

**Effect of accelerated storage temperatures on the shelf life limiting factors of apple
juice concentrate**

by

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Declaration

I, Siphiwe Dube declare that the dissertation, which I hereby submit for the degree Master of Science (Agriculture) Food Science and Technology at the University of Pretoria, is my work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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ABSTRACT

Apple juice concentrate amongst other uses is used to formulate concentrated and single strength fruit juice blends. An apple juice concentrate whose quality has deteriorated during storage poses problems for the processing industries as it limits its use in the formulation of certain products. A dark coloured apple juice concentrate cannot be used to formulate light coloured beverages while an apple concentrate contaminated with microbes can only be incorporated into products with a pasteurisation treatment. It is important to evaluate the deterioration in the quality of apple juice concentrate stored at normal and at accelerated temperatures.

The physicochemical parameters and microbial counts were monitored in apple juice concentrate stored at 10 °C (normal temperature) and at 25 °C and 35 °C (accelerated temperatures) over 12 weeks after which modelling of the experimental data was done. The Biolog system and (GTG)₅ PCR fingerprinting were used to identify isolated yeasts.

There was a significant change ($p \leq 0.05$) in L^* , b^* , a^* , hue, chroma, total colour difference (TCD), non-enzymatic browning index ($A_{420\text{nm}}$) and 5-hydroxymethylfurfural concentration with storage time and temperature. The Q_{10} values for L^* , b^* , non-enzymatic browning index ($A_{420\text{nm}}$) and 5-hydroxymethylfurfural were 1.53, 1.20, 1.81 and 5.25, respectively and activation energies were 8.17, 7.70 and 14.8 kcal mol⁻¹ for L^* , b^* and non-enzymatic browning index ($A_{420\text{nm}}$), respectively.

Microbial growth was not observed in apple juice concentrate stored at 10 °C while yeast and mould growth was observed after 5 weeks of storage at 25 °C and 35 °C. After fitting the yeast growth data into the Baranyi and Roberts model, no significant difference was observed in the lag phase for yeast growth at 25 °C and 35 °C while there was a significant difference $p \leq 0.05$ in the maximum specific growth rate. The theoretical minimum temperature of growth extrapolated from the Ratkowsky model was about 20 °C. The activation energy and Q_{10} values for the maximum specific growth rate were 39.95 kcal mol⁻¹ and 4.47, respectively. The isolated yeasts were identified as *Kluyveromyces delphensis*, *Saccharomyces dairensis*, *Zygosaccharomyces bailii*, *Rhodotorula glutinis* and *Metchnikowia reukaufii* and strain variability was observed for the species *K. delphensis* and *S. dairensis*. The higher Q_{10} and activation energy values for the maximum specific growth rate of yeasts in comparison with the Maillard browning parameters (L^* , b^* and A_{420nm}) signify that loss in quality due to yeast growth is more rapid compared to Maillard browning.

The results of this study show that under refrigerated storage temperatures deterioration in the quality of apple juice concentrate is due to Maillard browning while at accelerated storage temperatures quality loss is due to both Maillard browning and microbial growth of yeasts. At accelerated storage temperatures, darkening of the apple juice concentrate starts immediately and continues throughout the storage period. However growth of spoilage yeasts is only detected after 5 weeks of storage and proceeds rapidly thereafter resulting in spoilage.

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CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT

Apple juice concentrate is an important domestic and export product produced by concentration of apple juice to a total soluble solids content greater than 40 °Brix (US International Trade Commission, 2000; Department of Agriculture and Fisheries, 1980). South Africa is ranked 22nd worldwide in terms of apple juice concentrate exports with major destinations being Japan, United States, Canada, Australia, Spain and Netherlands (USDA Foreign Agriculture Service, 2011). Apple juice concentrate is mainly reconstituted to form ready-to-drink fruit juices whose consumption has increased substantially over the last few years, mostly due to the increasing demand for low calorific food products with fresh-like characteristics (Raybaudi-Massilia, Mosqueda-Melgar, Soliva Fortuny; Martin-Belloso, 2009). It is also used as a flavouring ingredient in carbonated and other beverages, as a sweetener in bakery products, cereal and health foods (US International Trade Commission, 2000). A small portion is used as an additive in cosmetics and various medicines (USDA Foreign Agricultural Services, 2004) and also for the correction of the taste of products whose acidity needs to be increased for example carrot puree-based pulp juices (Pierzynowska-korniak and Zywicka, 2004).

While the utilization of fruit products may occur throughout the year, processing of the fruits is seasonal and this necessitates the year long storage of fruit juice concentrates (Olubukola, Obashola and Ramokoni, 2011). While in storage, the quality of apple juice concentrate deteriorates primarily due to Maillard browning (Burdurlu and Karadeniz, 2003; Buedo, Elustondo and Urbicain., 2001; Toribio and Lozano, 1984) and the growth of yeasts and moulds (Stratford, 2006; Graumlich, Marcy and Adams, 1986).

Yeast growth reduces the sensory quality of apple juice concentrate as it is accompanied by the formation of alcohol which gives it a fermentative off-odour and the carbon dioxide released gives the apple juice concentrate a gassy, frothy appearance and causes the packed product to swell and explode (Patil, Valdramidis, Tiwari, Cullen and Bourke, 2011). Moulds appear as surface mats on the concentrate (Wareing and Davenport, 1998) and their growth is of serious concern as certain species of moulds such as *Byssoschlamys*, *Penicillium* and *Aspergillus* produce the toxic metabolite known as patulin (Duarte, Delgadillo and Gil, 2006). Maillard browning produces off-flavours and changes the amber-yellow colour of apple juice concentrate to an undesirable dark brown (Toribio and Lozano, 1984) and since

colour is one of the most important characteristics of fruit products influencing a customer's choice (Sadilova, Stintzing, Kammerer and Carle, 2009) browning limits the shelf life of the fruit concentrates.

The large quantities of apple juice concentrate produced during the apple season are stored in refrigerated tanks at 10 °C to control colour degradation and fungal growth. However when storage space is limited the apple juice concentrate is stored at ambient temperature resulting in a rapid deterioration of quality (Rhonde, 2011). Food deterioration occurs progressively during storage (Liu and Li, 2006) and increasing the storage temperature has an accelerating effect on the rate of deterioration. Spoilage of apple juice concentrate reduces the value and versatility of apple juice concentrate and poses a problem for the fruit juice industry as it leads to economic losses when some rework has to be done so that quality parameters are within specification (Rhonde, 2011). Rework entails a second pasteurisation to destroy yeasts and moulds that have grown and/or a second activated carbon treatment to remove excess brown pigments and restore the light brown colour of the apple juice concentrate (Rhonde, 2011).

The objectives of this study were to evaluate the changes in the shelf life limiting factors during storage of apple juice concentrate under accelerated and normal storage temperatures and to determine the parameter with the highest rate of change. This information will show manufacturers the behaviour of apple juice concentrate characteristics during storage enabling them to identify the most significant characteristic in decreasing the shelf life of apple juice concentrate. When the rate of deteriorative reactions is known, the storage and processing conditions can then be optimized by developing methods to counteract the loss in quality so that the apple juice concentrate does not have to be reworked before it is supplied to the customer.

CHAPTER 2: LITERATURE REVIEW

2.1 Fruit juice concentrate

Fruit juice concentrate is obtained by removing water from fruit juice to yield a product with a high sugar concentration typically around 65 to 85 °Brix (Department of Agriculture and Fisheries, 1980). Concentration reduces the volume and weight of the juice resulting in lower costs of packaging, storage and transportation (Onsekizoglu, Bahceci and Acar, 2010; Alvarez, Riviera, Alvarez, Coca, Cuperus, Bouwer, Boswinkel, Van Gemert, Veldsink, Giorno, Donato, Todisco, Drioli, Olsson, Tragardh, Gaeta and Panyor, 2000). In the concentrated form, juice can be held for extended periods and shipped throughout the world as a relatively stable product allowing year long utilization of perishable agricultural products (Assawarachan and Noomhorm, 2010; Alvarez *et al.*, 2000). In many developing countries, fruit concentrate processing is seen as a way to salvage some return from fruit that cannot be sold on the fresh market (USDA Foreign Agriculture Service, 2011). The consumption of apple juice is the main driver for the consumption of apple juice concentrate as a large part of apple juice concentrate is processed into apple juice (Oliveira, 2007).

2.2 Apple juice concentrate production in South Africa

South African fruit concentrate manufacturers have successfully exported fruit concentrates to a great number of international markets (Figure 1) due to the price competitiveness of their apple juice concentrate in European markets (USDA Foreign Agriculture Service, 2009). China is the largest producer and exporter of apple juice concentrate (Gale, Huang and Gu, 2010) while South Africa is ranked twenty second globally in terms of apple juice concentrate exports (USDA Foreign Agriculture Service, 2011). In 2010 there was an increase of fresh apples to the processing sector due to the increased demand for apple juice and higher monetary returns to producers which subsequently led to an increase in apple juice concentrate production (USDA Foreign Agriculture Service, 2011).

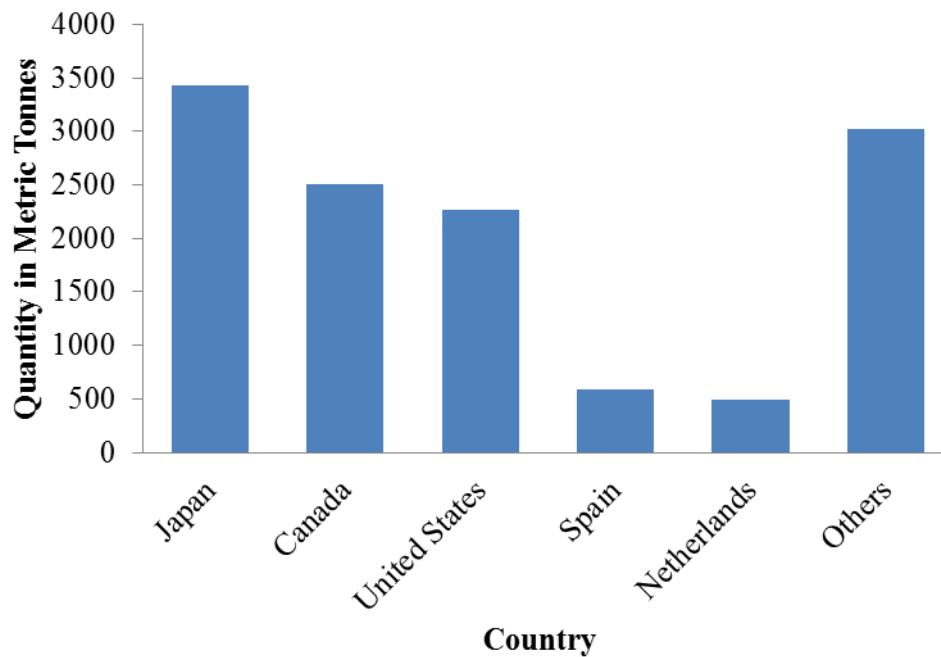


Figure 1. South African apple juice concentrate export statistics for January-December 2011 (USDA Foreign Agricultural Services, 2011).

2.3 Spoilage mechanisms in fruit concentrate

The major spoilage mechanisms of fruit concentrate are primarily microbial and chemical reactions which adversely affect their nutritional quality, colour and flavour (Graumlich *et al.*, 1986). Moulds and yeasts tolerate high-osmotic and low-pH conditions and grow at refrigeration temperatures and can therefore cause spoilage of processed products such as apple juice concentrate (Arias, Burns, Friedrich, Goodrich and Parish, 2002).

2.3.1 Microbial spoilage

2.3.1.1 Yeast spoilage

Yeasts are common contaminants of fruit concentrates and represent a major problem to industries that process fruit products (Davenport, 1996). The contamination of fruit juices by yeasts is a sign of contaminated raw materials, failure in juice pasteurisation, poor sanitation practices or due to the presence of resistant yeasts (Tribst, Sant'Ana and de Massaguer, 2009). Osmotolerant yeasts are the principal microorganisms responsible for the spoilage of concentrated fruit juice. The most common yeast contaminants isolated from fruit juice concentrates are *Candida* spp, *Rhodotorula* spp, *Dekkera* spp, *Debaryomyces hansenii*, *Lodderomyces elongisporus*, *Hanseniaspora* spp, *Issatchenkia orientalis*, *Kloeckera* spp,

Kluyveromyces marxianus, *Panomala*, *Saccharomyces* spp, *Torulaspora delbrueckii* and *Zygosaccharomyces* spp (Sancho, Gimenez-Jurado, Malfeito-Ferreira and Loureiro, 2000). Osmotolerant yeasts, *Zygosaccharomyces rouxii*, *Z. bisporus*, *Z. bailii* and *Saccharomyces cerevisiae* are the principal microorganisms responsible for the spoilage of concentrated fruit juice (Loureiro, 2000). In a study to determine the population and frequency of occurrence of yeasts in frozen apple, cherry, grape, orange and pineapple juice concentrates, Deak and Beuchat (1993) found the most frequently isolated yeasts to be *S. cerevisiae* (24.7 % of isolates), *Candida stellata* (22.1 %), *Z. rouxii* (14.3 %) followed by *Torulaspora delbrueckii*, *Rhodotorula mucilaginosa*, *Issatchenkia orientalis*, *Hanseniaspora occidentalis*, *Lodderomyces elongisporus*, *Kluyveromyces thermotolerans*, *Hanseniaspora guilliermondii*, *Candida glabrata* and *Pichia anomala* in decreasing order of frequency each representing 3-8% of isolates. Deak and Beuchat (1995) isolated nineteen yeast strains from beverage concentrates and identified them as *Z. bailii*, *Z. bisporus* and *Z. Rouxii* with *Z. Bailii* and *Z. rouxii* being dominant. The growth requirements of dominant species isolated from fruit juice concentrate with reference to temperature, pH, sucrose concentration and water activity are shown in Table 1.

Table 1. Comparison of growth responses of yeasts isolated from fruit concentrates (Fleet, 2011).

Yeast	Temperature (°C)	pH range	Maximum sucrose (% w/v)	Minimum a_w
<i>Debaryomyces hansenii</i>	0 – 37	2.5 – 9.0	50 - 60	0.81 – 0.91
<i>Hanseniaspora uvarum</i>	3 – 35	1.5 – 7.5	50 - 60	0.90 – 0.95
<i>Kluyveromyces marxianus</i>	1 – 47	2.5 – 8.0	50	0.96
<i>Pichia membranifaciens</i>	5 – 30	2.7 – 7.5	50	0.84 – 0.93
<i>Saccharomyces cerevisiae</i>	0 – 45	2.5 – 8.0	50 - 60	0.89 – 0.92
<i>Zygosaccharomyces bailii</i>	2 – 37	2.5 – 7.0	70	0.87 – 0.94
<i>Zygosaccharomyces rouxii</i>	4 – 40	2.5 – 7.5	70	0.65 – 0.86

The values represent a comparative guide only and absolute values vary with yeast strain and influence of other environmental factors.

Z. bailii is an effective spoiler of fruit concentrates particularly because of its exceptional tolerance to preservatives, high sugar, low acid and pasteurization regimes (Thomas and Davenport, 1985). *Z. bailii* is strongly fermentative and preferentially ferments fructose unlike most other yeasts (Adams and Moss, 2008). *Z. rouxii* is characterized by its ability to tolerate low water activity environments, being one of the most xerophilic microorganisms known as it can grow at a_w below 0.70 (Deak, 2004).

According to Veiga and Madeira-Lopes (2000), *Pichia membranifaciens* is usually isolated at lower frequency than other yeasts from fruit concentrates and its ability to form ascospores constitutes a problem when thermal inactivation is the major mode of microbial inactivation. There are two types of yeast spoilage: visible yeast spoilage, which is yeast on the surface of the product that may appear as pink and/or white patches and fermentative spoilage that normally results in an alcoholic odour with or without visible gas bubbles (Legan and Voysey, 1991; Smith, Daifas, El-Khoury, Koukoutsis, and El-Khoury, 2004). Yeast growth reduces the sensory quality due to off-odours and discolouration of the products (Deak, 2004).

2.3.1.2 Mould spoilage

Mould problems are caused by the growth of a variety of moulds due to poor hygiene within the factory or by the growth of heat-resistant moulds within heat-processed juices (Wareing and Davenport, 1990). Some species of moulds are xerophilic and are potential spoilage agents of foods with low water activity such as dried fruits and fruit juice concentrate (Splittstoesser, 1996). The spoilage of thermally processed fruit and fruit products by heat resistant moulds has been recognised in several countries (Beuchat and Pitt, 2000). The ability of heat resistant moulds to spoil fruit products is due mainly to their ability to survive the heat treatment of 85-105 °C for up to 45 seconds, to grow under low oxygen tension and to grow at low pH values (Sant'Ana, Dantigny, Tahara, Rosenthal and Massaguer, 2010; Tournas, Heeres and Burgess, 2006). *Byssochlamys fulva*, *B. nivea*, *Neosartorya fischeri*, *Talaromyces macrospores*, *Talaromyces bacillisporus* and *Eupenicillium brefeldianum* have been frequently encountered (Splittstoesser, 1996; Beuchat and Pitt, 2000). *N. fischeri* is one of the most frequently reported heat resistant moulds causing spoilage in fruit products (Esteve and Frigola, 2007). Heat resistant moulds are characterized by the production of ascospores which enable them to survive the thermal process given to fruit products (Splittstoesser, 1996). Germination of ascospores may result in visible growth of mycelia on

fruit products (Beuchat and Pitt, 2000) seen as surface mats, flavour alterations and phase separation due to the production of pectinolytic enzymes (Sant'Ana *et al.*, 2010).

2.3.1.3 Patulin

Patulin is a mycotoxin produced by certain species of *Penicillium*, *Aspergillus* and *Byssoschlamys* (Welke, Hoeltz, Dottori and Noll, 2010) of which *Penicillium expansum* commonly identified as the “blue mould rot” is the most important cause in apples (Murillo-Arbizu, Amezcua, Gonzalez-Penas and de Cerain, 2009). *Penicillium* and *Aspergillus* are the main species responsible for the production of patulin in the pre-processing stages while some species of *Byssoschlamys* are considered the main potential producers of patulin in the post pasteurization stages due to their capacity to survive heat treatments (Sant'Ana, Rossenthal and Massaguer, 2008). The contamination of apple concentrates and its products with mycotoxins is not only a health hazard but also results in economic losses (Fernandez-Cruz, Mansilla and Tadeo, 2010).

The maximum permitted levels of patulin are 50 µg/kg for concentrates, reconstituted fruit juices, fruit nectars, ciders and other fermented drinks derived from apples or containing apple juice; 25 µg/kg for solid apple products intended for direct consumption and 10 µg/kg for apple juice and solid apple products for infants and children (Bhat, Rai and Karim, 2010 and FDA, 2001) and its presence in excess is considered a good indication of the use of mouldy apples and unclean facilities (Dombrink-Kurtzman, 2008). The acute symptoms of patulin consumption in humans include convulsions, edema, ulceration, nausea, intestinal inflammation and vomiting (Barreira, Alvito and Almeida, 2010) and the chronic symptoms include genotoxic, neurotoxic, immunotoxic, immunosuppressive and teratogenic effects (Fernandez-Cruz *et al.*, 2010).

2.3.1.4 Alicyclobacilli species

Alicyclobacilli are thermo-acidophilic, non-pathogenic endospore forming bacteria that have been isolated from apple juice concentrate and several spoiled commercial fruit juices (Maldonado, Belfiore and Navarro, 2008). Spores of *Alicyclobacillus* have the ability to survive at pH < 4 and have D values of 16-23 minutes at 90°C which means that they might survive the thermal treatments applied during production of concentrates (Walker and Phillips, 2008; Steyn Cameron and Witthuhn, 2011). Apple juice concentrate is pasteurised at 85-89 °C for 45 sec (Rhonde, 2011). *Alicyclobacillus* is a soil borne organism that enters the

processing plant on the surface of the fruit that has come into contact with contaminated soil during harvesting. Considering the fact that *Alicyclobacillus* is soil borne, its association with water can very well be expected (Gouws, Gie, Pretorius and Dhansay, 2005). The presence of *Alicyclobacillus* in apple juice concentrate poses a serious problem for the juice industry (Vantarakis, Affifi, Kokkinos, Tsibouxi, and Papapetropoulou, 2011). Although the soluble solids content (<20 °Brix) of apple juice concentrate inhibits the germination of *Alicyclobacillus* endospores, these endospores retain their viability which upon dilution to single strength juice could multiply to numbers high enough to cause spoilage and product deterioration (Steyn *et al.*, 2011). Endospores of *Alicyclobacillus* species survive a pasteurisation temperature of 95 °C for over 2 minutes in apple juice; can grow at a pH of between 2.5 to 6.0 and at temperatures between 20 °C and 60 °C (Duvenage, 2006). Spoilage is manifested as off flavours due to methoxyphenol (guaiacol), 2, 6 bromophenol and 2, 6 chlorophenol and as a light sediment (Danyluk, Friedrich, Jouquand, Goodrich-Schneider, Parish and Rouseff, 2011).

2.3.2 Non-enzymatic Browning (NEB)

Although apple juices are bright and clear after clarification, change in colour is a common quality degrading side reaction that seriously compromises the acceptability of commercial apple juice (Gokmen and Serpen, 2002). Non-enzymatic browning is due to several causes including the Maillard reaction, caramelisation, Vitamin C decomposition and pigment destruction (Damasceno, Fernandes, Magalhaes and Brito, 2008). Both heating procedures and storage conditions show synergy in non-enzymatic browning reactions (Vaikousi *et al.*, 2008; Bozkurt, Gogus and Eren 1999). The accumulation of the brown colour in apple juice concentrate during storage is attributed to non-enzymatic reactions (Burdulu and Karadeniz, 2003; Garza, Ibarz, Pagan, and Giner, 1999). The most important cause of non-enzymatic browning in apple juice is considered as the Maillard reaction when concentrates stored at a relatively high temperature or for a long period of time (Burdulu and Karadeniz, 2003, Toribio and Lozano, 1984).

2.3.2.1 Chemistry of the Maillard reaction

The Maillard reaction takes place during thermal processing, cooking and storage of foods and it involves formation of brown pigments by condensation between carbonyl groups of reducing sugars, aldehydes or ketones and amine groups of amino acids, peptides or proteins or other nitrogenous compounds (Chawla, Chander and Sharma, 2007). A myriad of products is

formed which have a direct impact on the nutritional and sensory quality of foods (Chawla *et al.*, 2007).

The Maillard reaction is complex consisting of many parallel and consecutive reactions and various components (Vaikousi *et al.*, 2008). A simplified scheme of the chemistry of the Maillard reaction at pH < 7 is shown in Figure 2.

