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The use of nano-encapsulated plant extracts in inhibiting non-enzymatic browning in fruit canned in juice

by

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DECLARATION

I, Lusani Norah Vhangani, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

03/04/23

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ABSTRACT

Non-enzymatic browning (NEB) reactions occurring during processing and storage are critical to the quality of fruit and fruit-based products, particularly canned fruits.

This PhD work aimed to obtain more insight into inhibiting NEB reactions occurring in 'Golden Delicious' apples canned in fruit juice during storage by applying β-cyclodextrin (β-CD) encapsulated extracts of green rooibos.

The first approach was the optimisation of β-CD-assisted extraction of green rooibos. Extraction conditions of β-CD (0 – 15mM), temperatures (40 – 90°C) and time (15 – 60 min) resulted in optimal conditions of: 15 mM β-CD: 40°C: 60 min, yielding an extract with a total polyphenolic content (TPC) of 398.25 mg GAE.g^{-1}, metal chelation activity (MTC) 92.95%, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging of 1689.70 µmol TE.g^{-1}, ferric reducing antioxidant power (FRAP) of 2097.53 µmol AAE.g^{-1}, oxygen radical absorbance capacity (ORAC) of 11162.82 TE.g^{-1} and aspalathin content of 172.25 mg.g^{-1}. Strong positive correlations of TPC towards the antioxidant activity were observed R2 (0.929 – 0.978) at $p < 0.001$.

The physicochemical properties of optimal extract (β-GRE) in comparison to an aqueous counterpart (GRE) revealed that no differences ($p > 0.05$) were observed between the moisture content (MC) of GRE and β-GRE. However, the a_w of β-GRE was significantly ($p < 0.05$) lower at a value of 0.11 than that of GRE at 0.18. Regarding colour, β-CD resulted in increased lightness (L*) and reduced redness (a*) ($p < 0.05$), with no significant differences ($p > 0.05$) on the yellowness (b*) of green rooibos.

Thermogravimetric analysis (TGA) thermograms of β-CD, GRE and β-GRE revealed an initial loss in weight of 11, 2 and 6%, respectively. This loss was attributed to the evaporation of surface and adsorbed water. The thermal degradation of β-CD was observed between 340 – 375°C, while the GRE decomposed around 180°C. The thermogram of β-GRE was a superposition of GRE and β-CD, thus confirming the formation of inclusion complexes and improved stability with the degradation of β-GRE observed at 260°C. FT-IR Absorption spectra of β-CD and β-GRE samples overlapped at specific regions and showed certain spectral differences compared to the aqueous extract (GRE). Similarities between GRE and β-GRE were observed at 578, 1025, and 1154 cm-1. When the β-GRE inclusion complex formed, most characteristic peaks of GRE and β-CD disappeared or shifted in the newly formed complex.

Browning kinetics and activation energy (Ea) of AA-added canned apples was investigated at 5, 23, and 37°C for 24 and 60°C for 12 weeks, respectively. Brix (°B), pH, browning indices (A294 and A420 nm, lightness (L*value) and colour difference (ΔE*)), reactant consumption (reducing sugars (RS) and AA) and intermediate NEB reaction products (furfural and hydroxymethylfurfural (HMF)) were monitored.

The initial total sugar content comprised 66% fructose, 22% glucose and 12% sucrose. The °B ranged between 19.58 – 27.00 and pH 3.37 – 3.72. Overall, an increase and decrease in °Brix and pH were observed as the storage temperature and time increased, respectively, with no differences ($p > 0.05$) observed between (+AA) samples and those without added ascorbic acid (-AA). On the other hand, simultaneous sucrose hydrolysis and progression of the MR and sugar degradation resulted in no observed changes ($p > 0.05$) in RS for all samples.

In terms of browning indices, AA degradation, HMF and furfural formation, an increase ($p < 0.05$) in reaction rate constants (k_0 and k_1) was observed as the storage temperature increased. A clear indication that higher temperatures favour NEB reactions. Samples (+AA) exhibited faster progression browning, HMF and furfural content compared to -AA, as shown by higher ($p < 0.05$) reaction rate constants ($k0$ and $k1$). Ascorbic acid added samples (+AA) at 60°C after 12 weeks of storage exhibited the highest A_{294} nm (281.96), A_{420} nm (9.93) ΔE^* (61.88), lowest L^*-value (6.64), lowest AA (4.13 mg.L^{-1}) content, highest HMF (26.19 mg.100g-1) and furfural (64.31 mg.100g^{-1}). The furfural content was higher than HMF, and this was due to the high content of fructose in the sample. Regarding kinetics, A_{294} and A_{420} nm followed first-order kinetics at 5 – 37°C, and changed to zero-order at 60°C. The opposite was observed for L^*-value; meanwhile, ΔE^*, AA degradation, HMF and furfural were adequately described as zero-order for all temperatures.

The anti-browning capacity of β-GRE and GRE was described as inhibition (%I) and reduction in $k0$ of canned apples added with 0.25 and 0.5% extracts stored at 23 and 37°C for six months. Overall, β-GRE samples demonstrated superior inhibitory ($p < 0.05$) effect compared to GRE, and higher inhibitory was observed for samples stored at 23°C. For instance, β-GRE 0.25 and 0.5 exhibited the highest %I against browning development via L^*value (40.93 – 46.67%), β-GRE 0.25 for ΔE^* (46.67%) and β-GRE 0.25 and 0.5 for HMF (59.55 – 67.33%). In terms of furfural, no significant differences ($p > 0.05$) were observed between all GRE and β-GRE, although inhibition of furfural was reported at a range between 62.69 – 72.29%. The control sample at 23°C exhibited a high ($p < 0.05$) (k_0) compared to GRE and β-GRE for L^*value, ΔE^*, furfural and HMF. However, no significant differences ($p > 0.05$) were observed amongst all extracts, with the exception of HMF. Increased storage temperature of 37°C reduced ($p < 0.05$) the inhibitory efficacy of all extract types, resulting in comparable abilities between GRE and β-GRE. In some cases, β-GRE 0.5 exhibited less inhibition ($p < 0.05$) than GRE, and even exhibited pro-oxidant activity, i.e., -17.17% for ΔE^*. Higher Ea further confirmed the browning inhibition capacity of β-GRE in terms of colour development and HMF; however, GRE 0.25 proved superior against furfural formation.

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RESEACH OUTPUTS OF THE STUDY

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GLOSSARY

Terms/ Acronyms/ Abbreviation	Definition/Explanation
AA	Ascorbic acid
AG	Aminoguanidine
AGEs	Advanced glycation end-products
APSA	Agricultural Product Standard Act
CML	Carboxymethyl lysine
β-CD	Beta-cyclodextrin
BSA	Bovine serum albumin
CDs	Cyclodextrins
DHAA	Dehydroascorbic acid
DMHF	Dimethyl hydroxyl furanone
EC	Epicatechin
EGCG	Epigallocatechin gallate
GRE	Green rooibos extract
FAO	Food and Agriculture Organisation
FBDG	Food-based dietary guidelines
FDA	Food drug administration
FRAP	Ferric reducing antioxidant power
FT-IR	Fourier-transform infrared spectroscopy
HMF	Hydroxyl methyl furfural
IARC	International Agency for Research on Cancer
MC	Moisture content
MR	Maillard reaction
MRP's	Maillard reaction products
MTC	Metal chelation
NEB	Non-enzymatic browning
ORAC	Oxygen radical absorbance capacity
RSM	Response surface modelling
SPME	Solid phase micro-extraction
TGA	Thermogravimetric analysis
UN	United Nations
WHO	World Health Organisation

CHAPTER 1: MOTIVATION AND DESIGN OF THE STUDY

1.1 Introduction

The cause of diseases due to lack of proper nutrition in South Africa is characterised by under- and over nutrition. Conditions and risk factors related to nutrition amongst South Africans include vitamin A deficiency which results to impaired growth and development in children, and inadequate micronutrient intake in adults (Naude, 2013). These risk factors lead to conditions such as cancer and lifestyle diseases. To combat this issue, a lot of research has been undertaken to investigate the relationship between the intake of fruit and vegetable, and the prevalence of diseases of lifestyle. According to the World Health Organisation (WHO) report of 2014, low fruit and vegetable intake accounted for at least 19% of gastrointestinal cancer, 31% of ischemic heart disease and 11% of stroke worldwide (Mendis, 2014). As a result, extensive scientific evidence based on systematic reviews and meta-analysis studies have been gathered which supports the inclusion of fruit and vegetable in the diet in reducing the risk of lifestyle diseases, and micronutrient malnutrition (Schneider *et al.*, 2007; Aune *et al.*, 2017; Yip *et al.*, 2019; Wallace *et al.*, 2020). These properties are related to the relatively low energy, significant vitamins, minerals, phytochemicals, bioactive compounds, dietary fibre and ample water content of 95% in fruits and vegetables (Abbas *et al.*, 2017; Yip *et al.*, 2019). Thus, fruit and vegetables are an important component of a nutritious diet (Naude, 2013). However, the daily intake of South African children and adults is below the recommended intake of 400 g fruit and vegetables per day (Durst & Weaver, 2013; Pereira, 2015). This led to the South African government adopting the global Food-Based Dietary Guidelines (FBDG) as a national intervention (FAO, 2012). These guidelines include the phrase "eat plenty of fruits and vegetables every day" or the "5 a day" concept as a strategy to increase the consumption of fruits and vegetables (FAO, 2012). On the other hand, food security plays a major role in the afore-mentioned concept. If recommendations are made, government together with food producers have a responsibility to ensure that fruits and vegetables are adequately available and are also safe for consumption. However, like any other fresh produce, certain fruits and vegetables are only available seasonally (Wallace *et al.*, 2020). When fruits are in season they are harvested in large quantities, they typically undergo chemical, microbiological or physical spoilage if not properly handled, leading to wastage. Therefore, processing is imperative. Advances in agri-processing technologies ensured that certain seasonal products are available throughout the year, canning is amongst some of these techniques applied (Chen & Ramaswamy, 2012; Renard & Maingonnat, 2012).

Canning is a preservation method where food contents are sealed in a hermetic container and processed. In developed countries, canned food products are a significant component of the diet for most individuals. A study conducted by Rickman *et al.* (2007) found

that processed fruits and vegetables contain as much dietary fibre and vitamins compared to their fresh and frozen counterparts, and at times canned fruit and vegetable products contained more of the aforementioned nutrients (SAFVCA, 2022). However, it is difficult getting children and teens to consume fresh fruits and vegetables, while, this can be alleviated by consuming canned fruits which have similar or even better nutritional content (SAFVCA, 2022).

Ingredients, processing and storage conditions employed during canning favours non-enzymatic browning (NEB) reactions (Valero, 2017). The deleterious effects associated with this is that NEB reactions proceed via three different mechanisms, namely; Maillard reaction, ascorbic acid (AA) and acid-catalysed sugar degradation, were proven to take place simultaneously during food processing and storage (Rufian-Henares & Pastoriza, 2016; Paravisini & Peterson, 2019). NEB reactions taking place in food produce desirable flavours, aromas and colour, and in some instances, they enhance antioxidant properties of certain products, on the other hand, they may also produce toxic compounds, and result in reduced nutritional values of the food product they are formed in (Louarme & Billaud, 2012; Vhangani & Van Wyk, 2013; Aktag & Gokmen, 2021a)

The aforementioned is of great significance considering that food safety is a major concern for food manufacturers, consumers and health authorities. The International Agency for Research on Cancer (IARC), Food and Drug Administration (FDA) and the Joint Food Agricultural Organisation/World Health Organisation (FAO/WHO), identified some toxic compounds formed during food processing, some of these are proven as mutagenic or carcinogenic (Anese *et al.*, 2014; Cueto *et al.*, 2016; Rannou *et al.*, 2016). Such compounds form via through several pathways where lipids, carbohydrates, amino acids or AA react. The toxic compounds include furans, acrylamide, and other heterocyclic compounds. The concentration of these compounds increase with the increase in treatment temperatures, storage time and conditions (Valero, 2017; Agcam, 2022). These compounds are found in a variety of processed food products such as fruits, honey, juices (Pham *et al.*, 2020), milk, extruded cereals and bread (Hidalgo *et al.*, 2018; Lin and Zhou, 2018). Furans are known particularly for foods subjected to retorting in hermetically sealed containers which prevents its losses by volatilisation, thus permitting its accumulation (Anese *et al.*, 2014; Rannou *et al.*, 2016).

