the R^2 between ΔRGB and TTI at 10 °C with TVC and Y&M for all the three fruit samples. Both the TTIs (I and II) demonstrated good correlations with $R^2 > 0.7$ for all the three fruits. The regression equation obtained was therefore utilised for calculations of predicted TVC and Y&M counts for samples stored under abusive conditions. The prediction performance was judged by evaluating the overall accuracy factor between observed and predicted counts (Table 29).

Table 28: Regression equation between ΔRGB scores of selected TTI with TVC & Y&M at 10 ºC for pineapple, pomegranate and jackfruit.

TTI	Equations for TVC	R^2	Equations for Y&M	R^2
TTI-I with pineapple	y = 4.9964x + 2.4389	0.71	y = 5.8245x + 2.4611	0.7371
TTI-I with pomegranate	y = 3.5319x + 2.9154	0.86	y = 4.4492x + 1.4196	0.722
TTI II with jackfruit	y = 6.4453x + 2.2399	0.74	y = 5.8192x + 2.8017	0.8187

In case of minimally processed pineapple, TTI I demonstrated R^2 of 0.71 and 0.74 with TVC and Y&M counts, respectively (Table 28). The overall prediction performance for samples stored under abusive condition could be explained by the accuracy factor obtained as 17.9 and 20.9 % for TVC and Y&M, respectively (Table 29). Plot between observed and predicted counts is shown in Figure 31. It can be clearly observed that the deviation between observed and predicted counts is ± 1 Log_{10}CFU/g for pineapple samples (Figure 31A). In case of minimally processed pomegranate, TTI I gave R^2 of 0.74 and 0.81 for TVC and Y&M, respectively (Table 28). Prediction for Y&M counts using TTI I data was characterised by low prediction error with a higher accuracy factor of 11.7%. However, for TVC counts, accuracy factor of 24.09% was observed indicating higher prediction errors. The difference between predicted and actual counts was, however, within the range of ± 1 Log_{10}CFU/g (Table 29,

Figure 31B). TTI II (Ea 66.67 kJ/mol) demonstrated R^2 value of 0.86 and 0.72 for TVC and Y&M, respectively for minimally processed jackfruit (Table 28). The accuracy factor obtained was 14 and 26.3 % for TVC and Y&M, respectively and the deviation in the observed vs predicted counts were mostly within the range of ± 1 Log_{10} CFU/g (Table 29, Figure 31C).

In general, it can be observed from the results that selected TTI resulted in prediction of both TVC and Y&M counts in all the three fruits selected within a range of ± 1 Log_{10}CFU/g and had an acceptable limit for prediction of microbial spoilage [70]. The selected TTI for all the three fruit samples could show good performance even when the samples were stored under abusive storage conditions to mimic market conditions.

Table 29. Performance of the selected TTI's for minimally processed pineapple, pomegranate and jackfruit.

Fruit sample	Microbial counts (R^2)	TTI	Observed (Log_{10} CFU/g)	Predicted (Log_{10} CFU/g)	Prediction error (%)	Accuracy factor (%)
Pineapple	TVC (0.73)	I	3.57	2.46	31.09	17.9%
			5.28	5.92	12.10	
			6.90	6.54	5.25	
			7.11	6.59	7.32	
	Y&M (0.50)	I	3.91	2.43	37.91	20.7%
			5.30	5.39	1.72	
			6.86	5.93	13.54	
			7.21	5.97	17.19	
Pomegranate	TVC (0.74)	I	3.28	2.23	31.91	24.09%
			5.58	4.16	25.33	
			6.13	6.07	1.04	
			6.93	6.25	9.83	
	Y&M (0.81)	I	3.28	2.80	14.65	11.7%
			5.33	5.13	3.74	
			6.03	6.26	3.65	
			7.13	6.42	9.95	
Jackfruit	TVC (0.86)	II	3.09	2.91	5.89	14.53%
			4.02	5.04	25.32	
			6.28	5.37	14.47	
			7.27	6.58	11.00	
	Y&M (0.72)	II	2	1.41	29.02	26.3%
			3.42	4.38	28.07	
			3.90	4.62	18.35	
			4.32	5.15	19.08	