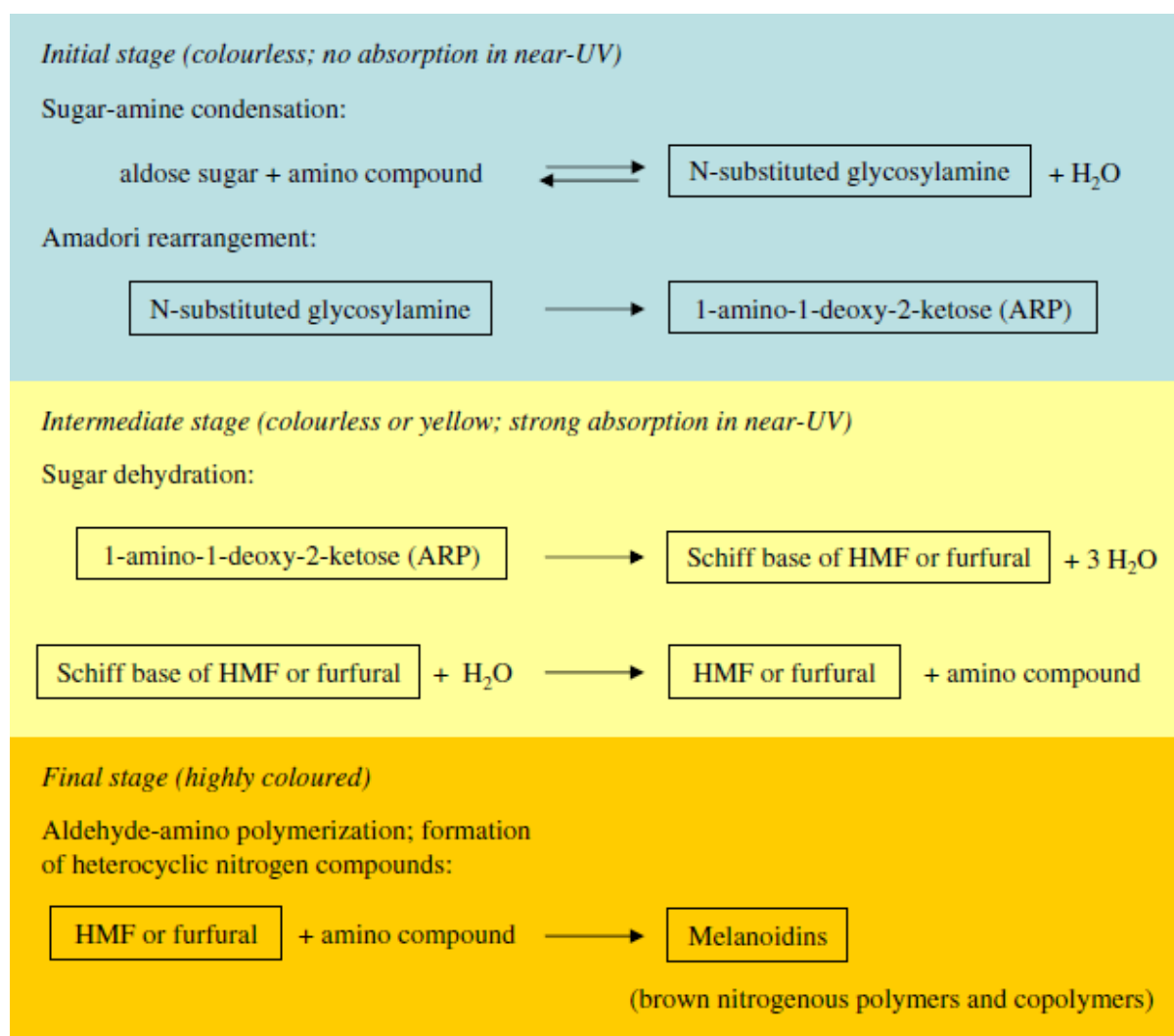


Figure 2. Simplified scheme of the Maillard reaction (pH < 7), adapted from Purlis (2010).

ARP: Amadori rearrangement product; HMF: 5-hydroxymethylfurfural.

Generally the reaction mechanism is divided into three stages. The first stage starts with the addition of an amine group to the carbonyl carbon of the reducing sugar in a condensation reaction. For aldoses such as glucose, the addition product is dehydrated to form an unstable Schiff base which cyclises rapidly into an N-substituted aldosylamine (Fennema, 2008). Irreversible Amadori rearrangement of the N-substituted aldosylamine follows to form the

Amadori Rearrangement Product (ARP), 1-amino 1-deoxy 2-ketose. Ketoses such as fructose react with amino groups to form amino aldoses in the Heyns reaction. Amino aldoses are not stable intermediates and readily react, forming 2-amino 2-deoxy 2-ketose. No browning reactions occur at this stage (Coca, Garcia, Gonzalez, Pena and Garcia, 2004).

In the second stage, the subsequent degradation of the ARP is dependent on the pH of the system. At pH 7 or below, the ARP undergoes mainly 1, 2-enolisation to yield intermediary dicarbonyl compounds known as 3-deoxysones which are powerful precursors of brown compounds. 3-deoxysones cyclise to form furfurals when pentoses are involved but hydroxymethylfurfurals (HMF) when its hexoses (Ruffian-Henares, Delgado-Andrade and Morales, 2009; Martins, Jongen and Van Boekel, 2001). In apple cider, 5-HMF is formed (Gentry and Roberts, 2004). 5-HMF is also formed from sugar degradation during thermal treatment (Sadilova *et al.*, 2009), the reaction being catalyzed by malic acid (Capuano and Fogliano, 2011; Gentry and Roberts, 2004; Burdulu and Karadeniz, 2003). In acidic conditions 5-HMF can form even at low temperatures although its concentration increases drastically as the temperature of thermal treatment or storage increases (Capuano and Fogliano, 2011). The initial HMF concentration before storage is important because it indicates the degree of heating of apple juice concentrate during processing (Garza, Ibarz, Pagan and Giner, 1999). Bozkurt, Gogus and Eren (1999) state that HMF formed during processing is a highly reactive intermediate which accelerates the rate of brown pigment formation during further storage. Manso, Oliveira, Oliveira and Frias (2001) further state that the accumulation of HMF in juice is an autocatalytic reaction, so its concentration at the beginning of a storage period may have an influence on its further increase.

The intermediary products from the second stage are highly reactive and take part in further reactions. Carbonyl groups can condense with free amino groups in a process known as Strecker degradation to incorporate nitrogen into reaction products (Ruffian-Henares *et al.*, 2009). In the advanced stage, polymerisation of the highly reactive intermediary products forms heterocyclic, high molecular weight brown pigments known as melanoidins (Zhu, Ji, Eum and Zude 2009; Fennema, 2008; Coca *et al.*, 2004).

2.3.2.2 Factors affecting the Maillard reaction

Although the Maillard reaction occurs between reducing sugars and amino acids, the initial rate of this reaction depends on many factors (Burdulu and Karadeniz, 2003). The factors that

influence the rate of the Maillard reaction during processing and storage of food include its composition, the time temperature conditions, pH, water activity, oxygen tension and the presence of promoters and inhibitors (Shen and Wu, 2004).

Water activity

The rate of browning increases from the dry state, starting at a critical a_w of 0.2-0.3 for most foods, to a maximum at a_w of 0.5 - 0.8 and then decreasing at higher a_w . At low water activity, solute mobility is limited below the monolayer due to the higher binding energy and glass state that forms. At a_w of 0.5 - 0.8, the solutes are more mobile and they can dissolve, water is more mobile and the system enters a rubbery state giving increased rate constants (Vaikousi *et al.*, 2008; Labuza and Baiser, 1992). The rate of browning is reduced at higher a_w (0.7 - 0.8) as a result of dilution effect on solute concentration (Vaikousi *et al.*, 2008; Troller and Christian, 1978).

pH

The rate and extent of the Maillard reaction increases with an increase in pH with an optimum pH range of 6 to 8 (Coca *et al.*, 2004). Alkaline conditions favour the reaction as high pH increases the availability of the acyclic form, a high reactivity form of reducing sugar (Thongraung and Kangsanant, 2010) and the unprotonated form of the amino group considered to be the reactive form (Martins *et al.*, 2001).

Temperature

An increase in temperature increases the rate of Maillard browning (Ames, 1990) as it leads to an increase of the reactivity between the sugar and the amino acid (Martins *et al.*, 2001). According to Simsek, Poyrazoglu, Karacan and Velioglu (2007), the rate of the Maillard reaction increases four-fold with every 10 °C increase in temperature.

Sugars

The most important monosaccharides in apple juice concentrate participating in the Maillard reaction are glucose and fructose. Sucrose is easily cleaved into glucose and fructose during heat treatments and so can participate in non-enzymatic browning quite easily. The reactivity of aldoses (glucose) is higher than ketoses (fructose) in browning systems (Fennema, 2008).

Amino acids

The type of amino acid also affects Maillard reaction browning with basic amino acids having more reactivity than acidic amino acids (Namiki, 1998).

Storage

Longer storage times result in formation of more brown pigments (Simsek *et al.*, 2007). The length of heating time is important as formation of melanoidins usually occurs at a rate which is proportional to the square of the reaction or storage time at any given temperature (Davies and Labuza, 2003).

2.3.2.1 Influence of Maillard reaction products on food properties

The Maillard reaction may be desirable during food processing as in the manufacture of coffee, tea, beer and baking of bread because it improves desirable sensory characteristics such as colour, aroma and flavour. However in fruit concentrates it is undesirable because the Maillard reaction occurs at its maximum rate at a_w of 0.6 to 0.7 typical of apple juice concentrate (Burdulu and Karadeniz, 2003; Eskin, 1990).

Melanoidins are heterogenous, nitrogen containing brown pigments produced by the Maillard reaction and are predominantly responsible for the characteristic brown colour in foods such as honey and fruit concentrates (Wang, Qian and Yao, 2011). Colour is the most relevant external characteristic of melanoidins with a great influence on consumer acceptance and shelf life of products (Rufian-Henares *et al.*, 2009). Apple juice concentrate develops an amber to brown colour during processing that becomes dark brown to black during storage decreasing its versatility and value (Cornwell and Wrolstad, 1981). In a study by Graumlich *et al.*, 1986), as the non-enzymatic browning (NEB) increased, taste panel scores decreased significantly with increasing storage temperature and time when orange concentrate was stored for twelve months at -17.7 to 4 °C.

Another consequence of the Maillard reaction is the loss of nutritive value of proteins attributed to a decrease in digestibility, destruction and/or biological inactivation of amino acids, inhibition of glycolytic enzymes and interaction with metal ions. Also proteins can be cross linked with Maillard reaction products (Martins, 2003).

The Maillard reaction not only has a direct impact on nutritional and sensory quality of foods (Chawla *et al.*, 2007) but also leads to reduced food safety due to the presence of intermediate compounds such as 5-hydroxymethylfurfurals (Zhu *et al.*, 2009). HMF at high concentrations (more than 75mg/kg) is cytotoxic, irritating to eyes, upper respiratory tract, skin and mucous membranes in studies done on rodents (Islam, Khalil, Islam and Gan, 2013; Capuano and

Fogliano, 2011). In vitro studies have also revealed some mutagenic, carcinogenic and cytotoxic properties of melanoidins (Dolphen and Thiravetyan, 2011).

2.4 Shelf life

Shelf life is defined as the maximum period of time that a food product can be stored under specific environmental conditions without any appreciable deterioration in quality and acceptability (Jena and Das, 2012). In other words, during this period, it should retain its desired sensory, chemical, physical, functional or microbiological characteristics and where appropriate comply with any label declaration of nutritional information when stored according to recommended instructions (Kilcast and Subramaniam, 2000). Every food product has and should be recognized as having a microbiological shelf life, a chemical shelf life and a sensory shelf life because all foods deteriorate at different rates (Man, 2002). Shelf life can be determined and subsequently predicted for individual food products based on some primary mode of deterioration (Fu and Labuza, 1993). The shelf life of food can be determined using either direct (Real time) or indirect methods (Accelerated Shelf Life Testing).

2.4.1 Direct/Real time shelf life testing

Direct shelf life testing involves storing the food under preselected conditions that mimic those it is likely to encounter during storage for a period of time longer than the expected shelf life and assessing the quality of the product at regular intervals (Singh and Cadwallader, 2004; New Zealand Food Safety Authority, 2005). The time to reach a predetermined level of the quality parameter will be considered to be the end-point or shelf life. Since it is advisable to leave a safety margin in setting the shelf life, generally 70 % of the time to spoilage is taken to be the storage life (Kilcast and Subramaniam, 2000). While the direct determination of shelf life is feasible for short shelf life products, it requires an unrealistically long time for long shelf life products. Consequently, Accelerated Shelf Life Testing (ASLT) is done to circumvent this problem (Fu and Labuza, 1993).

2.4.2 Accelerated Shelf life testing

Accelerated shelf life testing involves the use of abuse or stressful testing conditions (temperature, a_w and oxygen level) in food quality loss and shelf life experiments and extrapolation of the kinetic results to normal storage conditions (Singh and Cadwallader, 2004; Mizrahi, 2004). The basis of ASLT is that using stressful test conditions such as

elevated temperature will accelerate the deterioration process (Ellis, 1994) hence shortening the time required to estimate a shelf life (Man, 2002). With the effective use of ASLT, an experiment that normally takes a year can be completed in about a month if the testing temperature is raised by 20 °C (Mizrahi, 2004). ASLT is applicable to any chemical, physical, biochemical or microbial deterioration process that has a valid kinetic model (Mizrahi, 2000). One of the limitations of accelerated storage tests is that they are product specific and results have to be interpreted with care based on detailed product knowledge and sound scientific principles (Man, 2011). ASLT should always be supplemented by normal condition testing (direct determination) for confirmation since it is hardly possible to predict the storage performance of a product under normal conditions with certainty from its behaviour when it is ‘abused’ (Ellis, 1994). According to Hough (2010) other disadvantages of ASLT are that it may be costly, there may be a change in the physical state of the product as temperature rises, moisture loss can occur leading to false results, upon freezing reactants are concentrated in the unfrozen liquid resulting in unpredictable high reaction rates and different microorganisms grow at different temperatures. According to Taoukis, Labuza and Saguy (1997), the following steps outline the ASLT procedure:

- a. Define the accelerated testing conditions and decide on the type and frequency of testing.
- b. Store samples under those conditions and analyse changes in the predetermined parameters.
- c. Plot the data as it is collected to determine whether the testing frequency should be altered.
- d. Determine the reaction order and rate constant from each testing condition and make the appropriate Arrhenius plot.
- e. Extrapolate the straight line to the desired lower storage temperature. Substitute the rate constant in an appropriate equation to get the shelf life.

2.4.3 Factors influencing the shelf life of apple juice concentrates

The rate at which deteriorative changes occur in a food product depends on the initial raw material quality, intrinsic factors (product composition, pH, total acidity, water activity) and extrinsic factors (processing, thermal operations, hygiene and storage conditions) (Kilcast and Subramaniam, 2000; McMeekin and Ross, 1996).

Raw material quality

The quality of apples, consistency, level of contamination and storage of apples prior to processing will affect the quality and subsequently the shelf life of apple juice concentrate. According to Rhonde (2011), apples intended for juice manufacture are generally of lower physical integrity and hence have a higher frequency of contamination. High quality juice operations are dependent upon a source of high quality raw material. No matter how good the process is, starting with poor quality fruit for juice production will lead to a poor quality juice product (Mclellan, 1996) with a limited shelf-life. Since fruit processing is seen as a way to salvage some return from fruit that cannot be sold on the fresh market (USDA foreign agriculture service, 2011), the quality of the apples used to produce apple juice concentrate may be poor (Figure 3).



Figure 3. Quality of apples sometimes used to produce apple juice concentrate (2011).

Processing stages

Apple juice concentrate is manufactured as illustrated in the flow diagram in Figure 4 although some differences may exist between different manufacturers with respect to the sequence of stages, materials and techniques used. The stages in apple juice concentrate processing: raw material quality, washing, grading, sanitization, pasteurisation, activated carbon treatment, evaporation and storage (temperature and time) have an influence on the shelf life of apple juice concentrate.

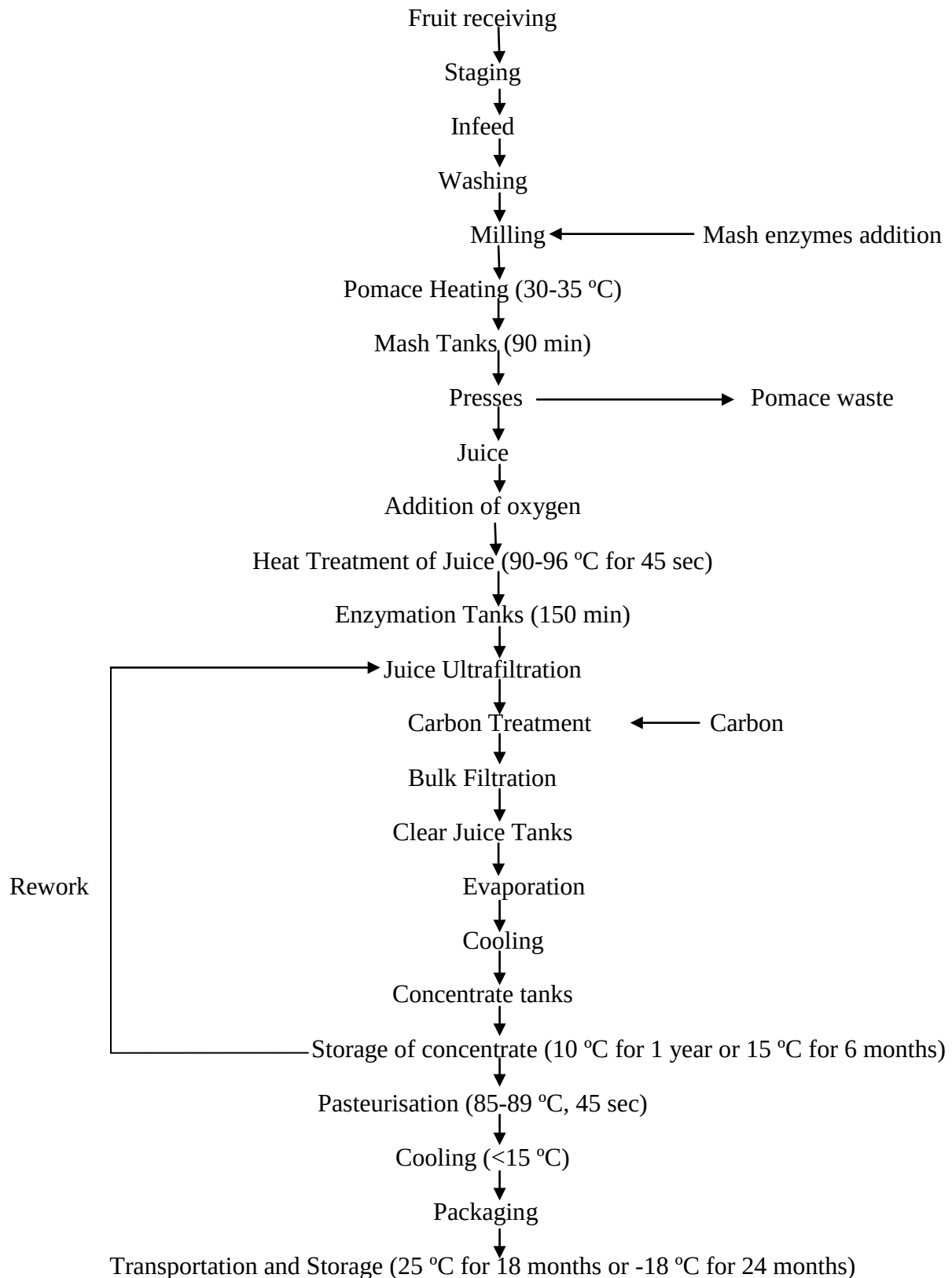


Figure 4. Process flow diagram for production of apple juice concentrate (Rhonde, 2011)

Washing and sanitization

Washing removes soil, microorganisms and pesticide residues. Spoiled fruit must be removed before washing and use of brushes during washing is effective in eliminating rotten portions of the apples thus preventing problems with microbial growth and mycotoxins (Lozano, 2006).

Activated carbon treatment

Adsorption using activated carbon is commonly used to remove the brown coloured pigments formed through enzymatic and non-enzymatic browning (Carabasa *et al.*, 1998) and to reduce the patulin levels (Rhonde, 2011). The amount of activated carbon added depends on legislation requirements and customer specifications for patulin (Rhonde, 2011).

Hygiene

Unsound, decomposed fruits are heavily contaminated with microorganisms and a small percentage of unwholesome fruit can “seed” operating equipment with spoilage microorganisms (Lawlor, Schuman and Simpson, 2009). Unit equipment in the preparation of fruit juices such as pipelines are significant sources of contamination as they are conducive to the formation of biofilms which can later on contaminate the fruit juice (Lawlor *et al.*, 2009). Starting with good quality sound fruit is important but so too is the cleanliness of the process operations (McLellan., 1996). Good hygienic practices and adherence to good manufacturing practices are the most effective control measures for microbiological contamination in the beverage industry as sticky sugars and fruit residues are ideal food sources for yeasts and moulds (Wareing and Davenport, 1998).

Thermal treatments

For acidic fruit juices (pH below 4.5) such as apple juice concentrate, the main purpose of pasteurisation is to inactivate enzymes and destroy spoilage microorganisms mainly yeasts, moulds and some aciduric bacteria (Raso, Calderon, Gongora, Barbosa-Canovas and Swanson, 1998; Molinari, Pilosof and Jagus, 2004) therefore reducing the microbial load substantially and extending the shelf life of the product (Tournas *et al.*, 2006). If the initial load is too high and/or the heat process is inadequate, some microorganisms will survive and subsequently cause spoilage (Tournas *et al.*, 2006).

Because pasteurisation utilises moderate temperatures <100 °C (Sukasih and Sektyadjit, 2008), only part of the microbes present are destroyed and the growth of any surviving spoilage

organisms is prevented by the additional hurdles employed such as low temperature during storage, high acidity and low water activity (Smith, 2011).

The temperature used in the first pasteurisation is chosen primarily with respect to the enzymes (polyphenolases and pectinases) to be inactivated and not the microorganisms to be destroyed. However, the treatment which inactivates the enzymes suffices, as a rule, for the destruction of the microorganisms (Stratford, 2006). The fruit juice industry usually uses a temperature of 90 °C for about 30 seconds (Stratford, 2006).

The resistance of yeasts to high temperatures is comparable to that of vegetative bacteria. However unlike bacterial endospores, the ascospores of yeasts may be only slightly more resistant to heat than vegetative cells (Deak, 2008). The microbial population of fruit juices is reduced rapidly at temperatures of 62.2 °C to 68.8 °C while the yeasts die off to a considerable extent at lower temperatures of 55 to 65 °C (Deak, 2008) leaving only the moulds which are also destroyed at 68.3 °C. Mould spores are more heat resistant as they normally require temperatures ranging between 75 to 80 °C for up to 15 minutes to be destroyed (Stratford, 2006). In a study by Raso *et al.* (1998), the $D_{60\text{ °C}}$ value for the vegetative cells of *Z. bailii* in apple juice concentrate at pH 4.1 was 3.02 minutes and 15.6 minutes for its ascospores.

The thermal inactivation rates of yeasts depend particularly on the solutes and their concentrations in heating medium (Truong-Meyer, Strehaiano and Riba, 1997). High concentrations of sugars protect cells of yeasts against thermal inactivation and this increased heat resistance is attributed to the dehydration of cells together with a reduction in pore size of the cell wall (Wareing and Davenport, 1998). Truong-Meyer *et al.* (1997) in his study confirmed *S. cerevisiae* as one of the most heat resistant strains in low pH sugared media solutions. In a study by Stecchini and Beuchat (1985), the heat resistance of *S. cerevisiae* was increased in puree containing added sugars and the order of enhanced protection against thermal inactivation was sucrose > glucose > fructose. Juven, Kanner and Weisslowicz (1978) in their study reported the heat resistance of *Saccharomyces chevalieri*, *Torulasporis magnoliae* and *Candida lambica* to be higher in orange juice concentrate at 50 °Brix than in the 30 and 12 °Brix juices. Removal of water by concentration increases the level of food acids and sugars in solution subsequently reducing the water activity (Potter and Hotchkiss, 1995).

Storage conditions

The storage temperature is important as it can slow down the rate of Maillard reaction and the growth of spoilage microorganisms in the apple juice concentrate and this is important to food quality and safety (New Zealand Food Safety Authority, 2005).