Prevention of NEB has been the main focus and mitigation strategies have been aimed at reduction of processing temperatures and time, substituting highly reactive ingredients with the less reactive. However, these interventions are not applicable to all product types, consequently this results in altered attributes such as taste, colour and flavour risking products of inferior quality (Lyu *et al.*, 2018; Pham *et al.*, 2021). In the canning industry, NEB reactions were heightened by changing medium and adoption of new packaging (Teixeira, 2019). Sugar syrup was substituted with fruit juice, retortable plastic cups were introduced to partially replace cans (Teixeira, 2019). The problem associated with these interventions was accelerating

oxygen diffusion through the plastic packaging, which increases AA degradation, in addition to increased AA content in fruit juice (Wibowo *et al.*, 2015a; Paravisini & Peterson, 2019; Pham *et al.*, 2019, 2020). Therefore, inhibition of non-enzymatic browning seems to be difficult to achieve.

Recently, plant extracts have attracted a great deal of attention in inhibiting NEB (Oral *et al.*, 2014: Liu *et al.*, 2015), the active component in the plant extracts being polyphenols. Furthermore, the use of plant extracts fulfils the current trend of using natural additives in food as an alternative to synthetic forms. Several mechanisms of NEB inhibition by plant extracts, or individual polyphenols contained in them have been confirmed and elaborated based on the different stages of each pathway. In an asparagine-fructose model system, chlorogenic acid and epicatechin blocked the formation of a Schiff base (Oral *et al.*, 2014). Guava leaf and fruit extracts at 100 µg.mL^{-1} exhibited inhibitory action against formation of Amadori rearrangement products (ARP), and α-dicarbonyl compounds formed via the MR and acid-catalysed sugar degradation (Wu *et al.*, 2009). NEB inhibition correlated with metal chelation activities, and inhibitory capacity was comparable to that of commercial aminoguanidine (AG). The inhibitory potency of guava extracts was due to the presence of four main polyphenols, namely; phenolic acids (ferulic and gallic acid) and flavonoids (catechin and quercetin). Comparisons between a plant extract and its individual primary polyphenols revealed that chokeberry exhibited the lowest 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging, ferric reducing antioxidant power (FRAP) and metal chelation (MTC) compared to cyanidin-3-galactoside (Cy-3-gal), chlorogenic acid (CA), epigallocatechin gallate (EGCG) and quercetin (QC), but, exhibited better 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity than CA (Zhao *et al.*, 2022). However, chokeberry extracts exhibited the highest inhibition against bovine serum albumin (BSA)-glucose mediated α-dicarbonyl at 48%, with the primary polyphenols' inhibition ranging between 15 – 44%. In real food systems, Favreau-farhadi *et al.* (2015) reported EGCG's capacity to reduce reduced browning apple sauce and bread rolls.

The indigenous South African plant species *Aspalathus linearis*, better known as rooibos, grows naturally in the Cederberg area in the Western Cape Province of South Africa (Joubert & DeBeer, 2011). Green rooibos extracts and infusions have been proven to exhibit significantly higher antioxidant activity than its red counterpart. Aspalathin and nothofagin are the major contributors to the total polyphenolic content, which in turn contributes more towards bio-functional properties (Villaño *et al.*, 2010; Monsees & Opuwari, 2017; Damiani *et al.*, 2019; Lawal *et al.*, 2019). Furthermore, of special interest to the present study in relation to possible anti-glycative properties of green rooibos, most of the polyphenols (quercetin, catechin and chlorogenic acid) whose anti-glycation activities were noted above are found in green rooibos, and it is reasonable to project that they would exhibit those properties in the heat-processed apple slices in fruit concentrate in the current study. Furthermore, it is worth to mention that

Joubert and DeBeer (2014) found that aspalathin's antioxidant capacity compares to that of EGCG.

The only drawback associated with the use of native plant extracts is that they may impart inherent flavour or pigments, as well as polymerisation reaction with other components in food systems. Moreover, high temperatures employed during common food preservation methods such as drying, pasteurisation and sterilisation may lead to degradation of polyphenols and other bioactives, thus reduced activity (Favreau-farhadi *et al.*, 2015; Hidalgo *et al.*, 2018). However, these undesirable flavours can simply be masked by means of encapsulation (Koteswara *et al.*, 2020). In addition, encapsulation of sensitive compounds provides improved stability during processing, and in the final product by preventing reaction with other components in food products such as oxygen or water (Nesterenko *et al.*, 2013). Common encapsulation material includes carbohydrates, protein and lipid polymers such as maltodextrin, inulin (Human *et al.*, 2020), soy protein isolates, sodium alginate and cyclodextrins (CDs), to mention a few. The choice of polymer is crucial as it affects how the active compound is released. For instance, Hidalgo *et al.* (2018) reported on elevated furosine levels when soy protein isolates were used to encapsulate beetroot pomace due to thermal treatments. Similarly, maltodextrin reacts with glycine to form browning compounds (DaSilva, 2018). Lavelli and Sri Harsha (2019) reported that sodium alginate-encapsulated grape skin extracts exhibited reduced potential to inhibit glycation of fructose-BSA and methylglyoxal (MGO)-BSA model systems, this was due to some phenolics interacting with alginate, resulting in a decreased release. Therefore, the encapsulating polymer should not participate in any chemical reaction during processing or storage. Cyclodextrins were the obvious choice since they were proven not to participate in an NEB reactions (DaSilva, 2018). Moreover, unlike other potential encapsulants, β-CD is also known to aid in extraction of these polyphenols from their sources, hence the term "β-CD assisted extracts" (Favre *et al.*, 2018; Maraulo *et al.*, 2021). Maraulo *et al.* (2021) proved that β-CD enhanced the physical properties of olive pomace extracts via improved heat stability and reduced hydroscopicity, in addition to increased antioxidant activity. In terms of β-CD encapsulated plant extracts inhibition of NEB reaction products, Favre *et al.* (2018; 2020) proved that β-CD-assisted extracts of thyme and green pepper were effective in retarding browning development (A_{420nm}) and HMF formation in glucose-BSA model systems, respectively.

Therefore, the aim of this study was to investigate the inhibitory potential of β-CD-assisted extracts of green rooibos against NEB reactions in apples canned in fruit juice during storage.

1.2 Statement of the research problem

Thermal processing, in this instance canning, ensures that food is palatable and shelf stable. However, as beneficial as it is, inherent properties of pH and sugar content, as well as

processing and storage conditions of canned fruits allow the formation of toxic compounds. These toxicants form as a result of non-enzymatic browning (NEB) reactions via ascorbic acid degradation, and acid-catalysed sugar degradation and the Maillard reaction. Recently, various interventions were introduced to sustain the fruit canning industry and to address consumer demands for convenience and healthier products. These included reduction in sugar content by substituting syrup with fruit juice, and introduction of easy to open retortable cups. However, contrary to the protection offered in cans or glass jars, an increase in oxygen permeation rate and increase in ascorbic acid (vitamin C) content in canned fruit creates a perfect environment for NEB reactions. Therefore, the aim of this study was to optimise the antioxidant potential of β-CD-assisted extracts of green rooibos, with a view to apply them as possible inhibitors of NEB reactions taking place in apples canned in fruit juice during storage.

1.3 Objectives of the research

1.3.1 Broad objectives

The aims of this study were: 1) to optimise the antioxidant and polyphenolic content of β-CD-assisted extracts of green rooibos (*Aspalathus linearis*).

2) To characterise the physicochemical properties of β-CD-assisted extracts of green rooibos.

3) To apply reaction kinetics to establish the NEB reaction taking place in apples canned in fruit juice during storage via reactant consumption and formation of intermediate furfural and HMF content.

4) To investigate the inhibitory potential of β-CD-assisted extracts of green rooibos against NEB reactions in apples canned in fruit juice during storage via reactant consumption and formation of intermediate furfural and HMF.

1.3.2 Specific objectives

To optimise the extraction of green rooibos in order to obtain extracts that exhibit the highest antioxidant activity and polyphenolic content using response surface methodology.

- This was achieved by applying encapsulation-assisted extraction using β-CD at varied concentrations, temperature and time.
- The resulting extracts were analysed for total polyphenolic content (TPC) and HPLC quantification of major flavonoids.
  - The antioxidant activity was determined via 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity, Ferric reducing antioxidant power (FRAP), metal chelation (MTC) and Oxygen Radical Absorbance Capacity (ORAC) assays.

To characterise the physicochemical properties of β-CD-assisted extracts of green rooibos with the highest total polyphenolic content and antioxidant activity.

- Determine the colour (L*a*b*), moisture content (MC) and water activity (a_w) of β-CD-assisted extracts of green rooibos.
- Confirm formation of inclusion complexes via Fourier-transform infrared (FT-IR) and thermogravimetric analysis (TGA).

To investigate the type of NEB reaction mechanism taking place in apples canned in fruit juice over time.

- Apples canned in fruit juice stored under 5, 23, and 37°C for 6 months and 60°C for three months.
- Common NEB reactants consumption was monitored prior and during storage. These include Brix, pH, browning development via L*a*b*, colour difference (ΔE*), absorbance at A_{294} and A_{420} nm, reducing sugar content and ascorbic acid degradation.
- Intermediate NEB reaction products HMF and Furfural were analysed.
- Kinetic modelling was applied to determine the rate constant for consumption of reactants and formation of intermediate products in order to determine the type of NEB reactions taking place.

To investigate the effectiveness of β-CD-assisted extracts of green rooibos extracts in inhibiting NEB in apple canned in fruit juice during storage.

- To evaluate the effect of encapsulation on the inhibition of NEB reactions, aqueous rooibos extracts GRE (0 mM β-CD: 40°C: 60 min) and β-GRE (15 mM β-CD: 40°C: 60 min) at 0.25 and 0.5% were added in apples canned in fruit juice stored under 23 and 37°C for 6 months.
- Inhibition browning development via L*a*b*, colour difference (ΔE*) was determined.
- Inhibition of formation of Intermediate NEB reaction products HMF and Furfural was monitored via percentage inhibition (%I), rate constant (k_0) and activation energy (Ea).

1.4 Hypotheses

Based on studies of previous studies of Favre *et al.* (2018; 2020), it was hypothesised that aqueous extracts of green rooibos extracts will yield lower antioxidant and polyphenolic content compared to β-CD-assisted extracts. Similarly, the antioxidant and polyphenolic content of green rooibos extracts will increase as the β-CD concentration increase.

β-CD-assisted extraction of green rooibos will facilitate formation of inclusion complexes between Green rooibos and β-CD, resulting in extracts with improved physicochemical properties (Koteswara *et al.*, 2020).

Due to the high acidic nature of 'Golden delicious' apples, NEB reaction via AA degradation will precede acid-catalysed sugar degradation and the Maillard reaction might take place. Consequently, AA degradation products and acid-catalysed reaction products will serve as reactants for the MR (Aktağ & Gökmen, 2020).

β-CD assisted extracts of green rooibos will exhibit higher inhibitory capacity against formation of furfural and HMF compared to aqueous extracts.

1.5 Delineation

Only the green (unfermented) rooibos *Aspalathus linearis* leaf was investigated in the present study. Three concentrations of β-CD were used for extraction, *viz*. 0, 7.5 and 15 mM. Three reaction temperatures (40, 65 and 90°C) and times (15, 30 and 60 min) were applied during extraction of green rooibos. Only 'Golden Delicious' apples were used in the study. Reconstituted apple juice was the only media used for canning.