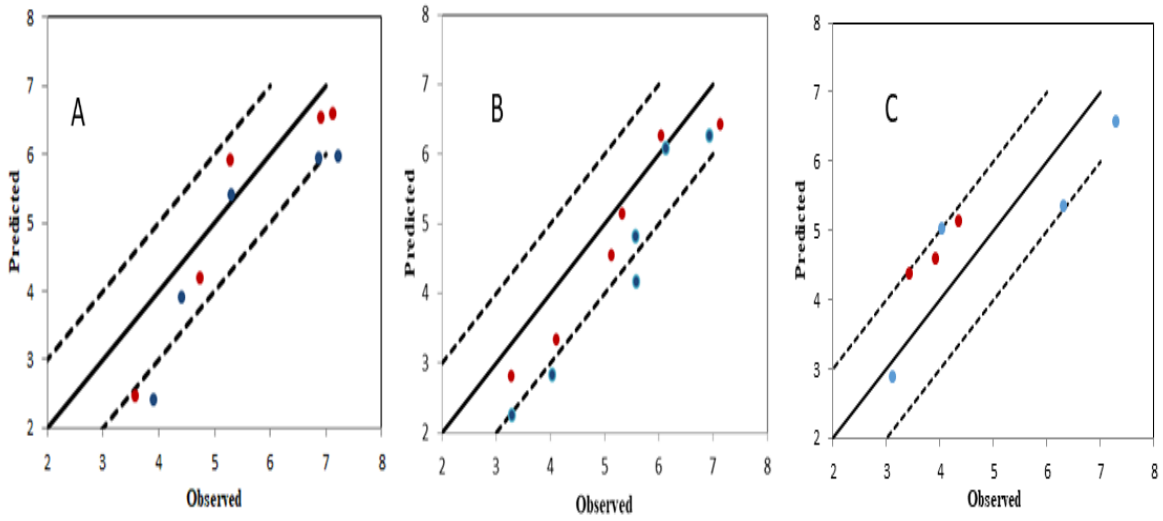


Figure 31: Observed Vs. predicted counts for minimally processed pineapple (A), pomegranate (B) and jackfruit (C) at abusive storage conditions. Blue dots (TVC); Red dots (Y&M)

3.2.5 Advantages of developed TTI for real time application

In present study, a very simple and cheap phenol based TTI was developed for application on minimally processed fruits packages. Microbial spoilage kinetics was evaluated for minimally processed pineapple, pomegranate and jackfruit. It was also observed that Ea of TTI can be varied based on concentrations of both the radical initiator (APS) and sodium carbonate. The chronochromic indicator can be readily tailored to obtain wider range of activation energies making TTI quite flexible and adjustable and can be specifically designed and formulated for each specific target process. Colour development of selected TTIs demonstrated good correlations with microbial growth in wide temperature range of 10-37 ^{o}C. Regression models correlating microbial growth with colour development were also developed using DSLR camera. Microbial status of samples stored under temperature abusive conditions was successfully evaluated using TTIs by observing colour change employing DSLR camera. The developed TTI is self-evolving and can indicate the product quality in a noncontact, non-destructive manner. When attached to product packages, the TTI can cover the entire

manufacturer-to-consumer custody chain of each individual product item with little or even no human supervision and give a reliable indication of product quality and residual shelf lives regardless of the temperature history.

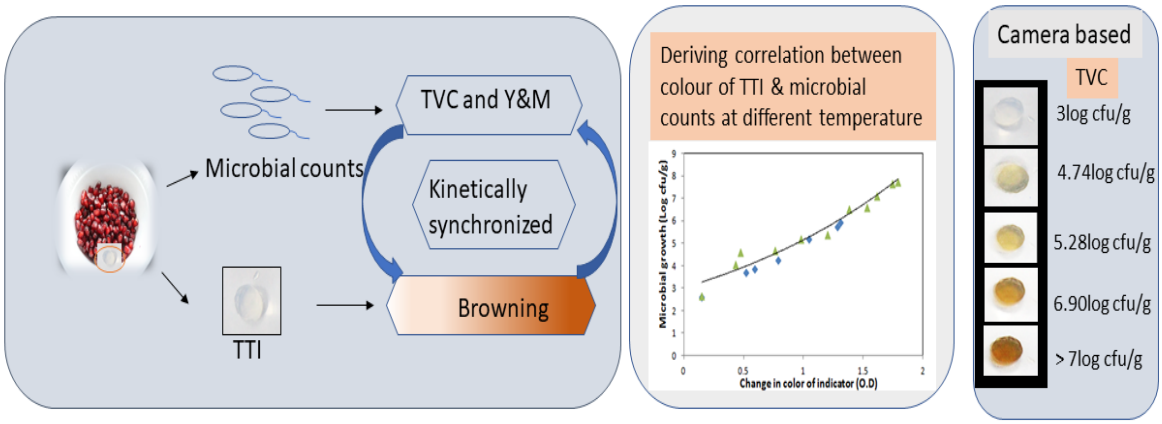


Figure 32: Schematics of TTI.