Composition of clarified apple juice concentrates

The major components of apple juice concentrate are carbohydrates, acids, nitrogen compounds, polyphenols, minerals and vitamins (Alvarez *et al.*, 2000). Apple juice concentrate is produced from a mixture of different apple varieties: Royal gala, Topred, Red delicious, Fuji, Braeburn, Early Red One, Starking, Golden Delicious, Pink Lady, Granny Smith and Sundowner whose composition varies (Greeff and Kotze, 2007). Sugars account for about 75-85 % of the total soluble solids in fruit juices. The major sugars present are fructose, glucose and sucrose with fructose being the primary sugar in apple juice accounting for more than 50 % of the total sugar content (Huang, Rasco and Cavinato, 2009). A variety of other sugars and sugar alcohols such as maltose, xylose and sorbitol are also present (Eisele and Drake, 2005).

Organic acids are the second most abundant soluble solids component in fruit juices and are typically present at about 1 % of the total weight of a fruit juice (Huang *et al.*, 2009). The pH/acidity of a product affects the range of organisms that can survive and grow (Stannard, 1997). Apple juice concentrate has a low pH in the range 1.6-3.8 which is due to acids naturally present in apples (Warczok *et al.*, 2004). Malic acid has been the only generally recognized acid in apple juice. Citric, tartaric, quinic, glycolic, succinic, lactic, galacturonic, citramalic, oxaloacetic, shikimic, glyoxylic and uronic acid are present in small amounts depending on variety, condition of fruit, growing conditions and location (Wu *et al.*, 2007; Moyer and Aitken, 1980). Yeast and mould counts are more relevant indicators of shelf life than bacteria for products with a low pH such as apple juice concentrate.

Water activity is a measure of the water available for microbial growth and a reduction in the water activity restricts the growth of many organisms (Bell, 2007). During the concentration of fruit juice, at least 50 % of the water content is removed producing concentrate with total soluble solids content between 65 and 71 °Brix (The Fruit juices and Fruit nectars Regulations, 2013). Only osmotolerant yeasts and moulds can grow at the relatively high soluble solids content of concentrates (Thomas and Davenport, 1985).

2.5 Hurdle technology in apple juice concentrate

The microbiological quality and safety of most foods is based on the intelligent application of hurdle technology a combination of several preservation factors which microorganisms present in the food are unable to overcome otherwise the food will spoil or even cause food poisoning (Leistner and Gould, 2002). To ensure the microbial stability in apple juice concentrate, the hurdles used to preserve it include high temperatures during processing (90-96 °C for 45 seconds), low storage temperature (10 °C), high acidity (pH 3 to 4) and reduced water activity of about 0.60 to 0.70 (Leistner, 1995). At reduced water activity less water is available in the cell for the microorganism to use for biochemical processes (Davidson and Critzer, 2012). A low pH renders the food less optimal as an environment for key enzymatic reactions and it also adversely affects the transportation of nutrients into the microbial cell (Rodrick and Schmidt, 2003). Thermal treatment destroys microorganisms by denaturing enzymes (Jay, Loessner and Golden, 2005). Maillard reaction products may also be considered as hurdles as they have antimicrobial properties and hence influence the quality of the apple juice concentrate (Leistner, 2000). The antimicrobial activity of melanoidins may be partially explained by the chelation of metal ions such as potassium which are essential for the growth of microorganisms (Pokorny, 2001). According to Leistner (1995), the different hurdles in a food do not just have an additive effect on stability but may act synergistically. A synergistic effect occurs if the different hurdles in a food affect, at the same time, different targets for example the cell membrane, DNA, enzyme systems, pH, a_w and redox potential within the microbial cell and thus disturb the homeostasis of the microorganism present in several aspects (Leistner, 2000). The repair of homeostasis as well as the activation of stress shock proteins becomes more difficult hence ensuring the microbial stability of the food product (Leistner, 2000).

2.6 Mathematical modelling of deterioration reactions

Modelling is a process whereby a system of mathematical equations is generated in order to represent “reality” as accurately as possible. The process comprises generation of initial hypothesis based on established reaction mechanisms, experimental design, modelling of data and model evaluation and criticism (Balagiannis *et al.*, 2010). According to Mizrahi (2000), the kinetic model is the most common method used for performing accelerated shelf life testing.

2.6.1 Kinetic modelling of the Maillard reaction

Kinetic modelling establishes that a process can be mathematically described by means of kinetic parameters with the aim of understanding, predicting and controlling quality changes in food processing and storage (Purlis, 2010; Van Boekel, 2008). Knowledge of the kinetic parameters such as reaction order, rate constant (k) and activation energy (E_a) is essential for predicting and controlling food quality attributes associated with the Maillard reaction (Martins and Van Boekel, 2005). The rate constant is a coefficient that relates the quality parameter with its rate of change with time and enables the calculation of the Q_{10} factor (Ruiz *et al.*, 2012). Activation energy is a measure of the energy barrier that molecules need to overcome in order to be able to react (Van Boekel, 2008).

2.6.1.1 Primary modelling

The reaction order and the rate constant are established by fitting simple kinetic models shown in Table 2 to one selected reaction such as colour formation, hydroxymethylfurfural formation, degradation of reducing sugars and amino acids (Vaukousi *et al.*, 2008; Martins and Van Boekel, 2005). Most of the chemical deteriorative reactions in shelf life studies follow either zero-order kinetics which represent a linear evolution (loss or gain) of the parameter or first-order kinetics which represent an exponential evolution of the parameters as illustrated in equation (Palazon, Perez-Conesa, Abellan, Ros, Romero and Vidal, 2009; Achour, 2006; Mizrahi, 2000). Numerous research studies on the storage of fruit concentrates have applied either zero or first order models (Table 3) to describe the degradation of colour due to the Maillard reaction.

Table 2. Primary models used to fit chemical reactions.

Model	Equation	Reference
Zero order	$P = P_0 + kt$	Martins <i>et al.</i> , 2001
First order	$P = P_0 \times \exp(kt)$	Martins <i>et al.</i> , 2001
Parabolic	$P = (P_0^{1/2} + kt)^2$	Buedo, <i>et al.</i> , 2001
Weibull	$P = P_{\max} + (P_0 - P_{\max}) \exp[-(kt)^\beta]$	Vaikousi <i>et al.</i> , 2008
Logistic	$P = P_0 + \frac{P_{\max} - P_0}{1 + \exp[-k(t - t_i)]}$	Vaikousi <i>et al.</i> , 2008

Where P is the parameter measured at time t , P_0 is the initial value of parameter ($t=0$), k is the reaction rate constant (week^{-1}), $P_{\max}$ is the maximum value of parameter, β is the shape constant in the Weibull model and t_i is the time when half of the maximum value of parameter is reached in the logistic model.

Table 3. Kinetics of non-enzymatic browning in fruit juice concentrates

Food System	Packaging	Storage time	Storage Temperature (°C)	Parameter	Reaction order	Activation Energy (kCal/mol)	Reference
Orange (61 °Brix) Lemon (44.5 °Brix) Grape(59 °Brix) Tangerine(59.5 °Brix)	Glass jars	8 weeks	28, 37 and 45	A _{420nm} L* b*	Zero First First	A _{420nm} (35.27, 17.60, 27.81 and 32.39) L* (28.99, 6.67, 6.74 and 27.84) b* (20.44, 3.90, 6.94 and 17.3)	Koca <i>et al.</i> , (2003)
Golden delicious (65, 70 and 75 °Brix) Amasya apple (65, 70 and 75 °Brix)	Glass jars	4 months	5, 20 and 37	A _{420nm}	Zero	21.3, 21,4 and 21.0 33.7, 32.7 and 32.5	Burdurlu <i>et al.</i> , (2003)
Red delicious (65, 70 and 75 °Brix) Granny smith (65, 70 and 75 °Brix)	Glass vials without head space	120 days	5, 20 and 37	A _{420nm}	First	16.4 – 19.3	Toribio <i>et al.</i> , (1984)
Peach (89, 80.7, 70, 60, 50, 40, 30, 20 and 12 °Brix)	Glass vials	118 days	5, 15, 30 and 37	A _{420nm}	First	32.16, 26.76, 24.72, 25.26, 25.29, 25.11, 24.04, 22.41 and 20.97	Buedo, <i>et al.</i> , (2001)

2.6.1.2 Secondary modelling

It is also important to know the effect of temperature on the browning kinetics and the use of accelerated storage studies allows this effect to be determined (Ruiz, Demarchi, Massolo, Rodoni and Giner, 2012). The activation energy (E_a) from the Arrhenius model and Q_{10} are the most important and widely used parameters for predicting the effect of temperature on any specific reaction (Liu *et al.*, 2008).

2.6.1.2.1 Arrhenius model

The Arrhenius model expressed as: $k = k_0 \exp\left(\frac{E_a}{RT}\right)$ (3)

is used to calculate the activation energy (E_a); where k is the rate constant, k_0 is the pre-exponential constant, R is the gas constant in kcal/mol in °K (1.986 kcal/molK) and T is the absolute temperature in K (273 + °C) (Fu and Labuza, 1993). The Arrhenius relationship can be determined by drawing a semi-log plot of rate versus inverse absolute temperature. By plotting the negative logarithm (-ln) of the rate constants (k) versus the reciprocal of absolute temperature, the slope value, E_a/R is used to calculate the activation energy for n-order reactions (Bostan and Boyacioglu, 1996). The activation energy for some parameters of non-enzymatic browning in different fruit concentrates is shown in Table 3.

2.6.1.2.2 Q_{10} concept

Q_{10} is another approach that can be used to study the effect of temperature on the degradation rate (Achour, 2006) and it is normally calculated for n-order type models provided the n-order is the same for each quality parameter at the two temperatures (Ruiz *et al.*, 2012; Vaikousi *et al.*, 2008). Q_{10} is the amount that the rate increases or decreases for every 10 °C change in temperature (Davies and Labuza, 2003).

$$Q_{10} = \frac{\text{Rate}(T + 10^\circ\text{C})}{\text{Rate}(T)} \quad (4)$$

Ruiz *et al.* (2012) state that, if the quality loss in a given food is caused by two reactions with different Q_{10} values, then the reaction with the higher Q_{10} would be the most damaging at high temperatures while that with the lower Q_{10} causes quality losses at lower temperatures.

2.6.1.2.3 Shelf life plots

Shelf life plots also known as time temperature plots are also a useful approach to quantify the effect of temperature on food quality (Dattatreya, Etzel and Rankin, 2007). Shelf life plots constructed by plotting shelf life against temperature are practical and easier to understand as one can read directly the shelf life of food at any storage temperature (Mizrahi, 2004). To construct a shelf life plot one needs some measure of loss of quality, some endpoint value for that quality parameter, data to measure the time to reach this endpoint and experiments to measure this loss for at least two temperatures so that the line can be constructed (Office of Technology Assessment, 1979).

Although shelf life plots are easier to use, they are applicable only to narrow temperature ranges (20 – 40 °C) (Robertson, 2000) and if a broad temperature range is required, the Arrhenius equation will predict stability more accurately (Bell, 2007). The higher the Q_{10} value or the steeper the slope, the more sensitive the reaction is to temperature change (Fu and Labuza, 1997).

2.6.2 Modelling of microbial growth

When data for the growth of the organism in the food system has been generated, the next stage involves mathematical analysis of the data to produce a model and mathematical validation to determine the quality of the data and goodness of fit of data to the model (Kilkast and Subramaniam, 2000). To model microbial growth, the growth curves for yeasts are fitted to primary models such as the modified Gompertz equation, logistic model and the Baranyi and Roberts's model (Man, 2002; Baranyi and Roberts, 1994; Xiong, Xie, Edmondson and Sheard, 1999). Primary level models describe changes in microbial numbers or other microbial response (e.g. acid production) with time to a single set of conditions (Buzrul, 2009; Zwietering, Jongenburger, Rombouts and Van't Riet, 1990). The maximum specific growth rate ($\mu_{\max}$) and the lag time (λ) are important growth parameters that can be established from the primary growth curves. According to Shimoni and Labuza (2000), since food becomes microbiologically unsafe before the stationary phase is reached, one should try to optimize analysis of the lag time and the growth rate in the exponential phase (Fu and Labuza, 1993). Zanoni, Pagliarini, Galli and Laureati (2006) in their shelf life study of fresh blood orange juice, used the Gompertz equation modified by Zwietering to model yeast growth.

Secondary level models describe the dependence of primary model parameters on environmental factors such as temperature, pH, organic acids and water activity. Temperature affects the duration of the lag phase, the rate of growth and the final cell numbers (Shimoni and Labuza, 2000). The Arrhenius model has been used successfully in describing the temperature dependence of many chemical reactions related to the shelf life of food. Since the replication of the gene during cell division is a chemical process, it seems logical that the growth rate of microorganisms would also follow the Arrhenius law for a certain temperature range (Shimoni and Labuza, 2000). The combination of primary and secondary modeling can be used to predict the microbial level and thus the shelf life and safety of food (Fu and Labuza, 1993).

2.7 Hypotheses and Objectives

2.7.1 Hypotheses

1. The physical (colour and non-enzymatic browning index) and chemical (5-hydroxymethylfurfural concentration) parameters of the apple juice concentrate will change with storage time at 10 °C due to Maillard browning while the microbial quality will remain stable. The growth of spoilage yeasts and moulds in apple juice concentrate will be inhibited due to the synergistic effect of low pH and low water activity which is enhanced at low storage temperatures (Betts, Linton and Betteridge, 1999). The hydroxymethylfurfural content will increase and the colour will darken as a result of Maillard reaction which still proceeds at low storage temperatures albeit at a lower rate. Maillard reaction occurs at a maximum rate in intermediate moisture foods with water activities of 0.6-0.7 typical of apple juice concentrate (Eskin, 1990).

2. Accelerated storage temperatures will increase the rate of both microbial growth and Maillard browning reaction. Storage at 35 °C will result in a more rapid loss of quality than storage at 25 °C. An increase in temperature will increase in the reactivity between the sugar and the amino group (Martins *et al.*, 2001) resulting in an increase in the Maillard reaction rate by 3-8 fold for every 10 °C rise in temperature (Davies and Labuza, 2003). For any microorganism the microbial growth rate increases with temperature only until the optimal temperature (Halasz and Lasztify, 1991). Since all metabolic reactions of microorganisms are enzyme catalyzed, increasing the storage temperature will increase the activity of enzyme systems associated with cell division resulting in an increase in microbial growth rate (Jay *et al.*, 2005). For most biological systems there is a 1.5-2.5 increase in the rate of reaction for each 10 °C increase in temperature (Jay *et al.*, 2005).

2.7.2 Objectives

1. To determine the changes with storage time in the physical (colour and non-enzymatic browning index), chemical (5-hydroxymethylfurfural concentration) and microbiological (yeasts and moulds, heat resistant moulds and *Alicyclobacillus acidoterrestris*) parameters of apple juice concentrate during refrigerated storage at 10 °C.

2. To determine the effect of accelerated storage temperatures of 25 °C and 35 °C on the rate of Maillard browning and growth of spoilage microorganisms (yeasts and moulds) in apple juice concentrate.

3. To determine the effect of storage temperature on the kinetics (reaction rate constant, activation energy and Q_{10}) of the Maillard reaction parameters (colour, non-enzymatic browning index and 5-hydroxymethylfurfural concentration) and microbial growth (lag phase and maximum specific growth rate of yeasts and moulds) during storage.
4. To identify the factor/parameter limiting the shelf life of apple juice concentrate during storage at 10 °C, 25 °C and 35 °C.

CHAPTER 3: RESEARCH

3.1 THE EFFECT OF STORAGE TEMPERATURE ON THE PHYSICOCHEMICAL CHARACTERISTICS OF APPLE JUICE CONCENTRATE

ABSTRACT

The aim of the study was to determine the quality changes in apple juice concentrate stored at refrigeration and accelerated temperatures. Apple juice concentrate was stored at 10 °C, 25 °C and 35 °C and the colorimetric parameters (L^* , b^* , a^* , hue, chroma and total colour difference), non-enzymatic browning index ($A_{420\text{nm}}$) and 5-hydroxymethylfurfural concentration were evaluated at fortnightly intervals. There was a significant change ($p \leq 0.05$) in all the Maillard reaction parameters with time and temperature. Hydroxymethylfurfural formation was more temperature dependant with a Q_{10} value of 5.25 in comparison to L^* , b^* and $A_{420\text{nm}}$ whose Q_{10} values were 1.53, 1.20 and 1.81, respectively. Results from accelerated storage testing were used to determine if this technique can be used to predict quality changes in apple juice concentrate when stored at lower temperatures. The predicted rate constant values for L^* , b^* and $A_{420\text{nm}}$ were 1.06, 1.70 and 1.63 times more than the actual values showing a good agreement between the predicted and actual reaction rate constant.

3.1.1 Introduction

The apple juice concentrate industry has experienced growth at a rate of about 6% per year as worldwide demand increases for more natural methods of sweetening processed foods (MGEX, 2013). Apple juice concentrate is one of the most consumed fruit juices in the world (Ceci and Lozano, 1998). Non-enzymatic browning takes place during storage of apple juice concentrate compromising its sensorial properties such as colour and flavour. Maillard reaction leads to the formation of a wide variety of end products including organic acids, furans, ketones and pyrroles some of which contribute to off-flavours in the juice (Fustier, St-Germain, Lamarche and Mondor, 2011). Colour is a product attribute which plays an important role in food acceptance since it is perceived immediately by the consumer (Ganjloo, Rahman, Bakar, Osman and Bimakr, 2009; Quintas, Brandao and Silva, 2007). A darkened apple juice concentrate leads to dissatisfaction or rejection of the product by the consumer which is a financial liability to the company (Vaikousi *et al.*, 2008 and Carabasa, Ibarz *et al.*, 1998). Maillard reaction products have also been shown to have mutagenic, carcinogenic and cytotoxic properties (Dolphen and Thiravetyan, 2011; Knol, Linssen and Van Boekel, 2010).

Browning in apple juice concentrate during storage is attributed to Maillard reaction which is a complex non-enzymatic reaction resulting from the condensation of an amino group and a carbonyl function (Laroque, Inisan, Berger, Vouland, Dufosse and Guerard, 2008; Burdurlu and Karadeniz, 2003). Factors that influence the Maillard reaction include the nature, concentration and proportion of reactants (amino and carbonyl groups), water activity, time, temperature, pH, buffer type and concentration, presence of oxygen, light and metal ions (Davies and Labuza; 2003). Maillard reaction occurs at a maximum rate in concentrated intermediate moisture foods with water activities of 0.6-0.7 such as apple juice concentrates (Vaikousi, Koutsoumanis and Biliaderis, 2008; Vercet, 2003). Quality in food is very important and deterioration during storage has to be controlled (Burdurlu and Karadeniz, 2003). Since apple juice concentrate is stored for long periods to provide yearlong availability, it is important to minimize the undesirable quality changes during storage.

Studies have been done on kinetic modelling of non-enzymatic browning reactions in apple juice concentrate during storage (Burdurlu and Karadeniz, 2003; Toribio and Lozano, 1984). Since apple juice concentrates are stored over long periods of time there is a need to ascertain if deteriorative changes can be reliably estimated within a short period of time using accelerated storage techniques. The objective of this study was to determine the effect of storage temperature using accelerated storage tests on the physicochemical attributes of apple juice concentrate. This information will show if accelerated storage testing can be used to estimate the rate of deteriorative reactions under normal storage temperature and serve as a guideline for the optimization of storage conditions for apple juice concentrate and establishment of its keeping quality.

3.1.2 Materials and methods

3.1.2.1 Samples and storage

The apple juice concentrate used in this study was sourced from a South African concentrate manufacturing company. Steps in the manufacture of the apple juice concentrate are outlined in Figure 5. Apple juice concentrate (45 litres) was taken from a concentrate tank (10 °C) and transported overnight in a Styrofoam cooler box with ice. On arrival at the University of Pretoria laboratory, the samples were mixed and immediately distributed aseptically into 9 sterile 5 litre glass bottles wrapped with foil paper. Three bottles were stored at 10 °C, three at 25 °C and three at 35 °C. For accelerated shelf life testing (ASLT), the storage

temperatures of 25 °C and 35 °C were used while 10 °C was the normal storage temperature for apple juice concentrate. Homogenous aliquots (200ml) were decanted aseptically from each of the nine bottles for analysis at fortnightly intervals over 12 weeks (Figure 6).

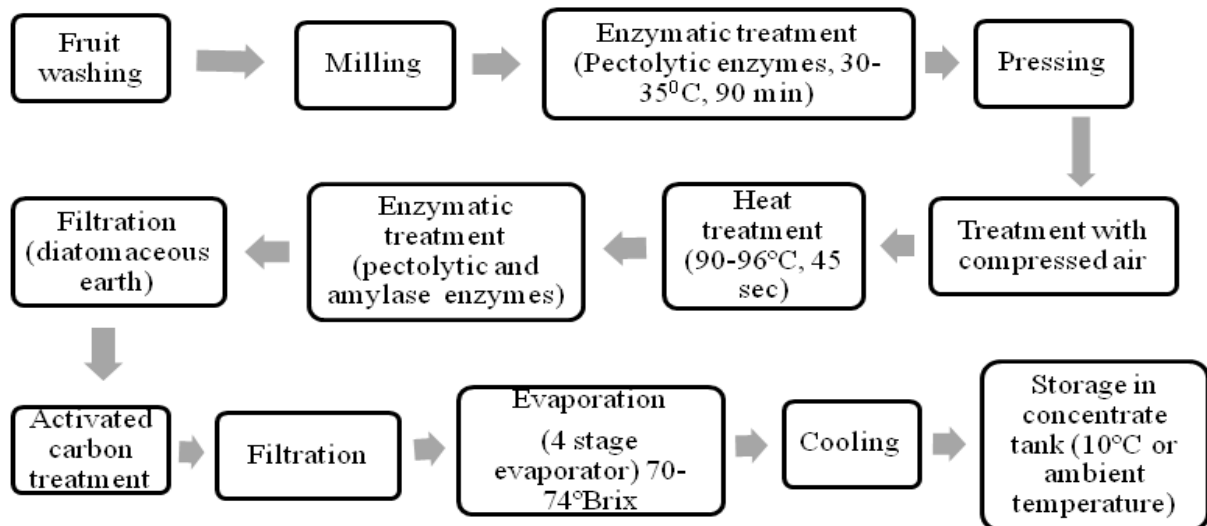


Figure 5. Flow chart of the manufacture of apple juice concentrate

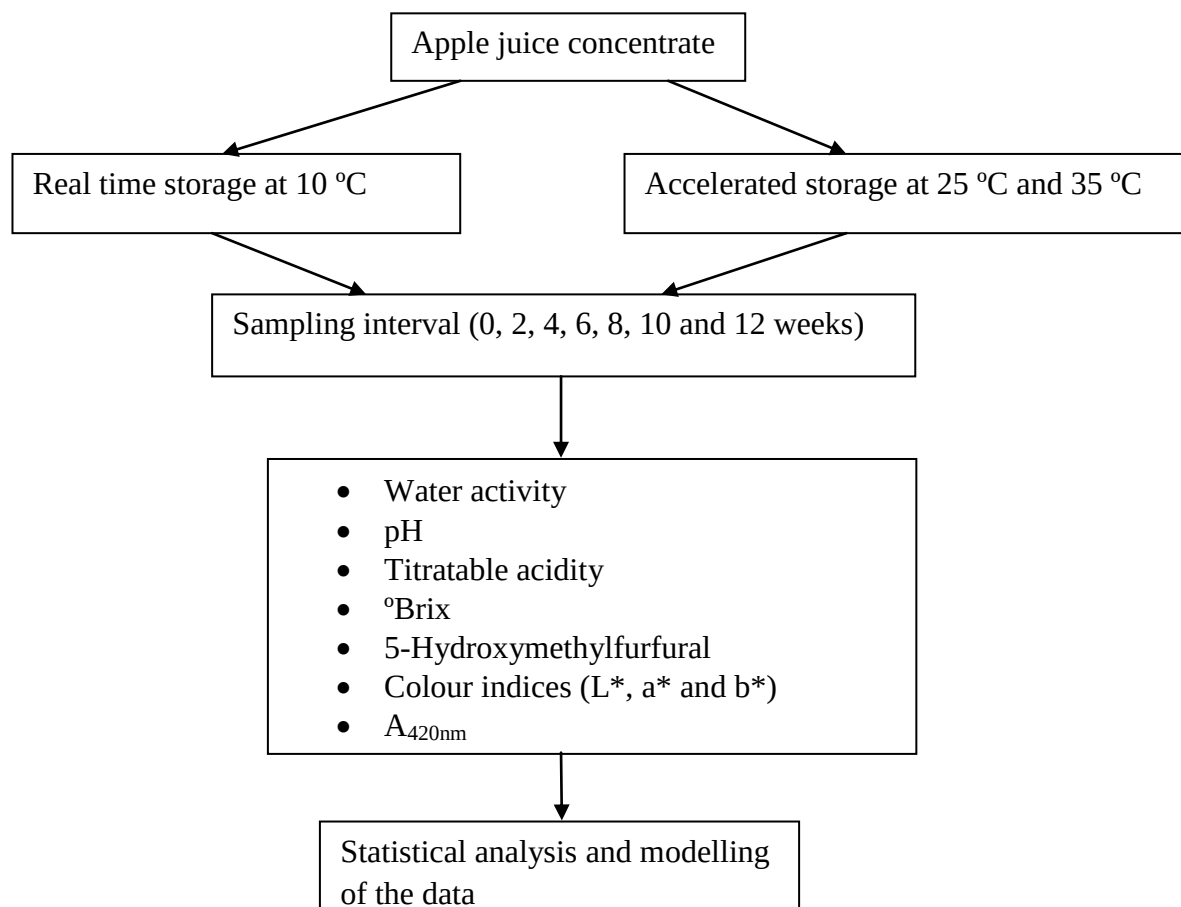


Figure 6. Schematic presentation of the experimental design

3.1.2.2 Water activity

The water activity (a_w) of apple juice concentrate was determined using a portable Pawkit water activity meter (Decagon devices, England).