1.6 Significance of the research

The move to reduce sugar consumption in South Africa was driven by the severely growing obesity pandemic. One of the strategies introduced was imposing of the current tax on sugar-sweetened beverages. The canning industry followed suit by moving from using traditional syrup as a canning medium to fruit juices. However, incorporation of juices resulted in the reduction in shelf-life due to browning of the final product, especially in permeable containers, e.g., plastic cups. Results generated from this research will be communicated to the canning industry as a possible solution to alleviate the current problem.

1.7 Thesis overview

This thesis consists of seven chapters structured in a format where each research chapter was produced as an individual manuscript (Figure 1.1). Chapter 1 titled "Motivation for and design of the study" introduced the research overview which included, the research problem, objectives, hypothesis, delineations of the research, as well as significance of the study.

Chapter 2: The literature review elaborated further on the background of the research study. An overview of the role of fruits and vegetables consumption in the diet was reviewed, including its importance and limitations. This then led to the discussion of food security and safety in relation to adequate supply of fruits and vegetable. One of the solutions to the above mentioned was canning of fruits and vegetables as a means of preservation. In this section, the canning process and specifications based on the South African legislation was briefly discussed. The pathways of three types of NEB reactions that take place in fruit and fruit-based products were discussed in detail, these included factors, as well end-products. Prevention of NEB reactions using plant extracts, and possible use of green rooibos was introduced. The inhibitory mechanisms of plant extracts and its constituents against NEB reaction was

discussed, as well as limitations thereof. Encapsulation as a possible intervention was introduced, placing emphasis on selection of a suitable encapsulant leading to discovering β-CD. Studies where encapsulated extracts were applied in preventing NEB reactions were discussed.

Chapter 3 was titled "Antioxidant activity of β-CD-assisted extraction of green rooibos (*Aspalathus linearis*)". In this research chapter, green rooibos extracts were obtained via β-CD-assisted extraction at various concentrations, reaction temperature and time. The resulting extracts were analysed for TPC, antioxidant activity via MTC, ORAC, FRAP and ABTS radical scavenging. Response surface modelling was performed to obtain the best combination in terms of TPC and antioxidant activity.

Chapter 4 was titled "Characterisation of physicochemical properties of β-CD-assisted extracts of green rooibos (*Aspalathus linearis*) plant extracts". In this research chapter the optimised combinations obtained in Chapter 3 were characterised by determining physicochemical properties that affect their functionality (L*a*b*, MC, a_w, TGA and FT-IR).

Chapter 5 was titled "Non-enzymatic browning of apples canned in fruit juice during storage". This chapter investigated the NEB reaction mechanism/s taking place in apples canned in fruit juice stored for six months. During the course of storage reactant consumption (reducing sugars, ascorbic acid) and formation of key NEB markers (HMF and furfural).

Chapter 6 was titled "Inhibition of NEB reactions in canned fruit using β-CD-assisted extracts of green rooibos". In this chapter, the best combination as determined by RSM (Chapter 3) was incorporated in apples canned in fruit juice at 0.25 and 0.5%, stored for six months at 23 and 37°C. During the course of storage, reactant consumption, inhibition of browning development via L*a*b* and ΔE* and formation of HMF and furfural were investigated.

Chapter 7 "General discussions and conclusions", was aimed at bringing all the results into context, concluding whether or not the hypotheses were accepted or not, as well as possible recommendations for future work.
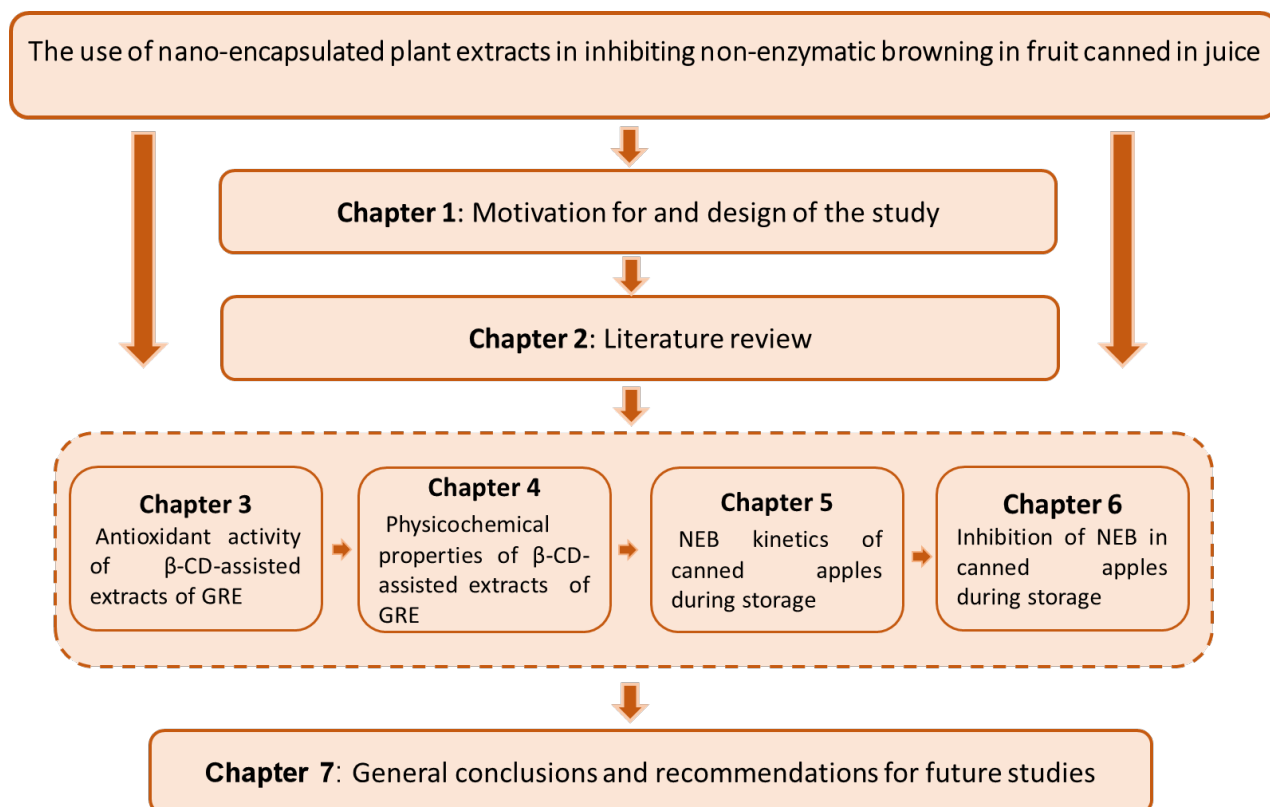
Figure 1.1: Thesis overview.

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CHAPTER 2: LITERATURE REVIEW

2.1 The role of fruits and vegetable consumption in the human diet

The nutrition-related disease risk profile in South Africa is characterised by both under- and over nutrition. Conditions and risk factors related to primary nutrition amongst South Africans, include stunting, underweight and vitamin A deficiency for children, and inadequate micronutrient intake in adults (Naude, 2013). These conditions lead to risk factors such as cancer, diabetes, chronic respiratory diseases, obesity, cardiovascular diseases, hypertension and hypercholesterolaemia. To combat this issue a lot of research was conducted to investigate the connection with reference to fruit and vegetable intake and the prevalence of diseases of lifestyle. The World Health Report found that the low intake of fruits and vegetables was responsible for 19% of gastrointestinal cancer, 31% of ischemic heart disease and 11% of stroke worldwide. As a result, extensive scientific evidence has been gathered which supports the importance of including fruit and vegetable in the diet in reducing the risk of diabetes, cardiovascular diseases and certain cancers, as well as micronutrient malnutrition (Shahidi & Ambigaipalan, 2015; Freedman & Fulgoni, 2016). These properties are related to the low energy, vitamins, minerals, phytochemicals, bioactive compounds, and dietary fibre content in fruits and vegetables, also not forgetting that they contain approximately 95% water (Abbas *et al.*, 2017). Thus, fruit and vegetables are an important component of a healthy diet (Naude, 2013). As a result of this, many countries have adopted the recommendation to eat at least 400 g fruit and vegetables per day as a preventative measure (Durst & Weaver, 2013). However, the daily intake of South African children and adults is below the stipulated intake. As a result, the South African government has adopted the global Food-Based Dietary Guidelines as a national intervention to educate the public and inform legislators about a healthy diet. They include the phrase "eat plenty of fruits and vegetable every day" or the "5 a day" concept as a strategy to increase the consumption of fruits and vegetables (Pereira, 2015).

Food security plays a major role in the concept of "eat plenty of fruits and vegetable every day". If recommendations for increased consumption are made, government together with food producers have to ensure that these fruits and vegetables are adequately available and are also safe for consumption. However, like any other fresh produce, certain fruits and vegetables are only available seasonally (Shahidi & Ambigaipalan, 2015). When fruits are in season they are harvested in large quantities. If not properly handled, they typically undergo chemical, microbiological or physical spoilage leading to wastage. Thus, processing has to be done to prevent spoilage. Recent advances in agricultural technology have contributed significantly to the improved production of fruits throughout the world. In addition, advances in fruit processing technologies, refrigeration, transportation, storage and distribution have made

it possible for consumers to enjoy these products year-round. Keeping raw fruits in cold storage and ripening with gas when needed, freezing, concentration, drying, fermentation and canning are amongst some of these techniques (Teixeira, 2019).

2.2 Fruit canning

Canning is a method of preserving food in which food contents are sealed in an hermetic container and processed. It is one of the age-old preservation methods aimed not at only extending the shelf-life of food, but also making seasonal produce available all year round (Teixeira, 2019). Canned food products are a significant component of the diet of most individuals in developed countries. The long shelf-life is attributed to the thermal processing which destroys spoilage microorganisms and those of concern to the public health (Valero, 2017). A typical canned food product can have a shelf-life ranging from one to five years, provided that the packaging integrity is maintained (Chen and Ramaswamy, 2012). Like any other invention, canning was also developed to solve a problem. Recent trends in canning also include the use of retortable plastic pouches or cups, which then resulted in further development such as aseptic processing, which requires fully automated agitating retorts (Chen and Ramaswamy, 2012; Boz & Erdoğdu, 2015)

Currently, a variety of food products are canned, such as meats, fish, poultry, fruits, vegetables and dairy products. In South Africa, canning companies such as Langeberg & Ashton Foods, Rhodes Food Group, Del Monte Foods South Africa, Boland Pulp and Summerpride, are those mentioned in SAFVCA (2022), known for producing quality canned products. Fruits are available for canning in a variety of forms: whole fruit, slices, halves, sauce, purée, juice, or in mixed fruit packs such as fruit cocktail (Rawson *et al.*, 2011). Peaches and pears are usually canned as halves, slices, or in fruit cocktail. Requirements for canned fruits are determined by the Agricultural Product Standards Act 119 of 1990. The Act requires that canned fruit should be presented as a regular pack which consists of fruit packed in a packing medium, a heavy pack which consist of crushed style fruit with or without sweetening ingredients and containing at least 73% drained mass and a solid pack which consist of only fruit with very little or no free flowing liquid (Anonymous, 2022).

Temperatures applied during heat processing are governed by the food type, components and type(s) of microorganism(s) that may grow to spoil the food, or those that are of concern to public health, in addition to type of packaging material used and heat transfer properties(Boz & Erdoğdu, 2015). Thermal processes used for canned food are designed to achieve three basic objectives, the most important being to reduce the number of microorganisms to very low levels. The second objective is to create an environment in the container which suppresses the growth or activity of spoilage type microorganisms by removing oxygen and controlling the pH (Chen & Ramaswamy, 2012). The third objective is to assure complete closure of the packaging to prevent contamination during processing and

storage. Furthermore, it should be stressed that thermal processing is not always required to achieve complete sterility of food. Application of high temperatures might lead to a shelf-stable product, but also result in diminished quality, especially heat sensitive nutrients.