Chapter 4

Summary and Future Perspective

4.1 Summary

In the present thesis, the rapid methods for microbial quality assessment of minimally processed fruits such as jackfruit, pomegranate and pineapple were developed using two approaches. In the first approach, the potential of instrumental techniques such as HS-SPME-GCMS and FTIR that allows rapid, reagent less and non-destructive means to analyse the microbial status of stored minimally processed fruit samples were explored. In the second approach, a simple, cheap, colour changing time temperature indicator was developed for real time microbial quality monitoring in minimally processed fruits

1. From the current study, it can be concluded that temperature played significant role in the microbial spoilage status of minimally processed fruits and is one of the deterministic factors for shelf life of these refrigerated perishables.
2. Both GCMS and FTIR aided in monitoring the metabolic profile during storage of fruit samples. It was observed that during storage alcohols such as ethanol, 3-methyl-1-butanol, 2-methyl-butanol and esters such as methyl acetate, ethyl acetate, 2-methyl-1-butanol acetate, 3-methyl-1-butanol acetate and other ethyl ester derivatives showed correlations with microbial spoilage. Microbial activity also leads to production of organic acids from sugars. Thus, both GCMS and FTIR data could be correlated with the microbial quality in terms of TVC and Y&M.
3. Chemometric tools such as Partial Least square regression and Artificial neural networks were successfully employed to generate prediction models for microbial quality estimation using FTIR and GCMS data. All the developed models had $R^2 \geq 0.8$ for all the generated models.
4. The results obtained in this study demonstrated that the instrumental techniques can be utilised in the industry for online and rapid quality monitoring of minimally processed

fruit, a suitable alternative to cumbersome, destructive and time-consuming conventional technique

5. In present study, a very simple and cheap phenol based Time Temperature Indicator was developed for application on minimally processed fruit packages. Colour development of selected TTIs for the fruit packages demonstrated good correlations with microbial growth in wide temperature range of 10-37 ^{o}C. Additionally, microbial status of samples stored under temperature abusive conditions was successfully evaluated using TTIs by observing colour change employing DSLR camera.
6. The developed TTI can indicate product quality, track the shelf life and promises general applicability to each single package of minimally processed fruits and is a suitable alternative for 'best before' date.

4.2 Future Perspective

The current study evaluated potential of instrumental technique and TTI for rapid monitoring of microbial quality in minimally processed fruits. Model fruit systems such as jackfruit, pomegranate and pineapple were selected in the current study in an attempt to identify biochemical markers correlating with microbial counts such as TVC and Y&M, however several aspects can be further explored to obtain in-depth insight of the current study.

1) Different microbial communities that contribute to TVC such as *Pseudomonas* species, LAB and *Enterobacteriacea* can be studied; Yeast and moulds can be studied separately to identify most dominant species contributing to spoilage by obtaining correlation at different storage temperature. Further, investigating the key metabolite compounds produced by dominant specific spoilage micro-organism will provide useful information.

2) Different packaging (aerobic, MAP), different storage conditions (temperature, humidity), different varieties and season of harvest may affect microbial association and hence the biochemical information. Thus, huge data considering all the variations should be included to build robust models. Further the efficacy of these models should also be tested with unknown market samples, samples kept under abusive temperature conditions, sample from different batch belonging to different origin.
3) Exploring different analytical tools such as HPLC can give qualitative and quantitative estimation of organic acids that were found to be affected from FTIR results. Other techniques such as Raman spectroscopy, hyperspectral imaging and e-nose can also be evaluated for their potentials for estimating microbial quality.
4) Exploring different machine learning tools such as least square-support vector machines and principal component regression to build models with better performance.
5) Data fusion approaches can be utilised for the complementary techniques that can help in building up accurate models with low prediction errors.
6) Several different fruit samples can be evaluated to undermine biochemical signature patterns or compounds that can assist in building models using these signature patterns, thus global models instead of sample specific models which will have better potential for industrial application.
7) Several different TTI prototype can be prepared having different days of final colour evolution thus broad activation energy can be achieved for TTI. Anti-oxidants such as quercetin or different free radical initiators instead of APS can be utilised to enhance the tunability of the developed TTI.
8) Other approaches for development of TTI that are cheap and printable should be explored for their applicability in industry.

STATEMENT BY AUTHOR

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Vanshika Adiani

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Vanshika Adiani

List of publication arising from the thesis

Publication in refereed journal:

1. V. Adiani, S. Gupta, P. S. Variyar, Microbial quality assessment of minimally processed pineapple using GCMS and FTIR in tandem with chemometrics, Sci. reports, 2020, 10, 6203.

2. V. Adiani, S. Gupta, R. Ambolikar, P. S. Variyar, Development of rapid method to assess microbial quality of minimally processed pomegranate arils using FTIR, Sensors and Actuators B: Chemical, 2018, 260, 800.

3. V. Adiani, S. Gupta, R. Padole, P. S. Variyar, A. K. Sharma, SPME-GCMS integrated with chemometrics as a rapid non-destructive method for predicting microbial quality of minimally processed jackfruit (*Artocarpus heterophyllus*) bulbs. Postharvest biol technol 2014, 98, 34.