3.1.2.3 Total soluble solids (°Brix)

An Atago PAL-3 pocket refractometer (Atago Japan) was used to measure the total soluble solids as °Brix in the apple juice concentrate.

3.1.2.4 pH

The pH of apple juice concentrate was measured using a pH 211 Microprocessor pH meter (Hanna Instruments), calibrated using pH 7.0 and 4.0 buffers.

3.1.2.5 Titratable acidity(%)

Titrateable acidity as malic acid was determined potentiometrically. A sample of apple juice concentrate (10 ml) was titrated with 0.1 N NaOH to an endpoint of pH 8.1 (AOAC, 2000).
% Titrateable Acidity (malic acid) = Titre value x 0.067.

3.1.2.6 Colour indices

A Konica Minolta Chroma meter CR-400 was used to measure the colour parameters L^* (lightness), a^* (redness), and b^* (yellowness) of apple juice concentrate after calibration using a white tile. The head of the colorimeter was placed against a 60 mm petri dish filled with apple juice concentrate and the three colour parameters L^* , a^* and b^* measured. Chroma, hue and total colour difference have also been suggested as effective means of predicting visual colour appearance over either L^* , a^* or b^* alone and they were calculated using equations 1-3 (Sadilova *et al.*, 2009).

$$\text{Total colour difference} = [(L^* - L^*_0)^2 + (a^* - a^*_0)^2 + (b^* - b^*_0)^2]^{1/2} \quad (1)$$

$$\text{Chroma} = (a^* + b^*)^{1/2} \quad (2)$$

$$\text{Hue} = \tan^{-1} (b/a) \quad (3)$$

3.1.2.7 Non-enzymatic browning Index (A_{420nm}) and % Transmission

Non-enzymatic browning index is commonly used to monitor the extent of Maillard reaction or development of brown pigments in food (Aguilo-Aguayo, Soliva-Fortuny and Martin-Belloso, 2009; Meydev, Sagy and Kopelman, 1977). The apple juice concentrate was diluted to 12 °Brix with distilled water and the absorbance and % transmission measured in 1 cm

cuvettes against distilled water at 420 and 440 nm respectively using a spectrophotometer (T80+ UV/VIS Spectrophotometer, PG Instruments Ltd). Both the $A_{420\text{nm}}$ and % Transmission were measured because in literature the non-enzymatic browning index is usually expressed as the absorbance at 420 nm but in some industries % transmission at 440 nm is measured with specifications for clarified apple juice concentrate being > 40 % transmission (Rhonde, 2011).

3.1.2.8 5-Hydroxymethylfurfural

5-Hydroxymethylfurfural (HMF) is an intermediate compound of the Maillard reaction used as an indicator of reduced quality in foods as a result of inappropriate long term storage (Vorlova, Borkovcova, Kalabova and Vecerek, 2006). 5-HMF was determined quantitatively using the method used by Cohen, Birk, Mannheim and Saguy (1998). The apple juice concentrate was diluted ten-fold before analysis and a calibration curve of HMF standards ranging from 0 – 25 mg/kg was used to quantify the HMF concentration in the apple juice concentrate.

3.1.2.9 Visual appearance

Photographs of apple juice concentrate were taken after diluting it with distilled water to 12 °Brix which is the solids content level of the commercial ready to drink apple juice. Differences in colour are also difficult to observe when the apple juice is in concentrate form.

3.1.2.10 Statistical analyses

Repeated measures analysis of variance using the General Linear Models (GLM) procedure in SAS/STAT software (SAS Institute, USA, 1989) was used to determine if significant differences existed among the samples stored at the three temperatures and results were expressed as the least square mean $\pm$ standard error. Means were considered to be different at 5% significance level.

Correlation coefficients between the parameters (lightness, redness, yellowness, absorbance (420nm) and 5-hydroxymethylfurfural, total colour difference, chroma and hue) were determined using STATISTICA windows version 10 (Statsoft Inc, Tulsa, Oklahoma, USA, 2011). All evaluations were based on a 5 % significance level.

3.1.2.11 Kinetic modelling

Knowledge on the quality changes including the reaction order, the reaction constant and the energy of activation are essential to predict quality losses during storage (Ganjloo *et al.*, 2009). The zero order, first order and parabolic models have been used to model non-enzymatic browning by several researchers (Wang, Hu, Chen, Wu, Zhang, Liao and Wang, 2005; Burdurlu and Karadeniz, 2003; Koca *et al.*, 2003; Buglione and Lozano, 2002; Toribio and Lozano, 1984; Beveridge and Harrison, 1984). The zero order (equation 5), first order (equation 6) and parabolic (equation 7) primary kinetic models were fitted to the experimental data using the non-linear estimation, user specified regression least squares method in STATISTICA windows version 10 (Statsoft Inc, Tulsa, Oklahoma, USA, 2011).

$$\text{Zero} \quad P = P_0 + kt \quad (5)$$

$$\text{First} \quad P = P_0 \times \exp(kt) \quad (6)$$

$$\text{Parabolic} \quad P = (P_0^{1/2} + kt)^2 \quad (7)$$

Where P is the parameter measured at time t , P_0 is the initial value of parameter ($t=0$), k is the reaction rate constant (week^{-1}).

The most appropriate model was selected on the basis of regression coefficients with higher values (R^2) providing the best fit. The reaction rate constant (k) was obtained from the best fit regression equations (Koca *et al.*, 2003).

Determination of the activation energy (E_a)

To determine the temperature dependence of the non-enzymatic browning reaction, activation energy and Q_{10} as shown in equation 8 and 9 were used

$$\text{Arrhenius model} \quad k = k_0 \exp\left(\frac{E_a}{RT}\right) \quad (8)$$

where k is the rate constant, k_0 is the pre-exponential constant, R is the gas constant in kcal/mol in °K (1.986 kcal/molK or 8.314 J/molK) and T is the absolute temperature in K ($273 + ^\circ\text{C}$) (Fu and Labuza, 1993). The negative logarithm of the rate constants ($-\ln k$) versus the reciprocal of absolute temperature ($1/T$) was plotted using Excel 2012 (Microsoft office) and the slope value represented E_a/R . To calculate the activation energy the slope value was multiplied by the gas constant (R) (Bostan and Boyacioglu, 1997).

Determination of Q_{10}

$$Q_{10} = (k_2/k_1)^{10/(T_2 - T_1)} \quad (9)$$

where T_1 and T_2 are the absolute temperatures ($^{\circ}\text{K}$), k_1 is the rate constant at T_1 and k_2 is the rate constant at T_2 (Koca *et al.*, 2003).

3.1.3 Results

3.1.3.1 Colour parameters

Change in lightness (L^*)

The L^* or lightness values (Figure 7) decreased with storage time and temperature. The decrease in L^* was significantly different ($p \leq 0.05$) with time throughout the storage period at all the three temperatures. However at 10°C , the decrease was not significant ($p > 0.05$) between weeks 0 and 2, weeks 4 and 6 and from week 8 until the end of storage at week 12. The decrease in L^* was significantly different ($p \leq 0.05$) between the temperatures. The change in L^* at 35°C within the first two weeks of storage (38.77 to 35.62) was greater than the change at 10°C after a 12 week storage period (38.77 to 36.26). L^* declined sharply during the first four weeks of storage at 35°C after which the change slowed down.

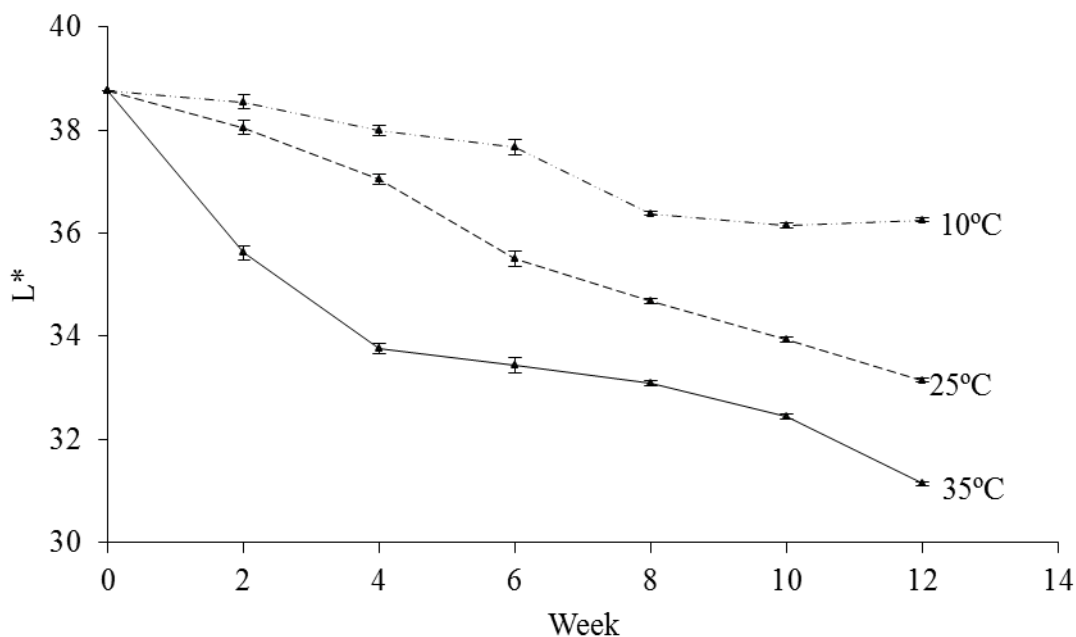


Figure 7. Change in lightness values of apple juice concentrate during storage at 10°C , 25°C and 35°C for 12 weeks ($n=3$)

Change in redness (a*)

The a* or redness values increased significantly ($p \leq 0.05$) with time from 15.41 to 16.75, 20.20 and 22.79 at 10 °C, 25 °C and 35 °C respectively (Figure 8). There was a significant ($p \leq 0.05$) time-temperature interaction in redness values. At 25 °C and 35 °C the increase in a* was exponential during the first six weeks of storage. a* values differed significantly ($p \leq 0.05$) between the temperatures.

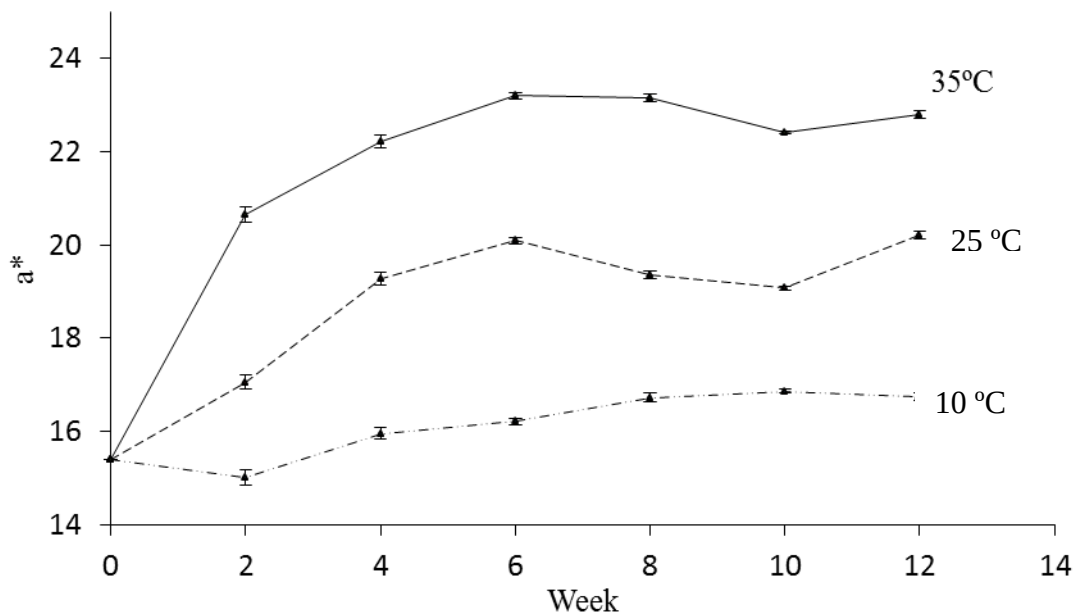


Figure 8. Change in redness values of apple juice concentrate during storage at 10 °C, 25 °C and 35 °C for 12 weeks (n=3)

Change in yellowness (b*)

Yellowness or b* values decreased significantly ($p \leq 0.05$) with storage time and temperature (Figure 9). The decrease in b* at 25 °C and 35 °C was higher during the first four weeks of storage (from 4.96 to -1.68 and - 4.14 at 25 °C and 35 °C respectively) while at 10 °C the change in b* was the lowest during the first four weeks of storage (4.96 to 4.39). Yellowness of the apple juice concentrate was significantly different ($p \leq 0.05$) among the three temperatures. There was a significant interaction ($p \leq 0.05$) between time and temperature.

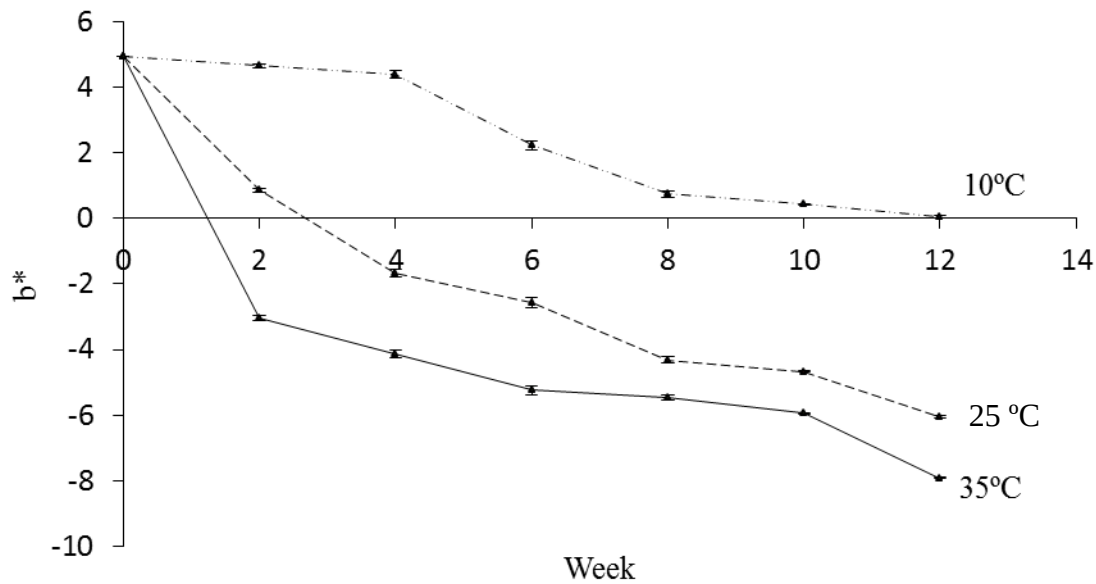


Figure 9. Change in yellowness values of apple juice concentrate during storage at 10 °C, 25 °C and 35 °C (n=3)

Total colour difference

Total colour difference is a colorimetric parameter that is extensively used to characterise the variation of colour in food as compared to the initial samples (Luo, Zhang, Wang, Guan, Jia, and Liao, 2008; Garza *et al.*, 1999). Total colour difference increased with time and temperature (Figure 10) and the increase was significantly different ($p \leq 0.05$) among the temperatures. According to Fonteles, Costa, Jesus, Fontes and Fernandes (2013) and Luo *et al.* (2008), a total colour difference value of 2 would be a noticeable difference in visual perception of many products. Within two weeks of storage at 25 °C and 35 °C, the total colour difference had surpassed 2 while at 10 °C it took about 5 weeks to exceed 2 (Figure 10). The total colour difference after 12 weeks of storage was 5.68, 13.27 and 16.69 at 10 °C, 25 °C and 35 °C, respectively.

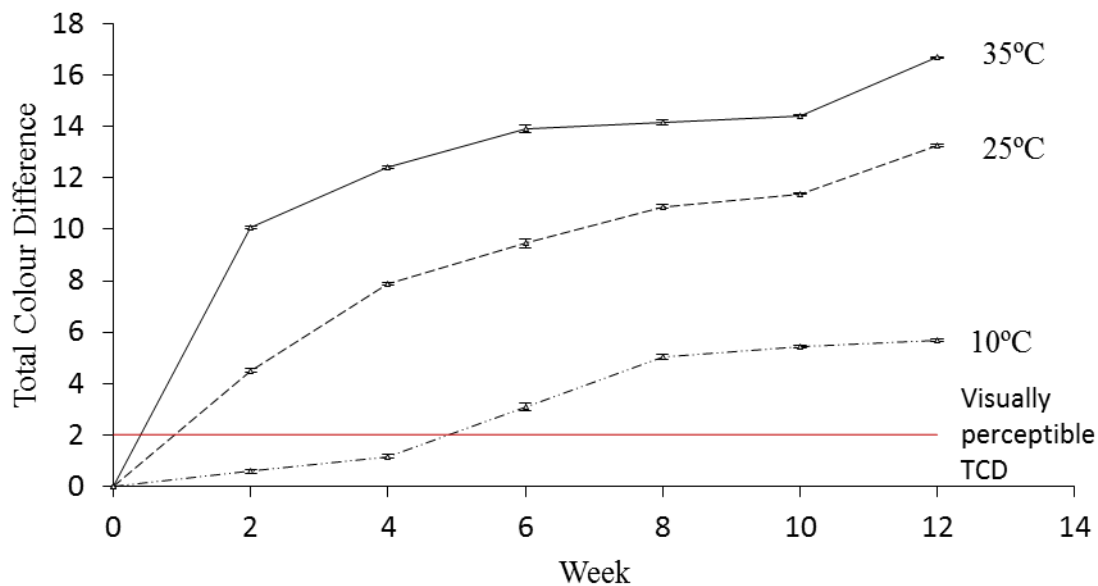


Figure 10. Total colour difference in apple juice concentrate stored at 10 °C, 25 °C and 35 °C for 12 weeks (n=3). TCD-Total colour difference

Hue

Hue is the attribute by which we recognise and therefore describe the colour as red, orange, yellow, green, blue or violet (Mohammadi, Rafiee, Emam-Djomeh and Keyhani, 2008). The hue angle is expressed on a 360 °grid where 0 °/360 ° = red, 90 ° = yellow, 180 ° = green and 270 ° = blue (Wrolstad, Durst and Lee, 2005; Bakker, Bridle and Timberlake, 1986). The initial hue value was 18 ° (Figure 11) corresponding to a red colour in the colour solid dimensions (Lee and Nagy, 1988). Upon storage of apple juice concentrate, the hue angle decreased significantly ($p \leq 0.05$) as a function of storage time and temperature showing a deepening of the red colour with storage. The difference in hue was not significantly different ($p > 0.05$) at 25 °C and 35 °C from week 8 but there was a significant difference ($p \leq 0.05$) between 25 °C and 35 °C in comparison to 10 °C.

Chroma

The chroma value indicates the degree of saturation of colour and is proportional to the strength of the colour (Saricoban and Yilmaz, 2010) with higher values corresponding to higher colour brilliance (Sadilova *et al.*, 2009). The chroma values increased significantly ($p \leq 0.05$) with storage time and temperature (Figure 12) by 0.56, 4.90 and 8.00 units at 10 °C,

25 °C and 35 °C, respectively. The time-temperature interaction was statistically significant ($p \leq 0.05$).