2.3 The role of canned fruits in the diet

There is a misconception that canned fruits and vegetables have an inferior quality and nutrition compared to the fresh counterparts. A study conducted by Rickman *et al.* (2007) and Durst and Weaver (2013), found that canned fruits and vegetables are as rich in dietary fibre and vitamins as the corresponding fresh or frozen foods, and in some cases the canned products are richer than their fresh or frozen counterparts. Moreover, the food-based dietary guidelines of the Food and Agriculture Organisation (FAO) of the United Nations recommend the consumption of plenty of fruits and vegetables. Furthermore, it is difficult getting children and teens to consume fresh fruits and vegetables, hence, this can be alleviated by consuming canned fruits which have similar nutritional composition and are perceived by children as a dessert or a treat (Freedman & Fulgoni, 2016). Nutritionists and Dieticians have always highlighted the important role of the human diet with reference to health. Food consumption is more than just satisfying hunger and nutritional requirements (Louarme and Billaud, 2012). In addition, foods have recently been recognised as protective agents as well. As mentioned, studies proved that a high intake of fruit and vegetables is linked to reduced risks of a number of chronic diseases (Freedman & Fulgoni, 2016). This is attributed to their provision of an optimal mix of phytochemicals, natural antioxidants, fibres and other bioactive compounds. Amongst them, antioxidants and vitamins present in fruit and vegetables are of utmost interest (Shahidi & Ambigaipalan, 2015). Although food composition tables rely mostly on food products consumed raw, they also take into consideration the fact that concentration of nutrients and their biological activity may change due to environmental variables and processing (Rickman *et al*., 2007). This aspect is of great importance, considering that only a small amount of fruit and vegetables are consumed in their raw state, whilst most of them need to be processed for safety, quality and economic reasons. As mentioned, fruits and vegetables are subjected to blanching, freezing, drying or canning and these preservation methods are generally but often erroneously believed to be responsible for depletion of naturally occurring nutrients in food (Rickman *et al.*, 2007).

However, while thermal processing preserves these food commodities against microorganisms, it may indeed also result in chemical reactions that also reduce their shelf-life (Bharate & Bharate, 2014; Valero, 2017). During preparation of so-called white fruits for canning, enzymatic browning takes place, and this is due to the contact of plant enzymes with phenols in the presence of oxygen, leading to the production of melanin (Du *et al*., 2012). However, pre-processing measures are undertaken to prevent this reaction. In addition, this reaction is completely inhibited once the contents are heat-processed resulting in deactivation

of all enzymes. Hence, although enzymatic browning might not be a problem in this context, non-enzymatic browning reactions (NEB) may also take place (Zhu *et al.*, 2009). Non-enzymatic browning control is more complex since there are four possibilities which are all favoured by key components as well as processing and storage conditions of canned fruits.

Non-enzymatic browning can namely be categorised as the Maillard reaction, ascorbic acid degradation, acid-catalysed sugar degradation, also or commonly known as caramelisation and to a large extent, lipid oxidation (Bharate & Bharate, 2014; Rufian-Henares & Pastoriza, 2016). The most important aspect of these reactions is that a compound formed via one mechanism serves as a key reactant in the other. Thus, all these reactions might be taking place in the same food product (Burdurlu *et al.*, 2006; Louarme and Billaud, 2012). Although non-enzymatic browning reactions produce desirable flavours, aromas and colour, and in some instances, they enhance antioxidant properties of certain food products, they produce toxic compounds and also result in reduced nutritional values of the food product they are formed in (Louarme and Billaud, 2012; Vhangani & Van Wyk, 2013).

This is of great significance, considering that food safety is a major concern for food manufacturers, consumers, and health authorities. In recent years, the International Agency for Research on Cancer (IARC), Food and Drug Administration (FDA) and the Joint FAO/WHO, identified some compounds that are generated in food as potentially toxic, mutagenic or even carcinogenic. Such compounds may be formed through multiple pathways in which mainly lipids, carbohydrates, amino acids or ascorbic acid are thermally degraded (Agcam, 2022). Some of these undesirable compounds include furfural (hydroxymethyl-furfural, methylfurfural, dimethyl hydroxyl furanone) acrylamide, heterocyclic compounds (furans, pyrroles and pyridines), whose concentration depends on the severity of the heat treatment and storage time and conditions (Anese *et al.*, 2014). Those compounds have been identified in a wide range of foods containing carbohydrates such as processed fruits, honey, juices (Pham *et al.*, 2020), milk, extruded cereals (e.g. breakfast and infant cereals), crackers and bread (Hidalgo *et al.*, 2018; Lin and Zhou, 2018).

Although the taste of food is one of the most important sensorial properties of food, colour is considered as the deciding factor as to whether a consumer will buy a certain food product or not. Since NEB has a significant influence on product quality, monitoring the extent of this reaction can be a valuable tool in food manufacturing processes. Thus, preventing browning in canned fruits is of vital significance (Valero, 2017). Possible interventions include lowering of processing temperatures and time and substituting sugar syrup with juice (Teixeira, 2019). However, most canned fruits lie in the high acid food range, thus minimal processing is required since the pH acts as a preservative. However, with ascorbic acid browning, pH in the acidic range is a pre-requisite and this reaction is accelerated at this pH (Pham *et al.*, 2020). Moreover, the canning industry recently moved from canning fruits in syrup to fruit juices, jellies and nectars as a means of reducing the sugar content of canned fruits, consequently reducing

the chances of non-enzymatic browning via caramelisation. However, commonly used juices include apple and pineapple juice, and these contain substantial amounts of vitamin C, which when present in higher quantities can undergo browning (Wibowo *et al.*, 2015a; Paravisini & Peterson, 2019). Another intervention is that the industry has moved from the use of cans to plastic cups. The oxygen ingress rate is much higher in the latter packaging, which results in oxidative degradation of ascorbic acid (Wibowo *et al.*, 2015a). Hence, inhibition of NEB seems to be a difficult task. The following sections will focus on NEB reactions and products formed in fruit and fruit-based products during processing and storage.

2.4 Non-enzymatic browning reactions

Non-enzymatic browning is a series of complex chemical reactions that take place during processing and storage of food products in the absence of enzymes. There are three major browning reactions, *viz*. Maillard reaction, ascorbic acid degradation and caramelisation (Agcam, 2022). The type of browning reaction taking place depends on the physico-chemical properties of the food in question. At times all these NEB reactions might take place in one product, however one pathway may dominate depending on intrinsic and extrinsic factors (Buvé *et al.*, 2021). These chemical reactions are known to be the contributing factor to food quality, consequently affecting consumer acceptability. Another lesser known consequence of NEB is the loss of nutrition and formation of toxic compounds (Rannou *et al.*, 2016). To maintain quality aspects of food, NEB reactions should be strictly monitored. As a result, product composition, observed changes and formed compounds are used as quality markers. The ingredient type, concentration before and after, already point us into the direction of which reaction to look out for. The most pronounced change in this case is browning measured by means of colour change (Wibowo *et al.*, 2015c,a). The last category relates to chemical compounds that form due to the type of ingredient and processing conditions. By following these three markers, the NEB pathway can be elucidated (Rufian-Henares & Pastoriza, 2016). This chapter gives a description of the chemistry of three major NEB reactions in fruit and fruit-based products. The factors affecting NEB reactions are discussed, and different methods applied to monitor NEB during processing and storage of fruit and fruit-based products are summarised.

2.5 Ascorbic acid degradation

Ascorbic acid, also known as vitamin C, is a water-soluble vitamin found abundantly in fruits and vegetables. The main physiological function of vitamin C is to form and maintain bones, blood vessels and collagen. Fruits and vegetables are known to be the main source of vitamin C, thus it is one of the indices used to measure nutritional quality of fruits (Mesías-García *et al.*, 2010). It also possesses health-promoting qualities such as use in the prevention of colds and flu and association with prevention of scurvy.

In food formulation, vitamin C is known for its role as an acidulant, flour improver in baking, and processing aid in preventing enzymatic browning (Laorko *et al.*, 2013). Chemically its lactone structure contributes to its reducing ability. Common processing and storage conditions of oxygen, heat, presence of enzymes and metals render vitamin C unstable (Mesías-García *et al.*, 2010). Therefore, it is crucial to fully describe and understand the underlying mechanism of vitamin C degradation.

In fruit and fruit-based products, degradation of AA during processing and storage has been found to occur via aerobic and anaerobic pathways, depending on factors such as temperatures, presence or absence of oxygen, pH, light, water activity and content. Based on these factors, oxidative degradation is more pronounced during processing due to constant aeration, whilst non-oxidative degradation occurs mainly during storage. Thus, at high oxygen concentration, aerobic degradation preceded the anaerobic pathway.

2.6 Aerobic degradation of ascorbic acid

The oxidative degradation of AA is initiated by oxidation to form dehydroascorbic acid (DHA). The oxidation step is reversible in the presence of a reducing agent. The presence of water results in hydrolysis of DHA to form 2,3-diketogulonic acid (DKG) which does not exhibit vitamin C activity. Decarboxylation of 2,3-diketo-gulonic acid leads to the formation of xylosone which is further dehydrated to form reactive carbonyl compounds such as 2-furoic acid, 3-hydroxy-2-pyrone and furfural, amongst many (Figure 2.1 as adopted from Buvé *et al. (*2021)). Due to their high reactivity, carbonyl compounds may participate in the MR via condensation with amino acids or proteins to form brown polymers.
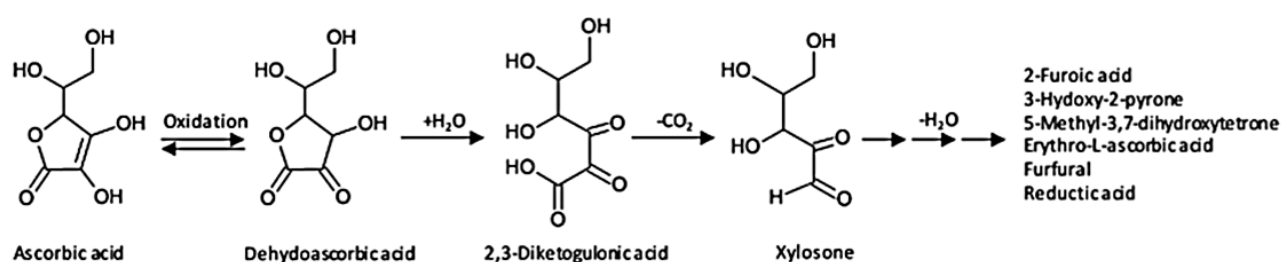
Figure 2.1: Proposed aerobic pathway of ascorbic acid degradation (as adopted from Buve *et al*. (2021).

2.6.1 Anaerobic degradation of AA

The initial step of the anaerobic pathway involves direct cleavage of the lactone ring of AA *via* hydrolysis to form 2,3-enegulonic acid, which is further decarboxylated to xylose (Figure 2.2

as adopted from Buvé *et al. (*2021)). The formed xylose undergoes intramolecular rearrangement and dehydrations to form furfural.
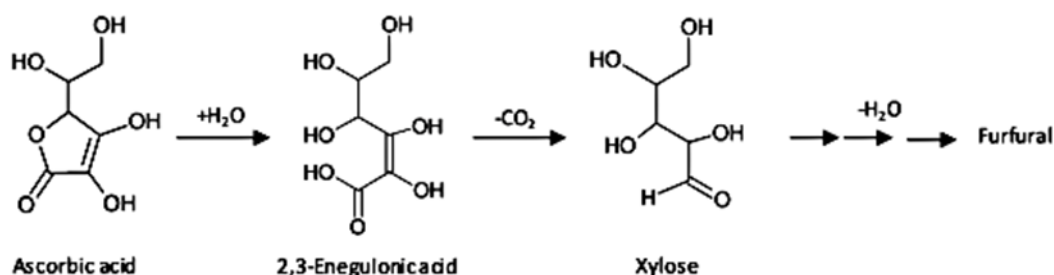
Figure 2.2: Proposed anaerobic pathway of ascorbic acid degradation as adopted from Buve *et al*. (2021).