Manuscript under review:

1. V. Adiani, S. Gupta, P. S. Variyar, Phenol based time temperature indicator for real time monitoring of microbial status of minimally processed fruits.

Conferences:

1. V. Adiani, S. Gupta, P. S. Variyar, "Rapid detection of microbial quality of minimally processed pomegranate using GC-MS and FTIR" presented at LSS-2015, 3-5 Feb, 2015 held at NUB, Anushakti Nagar, Mumbai.

2. V. Adiani, S. Gupta, P.S. Variyar, "Rapid detection of microbial quality to minimize postharvest losses of minimally processed pineapple (*Ananas comosus*) using Fourier

Transform Infrared Spectroscopy (FTIR)" presented at DAE-BRNS Life Science Symposium (LSS-2018) held at DAE convention centre, Anushakti Nagar, Mumbai.

Other:

1. J. Tripathi, **V. Adiani**, S. B. Ghosh, T. R. Ganapathi, A. K. Bauri, S. Chatterjee, et al., Identification of GTP binding nuclear protein Ran as an upregulation target in acetoin glucoside mediated plant growth enhancement. The Natural Products Journal, 2017, 7, 1-7.

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Vanshika Adiani

Dedicated to my Family

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Vanshika Adiani

Thesis Highlight

Name of the Student: Ms. Vanshika Adiani **Name of the CI/OCC**: B.A.R.C.

Enrolment No.: LIFE012015004004 **Thesis Title:** Biochemical changes as tools for rapid monitoring of microbial spoilage in minimally processed fruits

Discipline: Life Sciences **Sub-Area of Discipline**: Food Technology

Date of viva voce: 3rd Feb 2021

Fresh cut fruits have acquired their permanent places on shelves in food industry. Microbial quality is the deterministic factor of the overall quality of such produce having short shelf life. Conventional plate count technique takes 48-72h to determine microbial quality. Thus rapid methods for microbial quality assessment of minimally processed fruits (jackfruit, pomegranate and pineapple) were exploited using two approaches. In the first approach, instrumental techniques such as HS-SPME-GCMS and FTIR that allows rapid, reagent less and non-destructive means to analyse the microbial status of stored minimally processed fruit samples were explored. Both GCMS and FTIR aided in monitoring the metabolic profile during storage of fruit samples. It was observed that during storage, alcohols such as ethanol, 3-methyl-1-butanol, 2-methyl-butanol and esters such as methyl acetate, ethyl acetate, 2-methyl-1-butanol acetate, 3-methyl-1-butanol acetate and other ethyl ester derivatives showed correlations with microbial spoilage. Microbial activity also leads to production of organic acids from sugars. Chemometric tools such as Partial Least square regression and Artificial neural networks were successfully employed to generate prediction models for microbial quality estimation using FTIR and GCMS data. In the second approach, cheap phenol based Time Temperature Indicator (TTI) was developed for application on minimally processed fruit packages. Colour development of selected TTIs for the fruit packages demonstrated good correlations with microbial growth in wide temperature range of 10-37 ^{o}C. Additionally, microbial status of samples stored under temperature abusive conditions was successfully evaluated using TTIs by observing colour change employing DSLR camera. In summary, both the approaches aided in rapid microbial quality assessment in minimally processed fruit samples.

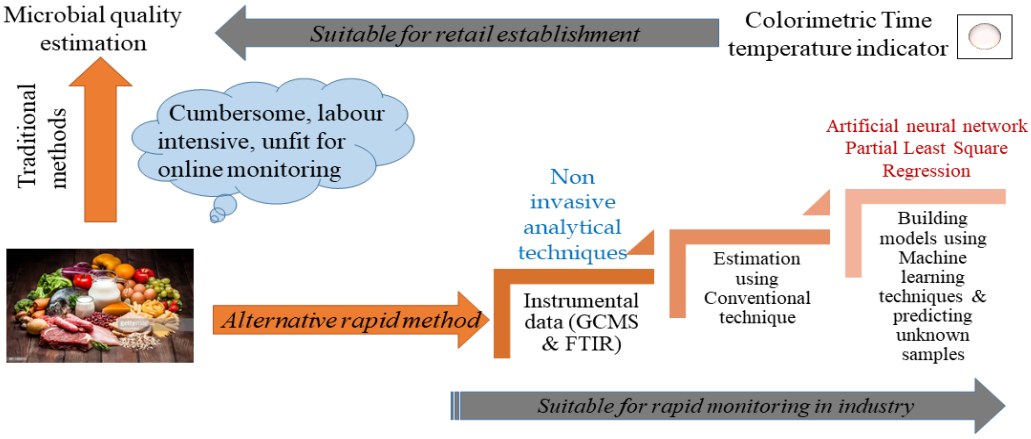

Figure: Schematic of the work carried out in thesis

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