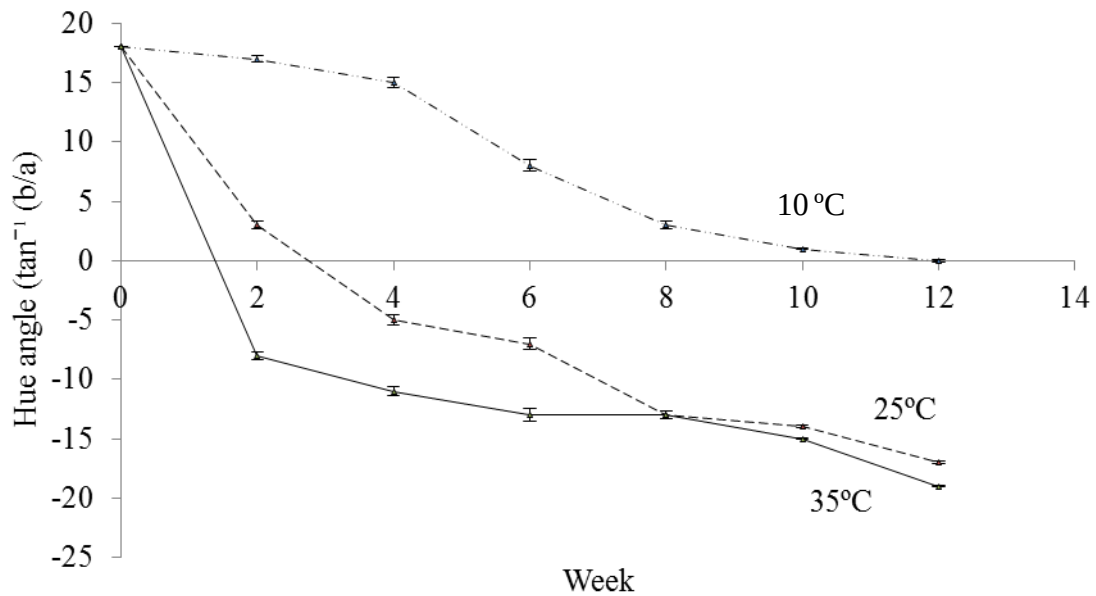


Figure 11. Changes in hue value of apple juice concentrate stored at 10 °C, 25 °C and 35 °C for 12 weeks (n=3)

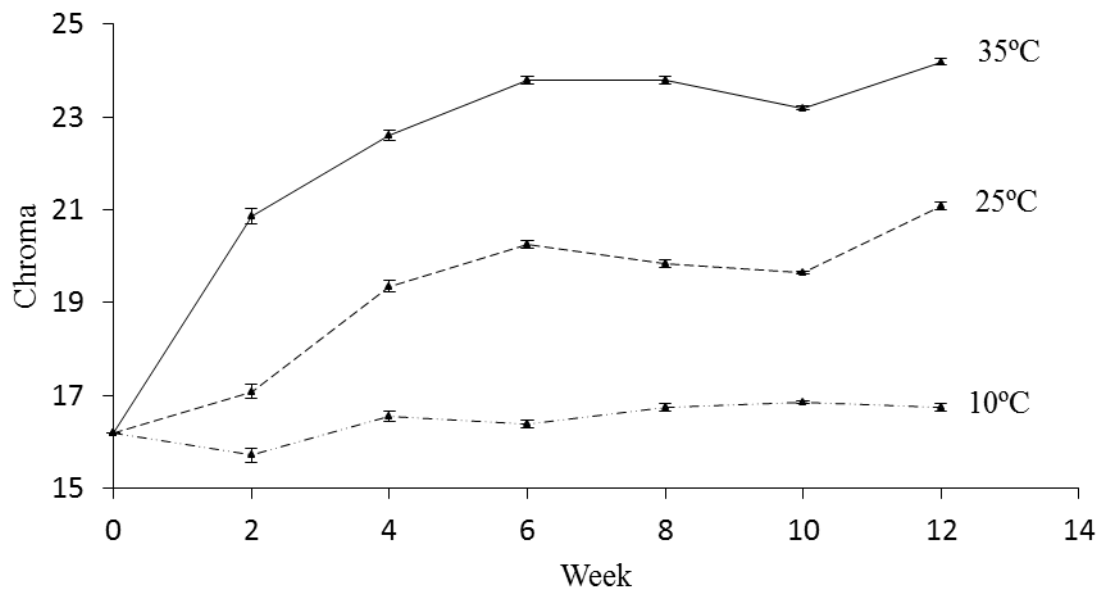


Figure 12. The effect of storage time on the chroma of apple juice concentrate stored at 10 °C, 25 °C and 35 °C

3.1.3.2 Non-enzymatic browning index and % Transmission

A significant ($p \leq 0.05$) increase in A_{420nm} (Figure 13) and conversely a significant ($p \leq 0.05$) decrease in % transmission with time (Figure 14) was observed at all the storage temperatures. The differences in the % transmission at 25 °C and 35 °C became less pronounced with storage time. There was a rapid initial drop in % transmission at 35°C from 40.6 to 6.4% during the first two weeks followed by a decrease in the rate of change. There was a significant ($p \leq 0.05$) time-temperature interaction for both A_{420nm} and % transmission. The changes in A_{420nm} and % transmission at 25 °C and 35 °C within the first two weeks of storage were greater than the changes at 10 °C after 12 weeks of storage.

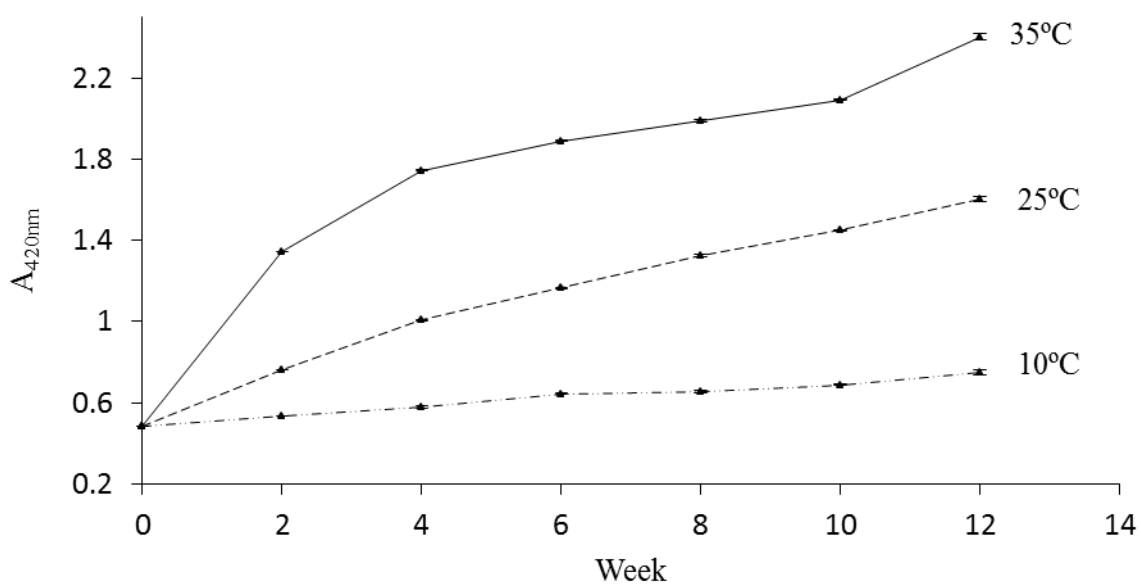


Figure 13. Non-enzymatic browning index (A_{420nm}) in apple juice concentrate ($n=3$) as a function of storage time and temperature

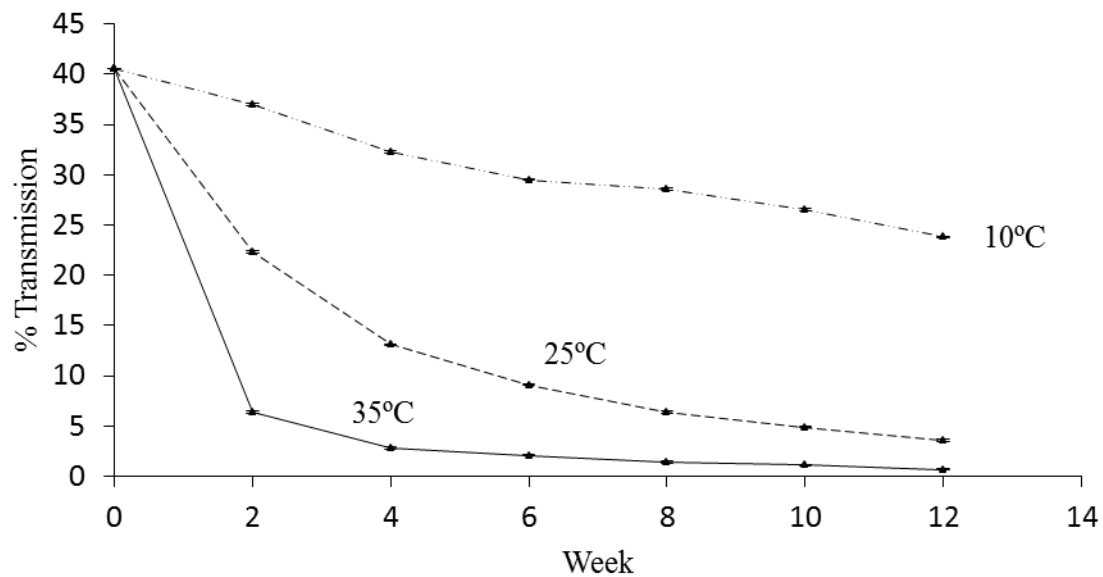


Figure 14. % Transmission in apple juice concentrate as a function of storage time and temperature (n=3)

3.1.3.3 Hydroxymethylfurfural

The International Federation of Fruit Juice Processors (IFFJP) states the HMF content in fruit concentrates must not exceed 25 mg/kg (Matic, Saric, Mandic, Milonovic, Jovanov and Mastilovic, 2009). In the initial sample of apple juice concentrate, the HMF content was 23 mg/kg and increased linearly with time at 25 °C and 35 °C (Figure 15) exceeding the limit of 25 mg/kg within 2 weeks of storage. The change in HMF content was significantly different ($p \leq 0.05$) between temperatures. The increase in the HMF content from the initial value of 23.17 mg/kg was 2.67 and 9.53 times at 25 °C and 35 °C, respectively after 12 weeks of storage. The HMF content at the lower storage temperature of 10 °C shows little fluctuation over the 12 week storage period.

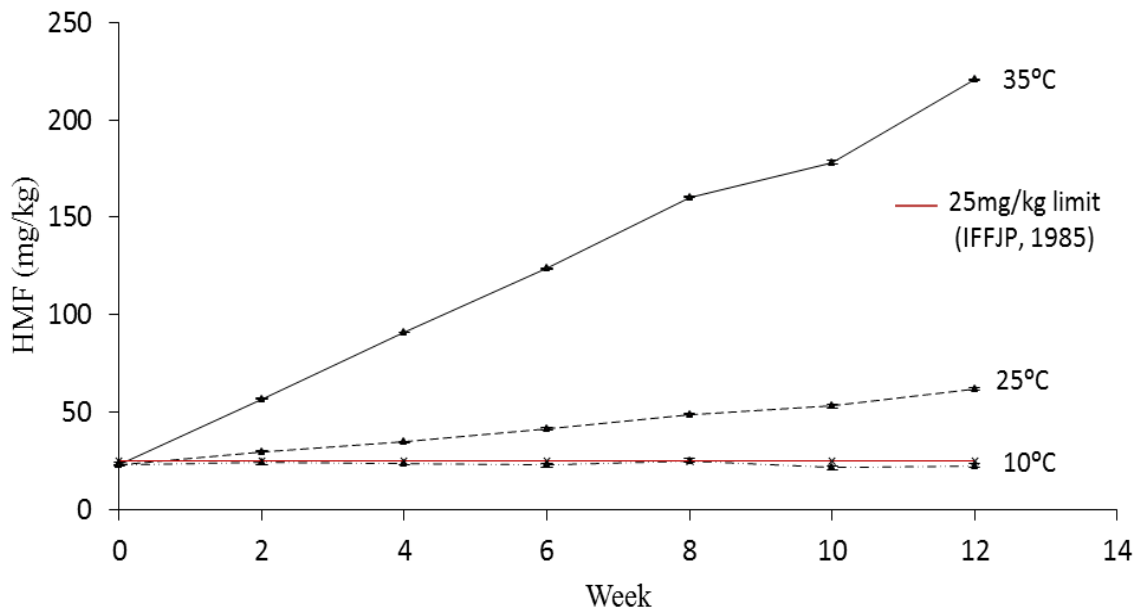


Figure 15. Effect of storage time on 5-hydroxymethylfurfural formation in apple juice concentrate stored at 10 °C, 25 °C and 35 °C (n=3)

3.1.3.4 pH, water activity, titratable acidity and °Brix

The average water activity of apple juice concentrate was 0.69 and the soluble solids content was 71 °Brix before storage and both of these parameters did not vary significantly ($p>0.05$) with storage time and temperature (results not shown). The initial average pH was 3.42 and titratable acidity was 1.66% and no obvious trend with storage time and temperatures was observed for these parameters (results not shown).

3.1.3.5 Visual assessment of colour change in apple juice concentrate

The colour of the diluted apple juice made from concentrate stored at 25 °C has an amber colour while that stored 35 °C for only 12 weeks is already dark brown (Figure 17). At 10 °C the change in colour of reconstituted apple juice concentrate is not easily discernible with the naked eye although there is a change in the browning index (A_{420nm}).

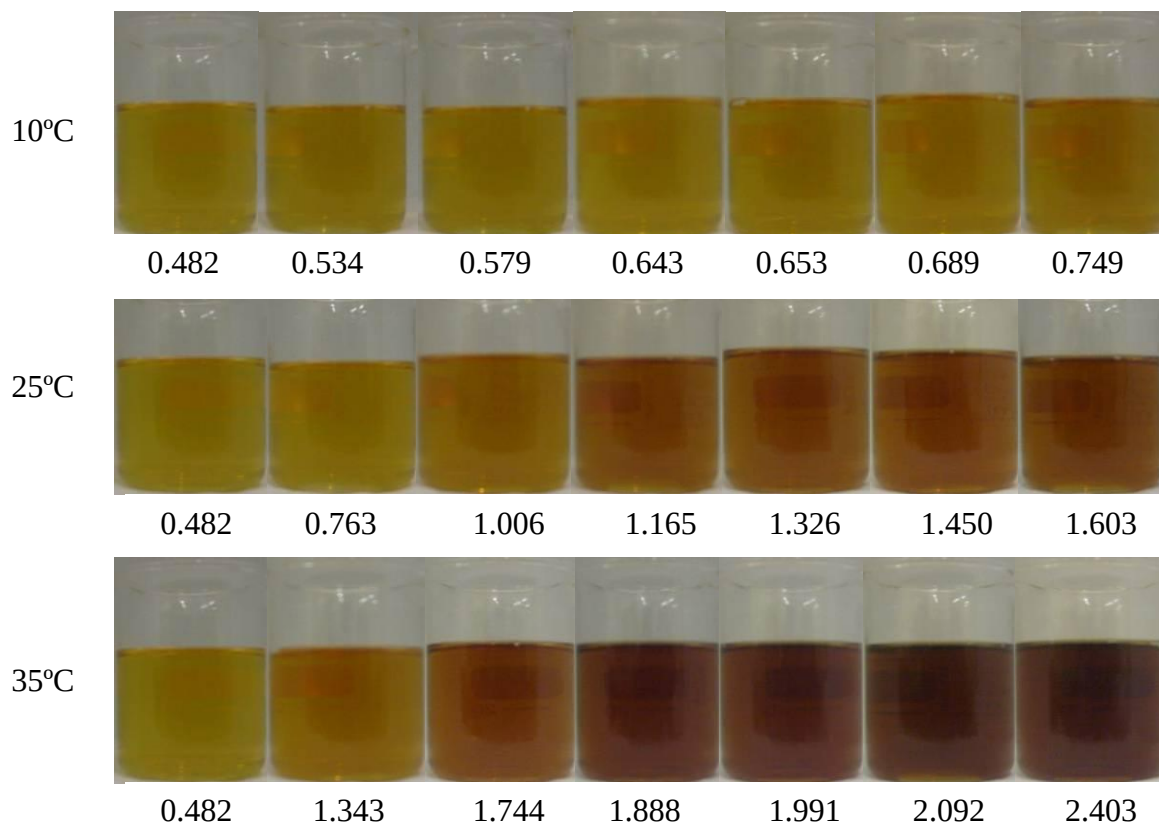


Figure 16. Visual colour change and the corresponding non-enzymatic browning index (Absorbance_{420nm}) of apple juice concentrate (diluted to 12 °Brix) after storage at different temperatures for 0, 2, 4, 6, 8, 10 and 12 weeks respectively

3.1.3.6 Relationship between the surface colour values (L*, a* and b*), browning index (A_{420nm}) and 5-hydroxymethylfurfural concentration

In order to assess the relationship between the Maillard reaction parameters, the correlation coefficients were determined (Table 4). The correlations between the parameters are highly significant ($p \leq 0.05$) at all the temperatures. However at the lower storage temperature of 10 °C the correlation between HMF and all the other parameters is not significant ($p > 0.05$).

Table 4. Correlation coefficients for lightness, redness, yellowness, A_{420nm} , HMF, TCD, chroma and hue in apple juice concentrate stored at 10 °C, 25 °C and 35 °C for 12 weeks

Temp (°C)	Parameters	Lightness	Redness	Yellowness	A_{420nm}	HMF	TCD	Chroma
10	Hue	0.96*	-0.93*	1.00*	-0.94*	0.37	-1.00*	-0.77*
	Chroma	-0.80*	0.95*	-0.75*	0.73*	-0.30	0.77*	
	TCD	-0.98*	0.92*	-1.00*	0.95*	-0.36		
	HMF	0.33	-0.34	0.37	-0.41			
	A_{420nm}	-0.92*	0.88*	-0.94*				
	Yellowness	0.96*	-0.91*					
	Redness	-0.92*						
25	Hue	0.93*	-0.92*	1.00*	-0.97*	-0.92*	-0.99*	-0.93*
	Chroma	-0.89*	0.98*	-0.94*	0.93*	0.88*	0.96*	
	TCD	-0.95*	0.93*	-1.00*	0.99*	0.95*		
	HMF	-0.99*	0.80*	-0.95*	0.98*			
	A_{420nm}	-0.99*	0.88*	-0.99*				
	Yellowness	0.95*	-0.92*					
	Redness	-0.82*						
35	Hue	0.95*	-0.96*	1.00*	-0.96*	-0.81*	-0.99*	-0.97*
	Chroma	-0.95*	0.99*	-0.98*	0.97*	0.82*	0.99*	
	TCD	-0.98*	0.97*	-1.00*	0.98*	0.85*		
	HMF	-0.93*	0.75*	-0.86*	0.93*			
	A_{420nm}	-1.00*	0.93*	-0.98*				
	Yellowness	0.97*	-0.96*					
	Redness	-0.91*						

*Correlations are significant at $p \leq 0.05$

TCD - Total colour difference

A_{420nm} - Absorbance (420nm)

HMF - Hydroxymethylfurfural

3.1.3.7 Kinetic parameters of Maillard browning from primary modelling

The zero order, first order and parabolic models were used to model the experimental data for L^* , a^* , b^* , $A_{420\text{nm}}$ and HMF (Table 3). No fitting kinetic model was found for a^* and HMF while for b^* and $A_{420\text{nm}}$ the zero order kinetic model provided the best fit. HMF formation at 25 °C and 35 °C followed a zero order equation while the change in a^* followed a zero order reaction at 10 °C and 25 °C with rate constants and regression coefficients shown in Table 5. The zero order, first order and parabolic kinetic models were all sufficient in describing the change in L^* values with time as shown by the regression coefficients which are similar (Table 5). For all the parameters the rate constant is increasing with temperature.

Table 5. Reaction rate constants and regression coefficients for lightness, redness, yellowness, absorbance (420nm) and 5-hydroxymethylfurfural in apple juice concentrate stored at 10 °C, 25 °C and 35 °C for 12 weeks.

Parameter	Model	Temp	Rate constant(k) $\pm$ SD week ⁻¹	Regression Coefficient (r)
L*	Zero	10	-0.0237 $\pm$ 0.011	0.95
		25	-0.4850 $\pm$ 0.001	0.99
		35	-0.7106 $\pm$ 0.007	0.85
	First	10	-0.0062 $\pm$ 0.000	0.95
		25	-0.0133 $\pm$ 0.000	0.99
		35	-0.0204 $\pm$ 0.000	0.88
	Parabolic	10	-0.0193 $\pm$ 0.001	0.95
		25	-0.0402 $\pm$ 0.000	0.99
		35	-0.0602 $\pm$ 0.001	0.86
a*	Zero	10	0.1303 $\pm$ 0.010	0.89
		25	0.4745 $\pm$ 0.011	0.69
		35	0.8380 $\pm$ 0.012	0.26
b*	Zero	10	-0.4314 $\pm$ 0.012	0.95
		25	-1.0516 $\pm$ 0.001	0.91
		35	-1.2658 $\pm$ 0.002	0.64
A ₄₂₀	Zero	10	0.0223 $\pm$ 0.000	0.99
		25	0.1007 $\pm$ 0.000	0.98
		35	0.1825 $\pm$ 0.002	0.83
HMF	Zero	10	-	-
		25	3.1206 $\pm$ 0.040	0.98
		35	16.3694 $\pm$ 0.123	0.99

SD - standard deviation

HMF - Hydroxyl methylfurfural

3.1.3.8 Influence of temperature on the reaction rate constant

Temperature quotient (Q_{10})

Temperature quotients (Q_{10}) are used to estimate the deteriorative change that would occur in a parameter over a longer period of time at lower storage temperatures (Ruiz *et al.*, 2012). Q_{10} values for L^* , b^* , $A_{420\text{nm}}$ and HMF calculated from the rate constants (Table 5) at 25 °C and 35 °C ranged from 1.20 to 5.25 (Table 6). The rate of HMF formation was more sensitive to an increase in temperature than L^* , b^* and $A_{420\text{nm}}$ as it increased 5.25 times at accelerated storage temperatures.

Table 6. Q_{10} and activation energy values for apple juice concentrate

Parameter	Q_{10}	E_a (kcal mol ⁻¹)
L^*	1.53	8.17
b^*	1.20	7.7
A_{420}	1.81	14.8
HMF	5.25	-

HMF - Hydroxymethylfurfural

E_a - Activation energy

Activation energy (E_a)

Arrhenius plots of $-\ln k$ versus $1/T$ were drawn for the parameters L^* , b^* and $A_{420\text{nm}}$ (Figures 17-19). Although the data points for the arrhenius plots did not fit exactly on the solid straight line for b^* and $A_{420\text{nm}}$ (Figures 18 and 19), their regression coefficients of 0.951 and 0.988 respectively are still quite high. L^* gave an almost exact fit with a regression coefficient of 0.9995. This means that the Arrhenius model can be used to explain the effect of storage temperature on these parameters. The activation energy (E_a) calculated from the slope of the Arrhenius plot was 7.70, 8.17 and 14.80 kcal/mol for b^* , L^* and $A_{420\text{nm}}$ respectively (Table 6). Arrhenius plots for a^* and HMF could not be drawn because of the low regression coefficients at 35 °C for a^* and at 10 °C for HMF.

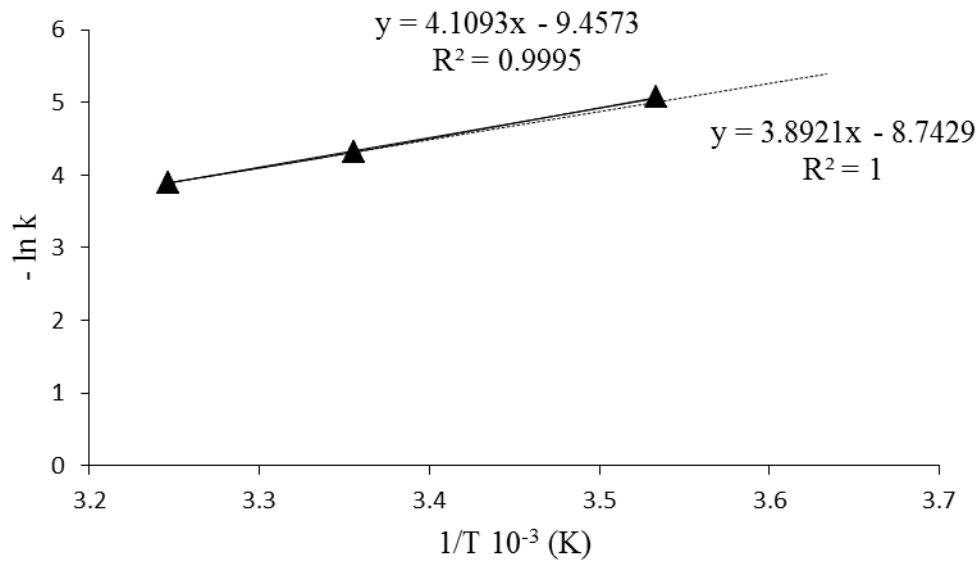


Figure 17. Arrhenius plot for the rate of change in lightness (L^*) during storage of apple juice concentrate for 12 weeks. The dashed line represents the projection of the two higher storage temperatures (25 °C and 35 °C) to the lower temperature 10°C.