2.6.2 Ascorbic acid degradation in fruit and fruit-based products.

Oxygen is present in food products via incorporation during preparation and processing, especially aeration during mixing. This is then transferred into the packaged food, present as dissolved and headspace oxygen. In addition, depending on the barrier properties of packaging, oxygen can diffuse through the package from the environment (Wibowo *et al.*, 2015c). Pham *et al.* (2019) observed a continuous diffusion of oxygen in orange juice packed in high oxygen permeable polyethylene terephthalate (PET) bottles during storage. On the other hand, Valero (2017) found that residual oxygen dissolved in the syrup and trapped in the headspace was found in canned peaches although preheating and exhausting was performed. Various authors reported on aerobic degradation of AA taking precedence over the anaerobic pathway, especially in the presence of high oxygen concentrations. These authors observed that as the O_2 concentration depleted to lower levels, anaerobic AA degradation takes over. Therefore, aerobic AA degradation takes place during processing and few days or weeks of storage, after which anaerobic takes place during the latter stage of storage. This is usually observed via imbalances in faster and greater depletion in AA, and smaller increases in DHA formation (Pham *et al.*, 2019). Low oxygen levels, thus lower conversion of AA to DHA. For instance, Wibowo *et al.* (2015b) reported on 75% loss of AA in orange juice after 8 and 32 weeks of storage at 42 and 20°C,respectively, and this was attributed to the drastic loss of dissolved and headspace oxygen observed in the first two weeks of storage. Consequently, furfural was detected at week 2 for samples stored at 42°C, which then continued to increase throughout 32 weeks of storage due to the anaerobic degradation pathway taking over when oxygen concentration was low. The oxygen of orange juice supplemented with AA and citric acid (CA) reached extremely low levels after 2 to 4 weeks of storage, with samples with the

highest AA content resulting in faster consumption rate of oxygen (Wibowo *et al.* 2015a). Similarly, furfural was also reported after 2 weeks storage for juice+CA+AA and 6 weeks for juice+AA. Therefore, implying that furfural formation via AA degradation is favoured at lower pH values. Lyu *et al.* (2018) also observed a decrease of AA in peach juice of approximately 29, 40 and 47% after 40-day storage at 4, 25 and 37 °C, respectively. However, instead of furfural, they reported HMF as a product of aerobic degradation. Burdurlu *et al.* (2006) also reported HMF as the main degradation product of AA degradation of citrus juice concentrates (orange, tangerine, lemon and grapefruit), although they also found that sugar degradation also took place, although it was preceded by the former reaction. Using precursor based studies, Agcam (2022) proved that AA-enriched fruit-based medium was superior in inducing furfural formation compared to sugar and amino acid-enriched samples. Furthermore, they found that no HMF formation was detected in the fruit juice-based medium enriched with AA only. Louarme and Billaud (2012) investigated AA degradation during conventional and ohmic heating treatment of chunky peach apple dessert. They confirmed that oxidative thermal degradation of AA preceded sugar degradation during the three-step production process involving apple puree, peach dice and apple puree-peach dessert. They reported approximately 33% loss in AA (supplemented in the puree) resulting in formation of 3-hydroxy-2-pyrone ($3H_2P$) (2 mg.100g^{-1}) at six and four times the levels of furoic acid (FA) and furfural. This was attributed to the wide pH range of the dessert which favoured formation of $3H_2P$, meanwhile the formation of FA and furfural are known to be favoured by high acid medium of pH (≤2).

2.7 Acid catalysed sugar degradation

Fruit and fruit-based products are classified according to acidity levels, pH > 4.5 as low, medium (3.7 – 4.5) and high acid < 3.7 (Agcam, 2022). Under these acidic conditions and other factors such as high processing temperatures and low a_w, sugar content and prolonged storage, sugars decompose to form reactive intermediates that take part in browning formation (Aktağ and Gökmen, 2020). Acid-catalysed degradation of sugars starts with hydrolysis of a disaccharide (sucrose) or complex carbohydrate (starch) to its reducing forms (fructose and glucose) (Figure 2.3 as adopted from Buvé *et al. (*2021)). Glucose and fructose undergo enolisation to form an 1,2-enediol intermediate. However, fructose can directly dehydrate, resulting in losing three molecules of water to form HMF (Agcam, 2022). The enediol is dehydrated and oxidised to form 3-deoxyglucosone (3-DG) and glucosone, respectively. The 3-DG compound degrades further via dehydration by losing two molecules of water to form HMF or undergoes retro-aldolisation to form MGO. On the other hand, in the presence of metals, retro-aldolisation of glucosone yields threosone and glyoxal (Pham *et al.*, 2020). At this point of the reaction, the formed intermediate compounds are highly reactive and might

polymerise with each other or react with amino acids to form brown polymers (Paravisini & Peterson, 2019).
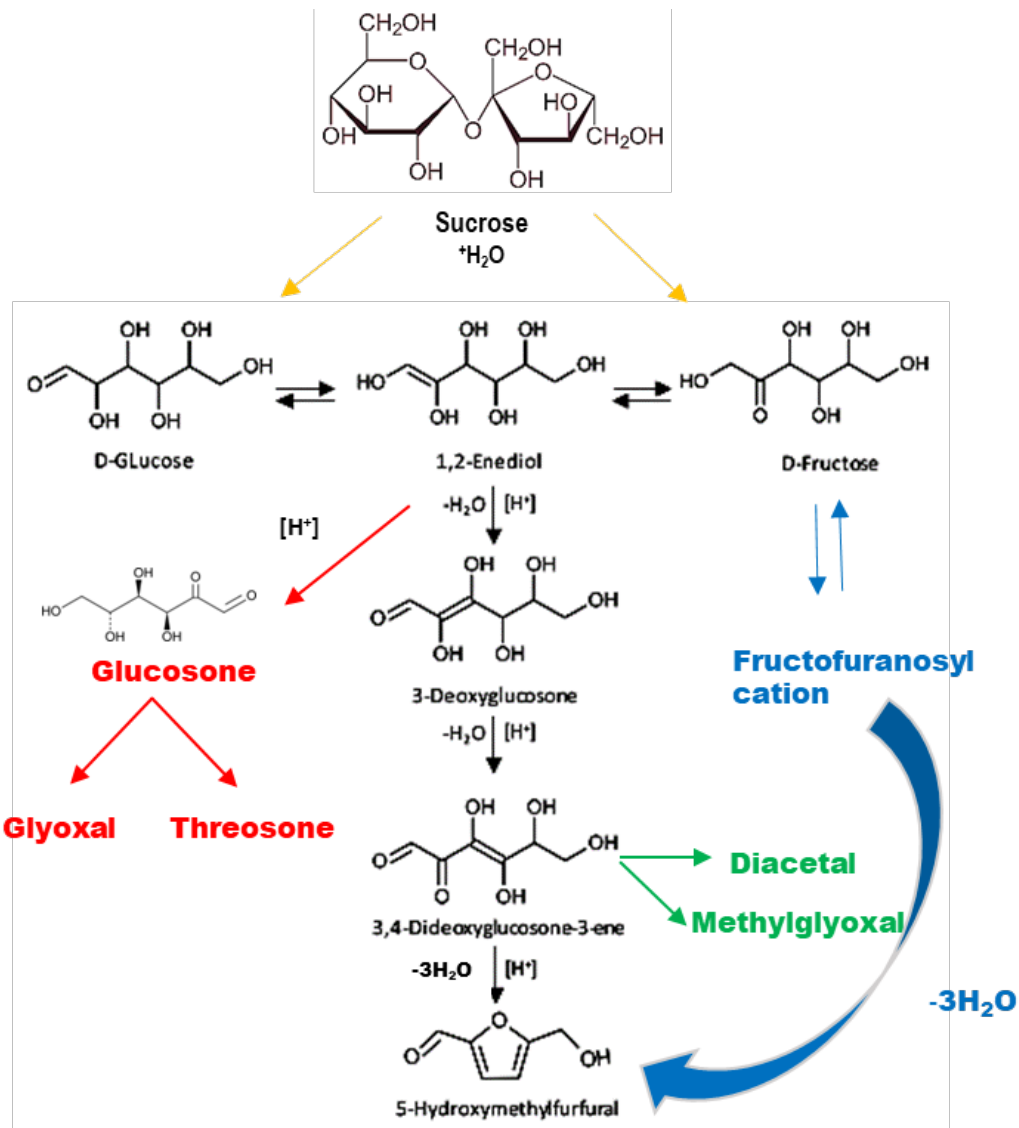
Figure 2.3: Proposed pathway of acid-catalysed degradation of sugars as adopted from Buve *et al.* (2021).

2.7.1 Acid-catalysed sugar degradation in fruits and fruit-based products

Fruit and fruit-based products, like apple and pear purees and orange juice, contain mainly three sugars: namely glucose, sucrose and fructose, with other complex carbohydrates like pectin, hemicellulose, oligosaccharides, etc. (Garza *et al.*, 2000; Pham *et al.*, 2020). In Food Science literature the concept of sugar concentration is commonly referred to as total sugar content, total soluble solids, or total dissolved solids expressed as °Brix (°B) (Wibowo *et al.*, 2015c; Pham *et al.*, 2019; Somjai *et al.*, 2021). Alternatively, the reducing sugar content can be measured, however, the limitation in this is that the contribution of sucrose is overlooked. Therefore, reducing sugars must be determined in conjunction with total sugar content as

determined by Liao *et al.* (2020) and Aktag and Gokmen (2021). Acid-catalysed degradation of sugars can be monitored by tracking sugar consumption *via* changes in °B, total or reducing sugar content of the product. However, to obtain an accurate measure of NEB, changes in sugar content must be done in conjunction with identification of specific intermediate or end-products.

Garza *et al.* (1999, 2000) reported variations in sucrose, fructose and glucose in peach and apple puree heated at 80 – 98°C for 8 hrs. An initial increase in fructose and glucose was due to sucrose hydrolysis, which was counteracted as the two participated in acid-catalysed dehydration to form HMF. Pham *et al.* (2019) also highlighted the process of sucrose hydrolysis as a step required prior to acid degradation of sugars to form HMF during storage of orange juice. Contrary to findings of Garza *et al.* (1999, 2000), Pham *et al.* (2019) observed that sucrose reduction did not coincide with an increase in fructose and glucose, alluding to the fact that complex carbohydrates such as pectin and hemicellulose were also hydrolysed to yield fructose and glucose. Based on the anomalies stated above, Buvé *et al.* (2021) cautioned against making conclusions about sugar degradation solely based on changes in total and reducing sugar content.