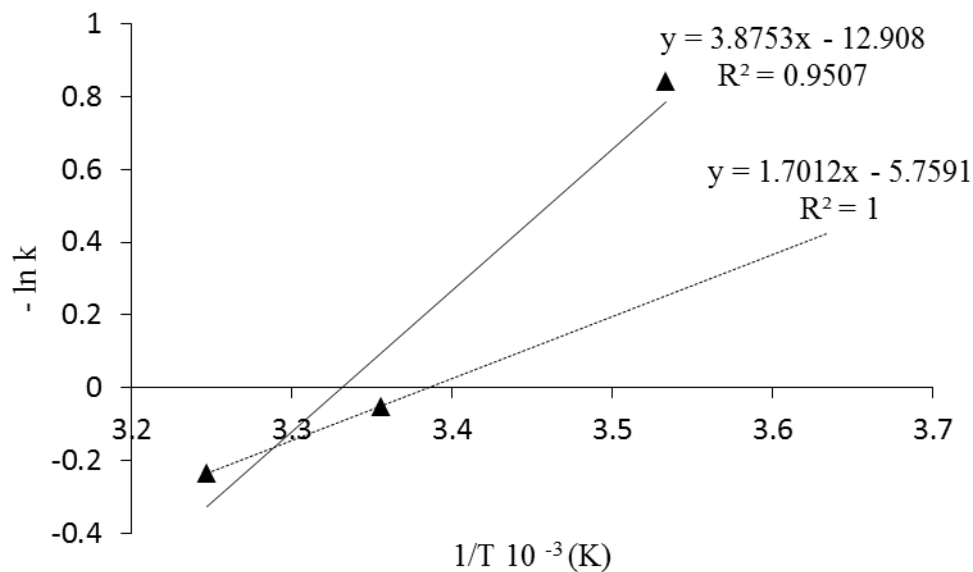


Figure 18. Arrhenius plot for the rate of change in yellowness (b^*) during storage of apple juice concentrate for 12 weeks. The dashed line represents the projection of the two higher storage temperatures (25 °C and 35 °C) to the lower temperature 10 °C.

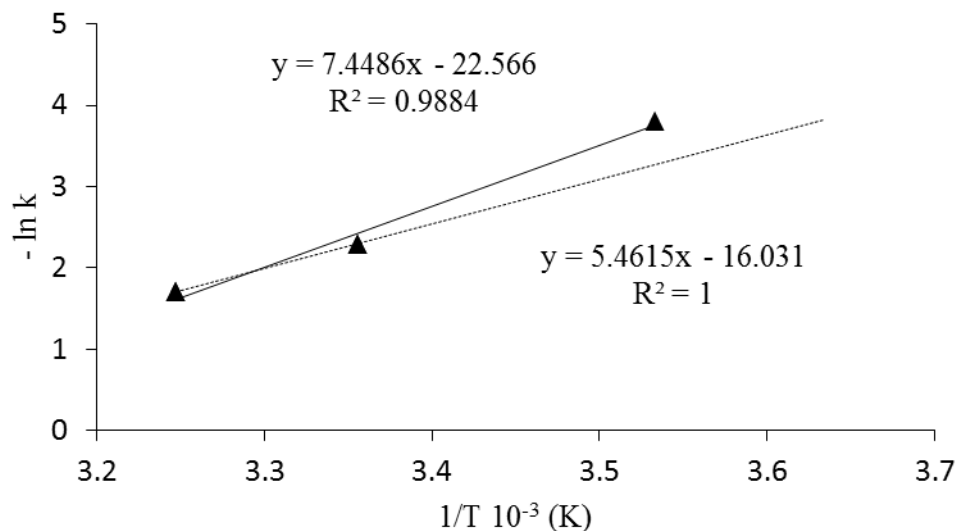


Figure 19. Arrhenius plot for the rate of brown pigment formation ($A_{420\text{nm}}$) during storage of apple juice concentrate for 12 weeks. The dashed line represents the projection of the two higher storage temperatures (25 °C and 35 °C) to the lower temperature 10 °C

3.1.3.9 Comparison of the actual and predicted reaction rate constant

The predicted rate constants for L^* , b^* and $A_{420\text{nm}}$ at 10 °C were determined by extrapolation from the dashed line of the Arrhenius plot passing through the accelerated temperatures (25 °C and 35 °C) whereas the actual rate constant was determined by extrapolation from the solid line passing through all the three temperatures of 10 °C, 25 °C and 35 °C (Figures 17-19).

Table 7. Predicted and actual rate of change for L^* , b^* and $A_{420\text{nm}}$

Parameter	Actual k (week ⁻¹) ^a	Predicted k (week ⁻¹) ^b	Predicted k/Actual k
L^*	-0.0063	-0.0067	1.06
b^*	-0.4558	-0.7771	1.70
$A_{420\text{nm}}$	0.0234	0.0381	1.63

^a k value determined from the solid line passing through all temperatures (10, 25 and 35 °C)

^b k value determined from the dashed line passing through the accelerated temperatures (25 and 35 °C)

The predicted rate constants from accelerated testing were 1.06, 1.70 and 1.63 times higher for L^* , b^* and $A_{420\text{nm}}$ respectively in comparison with the actual rate constants. The predicted

rate constant is almost similar to the actual value for the parameter L^* . For all the three parameters L^* , b^* and A_{420nm} , the dashed straight line passing through only the accelerated temperatures is below the solid straight line passing through all the three temperatures (Figures 17-19). This means that if used to predict the rate constant it will give a rate constant which is higher than the real rate constant.

3.1.4 Discussion

3.1.4.1 Colour parameters

The decrease in the L^* (lightness) of apple juice concentrate at all temperatures shows that the colour of the apple juice concentrate was changing from light brown and becoming darker as non-enzymatic browning progressed. Burdurlu and Karadeniz (2003) and Koca *et al.* (2003) also reported a decrease in L^* for apple juice and citrus (orange, lemon, grapefruit and tangerine) concentrates, respectively. The increase in a^* (redness) with an increase in time and temperature is due to the appearance of brown coloured melanoidins which increase the reddish tone of the apple juice concentrate. An increase in a^* with time and temperature has also been reported by Tosun (2004) for zile pekmezi (concentrated grape juice with a Brix content of 83.2 °Brix). The decrease in b^* values in apple juice concentrate is associated with a decrease in yellowness of apple juice concentrate as more brown pigments are formed with an increase in storage time and storage temperature. The b^* values also decreased as a function of time and temperature in a study on citrus concentrates (orange, lemon, grapefruit and tangerine) during 8 weeks of storage at 28, 37 and 45 °C. The decrease in L^* value correlates well with the increase in A_{420nm} and a^* and the decrease in b^* .

After 5 weeks of storage, a colour difference could be perceived in the apple juice concentrate at all the three temperatures. In a study by Lee and Nagy (1988) on orange juice concentrate, the total colour difference at 20 °C was non-perceptible as it changed by less than 0.5 of a unit while at 30 °C the total colour change was perceptible as it changed by more than 8 units after 21 days of storage. In comparison, in this study, TCD changed by about 6 units at 25 °C while at 35 °C the increase was about 11 units after 21 days. The higher increase in TCD in this study could be attributed to the higher storage temperature.

The decrease in hue value with an increase in temperature is due to the colour of apple juice concentrate becoming redder and less yellow. This decrease in hue is in accordance with

what is stated by Esteve and Frigola (2007) that the hue value depends on the relative amount of red and yellow colour and a decrease in hue is translated into a colour that is redder and less yellow.

The increase in chroma values of apple juice concentrate with storage time and temperature is a result of the brown colour becoming more vivid or intense as the concentration of the brown pigments increases. The higher the chroma value the more saturated or intense a colour is (Wrolstad *et al.*, 2005).

At time zero there was already an initial reading of 0.482 for $A_{420\text{nm}}$ which could be attributed to brown pigments being formed by both caramelization and Maillard reaction during processing of the apple juice concentrate (Lan, Liu, Xia, Jia, Mukunzi, Zhang, Xia, Tian and Xiao, 2010). Some of the brown colour may also have been generated before thermal treatment of apple juice through oxidation of phenolic compounds by polyphenol oxidases to o-quinones and a subsequent polymerisation to insoluble brown pigments of melanins (Komthong, Katoh, Igura and Shimoda, 2006). The increase in $A_{420\text{nm}}$ with storage time and temperature is because of the increase in the reactivity between the amino acids and reducing sugars. The observed increase in $A_{420\text{nm}}$ is in agreement with what was found for peach concentrate (Buedo *et al.*, 2001), orange juice concentrate (Roig, Bello, Rivera and Kennedy, 1999), grape juice concentrate (Buglione and Lozano, 2002) and pear concentrate (Beveridge and Harrison, 1984).

The colour of concentrated apple juice after reconstitution must be bright and transparent and of a light golden brown appearance (United States standards for Grades of frozen concentrated apple juice, 1975). Only the apple juice concentrate stored at 10 °C (Figure 16) conformed to this specification.

3.1.4.2 Hydroxymethylfurfural

The presence of the high initial HMF concentration (23.17 mg/kg) in the apple juice concentrate may be attributed to severe thermal treatments in the processing stages with both the Maillard reaction and caramelisation contributing to HMF formation. Tonelli, Errazu, Porras and Lozano (1995) reported simulated values for HMF concentration to be in the range 5-20 mg/kg for apple juice concentrate after evaporation and further stated that these

values were within the usual range obtained under industrial conditions. Koca *et al.* (2003) reported initial HMF values of 1.13, 0.37, 2.64 and 0.37 mg/kg for orange, lemon, grapefruit and tangerine concentrate respectively while Burdurlu and Karadeniz (2003) reported initial values of 0.17-0.66 mg/kg for apple juice concentrate.

In this study the HMF levels at 10 °C fluctuated around the initial level. This could be due to the slow rate of formation of HMF at low temperatures and the rate of formation of HMF being similar to the rate at which it is being converted to melanoidins. Similar results were obtained by Burdurlu and Karadeniz (2003), who found that the concentration of 5-HMF did not change significantly ($p \leq 0.05$) in all the apple juice concentrates stored at 5 °C during a 4 month storage period except for the 70 °Brix golden delicious apple juice concentrate sample. Sharma, Kaushal and Sharma (2004) in their study on lemon concentrate stored at 3 – 7 °C found a 4.86 fold increase to 0.55 mg/kg in HMF content.

The observed increase in HMF concentration with temperature is due to the non-enzymatic browning reaction proceeding faster at higher storage temperatures. Wang *et al.* (2005) in their study on carrot concentrate also found that temperatures of 25 °C and 37 °C had a significant effect ($p \leq 0.05$) on HMF formation while a lower storage temperature of 0 °C had less effect. Burdurlu, Koca and Karadeniz (2006) in their study on citrus concentrates reported the HMF content after 8 weeks of storage at 28 °C to range between 3.01 to 28.32 mg/kg while at 37 °C the HMF values ranged between 521.52 and 1141.99 mg/kg which was much higher than what was found in this study (160.32 mg/kg after 8 weeks of storage at 35 °C). This could be attributed to the slightly higher storage temperature of 37 °C in comparison to 35 °C. In citrus concentrates HMF is not only formed through Maillard reaction but also from ascorbic acid degradation.

3.1.4.3 Relationship between the surface colour values (L^* , a^* and b^*), browning index (A_{420nm}) and 5-hydroxymethylfurfural concentration

The high correlation coefficients between the parameters indicate the suitability of all the Maillard browning parameters as quality indicators during storage. It also means that one parameter can be measured and used to predict the value of another Maillard browning parameter. Since HMF is an intermediate of oligomeric and polymeric browning compounds (melanoidins), its correlation with browning index (A_{420nm}) and surface colour values is

important (Sadilova *et al.*, 2009). The poor correlation between HMF and the other parameters at 10 °C means that HMF has a minor influence on how the other parameters change at the low storage temperature. HMF concentration may not be measured and used alone to reliably predict the changes in the colour of apple juice concentrate at low storage temperatures of 10 °C. Burdurlu and Karadeniz (2003) also found no significant correlation ($p \leq 0.05$) between $A_{420\text{nm}}$ – HMF and L^* – HMF in apple juice concentrate stored at 5 °C for 4 months. At 25 °C and 35 °C any one of the parameters may be used as an index of quality deterioration for routine quality control purposes during storage of apple juice concentrate.

3.1.4.4 Kinetic parameters of non-enzymatic browning during storage of apple juice concentrate

Kinetic models are used to describe the changes in the quality of food with time and the influence of temperature on the rate of change (Sousa-Gallagher, Mahajan and Yan, 2011; Van Boekel, 2008). Zero order kinetics signifies a linear change in a parameter with time and an exponential change with time for first order kinetics (Garza *et al.*, 1999). Non-enzymatic browning formation has been reported as following zero order kinetics by Bozkurt *et al.* (1999) for grape juice concentrate, Burdurlu and Karadeniz (2003) for apple juice concentrate, Koca *et al.* (2003) for citrus concentrates and Beveridge and Harrison (1984) for pear juice concentrates, first order kinetics by Shan-guang and Nong-xue (2010) for apple juice concentrate and parabolic kinetics by Buedo *et al.* (2001) for peach concentrate. The zero order model provided the best fit for HMF formation at 25 °C and 37 °C similar to what Burdurlu and Karadeniz (2003) found for citrus concentrates. Wang *et al.* (2006) however in their study on carrot concentrate found HMF formation and non-enzymatic browning index to follow a first order reaction well at higher storage temperatures of 25 °C and 37 °C while at -18 °C and 0 °C these parameters did not conform to first order. No fitting kinetic model was found for the change in L^* in apple juice concentrate (Burdurlu and Karadeniz, 2003) while first order kinetics were used in citrus concentrates (Koca *et al.*, 2003). First order kinetics were used to evaluate b^* values in citrus concentrates (Koca *et al.*, 2003). Absorbance has been modelled using the parabolic model (Buedo *et al.*, 2001) and zero order kinetics (Burdurlu and Karadeniz, 2003).

Researchers have stated that the behaviour of some parameters changes with temperature from linear functions at lower temperatures to higher polynomial functions at higher

temperatures (Manso *et al.*, 2001). The results from this study are in agreement with this as seen in Figure 13. The change in $A_{420\text{nm}}$ is linear at 10 °C and 25 °C while at 35 °C it becomes sigmoidal. This is because at the higher storage temperature of 35 °C the rate of Maillard browning is accelerated and $A_{420\text{nm}}$ changes at a higher rate. With time however, the reaction slows down as the reactants get depleted giving the curve (Figure 13) a sigmoidal shape. The increase in the rate constant with temperatures for all parameters confirms that increasing the storage temperatures favours non-enzymatic browning (Garza *et al.*, 1999).

The higher Q_{10} value for HMF (5.25) compared to $A_{420\text{nm}}$ (1.81) indicates that formation of HMF which is an intermediate product in the Maillard reaction is more sensitive to an increase in storage temperature in comparison to the evolution of water soluble brown pigments (as measured by $A_{420\text{nm}}$) which are end products of Maillard reaction. Benzing-Purdie, Ripmeester and Ratcliffe (1985) and Labuza (1994) state that the Q_{10} for brown pigment formation has been determined to be within 2 and 8. According to Bostan and Boyacioglu (1997), in foods containing fructose, the increase in the rate of browning may be 5 to 10 times higher for each 10 °C rise in temperature. The Q_{10} values for HMF formation and $A_{420\text{nm}}$ are within this range but below 2 for L^* , a^* and b^* . This may be attributed to the fact that foods are complex systems and variance of Q_{10} is expected as it depends on the matrix of the food system.

The typical activation energy range observed for non-enzymatic browning reactions is between 105 and 210 kJ/mol (25.10 - 50.19 kcal/mol) (Bostan and Boyacioglu, 1997). Activation energies in this study were lower and ranged from 7.70 - 14.80 kcal/mol. Lower activation energy indicates a lower sensitivity of the reaction rate to temperature changes (Mann, 2009). According to Cohen *et al.* (1998), the differences in the activation energies are expected and could be attributed to differences in the food systems and pertinent process conditions.

3.1.4.5 Comparison of the actual and predicted reaction rate constant

Since the predicted rate constants from accelerated testing (25 °C and 35 °C) are comparable to the actual rate constants obtained from storage at 10 °C, this means that accelerated testing can be used reliably to predict the change in L^* , b^* and $A_{420\text{nm}}$. Since accelerated testing

projects the reaction rate constants to be higher than what it would be if storage tests are done under normal testing conditions, this gives a built in safety factor for the processor.

3.1.5 Conclusions

Redness, absorbance, chroma and total colour difference increased while lightness, yellowness and hue decreased during storage for 12 weeks at all the temperatures. These parameters can be used to monitor Maillard browning at 10 °C, 25 °C and 35 °C. HMF formation can only be used as an indicator of Maillard browning at accelerated storage temperatures of 25 °C and 35 °C and not at 10 °C.

The Q_{10} factor can however be used to calculate the kinetic constant for parameters such as HMF formation at a lower storage temperature. The value of the kinetic constant can then be substituted into the equation of the primary model (zero or first order) that provides the best fit for the experimental data. The predicted change in the parameter with time at that lower storage temperature can then be plotted with time. The shelf life will be the time it takes for the parameter to reach the cut off limit.

Since the predicted rate constants from accelerated testing (25 °C and 35 °C) are comparable to the actual rate constants obtained from storage at 10 °C, this means that accelerated testing can be used reliably to predict the change in L^* , b^* and A_{420nm} at lower storage temperatures and thus the quality of stored apple juice concentrate.

3.2 EFFECT OF STORAGE TEMPERATURE ON THE MICROBIOLOGICAL QUALITY OF APPLE JUICE CONCENTRATE AND CHARACTERISATION OF THE SPOILAGE MICROBES

ABSTRACT

The aim of this study was to determine the microbiological changes in apple juice concentrate stored at refrigeration temperature (10 °C) and accelerated storage temperatures (25 °C and 35 °C) and to isolate and identify the spoilage microbes. The Baranyi and Roberts equation was used for primary modelling and the temperature dependence was described using the square root model, Arrhenius model and temperature coefficient (Q_{10}). Yeast identification was done using a physiological method (Biolog system) and a molecular method ((GTG)₅ PCR fingerprinting). Coliforms, lactic acid bacteria, heat resistant moulds and *Alicyclobacillus* species were not detected at all storage temperatures. Yeasts were detected in apple juice concentrate at 25 °C and 35 °C after 5 weeks of storage. The lag phase was 4.67 ± 0.81 and 4.42 ± 2.64 weeks while the maximum specific growth rate was 0.40 ± 0.03 and 1.78 ± 1.63 log (cfu/g)/week at 25 °C and 35 °C, respectively. Although there was no significant difference ($p > 0.05$) in the lag phase at 25 °C and 35 °C, the maximum specific growth rate of yeast increased significantly ($p \leq 0.05$) with an increase in storage temperature. A 10 °C increase in storage temperature increased the maximum specific growth rate 4.47 times (Q_{10}). The yeasts isolated from apple juice concentrate were identified as *Kluyveromyces delphensis*, *Saccharomyces dairensis*, *Zygosaccharomyces bailii*, *Rhodotorula glutinis* and *Metchnikowia reukaufii*. PCR fingerprinting was able to distinguish the basidiomycetous yeast *R. glutinis* from the ascomycetous yeasts: *K. delphensis*, *S. dairensis*, *Z. bailii*, and *M. reukaufii*. Strain variability was observed for *K. delphensis* and *S. dairensis*. The presence of these yeasts indicates that the heat treatment may not have been effective or there was post process contamination and if storage temperature is not low their growth will subsequently result in spoilage of the apple juice concentrate. Good manufacturing practices are essential to prevent pre and post process contamination.

3.2.1 Introduction

Fruit juice concentrates are of great economic significance as much of the world trade in fruit juices involves concentrates (Stratford, Hofman and Cole, 2000). South Africa exports apple juice concentrate to a great number of international markets such as Japan, Canada and United States (USDA Foreign Agricultural Service, 2013). During storage of apple juice concentrate deterioration in quality may occur resulting in its rejection by potential buyers and consumers (Brugnoni, Pezzutti and Gonzalez, 2013; Beuchat and Pitt, 2000; Steels, James, Roberts and Stratford, 1999). Considering the large scale at which apple juice concentrates are made, the consequence of spoilage is a severe economic loss to the company (Loureiro, 2000) and as such it is desirable that microbiological deterioration is reduced to a minimum during storage (Brugnoni *et al.*, 2013).

In industrial practice, fruit concentrates are stored in a refrigerated or frozen state as an effective means of controlling yeast spoilage (Deak and Beuchat, 1993). Stratford, Hofman, and Cole (2000) also state that fruit concentrates are microbiologically stable if sufficiently concentrated and chilled. However when refrigerated storage space is not adequate, room temperature storage is also utilised (Rhonde, 2011). According to Silva, Gibbs, Vieira and Silva (1999), a pasteurisation treatment at 85 to 95 °C for 45 seconds should be adequate for the microbial stabilisation of fruit concentrates at room temperature. Toribio and Lozano (1984) also state that concentrates containing more than 65% total solids are normally stable against fermentation.

Although fruit concentrates are regarded as microbiologically stable (Groenewald, 2009), cases of spoilage have been documented. The presence of off odours, off flavours, discolouration, visible growth of mycelium and fermentation due to yeast and mould growth diminishes the quality of apple juice concentrates (Stratford, 2006). Apple juice concentrate are characterised by low pH, low water activity, high sugar concentration, high viscosity, reduced aeration capacity and reduced dissolved oxygen (Groenewald, 2009). Heat treatment during processing is also considered sufficient to destroy most non spore forming microorganisms.

It is known that storage temperature and time affect the microbiological stability of apple concentrates (Brugnoni *et al.*, 2013; Tournas *et al.*, 2006). Numerous studies have been done

to identify spoilage yeasts isolated from fruit concentrate but no study has been done to show the influence of time and temperature of storage on the growth of yeasts in apple juice concentrates.

The objectives of this study were to enumerate the microorganisms present in apple juice concentrate during storage and use predictive modelling to evaluate the influence of different storage temperatures (10, 25 and 35 °C) on their growth. The identity of yeasts isolated from apple juice concentrate was confirmed using physiological tests and molecular analysis of the identified yeasts used to understand the interrelationship among the yeast species.

3.2.2 Materials and methods

3.2.2.1 Sampling and sample preparation

The sampling and analysis intervals for the different microbiological tests were:

Coliforms and Lactic acid bacteria	} Week of storage: 0, 1, 2
Aerobic plate counts, heat resistant moulds and <i>Alicyclobacillus acidoterrestris</i>	} Week of storage: 0, 2, 4, 6, 8, 10, 12
Yeast and moulds	} Week of storage: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12

Ten grams of apple juice concentrate was weighed and diluted in 90ml of 0.1% buffered peptone water (Oxoid). A Stomacher Lab blender 400 (Seward Laboratory, London, UK) was used to homogenise the sample for 4 minutes. Serial dilutions up to 10^{-6} were prepared using the same diluent and all plating was done in duplicate.

3.2.2.2 Coliforms

Dilutions were spread plating on Violet Red Bile Agar (Oxoid CM 107) and plates incubated at 37 °C for 24 hours (Mahale, Khade and Vaidya, 2008).

3.2.2.3 Lactic acid bacteria

The pour plate method and de Man Rogosa and Sharpe (MRS) agar (Merck 1.10660) were used. Plates were inverted before incubation anaerobically at 30 °C for 48-72 hours (De Man, Rogosa and Sharp, 1960).

3.2.2.4 Aerobic plate counts

Pour plating of the dilutions was done and Plate Count Agar (Oxoid 0325) used. Plates were incubated at 35 °C for 24 hours (Suarez-Jacobo, Gervilla, Guamis, Roig-Sagues and Saldo, 2010). Manufacturer's recommendations for time/temperature were followed.