Other crucial aspects in sugar degradation relating to sugar, are the concentration, type and most importantly the pH of the food product. Concentration has a direct effect on the content of NEB reaction products formed. An increase in sugar has been proven to have a positive correlation with HMF, furfural and other dicarbonyl compounds. The HMF content of red and white grape juice concentrates increased as the total sugar increased from 15 to 45, and then 65°B. Hydroxymethyl furfural was more pronounced in white than in red grape concentrates (Simsek *et al.*, 2007). Although red grape juice contained 3% more sugar, its pH averaged around 3.7, while white grape juice was lower at 3.3. In this case, pH was the rate-determining factor. The effect of low pH on HMF formation *via* sugar degradation was reported by Burdurlu *et al.* (2006) on fruit juice concentrates of pH ranging from 1.82 – 3.23, and the lowest activation energy (Ea) of 43 kcal.mol^{-1} for HMF formation was reported for lemon juice at pH 1.82, increasing to 80.02 kcal.mol^{-1} for orange at pH 3.2. In addition to sugar degradation, Burdurlu *et al.* (2006) attributed HMF formation to both AA and acid-catalysed degradation. Pham *et al.* (2020) noticed negligible changes in sugars of control samples at pH 3.8 and 4.5 of pasteurised orange juice, while drastic hydrolysis of sucrose was observed for samples adjusted to pH 1.5 and 2.5. It is worthy to mention that sucrose hydrolysis was complete after 1 day and 2 weeks of storage for the pH 1.5 and 2.5 samples, respectively. Furthermore, these authors concluded that lowering the pH from 3.8 to 1.5 increased the degradation rate constant of AA, sucrose inversion, and the formation rates of furfural and HMF. Regarding sugar type, it has been established that monosaccharides react faster than disaccharides, since the latter requires the step of hydrolysis. In addition, the ketohexoses react faster than aldohexoses (Agcam, 2022). For instance, HMF can be formed via direct dehydration of fructose, meanwhile

glucose requires the steps of enolisation to 1,2-enediol, dehydration to 3-deoxyglucosone and then further dehydration to form HMF (Agcam, 2022). Hence, expectedly, Gürsul Aktağ and Gökmen (2020) found that HMF formation via dehydration of the fructofuranosyl cation was 2, 4 and 7 times higher in peach nectar, apple and orange juice compared to the 3-deoxyglucosone pathway due to a high fructose content. The fast reactivity of fructose is due to it being available as an open chain in aqueous media, thus making it more readily available to react compared to glucose's reactivity which is limited by its ring opening step (Paravisini & Peterson, 2018).

2.8 The Maillard reaction (MR).

The MR is the most common type of NEB reaction occurring in food during processing and storage of food, but not fruits and fruit-based products. This is mainly due to the acid nature of these products. However, when it does take place, it is usually preceded by ascorbic acid (AA) degradation. The MR occurs when a carbonyl-containing compound, namely reducing sugars, aldehydes or ketones condenses with a free amino group of amino acids, peptides or proteins during processing and prolonged storage of food products. The reaction is divided into early, intermediate and advanced stages with each resulting in a myriad of reaction products termed Maillard reaction products (MRPs). Processes such as roasting, baking or frying result in the formation of colour and flavour which are amongst the favourable effects of the MR, on the other hand, during drying, pasteurisation, sterilisation and storage the occurrence of the MR is unfavourable due to discolouration, off-flavour formation as well as reduction in nutritional value. Owing to this, it is essential to better understand the MR progression in order to control the formation of desirable attributes, while ensuring and preventing or minimizing undesirable effects.

The first step of the MR involves the condensation of the carbonyl group of a reducing sugar and a free amino group of an amino acid (Rufian-Henares & Pastoriza, 2016). This forms a reversible Schiff base adduct *N*-substituted glycosylamine, which due to its instability further rearranges to form a stable 1-amino-1-deoxy-2-ketose or 2-amino-2-deoxy-1-aldose, respectively, also known as Amadori or Heyns products (Figure 2.4 as adopted from (Rufian-Henares & Pastoriza, 2016)). Depending on which reducing sugar participated in the Maillard reaction, the rearrangement is either called Amadori, if an aldose sugar like glucose participated, or Heyns rearrangement if a ketose like fructose participated in the reaction. These products are colourless or slightly yellow compounds and are known as intermediate Maillard reaction products (IMRPs) and are referred to as pre-melanoidins or low molecular weight fractions (LMWF). As the reaction proceed, more complex dark brown and fluorescent products are formed. The colour changes and intermediate products formed are used as an indicator of the progression of the reaction and to identify the stages of the MR (Rufian-Henares & Pastoriza, 2016).
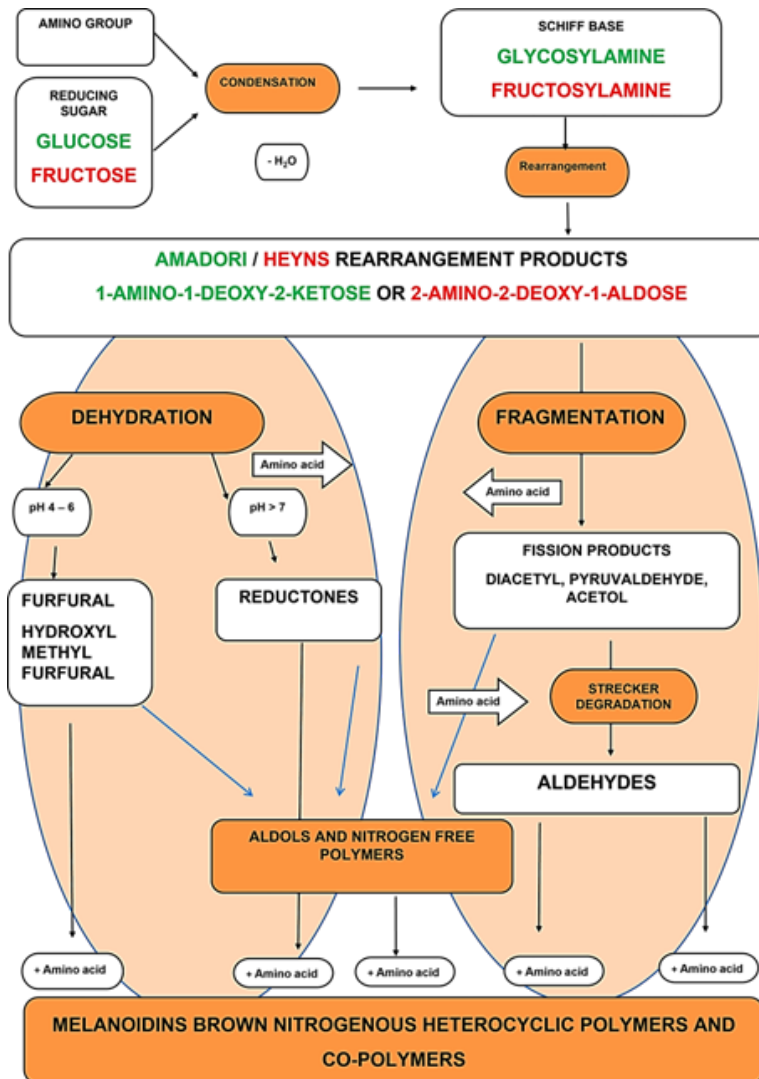
Figure 2.4: Proposed Maillard reaction schematic as adopted from (Rufian-Henares & Pastoriza, 2016).

The intermediate stage begins when Amadori and Heyns products degrade, resulting in the formation of new molecules *via* dehydration, fission and Strecker degradation. The newly formed compounds contribute to the flavour and aroma and vary in their characteristics depending on the conditions employed. At this stage, pH and reactant type are the important rate-determining factors. The sugar moiety of the Amadori/Heyns products is degraded into deoxyosones and various carbonyl compounds such as methylglyoxal and glyoxal, resulting in the release of the amino group. The subsequent pathways of deoxyosones are mainly governed by the pH. At a pH below 5, their fragmentation leads to the formation of 3-deoxyosone, but if the pH is above 7, the pathway involving the 1- and 4-deoxyosones prevails. However, most food products are in the pH range 4 – 6 where the 3-deoxyosones route predominates. Furthermore, dehydration of 1-deoxyosone and 3-deoxyosones leads to the

formation of reductones and furfurals, respectively. Specifically, 3-deoxyosones forms furfural when a pentose sugar is involved or hydroxyl methyl furfural (HMF) when a hexose sugar is involved. The Amadori rearrangement products have been reported to be less reactive than reductones and methylglyoxal, but 10 – 100 times more reactive than the parent reducing sugars (Wang *et al.*, 2009). During the intermediate stage, amino acids act only as catalysts, but in the final stage they participate in the Strecker degradation reaction. In this reaction, the amino acids are degraded by pre-formed dicarbonyl compounds leading to the oxidative deamination and decarboxylation of amino acids to form aldehydes with one carbon atom less than the original amino acid. Furthermore, Strecker aldehydes are major contributors to the aroma of thermally processed food. Some examples include 3-methyl butanal, methional, and phenylacetaldehyde which have been identified in yeast extracts, boiled chicken and beef, roasted coffee, chocolate and various other food products. These are potent aroma components with extremely low odour threshold (Rufian-Henares & Pastoriza, 2016).

In the final stage of the reaction, aldehydes, reductones and other dicarbonyls polymerise in the absence of amino acids to give aldols and high molecular weight nitrogen-free polymers, while in the presence of amino acids, brown-coloured nitrogen polymers and co-polymers, called melanoidins, are formed. Although the structure of melanoidins is not well understood, it is known that it is the main contributor of colour in food (Pérez-Burillo *et al.*, 2019).

2.8.1 Maillard rection in fruit and fruit-based products.

The high sugar content and low pH conditions of fruits juice concentrates are usually synonymous with AA degradation and sugar degradation (caramelisation), however, Wang *et al.* (2006) solely attributed the browning and HMF development in carrot juice concentrates during storage to the MR. Zhu *et al.* (2009) reported the formation of HMF in pasteurised apple juice (95°C:30 and 60 min) *via* dehydration of sugars, However, a sudden decrease after 6 days of storage at 20°C was observed due to condensation between HMF with available amino acids taking place *via* the MR. In another study, the HMF of orange juice heated at 90, 105 and 120°C was lower for the latter two temperatures. They found that HMF quickly decomposed in fruit juice-based medium containing amino acids *via* the MR. Moreover, they concluded that fructose had remarkably higher potential to induce HMF. Canned peaches in syrup with a final pH of 3.7 were found to have simultaneously undergone AA and acid-catalysed sugar degradation during the initial stages of storage, followed by the MR in the later stages of the 365 days storage at 30°C. The reason for this is that, during initial storage days, the acidic pH 2.7 favoured the former reactions. Although it is common knowledge that the classical MR *via* condensation of sugar and amino acid is not favoured by pH < 4.0, it is the reactive carbonyl species (RCS) originating from AA and sugar degradation that condense with amino acids, and these are termed Maillard-associated reactions (Pham *et al.*, 2020).

Testament to this, Gursul Aktag and Gokmen (2021) reported a decrease in free amino acids of apple at 33, 58 and 77% and pomegranate concentrate at 37, 67 and 87% as the sugar increased from 30 to 50, and 65°B. In addition, the same study also reported 71, 83 and 76% decrease in free amino acids of blueberry, raisins and dates stored at 20°C for 6 months. The changes observed in free amino acid content was presumptive of the MR. A negative correlation of 0.861 between the total free amino acid and of 3-DG content of dates during storage led to the MR. Considering that the MR is favoured at neutral pH, it is important to mention that that the initial pH of dates was the highest at 6.61, compared to 3.79 and 2.64 of raisin and blueberry, respectively. Furthermore, mass spectra of Schiff base and adducts between amino acid asparagine and RCS (HMF and 3DG) proved the occurrence of the MR during storage of apple concentrate and raisins. Notwithstanding their highly acidic intrinsic nature, the MR could take place in dried fruits and juice concentrates due to a favourable a_w of 0.6 – 0.8. Contrary to the above findings, earlier work of Aktağ and Gökmen (2020) observed no significant changes in free amino acids of peach, apple and orange juice stored at similar conditions as concentrates. The most probable explanation could be that the a_w of 0.9 of juice was a rate limiting factor for the MR. The effect of the MR on browning of fruit juice has been long underestimated. This might be due to all NEB reactions taking place simultaneously in a product and based on the conditions of pH, a_w composition, the MR being ruled out erroneously. As a result, reactant isotope labelling was sought and suggested as an ideal approach to track the precursors and intermediate compounds of NEB, in particular the MR (Pham *et al.*, 2021; Agcam, 2022). With isotope labelling, Paravisini and Peterson (2019) estimated that the MR generated up to 70% and AA 30% of the total browning of orange juice, with fructose being the main precursor of 3-DG (62%), MGO (75), GO (78%) and AT (26%).

2.9 The multidimension of NEB reactions

Numerous authors have reported on the contribution of AA, acid-catalysed sugar degradation and the MR on NEB reactions in fruit and fruit-based products. To a large extent NEB-related research focus on the quantitative changes in the initial reactants (reducing sugars, amino acids, or ascorbic acid), changes in browning indices as well as formation of chemical markers (intermediate RCS). Unfortunately, these NEB reactions rely on a pool of similar reactants, environmental conditions, and may form the same intermediate compounds. In complex food systems these reactions may take place simultaneously. For instance, Liao *et al.* (2020b) observed that the browning rate as measured by 420 nm was faster than HMF formation which revealed that more than one NEB reaction was taking place (Liao *et al* 2020). The interdependency of various NEB factors, as well as transient nature of intermediate compounds may lead to under-and overestimating the effect of one variable or pathway over another. Hence, the application of kinetics in conjunction with isotope labelling can solve the problem relating to the effect of precursor on the identification of specific compound formation.

NEB related reactions in fruit and fruit-based products and compounds formed as a result have been successfully defined using kinetic modelling. Garza *et al.* (2000) established that sucrose reduction in apple puree followed first-order kinetics, whilst HMF formation was characterised by both first and second-order. Similarly, first-order kinetic modelling was established for browning and HMF development in carrot juice concentrate during storage (Wang *et al.*, 2006), ascorbic acid degradation in citrus juice concentrates (Burdurlu *et al.*, 2006), orange juice (Tiwari *et al.*, 2009) and pasteurised mango juice (Wibowo *et al.*, 2015a). However, in the same study Wibowo *et al.* (2015a) classified colour and HMF formation as best described by zero-order models. This happens to contradict the afore-mentioned results in terms of the modelling using kinetics. This is a common occurrence in NEB related research and is usually due to the complexity of the reactions as well as composition of the food system.

2.10 The role of plant extracts in inhibiting NEB reactions

Recent trends in alleviating thermally induced toxic compounds focus on the utilisation of plant extracts (Wu *et al.*, 2009; Oral *et al.*, 2014; Favre *et al.*, 2020). This follows the current trend of using natural ingredients as an alternative to synthetic additives, and optimisation of production processes for improving safety of processed and stored foods. Chemical inhibitors are the most preferred choice due to low cost and high performance. Amongst them, sulphites have proven to be superior in inhibiting NEB reactions, in particular the MR. Sulphites are known to exhibit their mode of action via blocking the initial condensation step by attaching to the carbonyl of sugars, as well as stabilising the intermediate HMF from reacting further (Bharate & Bharate, 2014). Another synthetic AGE inhibitor, aminoguanidine (AG), is known as an excellent trapper of MGO (Khan *et al.*, 2020). However, both these have been implicated in terms of safety when incorporated in food products. Sulphites have been subjected to increased regulatory scrutiny due to association with initiating reactions in asthmatic individuals (Vally *et al.*, 2009) and that it reduces the uptake of the B vitamin thiamine. On the other hand, AG suffers from safety concerns due to possible side effects (Wu *et al.*, 2009) related to weakened liver, anaemia, vomiting, gastrointestinal disorders, diarrhoea, dizziness, headache and flu (Wang *et al.*, 2016; Khan *et al.*, 2020).

Due to the critical role that NEB reaction end-products play in food quality and glycation, there is a need to find natural additives which exhibit both anti-browning and antiglycation properties to be delivered through the diet. Therefore, the best alternative is the utilisation of the plant extracts (Yeh *et al.*, 2017). Many plants extracts, particularly medicinal types, have been studied for their antioxidant capacity due to their versatile bioactive constituents, such as vitamins, minerals, and polyphenols. Intake of foods with antioxidants have beneficial effects on human health, showing strong evidence that regular dietary intake of inherent antioxidants is associated with lowering risks of degenerative diseases, particularly cardiovascular diseases

and cancer (Pinho *et al.*, 2014). In this study, the antioxidant and anti-browning properties of crude extracts derived from the green rooibos plant were investigated.

The indigenous South African plant species *Aspalathus linearis*, better known as rooibos, is native in the Cederberg of Western Cape (South Africa) (Joubert & DeBeer, 2011). *Aspalathus linearis* is known for its commercial use as rooibos tea, where it is commercialised in two different forms: unfermented (green) and fermented (red) rooibos. *Aspalathus linearis* infusions have received great attention in recent years due to their bio-functional properties, with numerous studies having been conducted on health-promoting properties associated with rooibos. These include antioxidant (Villaño *et al.*, 2010), anti-inflammatory (Ku *et al.*, 2014), anti-carcinogenic (Marnewick *et al.*, 2005), antimicrobial, anti-obesity and hypoglycaemic (Beltrán-Debón *et al.*, 2011) activities. These authors reported the presence of polyphenols as the responsible compounds for the above-mentioned properties (Joubert & DeBeer, 2011).

2.10.1 Polyphenols as major bioactives in plant extracts

Polyphenols are secondary plant metabolites involved in defence against ultraviolet radiation or attack by pathogens (Pinho *et al.*, 2014). They are found largely in fruits, vegetables, cereals, and beverages (Shahidi & Ambigaipalan, 2015). In food products, polyphenols may impart sensory attributes such as bitterness, astringency, colour, flavour, odour, and in terms of functionality, stability against spoilage related to microbes and oxidation (DeBeer *et al.*, 2019). Of the prior mentioned varieties, green rooibos extracts and infusions have been proven to exhibit significantly higher antioxidant content compared to their red counterpart. This is mainly due to the decrease of the chief polyphenols aspalathin and nothofagin during fermentation. Evident to this were findings of Joubert *et al*. (2004), who reported a reduction in aspalathin content in ethyl acetate-soluble fractions of green rooibos extracts from 547 to 36.4 mg.g^{-1} after fermentation. Aspalathin and nothofagin are the major contributors to the total polyphenolic content, which in turn contributes the most towards bio-functional properties (Villaño *et al.*, 2010; Monsees & Opuwari, 2017; Damiani *et al.*, 2019; Lawal *et al.*, 2019). These two polyphenols fall under flavonoids, which is one of the four major classes of polyphenols, with the other three: phenolic acids, stilbenes and lignans. Not only flavonoids, but also phenolic acids are of interest in the present study, since they have been proven to exhibit anti-glycation activity *in vitro* (model and real food systems), as well as *in vivo* (Yeh *et al.*, 2017).

Many studies have been conducted and confirmed that different polyphenolic substances possess anti-glycation activities *in vivo* and *in vitro*, and that they could also prevent RCS production during food processing and storage (Wang *et al.*, 2016; Yeh *et al.*, 2017; Khan *et al.*, 2020). The structure and type of polyphenols is greatly associated with the extent of anti-glycation activities (Khalifa *et al.*, 2019). For instance, the more the hydroxyl

groups, the better the activity. Phenolic acids with three or more hydroxyl (OH) groups exhibited higher MGO trapping abilities (Yeh *et al.*, 2017).

2.10.2 Plant extracts mechanism of inhibition of NEB reactions using plants extracts

Several mechanisms of inhibition have been confirmed and elaborated based on the different stages of glycation. During the initial stages of the MR, polyphenols block the carbonyl or dicarbonyl groups of sugars preventing the production of Schiff bases (Wang *et al.*, 2016), and consequently the Amadori products (Khalifa *et al.*, 2019). When Schiff bases are formed, they become prone to oxidation and produce free radicals and RCS, with epigallocatechin gallate (EGCG) having been found to scavenge these free radicals (Wang *et al.*, 2016) and significantly reduced browning in a glucose-glycine model system, apple sauce and bread roll formulation, compared to the control sample (Favreau-farhadi *et al.*, 2015). Furthermore, of special interest to the present study in relation to possible anti-glycative properties of green rooibos based on the above finding, it is worth to mention that Joubert and DeBeer (2014) found that aspalathin's antioxidant capacity compares to that of EGCG. Moreover, most of these polyphenols whose anti-glycation activities are noted are constituents of green rooibos, and it is reasonable to project that they would exhibit these properties in apples canned in fruit juice in the current study. Moreover, the said oxidation is usually catalysed by metals. In addition, quercetin was found to inhibit AGE production through trapping and blocking MGO and GO, as well scavenging free radicals (Yeh *et al.*, 2017). Ferulic acid has been found to inhibit dicarbonyl (Wu *et al.*, 2009; Yeh *et al.*, 2017), formation and prevent CML and CEL developments in model systems, however, its efficacy gravitates more toward prevention of late stage glycation (Srey *et al*., 2010). The anti-glycation action was credited to the antioxidant property of FA.

In addition, authors who studied the anti-glycative effect of plant extracts proved that these plant extracts exhibited considerably better anti-glycation than the identified individual polyphenols contained in the said extracts (Favreau-farhadi *et al.*, 2015). A case in point, the aforementioned study of Wu *et al.* (2009) also reported that guava leaves and fruit extracts exhibited excellent metal chelating (MTC) activities, as well as inhibiting the progression of early, intermediate and final stages of the MR. Amongst the extracts, Shi Ji Ba, Ta Ba guava leaves and whole fruit extracts at 100 μg.mL^{-1} exhibited 7, 14 and 19% inhibitory against Amadori rearrangement products (ARP), and 28, 36 and 14% against formation of bovine serum albumin BSA-glucose α-dicarbonyl, respectively. In addition, these guava extracts were compared to commercial AG which exhibited 39% inhibition against glucose-BSA α-dicarbonyl. The inhibitory potency of guava extracts was due to the presence of four main polyphenols, phenolic acids (ferulic and gallic acid) and flavonoids (catechin and quercetin). Zhao *et al.* (2022) made comparisons between a plant extract and their individual primary polyphenols. They revealed that chokeberry extracts exhibited the lowest 2,2'-azino-bis(3-

ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging, Ferric reducing antioxidant power (FRAP) and MTC compared to cyanidin-3-galactoside (Cy-3-gal), chlorogenic acid (CA), epigallocatechin gallate (EGCG) and quercetin (QC), nonetheless, exhibited better DPPH radical scavenging activity than CA. However, chokeberry extracts exhibited the highest inhibition against BSA-glucose α-dicarbonyl at 48%, with the primary polyphenols' inhibition ranging between 15 – 44%.

2.10.3 Limitations of plant extracts

Although these plant extracts prove to exhibit excellent anti-browning activities *in vitro*, the final intended application is in food processing and storage. However, crude plant extracts or purified individual polyphenols thereof have been reported to affect colour changes when incorporated in food or model systems. This might be influenced by either natural pigmentation, or reaction of polyphenols with food components resulting in colour augmentation. These changes are also accelerated by high temperatures employed during common food preservation methods such as drying, pasteurisation and sterilisation, leading to reduced bioactivities.

Concerning pure polyphenols, at concentrations of 1 – 1.5% EGCG successfully reduced the browning rate of bread rolls. However, at increased concentrations of 3% it exhibited pro-oxidative activity (Favreau-farhadi *et al.*, 2015). Qi *et al.* (2018) also reported the efficacy of 0.01 – 0.1 mg.L^{-1} flavan-3-ols against the MR, however at 0.3 – 1 mg.L^{-1} darkening occurred due to autoxidation at high temperatures.