3.2.2.5 Heat resistant moulds

Apple juice concentrate (20 g) was diluted with 20 ml of 0.1 % buffered peptone water and homogenised. Four 10ml dilutions were measured into 4 test tubes. A thermometer was put in one of the tubes and all tubes heated in a water bath at 70 °C for 1 hour. After heating, the tubes were cooled to room temperature and the contents mixed with an equal volume of molten double strength Potato Dextrose Agar in petri dishes and allowed to solidify. The plates were incubated at 25 °C for 3-5 days (Kotzekidou, 1997).

3.2.2.6 *Alicyclobacillus acidoterrestris*

A heat shock treatment to promote endospore germination and eliminate vegetative cells was done by subjecting the serial dilutions to a temperature of 80 °C for 10 minutes. Growth was examined by spread plating the serial dilutions onto acidified Potato Dextrose Agar (pH 3.70) and incubating at 45 °C for 5 days (Witthuhn, Duvenage and Gouws, 2007; Groenewald, Gouws and Witthuhn, 2009).

3.2.2.7 Yeasts and moulds

Serial dilutions for enumeration of yeasts and moulds were performed in 0.1 % buffered peptone water containing 10 % glucose to avoid osmotic shock when culturing low water activity foods such as fruit juice concentrates (Andrews, de Graaf and Stamation, 1997). Potato Dextrose Agar acidified to pH 3.5 with sterile 10% tartaric acid (2ml per 100ml) and containing 10% glucose was used. Spread plates were incubated at 25 °C for 3-5 days.

3.2.2.8 Isolation and purification of yeasts

After enumeration of yeasts and moulds, 10 yeast colonies with different morphologies were selected and purified by repeated streaking on acidified Potato Dextrose Agar containing 10 % glucose. Isolates were from apple juice concentrate stored at both 25 °C and 35 °C over the 12 week storage period. Pure yeast colonies were stored frozen on Cryobank™ beads (Copan Diagnostics, Inc, USA).

3.2.2.9 Identification of yeast using the Biolog system

Yeasts were identified based on their physiological attributes using the BIOLOG® microbial identification system (Biolog Inc., Hayward, California) (Mankowski and Morrell, 2004).

3.2.2.10 Extraction and purification of yeast DNA

The Purelink™ Genomic DNA Purification kit (Invitrogen by Life technologies) was used for the extraction and purification of yeast DNA. The yield and quality of DNA after purification was analysed using the Nano drop spectrometer (Genova Plus, Bibby Scientific LTD, UK).

3.2.2.11 Microsatellite PCR fingerprinting

For the PCR reaction 2 µl of the DNA sample (25 ng/µl) was mixed with 24 µl of the Platinum® PCR super mix (Invitrogen by Life technologies), 4 % Dimethyl Sulfoxide (DMSO) and 1.00 µl of (GTG)₅ primer (0.6 µg/µl) (Integrated DNA Technologies, Inc). The composition of the super mix was 22 U/ml complexed recombinant Taq DNA polymerase with Platinum Taq antibody, 22 mM Tris HCl pH 8.4, 55 mM KCl, 1.65 mM MgCl₂, 220 uM dGTP, 220 uM dATP, 220 uM dTTP , 220 uM dCTP and stabilizers. The PCR programme was: 94 °C for 5 min followed by 40 cycles at 94 °C for 15 sec, 55 °C for 45 sec and 92 °C for 90 sec. The final heating was 72 °C for 4 min and the mixture subsequently cooled to 4 °C (Couto, Eijsima, Hofstra, Huis in't Veld and Van der Vossen, 1996). The PCR amplified DNA was separated on 1.50 % agarose gel (containing the Invitrogen SYBR® Safe Gel stain) by electrophoresis and visualised on a UVITEC Cambridge Imager under UV light. The Invitrogen 100 base pair molecular marker was used to estimate the length of the amplicons.

3.2.2.12 Statistical analysis

Analysis of variance (ANOVA) was used to determine the effects of time, temperature and time-temperature interaction on the growth of yeast and moulds in apple juice concentrate during storage. STATISTICA software for windows version 10 (Statsoft Inc, Tulsa, Oklahoma, USA, 2011) was used to carry out ANOVA. The level of significance was $\alpha=0.05$ and $n=3$.

3.2.2.13 Microbial growth modelling

To describe the growth of yeasts and mould with time, the counts were fitted into the Baranyi and Roberts's primary model (Equations 10 - 12) using the ComBase DMFit Web Edition statistical software package. The parameters $\mu_{\max}$ and λ were obtained.

$$N(t) = N(0) + \mu_{\max} A(t) - \ln \left(1 + \frac{e^{\mu_{\max} A(t)} - 1}{e^{(N_{\max} - N(0))}} \right) \quad (10)$$

$$\text{with } A(t) = t + \frac{1}{\mu_{\max}} \ln \left(\frac{e^{(-\mu_{\max} t)} + q(0)}{1 + q(0)} \right) \quad (11)$$

$$\text{and } \lambda = \ln \left(\frac{1 + \frac{1}{q(0)}}{\mu_{\max}} \right) \quad (12)$$

where $\mu_{\max}$ is the maximum specific growth rate (weeks^{-1}), λ the lag phase (weeks), $N(0)$ the initial microbial population ($\log_{10}$ cfu/g), $N_{\max}$ the maximum population density ($\log_{10}$ cfu/g) and $q(0)$ the concentration of substance critical to the microbial growth and is related to the physiological state of the cells (Patil *et al.*, 2011).

The square root model (Equation 13) was used to model the maximum specific growth rate as a function of storage temperature.

$$\mu_{\max} = b (T - T_{\min})^2 \quad (13)$$

where b is the slope of the line of $\sqrt{k}$ versus temperature, T is the storage temperature ($^{\circ}\text{C}$) and $T_{\min}$ is the microbial growth temperature where the line cuts the temperature axis at $\sqrt{k} = 0$. $T_{\min}$ is considered the theoretical minimum temperature for the growth of the microorganism (Shimoni and Labuza, 2000).

The Arrhenius model was used to describe temperature dependence of the growth rate of yeasts.

$$\text{Arrhenius model} \quad k = k_0 \exp \left(\frac{E_a}{RT} \right) \quad (14)$$

where k is the microbial growth rate constant, k_0 is the pre-exponential constant, R is the gas constant in kcal/mol in °K (1.986 cal/molK or 8.314 J/molK) and T is the absolute temperature in K (273 + °C) (Shimoni and Labuza, 2000).

Another way to evaluate the effect of temperature on microbial growth is with the Q_{10} value.

$$Q_{10} = (\mu_2 / \mu_1)^{10 / (\theta_2 - \theta_1)} \quad (9)$$

Where μ_1 and μ_2 are growth rates at two temperatures θ_1 and θ_2 respectively (Ortiz-Muniz, Carvajal-Zarrabal, Terrestiana-Sanchez and Aguilar-Uscanga, 2010 and Montagnes, Kimmance and Atkinson, 2003).

3.2.2.14 Cluster analysis of fingerprinting data

The banding patterns were analysed using the Gel Compar II software version 6.5 (Applied Maths). The similarity matrix among the amplification pattern was prepared using Jaccard's coefficient and subjected to pairwise comparison by the Unweighted Pair Group Method with Arithmetical Average (Silva-Filho, Santos, Resende, Morais, Morais and Simoes, 2005).

3.2.3 Results

3.2.3.1 Microbial growth

Alicyclobacillus acidoterrestris, heat resistant moulds, coliforms, lactic acid bacteria and aerobic plate counts were not detected during storage. For the yeast and mould counts, the frequency of isolation of moulds was very low in comparison to yeasts and when the moulds were isolated it was only one or two colonies. The growth response of yeasts in the replicates was different with growth being detected at different times (Figures 20A and 20B) for the same storage temperature and in some replicates growth was not detected during the twelve weeks of storage (results not shown).

Yeast and moulds were not detected in apple juice concentrate stored at 10 °C throughout the 12 weeks of storage while at 25 °C and 35 °C they were detected from week 5 until the end of storage (Figure 20C). For apple juice concentrate stored at 25 °C and 35 °C, the yeast and mould counts increased significantly ($p \leq 0.05$) with time. The time-temperature interaction did not significantly ($p > 0.05$) affect the growth of yeasts and moulds.

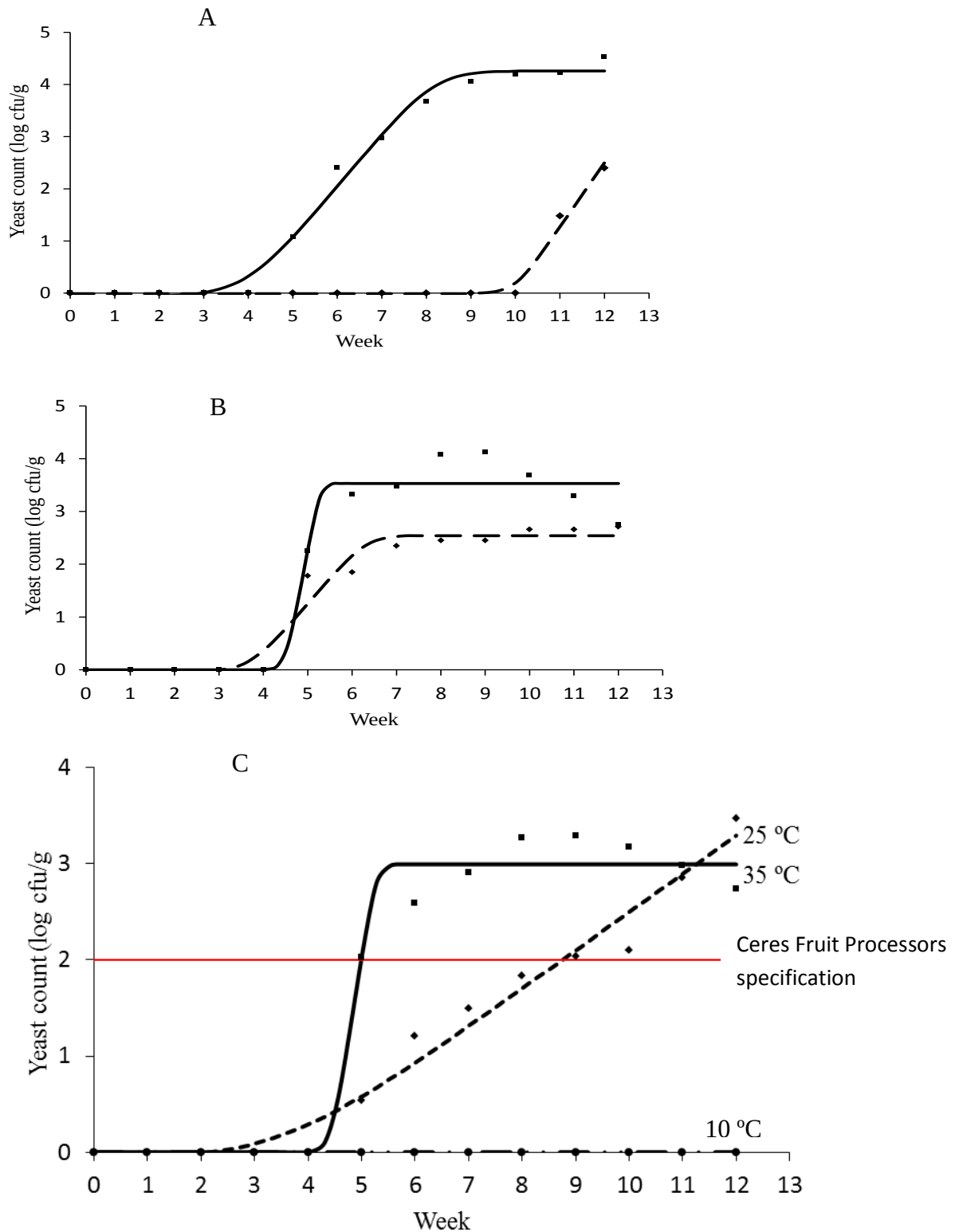


Figure 20. Growth data and Baranyi and Roberts model fitted curves of yeast growth in apple juice concentrate for (A) individual replicates stored at 25 °C (B) individual replicates stored at 35 °C and (C) mean data at 10 °C, 25 °C and 35 °C. In Figures A and B the different lines/symbols indicate different replicates and in Figure C different temperatures.

Although the yeast and mould counts in apple juice concentrate stored at 35 °C were greater than the counts at 25 °C until week 10, the differences were not significant ($p \geq 0.05$). At week 10 the counts at 35 °C had declined while they continued to increase in apple juice concentrate stored at 25 °C (Figure 20C). The maximum yeast count at 35 °C was reached at week 8 while the same count was reached after 10 weeks in apple juice concentrate stored at 25 °C. The difference between the yeast and mould counts at week 5 ($0.54 \log_{10}\text{cfu/g}$) and at week 12 ($3.47 \log_{10}\text{cfu/g}$) were significantly different ($p \leq 0.05$) in apple juice concentrate stored at 25 °C. Andrews (1992) states that a total yeast population of less than 10 cells per ml is generally considered as an appropriate limit to evaluate the quality of unfrozen grape juice concentrate. Ceres Fruit Processors has set the limit for yeasts and mould counts at less than 100 cfu/g of apple juice concentrate. After 12 weeks of storage at 10 °C the counts were still below the specification of 2 $\log_{10}$ set by Ceres Fruit Processors but this limit was surpassed at week 5 for concentrate stored at 35 °C and at week 6 and 9 for the two replicates stored at 25 °C.

3.2.3.2 Modelling of yeast growth in apple juice concentrate during storage

Modelling was based on two replicates where growth was observed at 25 °C and 35 °C. A typical sigmoidal curve with three distinct phases (lag, exponential and stationary phase) was obtained for the growth of yeasts and moulds in apple juice concentrate stored at 35 °C. However, at 25 °C the growth curve consisted of only two phases (lag and exponential phase). The high regression coefficient values of 0.94 and 0.98 for the predicted growth curves at 25 °C and 35 °C, respectively, show that the Baranyi and Roberts model fits the experimental data well. The maximum specific growth rate of yeast increased significantly ($p \leq 0.05$) with an increase in temperature but there was no significant difference ($p > 0.05$) in the lag phase (λ) at 25 °C and 35 °C (Table 8).

3.2.3.3 Effect of temperature on microbial growth

Temperature had a marked effect on specific growth rate. As the temperature increased, the specific growth rate also increased (Figure 21). $T_{\min}$ is a useful parameter as it helps to predict the temperature at which yeast would be expected to grow in the apple juice concentrate. $T_{\min}$ for yeasts in apple juice concentrate is about 20 °C. The Arrhenius plot enables one to calculate the activation energy needed by the yeast cells for growth to start (Serra, Strehaiana and Taillandier, 2005). The activation energy calculated from the slope of the Arrhenius plot (Figure 22) was 39.95 kcal/mol. Ortiz-Muniz *et al.* (2010) in their study on kinetics of

fermentation calculated the activation energy to be 15.6 kcal/mol at temperatures between 27 and 39 °C.

Table 8. Kinetic parameter estimates for yeast and mould growth in apple juice concentrate stored at 10 °C, 25 °C and 35 °C

Temperature (°C)	Lag phase (λ) (weeks)	Maximum growth rate ($\mu_{\max}$) log (cfu/g)/week	R ²	SE ^b of fit
10	-	-	-	-
25	4.67 (0.8090) ^a	0.3973 (0.0310)	0.9712	0.2050
35	4.42 (2.6404)	1.7764 (1.6338)	0.9782	0.1099

^a Values in parentheses are the standard errors of the parameters

^b SE (Standard error)

- Not determined as there was no growth of yeast

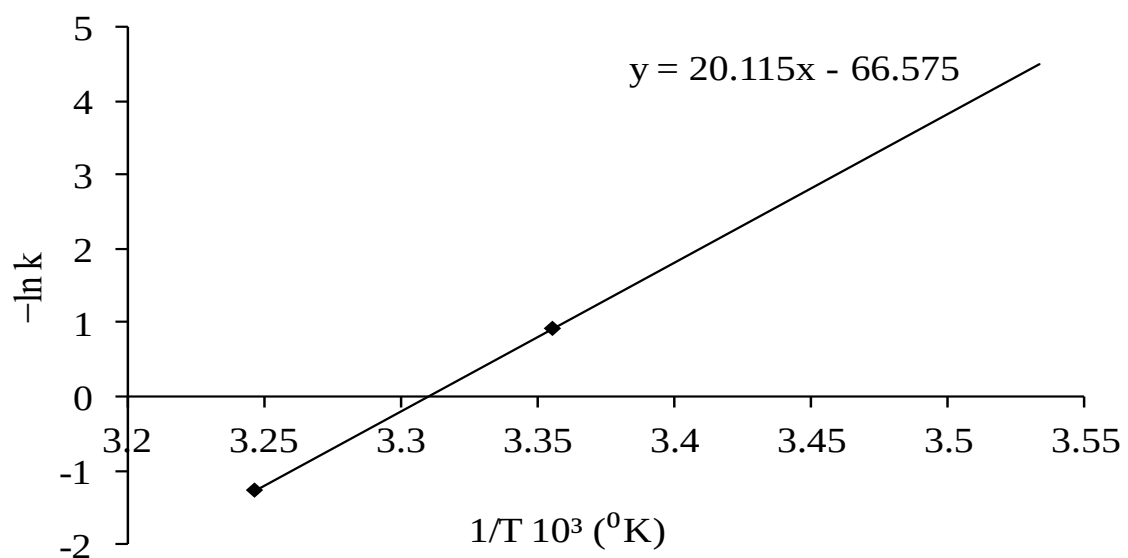


Figure 21. Arrhenius plot for the change in specific growth rate of yeast during storage of apple juice concentrate for 12 weeks at 25 °C and 35 °C

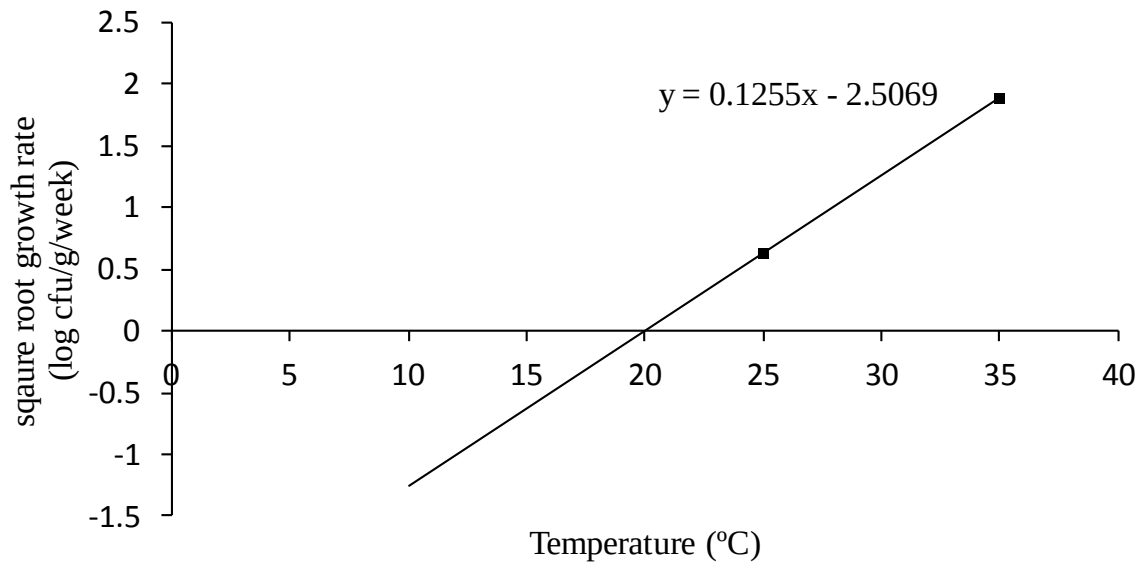


Figure 22. Square roots of specific growth rate data as a function of temperature. Ratkowsky equation is $\sqrt{k} = 0.1255 (T ^\circ\text{C} - 19.97 ^\circ\text{C})$

The Q_{10} calculated from the maximum specific growth rate of yeast at was 4.47. According to Drotz (2012), the Q_{10} value for microbial growth rate among other reactions is usually around 2.

3.2.3.4 Visual assessment of microbial spoilage in apple juice concentrate

There were no visual signs of spoilage in apple juice concentrate stored at 10 °C (Figure 23).



Figure 23. Apple juice concentrate with no visible sign of spoilage at 10 °C



Figure 24. Apple juice concentrate showing visible signs of spoilage at 35 °C

Yeasts were detected after 5 weeks of storage in apple juice concentrate stored at 25 °C and 35 °C. Spoilage was however observed visually after 7 and 6 weeks of storage at 25 °C and 35 °C, respectively. There was gas production in the bottles as evidenced by bubbles on the surface of apple juice concentrate (Figure 24). An alcoholic off odour was also detected when the bottles were uncapped for sampling. The results from the counts also indicate that yeast cells have to be more than 1000 cfu/g for spoilage to become evident in apple juice concentrate.

3.2.3.5 Yeast identification and microsatellite PCR fingerprinting

Of the ten isolates isolated from apple juice concentrate, 3 were identified as *Kluyveromyces delphensis*, 3 as *Saccharomyces dairensis*, 2 as *Zygosaccharomyces bailii*, 1 as *Rhodotorula glutinis* and 1 as *Metchnikowia reukaufii*. *S. dairensis*, *K. delphensis* and *Z. bailii* were isolated from concentrate stored at 25°C and 35 °C whereas *R. glutinis* and *M. reukaufii* were isolated only in apple juice concentrate stored at 25°C. The presence of *R. glutinis* and *M. reukaufii* has been described by Davenport (1996) as indicators of poor hygiene. Microsatellite PCR fingerprinting was used to characterise the yeast population found in apple juice concentrate as it enables differentiation of yeast at species and strain level (Silva-Filho *et al.*, 2005). The PCR fingerprint patterns for the ten isolates were grouped into 4 clusters (Figure 25).

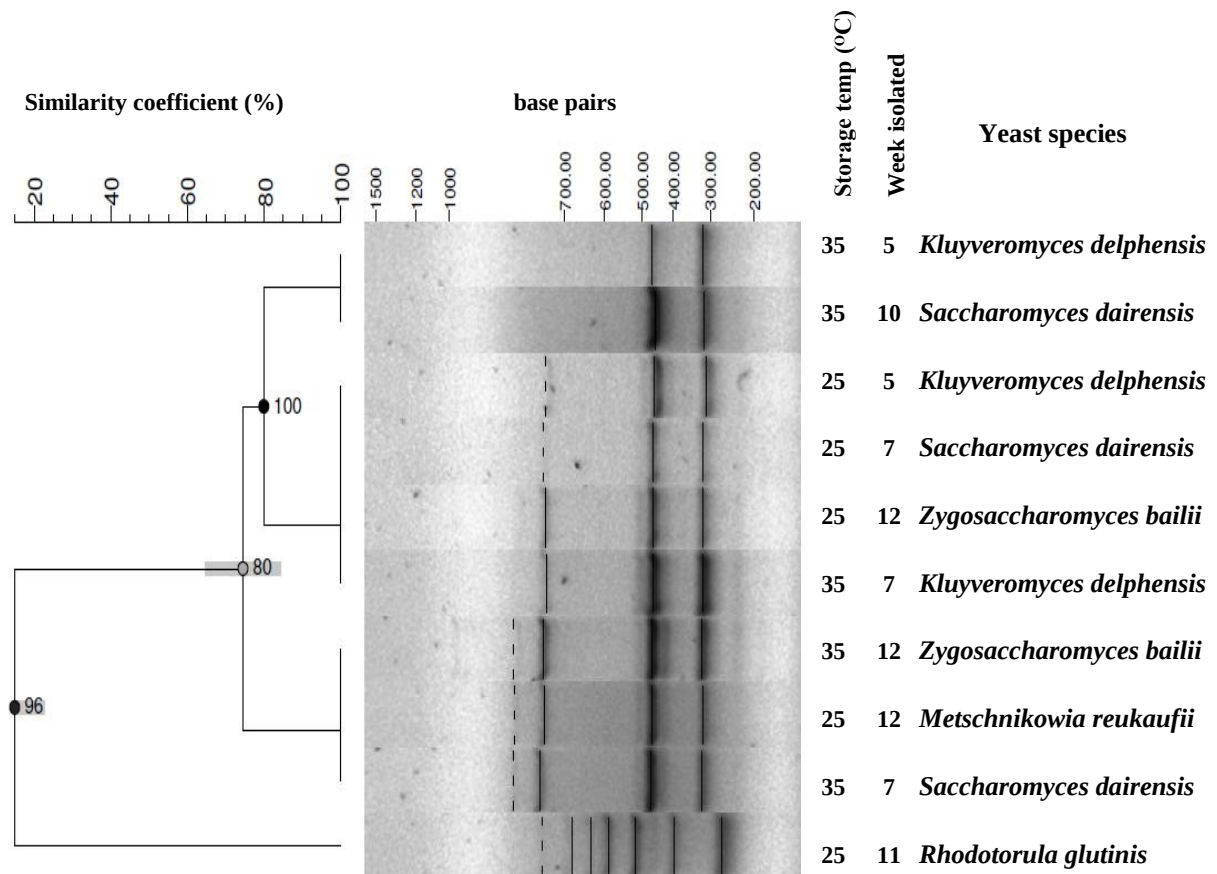


Figure 25. PCR fingerprints using (GTG)₅ microsatellite primer of yeasts isolated from apple juice concentrate and cluster analysis showing the similarity values among the 10 isolates

The similarity coefficient for each cluster was 100 % and the banding pattern in each of the clusters was the same even though the yeast species in these clusters were different. *R. glutinis* gave a unique banding pattern which significantly separated it from the other yeast species as shown by the similarity coefficient of less than 20 % (Figure 25). The nine isolates belonging to the species *K. delphensis*, *S. dairensis*, *Z. bailii*, and *M. reukaufii* showed a 75 % similarity and had two bands (325 and 460 base pairs) which were common in their banding patterns. *K. delphensis* and *S. dairensis* each generated two different banding patterns thus showing strain variability in these microbial species. The strains within the same species differed in the number of bands otherwise the amplification patterns were similar. *K. delphensis*, *S. dairensis* and *Z. bailii* were isolated in both apple juice stored at 25 °C and 35 °C.

3.2.4 Discussion

3.2.4.1 Microbial growth

The absence or enumeration of only a few moulds in the yeasts and mould counts means that they were not an important spoilage microorganism in this apple juice concentrate despite their potential to cause spoilage. Heat resistant moulds were not detected in apple juice concentrate from all three storage temperatures because the concentration of the spores may have been too low or they were absent. Murdock and Hatcher (1976) state that the outgrowth of heat resistant moulds usually occurs 3 to 5 days if the fruit concentrate contains 100 or more spores per gram and if the concentration of spores is extremely low it may take as long as a month before colony formation appears.

Alicyclobacillus species are now considered a microbiological problem of juice concentrates (Brugnoni *et al.*, 2013). The absence of *Alicyclobacillus* spores in this study suggests that good manufacturing practices were followed and the apples used to manufacture the apple juice concentrate were not contaminated with this bacterium. An uncertainty also exists about which media is most effective for the isolation of *Alicyclobacillus* spores (Brugnoni *et al.*, 2013). The media used in the current study which was acidified potato dextrose agar may have failed to isolate *Alicyclobacillus* spores. Brugnoni *et al.* (2013) did not isolate *Alicyclobacillus* spores using three types of media (Yeast extract starch glucose agar, K agar and acidified potato dextrose agar). On the contrary, *Alicyclobacillus* spores were found in pear concentrate using acidified potato dextrose agar (Steyn *et al.*, 2011) and in mango concentrate using yeast extract starch glucose agar (Gouws *et al.*, 2005). Although Groenewald (2009), isolated *Alicyclobacillus* spores from pear concentrate using orange serum agar, yeast extract starch glucose agar and acidified potato dextrose agar; yeast extract starch glucose agar plates contained the highest number of colony forming units.

Lactic acid bacteria are typical micro biota in conditions prevailing in apple juice concentrate (pH 3.0 – 4.0, and high sugar content) (Brugnoni *et al.*, 2013). Their absence in this apple juice concentrate suggests that they if they were present in the apple juice they may have been eliminated through pasteurisation.

The absence of growth of yeasts at 10 °C may be due to the low storage temperature working synergistically with low water activity (0.69) and low pH (3.42) to suppress its growth. Secondly the storage time may not have been sufficient for yeasts to start growing. According to Betts *et al.* (1999), the time for visible growth of yeasts to occur is remarkably longer at low temperatures (8 °C) in comparison to higher temperatures (22 °C) as yeasts need more time to adapt to the environment of the apple juice concentrate (Patil *et al.*, 2011). Martorell *et al.*, (2007) also did not observe the growth of *Zygosaccharomyces bailli* and *Zygosaccharomyces rouxii* at 4 °C.

Yeasts and moulds were however able to grow in apple juice concentrate stored at 25 °C and 35 °C because increasing the storage temperature enhances the growing capability of yeast. Temperature has an important effect on yeast growth and that is the starting of fermentation, reaching maximal cell population and the decline of yeast population (Reddy and Reddy, 2011). At higher temperatures, the maximal yeast load is reached within a shorter period of time and fermentation ends faster (Reddy and Reddy, 2011; Viljoen, Lourens-Hattingh, Ikalafeng and Peter, 2003). This study is in agreement with this as the maximal yeast load at 35 °C was reached faster than at 25 °C and also whereas the yeast count started declining at 35 °C, at 25 °C there was no decline but the yeast population continued to increase. According to Torija, Rozes, Poblet, Guillamon and Mas (2003), at the higher storage temperature of 35 °C, the decline in yeast population could be due to the greater accumulation of intracellular ethanol causing cell toxicity and altering the cell membrane thereby decreasing its functionality.

The lag phase is the time required for microbial cells to adapt to their environment by synthesizing ribosomes and enzymes needed to establish growth at a higher rate (Malo, 2013). The first four weeks of storage at 25 °C and 35 °C constituted the lag phase and yeast counts in the apple juice concentrate were zero. The absence of counts may have been a result of the yeast existing in a viable but non-culturable state. The change in counts at 5 weeks and thereafter may have been due to the yeasts exiting the viable but non-culturable state (VBNC) resulting in their enumeration. The lack of a stationary phase in apple juice concentrate stored at 25 °C may be a result of the storage time used in this study not being long enough to show the arrival at the stationary phase.

Fleet (2011) states that the temperature range for the growth of *Z. bailii* is 2 °C to 37 °C. The high minimum theoretical temperature (20 °C) may be attributed to the effect of sugar. The minimum growth temperature varies with temperature increasing as the glucose concentration also increases.

3.2.4.2 Yeast identification microsatellite PCR fingerprinting

All the yeast species isolated from the apple juice concentrate are considered typical inhabitants of a fruit juice manufacturing environment. *K. delphensis* has been isolated from grape juice concentrate (Combina, Daguerre, Massera, Mercado, Sturm, Ganga and Martinez, 2008) and dried figs (Lachance, 2011). *Z. bailii* has been implicated as an effective spoiler in fruit concentrates (Thomas and Davenport, 1985) and other products with high sugar content such as candied fruit nougats (Martorell *et al.*, 2005). Mantynen (1999) in a study of orange and apple juice concentrate found *S. dairensis* to be one of the contaminants. Although the *Zygosaccharomyces* and *Saccharomyces* genera are considered the dominating groups of spoilage yeasts and are highly fermentative and osmotolerant, *S. dairensis* is not considered dangerous to product stability (Loureiro, 2000). This may suggest that the yeast species dominantly responsible for observed fermentative spoilage was *Z. bailii*.

The presence of *Z. bailii*, *S. dairensis* and *K. delphensis* in apple juice concentrate stored at 25 °C and 35 °C implies that they are able to grow within this temperature range. According to Spiller (1987), growth of *S. dairensis* occurs at temperatures above 25 °C but below about 45 °C. The presence of *Rhodotorula glutinis* and *Metschnikowia reukaufii* is suggestive of poor hygiene conditions in the plant resulting in post process contamination of apple juice concentrate.

PCR fingerprinting using the (GTG)₅ microsatellite primer was only able to distinguish *R. glutinis* a basidiomycetous yeast from the ascomycetous yeasts: *K. delphensis*, *S. dairensis*, *Z. bailii*, and *M. reukaufii*. The same amplification pattern for different yeast species may indicate a common ancestry. Kurtzman (2003) states that there are relatively high levels of genetic relatedness among species now assigned to the genera *Saccharomyces*, *Kluyveromyces*, *Zygosaccharomyces* and *Torulaspora*. Cai, Roberts and Collins (1996) in their study also showed that *Kluyveromyces* displays a marked phylogenetic heterogeneity and among other species of *Kluyveromyces*, *K. delphensis* was phylogenetically intermixed with

species of the genera *Zygosaccharomyces*, *Saccharomyces* and *Torulaspora*. Lopandic, Prillinger, Molnar and Gimenez-Jurado (1996) conducted a study to determine the potential molecular similarity of *Metchnikowia* species with other ascomycetous yeasts and they found 10 species of the genus *Metchnikowia* including *M. reukaufii* to cluster within the *Saccharomyces* genus. Couto *et al.* (1996) were able to differentiate the species *Z. bailii* from *Z. bisporus* and also discriminate different strains within the *Z. bailii* species.

3.2.5 Conclusion

These results show that temperature has an important effect on the microbiological quality of apple juice concentrate as seen by fermentative spoilage which occurred at storage temperatures of 25 °C and 35 °C. The lack of microbial growth at 10 °C indicates that if apple juice concentrate is stored at this temperature it will remain stable and retain its microbiological quality. However if storage temperature is abused, microbial spoilage may occur even though concentrates are considered relatively stable even at room temperature. Although PCR fingerprinting using the (GTG)₅ microsatellite primer could not adequately differentiate yeast species, it is an important tool in showing strain variability in a species.

CHAPTER 4: GENERAL DISCUSSION

Apple juice concentrate is produced seasonally and when refrigerated storage space is not enough it is common practice for it to be stored in tanks at ambient temperatures. The objectives of this study were to determine the quality parameter(s) limiting the shelf life of apple juice concentrate under refrigerated storage and also at ambient temperatures (accelerated temperatures). The second objective was to determine the rate of change in the physical, chemical and microbiological quality parameters when the apple juice concentrate is stored at accelerated temperatures and subsequently use the data to determine if accelerated storage techniques can be used to predict the rate of quality change at lower storage temperatures.

4.1 Review of Methodology

The initial aim of the study was to determine the shelf life of apple juice concentrate using accelerated shelf life testing. However because the quality of the apple juice concentrate supplied was such that parameters such as % transmission and 5-hydroxymethylfurfural concentration were almost out of specification, the aim of the study was changed to the determination of the quality changes in apple juice concentrate stored at refrigeration and accelerated storage temperatures.

Although the analysis of apple juice concentrate stored at 10 °C was supposed to be for six months since deterioration in quality occurs at a slow rate, the study was terminated after 13 weeks of storage when the refrigerator malfunctioned and because of time constraints the study could not be repeated. For future storage studies, temperature sensors can be installed in the fridges and incubators to detect such.

The variations in the quality of apples used in the manufacture of apple juice concentrate means that the quality will differ with batches. A model developed using an inconsistent product has to be used carefully and only as a guide because of the batch to batch variability. More studies have to be done to determine the influence of the initial value of parameters such as HMF concentration and $A_{420\text{nm}}$ on the reaction rate constant and activation energy.

Microbiological analysis was based on the microorganisms that are inherently present in apple juice concentrate. Although the apple juice concentrate was mixed so that there is a homogenous distribution of any microbes present, the growth response varied between

replicates. No growth was detected in some replicates and where growth was detected the kinetic parameters were significantly different $p \leq 0.05$ among the replicates. Couto *et al.* (2005) state that large volumes of beverages will always represent a challenge in terms of microbiological sampling as low levels of contamination or localised pockets of unmixed beverage might lead to the non-detection of viable cells even in beverages which will eventually become spoiled. To improve on this a preliminary storage study at accelerated temperature could have been done to grow and isolate spoilage yeasts. The same amount of spoilage yeast could then have been inoculated into replicates of sterile apple juice concentrate in a challenge study. Panagou, Karathanassi, Le Marc and Nychas (2009) in their study to develop a model for the spoilage of pasteurised fruit juices inoculated *Saccharomyces cerevisiae* that was isolated from spoiled packages of fruit juices.

Although five yeast species were isolated from apple juice concentrate it is difficult to conclude with certainty as to which species actually caused the spoilage. A follow up study could be done by inoculating each isolated yeast species separately into apple juice concentrate to determine the yeast species most predominant in causing spoilage.

Molecular techniques such as PCR fingerprinting of repeat primers such as (GTG)₅ are now being used to differentiate yeasts at species level. The correlation between yeast species and a specific genetic pattern can facilitate a fast and reliable recognition of contaminating yeast (Capece, Salzano and Romano, 2003). The biolog system analyses the physiological activities of the yeast isolates thus providing a metabolic fingerprint which can be used to characterise and identify the yeast (Mantynen, 1999). In this study the biolog system was able to identify all the isolates into five species belonging to five genera but PCR fingerprinting using the (GTG)₅ primer failed to distinguish the basidiomycota yeasts which were *Z. bailii*, *S. dairensis*, *K. delphensis* and *M. reukaufii*. Other PCR based methods such as ITS sequence analysis can be used to identify yeasts to species level but this could not be done because of cost implications. Combina *et al.* (2008) used it for grape concentrate, Ridawati, Ita and Wellyzar (2010) for foods with a high concentration of sugars and Arias *et al.* (2002) for orange juice. It is necessary to confirm the identity of yeast using another identification method.

Analysis of the physicochemical parameters was done on a weekly basis although modelling was based on the fortnightly results for statistical reasons. For the colour parameters

(lightness, redness, yellowness) and absorbance (420nm), there was a rapid increase or decrease during the first two weeks of storage followed by a decreased rate of change over the subsequent weeks. For such parameters the sampling frequency can be increased from weekly to daily during that period where the rate of change is high making it easier to determine the exact cut off point for parameters with specifications. Fu and Labuza (1997) state that if the interval between sampling is too long the risk of under- or over estimating parameters increases and the more analysis that are completed, the more accurate will be the determination of the kinetic parameters.

The most commonly used method for the quantification of HMF in concentrates is the Winkler method (Simsek *et al.*, 2007; Tosun, 2004; Burdulu and Karadeniz, 2003; Koca, Burdurlu and Karadeniz, 2003; Babsky, Toribio and Lozano, 1986). This is a colorimetric method based on the reaction between barbituric acid, p-toluedine and HMF to form a red coloured complex whose intensity is measured with a spectrophotometer at 550nm. It is recommended that the Winkler method should not be used if other methods are available since p-toluedine may be carcinogenic. The chromatographic methods although more sensitive and more accurate than the colorimetric methods, they were not used because they are expensive. The colorimetric method (Keeney and Bassette, 1959) also used by Cohen *et al.*, (1998) which measures the intensity of the yellow complex formed by the reaction of HMF and thiobarbituric acid was used for this study. The amount of HMF was quantified using a calibration curve ranging from 0-25mg/kg since the recommended limit for HMF in concentrates is 25mg/kg according to IFFJP (Matic *et al.*, 2009). However, because the quantity of HMF in apple juice concentrate stored at 25 and 35 °C exceeded this limit in the first week of storage, serial dilutions had to be carried out before analysis.

4.2 Quality changes in apple juice concentrate during storage

Apple juice concentrate has intermediate water activity, low pH and high total soluble solids content providing an ideal matrix for Maillard browning and yeast growth. The first objective of this study was to determine the quality changes in apple juice concentrate stored at 10 °C. During this study, it was seen that when apple juice concentrate was stored at a refrigeration temperature of 10 °C, the microbiological quality remained stable but colour changes were observed although at a low rate. This means that Maillard browning would be the shelf life limiting factor in apple juice concentrate stored at low temperatures. Although the rate of

change in HMF at the lower storage temperature of 10 °C could be predicted using the accelerated storage temperatures of 25 °C and 35 °C, it was difficult to compare it with the actual rate of change at 10 °C because there was no fitting kinetic model for the change in HMF concentration at 10 °C. More storage temperatures such as 15 °C, 20 °C and 30 °C could have been used to avoid such a problem.

Since Maillard browning still occurs at the lower storage temperatures, other measures can be implemented to reduce Maillard browning. In a study by Pacheco, Christian and Feng (2012), ascorbic acid, cysteine and nicotinic acid were found to have inhibitory effects on the Maillard reaction during sugar cane processing by acting as antioxidants preventing formation of intermediate brown compounds. Cysteine is a sulphur containing amino acid which forms stable Amadori compounds which are less susceptible to browning reactions (Narayan, 1998). Enzymes have also been used that modify the amino group to an amide group through acetylation hence preventing the amino acid from participating in the Maillard reaction (Friedman, 1996). The reducing group of the sugar can also be oxidised by enzymes such as hexose oxidase making the sugar unavailable to undergo the Maillard reaction (Soe and Petersen, 2005)

The second objective of the study was to determine the effect of accelerated storage temperatures of 25 °C and 35 °C on the rate of Maillard browning and growth of spoilage microorganisms (yeasts and moulds) in apple juice concentrate.

The accelerated temperatures chosen for this study had to accelerate both microbial growth and physicochemical reactions. The minimum, optimum and maximum growth temperature of yeasts is species dependant and also growth condition dependant (Deak, 2004), although generally they grow from 0 to 37 °C (Lachance, 1990). Walker (1998), also states that when storage temperatures are increased to the maximum growth temperature, yeast cell viability rapidly declines. The highest temperature selected was 35 °C as mesophilic yeasts and thermophilic yeasts are able to grow at this temperature. Selecting a higher temperature would have affected the viability of mesophilic yeasts.

Since it is normal practice to store apple juice concentrate at ambient temperature if refrigerated storage space is not available; seeing that the lag phase for yeast growth is

prolonged and spoilage prevented in apple juice concentrate stored at 10 °C, it would be advisable to change storage conditions from high to low temperatures should the space become available. Studies have been done on dynamic storage temperatures that have shown that decreasing the storage temperature alters the physiological state of microorganisms resulting in an additional lag phase that will delay or stop spoilage. If cells are still in the lag phase, growth might stop but if cells are in the exponential phase then growth will only be delayed. It would be worthwhile to carry out a study to find out the exact effects of changing temperatures during storage of apple juice concentrate on both the lag phase and the maximum specific growth rate of yeasts.

The activation energy is the minimum energy necessary for a specific reaction to occur. The energy required for Maillard browning to occur was 7.70, 8.17 and 14.80 kcal/mol for yellowness, lightness and non-enzymatic browning index respectively and that required for yeast growth to occur was 39.95 kcal/mol (Figure 26). This means that the growth of yeast is affected by temperature more than the Maillard reaction. Yeast growth will be slow at low temperatures. However, when storage temperature is increased or abused; yeasts will grow rapidly up to an optimum subsequently causing spoilage within a short period of time whereas the change in the parameters of the Maillard reaction will not be as appreciable.

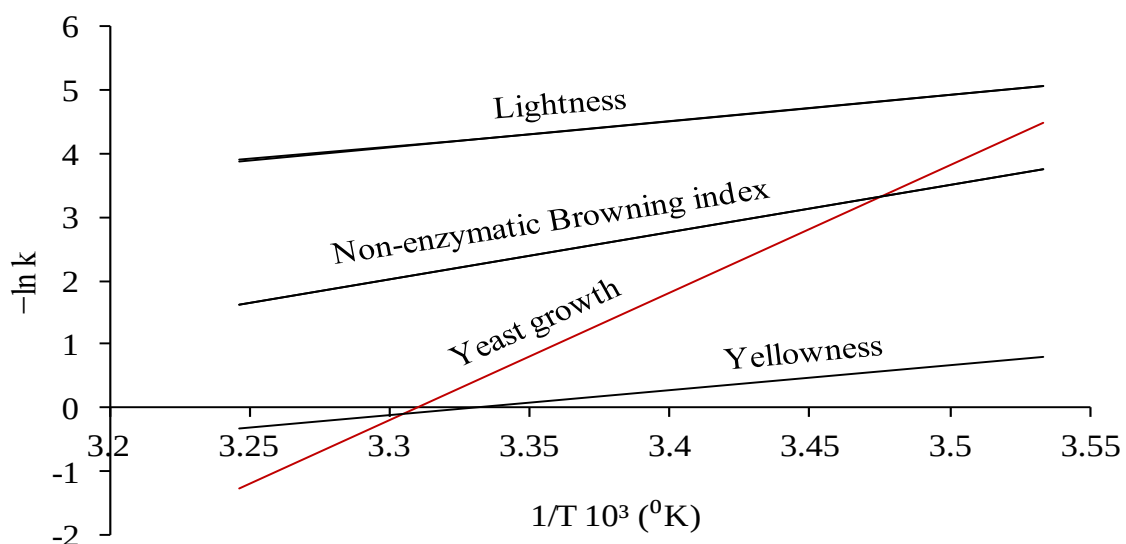


Figure 26. Temperature dependence of yeast growth and Maillard reaction

The Q_{10} value for microbial growth was 4.47 which was higher than the Q_{10} values for Maillard browning parameters which were 1.53, 1.20 and 1.81 for lightness, yellowness and the non-enzymatic browning index, respectively. These results are consistent with what was found for activation energy. Microbial growth will increase about 4.5 times for a 10 °C increase in temperature. However for Maillard reaction the rate will not increase by more than double for the same temperature increase. The Q_{10} value for hydroxymethylfurfural formation was 5.25 which is higher than 4.47 calculated for microbial growth.

It is important to minimise both Maillard browning and yeast spoilage in apple juice concentrate. An apple juice concentrate with a dark colour will impact negatively on the colour of other concentrate formulations where it is used. Yeast growth despite a second heat treatment will alter the flavour of apple juice concentrate compromising its acceptability to the consumer.

4.3 Conclusions and Recommendations

It can be concluded that Maillard browning is the shelf life limiting factor when apple juice concentrate is stored at refrigeration temperatures (10 °C). However at higher storage temperatures (25 °C and 35 °C), although deterioration in the quality of apple juice concentrate is a result of both yeast growth and Maillard browning, yeast growth seems to be more temperature dependant. In spite of the fact that apple juice concentrate is generally considered as having a long shelf life even at ambient temperatures, keeping the storage temperature low minimises the growth of any surviving yeast which may grow and subsequently cause spoilage if the storage temperature is abused.

Five species of yeast from five different genera were isolated from apple juice concentrate showing that yeast can survive and grow in an osmotic environment. Although the presence of *Rhodotorula glutinis* and *Metchnikowia reukaufii* is not expected to result in spoilage of a product, their presence in apple juice concentrate reflects badly on the hygiene status of the factory. Good manufacturing practices are essential to prevent pre- and post-process contamination.

Since it is a common practice that if refrigerated storage space is not available apple juice concentrate is stored at ambient temperature until refrigerated storage becomes available;

further studies can be done to determine the quality changes if apple juice concentrate previously stored at ambient temperatures is changed and stored at refrigeration temperatures.

Accelerated testing predicted the reaction rate constants for some Maillard reaction parameters to be higher than what they would be if storage tests were done under normal testing conditions. This provides a built in safety factor for the processor as it is better to think that quality will deteriorate at a faster rate.

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