2.11 Encapsulation of plant extracts

The aforementioned instabilities of polyphenols during heat processing and storage may pose a challenge against using native plant extracts (Albahari *et al.*, 2018). Consequently, development of encapsulation methods and delivery systems containing specific compounds can improve some physicochemical properties of these extracts in food products. Encapsulation of sensitive compounds provides improved stability during processing, and in the final product by preventing reaction with other components in food products such as oxygen or water (Nesterenko *et al.*, 2013). The release of microparticle content at controlled rates can be triggered by shearing, solubilisation, heating, pH or enzyme activity of these agents naturally present in certain plants, ensuring their stability. Common encapsulation material includes carbohydrates, protein and lipid polymers such as maltodextrin, inulin (Human *et al.*, 2020), soy protein isolates, sodium alginate and cyclodextrins to mention a few. The choice of polymer is crucial as it affects how the active compound is released. For instance, Hidalgo *et al.* (2018) reported on elevated furosine levels in soy protein isolates applied to encapsulate beetroot pomace due to thermal treatments. Similarly, maltodextrin reacts with glycine to form browning compounds (DaSilva, 2018). Lavelli and Sri Harsha, (2019) reported that sodium

alginate encapsulated grape skin extracts exhibited reduced potential to inhibit glycation of fructose-BSA and MGO-BSA model systems, this was due to some phenolics interacting with alginate, resulting in a decreased release. Therefore, the encapsulating polymer should not participate in any chemical reaction during processing or storage. Cyclodextrin were the obvious choice since they were proven not to participate in NEB reactions (DaSilva, 2018). Moreover, unlike other potential encapsulants, β-CD is also known to aid in extraction of these polyphenols from their sources, hence the term "β-CD assisted extracts" (Favre *et al.*, 2018; Maraulo *et al.*, 2021).

Many techniques applied to recover antioxidants from plants, such as Soxhlet extraction, are coupled with maceration, and are also dependent on the extracting solvent used for extraction. Water is the safest solvent that may be used for extraction of bioactive compounds from plant material, however its capability is limited to polar compounds, and also results in lower extraction efficiency (Miller, 2016). Polar organic solvents, on the other hand, are frequently applied for recovery of polyphenols from plant material. Solvents frequently used contain an aqueous mixture of ethanol, methanol, acetone, or ethyl acetate. Although these solvents are effective in increasing the extraction yield, enormous volumes are used, and they are not environmentally friendly, by contributing to pollution. Hence "greener" extraction procedures are sought to reduce the environmental impact caused by these solvents (Cai *et al.*, 2018; Wang *et al.*, 2019).

2.11.1 Beta-cyclodextrin assisted extraction of plant extracts.

Alternative green extraction methods, such as supercritical fluid and water extraction, ultrasound and pulsed electric field-assisted extraction are considered to reduce the use of organic solvents (Albahari *et al.*, 2018). However, the cost associated with procurement of these alternative technologies might prohibit their implementation. It is in the light of this information that the present study focused on exploring the combination of water and beta-cyclodextrin (β-CD).

Cyclodextrins are made up of cyclic oligosaccharides consisting of a number of α (1→4) linked D-glucose subunits with a truncated cone spacial geometry. The most common forms, alpha (α), beta (β) and lambda (γ-CDs), are composed of six, seven and eight glucose units, respectively (Cai *et al.*, 2018). These molecules are widely used in the food, pharmaceutical and chemical industries for their ability to form host-guest inclusion complexes with a wide range of bioactive compounds (Haidong *et al.*, 2011; Cai *et al.*, 2018). This results in modification of the physicochemical properties of the encapsulated compound leading to improved rheological and structural properties such gelling, viscosity, solubility and stability (Navarro-orcajada *et al.*, 2020). Maraulo *et al.* (2021) proved that β-CD enhanced the physical properties of olive pomace extracts via improved heat stability and reduced hydroscopicity, in addition to increased antioxidant activity. Numerous studies have been conducted where the

ability of CDs to improve the extraction of polyphenols from plant matrices was investigated. In these studies, extraction parameters, such as type of CD and concentration, temperature, and time are likely to influence the process, having possible interactions among the variables (Ratnasooriya & Rupasinghe, 2012; Aree & Jongrungruangchok, 2016; Cai *et al.*, 2018; Mourtzinos *et al.*, 2018). For instance, the extraction of some phenolic compounds from plants with different aqueous CD solutions has been demonstrated to be an efficient and eco-friendly extraction process (Parmar *et al.*, 2015). A study by Ratnasooriya and Rupasinghe (2012) proved that amongst α-CD, γ-CD and β-CD, on average the latter was the most effective in recovering stilbenes, flavonols, and flavan-3-ols from grape pomace. In another study, Rajha *et al.* (2015) compared the efficacy of solvents, namely water, methanol and hydro-ethanol with that of a β-CD-water solution (37.7 mg.mL^{-1}) to extract polyphenols from vine shoots. Results showed that it took ten hours' extraction time to attain the same polyphenolic yield with pure water, compared to five hours using the β-CD solution. In addition, β-CD-assisted extraction also resulted in higher content of polyphenols than hydro-ethanol. The encapsulation role of β-CD extracted polyphenols played a role in increasing stability against degradation by oxidation compared to methanolic extracts. Diamanti *et al.* (2017) reported similar results where β-CD enhanced the total phenolic content and the radical scavenging activity of whole pomegranate extracts.

Cyclodextrins have been proven to effectively enhance extraction of polyphenolic compounds from plant material. Numerous authors who investigated application of β-CD to enhance the extraction and encapsulation of polyphenols from plant materials used response surface methodology (RSM) to select optimal extraction parameters that resulted in high yield and improved functional properties (Haidong *et al.*, 2011). Response surface methodology is a collection of statistical and mathematical techniques useful for development, improvement, and optimisation of products and processes (Miller, 2016; Favre *et al.*, 2018), in this case the yield of bioactive compounds. This technique is applied particularly when several input variables can potentially influence some performance measures or quality characteristics of the product/process. In studies conducted by the above-mentioned authors, some of the parameters that were optimised include β-CD concentration, solvent to plant material/polyphenol ratio (Koteswara *et al.*, 2020), extraction temperature and time (Rajha *et al.*, 2015; Mourtzinos *et al.*, 2018; Wang *et al.*, 2019). In instances where extraction was used in conjunction with a technology such as ultrasound, parameters associated with the specific technology were also optimised (Albahari *et al.*, 2018; Favre *et al.*, 2018).

In terms of h β-CD encapsulated plant extracts inhibition of NEB reaction products, Favre *et al.* (2018;2020) proved that β-CD-assisted extracts of thyme and green pepper were effective in retarding browning development (A$_{420nm}$) and HMF formation in glucose-BSA model

systems, correspondingly. Furthermore, the inhibition of browning development and HMF formation was correlated with antioxidant activity.

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CHAPTER 3: ANTIOXIDANT ACTIVITY OF BETA-CYCLODEXTRIN-ASSISTED EXTRACTS OF GREEN ROOIBOS (*ASPALATHUS LINEARIS*)

3.1 Abstract

The present study investigated the use of aqueous solutions of beta-cyclodextrin (β-CD) to optimise the extraction of green rooibos. A Taguchi orthogonal array design L_9 (3^3) with β-CD concentrations (0 – 15 mM), extraction temperatures (40 – 90°C) and time (15 – 60 min) together with response surface methodology was applied to obtain crude extracts with the highest total polyphenolic content and antioxidant activity. The optimal conditions of green rooibos extraction were: 15 mM β-CD, temperature of 40°C and time of 60 min, which yielded a total polyphenol content (TPC) of 398.25 mg GAE.g^{-1}, metal chelation activity (MTC) 92.95%, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging of 1689.70 µmol TE.g^{-1}, ferric reducing/antioxidant power (FRAP) of 2097.53 µmol AAE.g^{-1}, oxygen radical absorbance capacity (ORAC) of 11162.82 TE.g^{-1} and the flavonoids aspalathin 172.25, hyperoside 29.27, orientin 8.86, iso-orientin 7.93, vitexin 2.53, iso-viten 2.31, quercetin 0.137, luteolin 0.070 and chrysoeriol 0.060 mg.g^{-1}. To ascertain the contribution of TPC towards the antioxidant assays, positive strong correlations were observed with MTC ($r^2 = 0.929$), ABTS ($r^2 = 0.961$), FRAP ($r^2 = 0.978$) and ORAC ($r^2 = 0.956$) all at $p < 0.001$. Furthermore, correlations between antioxidant assays were also revealed (r^2 0.839 – 0.955).

Keywords: Green rooibos; β-cyclodextrin extraction, Antioxidant activity, Polyphenolic content; Encapsulation.

3.2 Introduction

Many plants, particularly medicinal plants have been studied for their antioxidant capacity. Intake of foods with antioxidants have beneficial effects on human health, showing strong evidence that regular dietary intake of inherent antioxidants is associated with lowering risks of degenerative diseases, particularly cardiovascular diseases and cancer (Pinho *et al.*, 2014). The indigenous South African plant species *Aspalathus linearis*, better known as rooibos, grows naturally in the Cederberg area in the Western Cape Province of South Africa (Joubert & DeBeer, 2011). *Aspalathus linearis* is known for its commercial use as rooibos tea, where it is commercialised in two different forms: unfermented (green) and fermented (red) rooibos. *Aspalathus linearis* infusions have received great attention in recent years due to their bio-functional properties, with numerous studies having been conducted on health-promoting properties associated with rooibos. These include antioxidant (Villaño *et al.*, 2010), anti-inflammatory (Ku *et al.*, 2014), anti-carcinogenic (Marnewick *et al*., 2005), antimicrobial, anti-

obesity and hypoglycaemic (Beltrán-Debón *et al.*, 2011) activities. These authors reported the presence of polyphenols as the responsible compounds for the above-mentioned properties (Joubert & DeBeer, 2011).

Polyphenols are secondary plant metabolites involved in defence against ultraviolet radiation or attack by pathogens (Pinho *et al.*, 2014). They are found largely in fruits, vegetables, cereals, and beverages (Shahidi & Ambigaipalan, 2015). In food, polyphenols may contribute to the bitterness, astringency, colour, flavour, odour and oxidative stability (DeBeer *et al.*, 2019). Of the prior mentioned varieties, green rooibos extracts and infusions have been proven to exhibit significantly higher antioxidant content compared to their red counterparts. This is mainly due to the reduction of the chief polyphenols aspalathin and nothofagin during fermentation. Evident to this were findings of Joubert *et al*. (2004), who reported a reduction in aspalathin content of ethyl acetate-soluble fractions of green rooibos from 547 to 36.4 mg.g^{-1} after fermentation. Aspalathin and nothofagin are the major contributors to the total polyphenolic content, which in turn contributes more towards bio-functional properties (Villaño *et al.*, 2010; Monsees & Opuwari, 2017; Damiani *et al.*, 2019; Lawal *et al.*, 2019). These two polyphenols fall under flavonoids, which is one of the four major classes of polyphenols, with the other three: phenolic acids, stilbenes and lignans. Not only flavonoids, but also phenolic acids are of interest in the present study, since they have been proven to exhibit anti-glycation activity *in vitro* (model and real food systems), as well as *in vivo* (Yeh *et al.*, 2017).

For instance, Wu *et al.* (2009) investigated the effect of phenolic acids (ferulic and gallic acid) and flavonoids (catechin and quercetin), in comparison with aminoguanidine (AG) against the formation of dicarbonyl compounds and advanced glycation end-products (AGE) in a glucose-bovine serum albumin (BSA) model system. They found that the order of inhibition increased from ferulic acid < AG < catechin < gallic acid < quercetin. In this case, it is worthy to note that AG is a well-known synthetic anti-glycation agent (Yeh *et al.*, 2017; Lavelli & Sri Harsha, 2019; Khan *et al.*, 2020).

Epigallocatechin gallate (EGCG) significantly reduced browning in a glucose-glycine model system, apple sauce and bread roll formulation, compared to the control sample (Favreau-farhadi *et al.*, 2015). Furthermore, of special interest to the present study in relation to possible anti-glycative properties of green rooibos based on the above finding, it is worth to mention that Joubert and DeBeer (2014) found that aspalathin's antioxidant capacity compares to that of EGCG. Moreover, all these polyphenols whose anti-glycation activities were noted above, are found in green rooibos, and it is reasonable to project that they would exhibit those properties in the heat-processed apple slices in fruit concentrate in the current study. Similar observations were made by authors who studied the anti-glycative effect of plant extracts, and they proved that these plant extracts exhibited considerably better anti-glycation than the identified individual polyphenols contained in the said extracts (Favreau-farhadi *et al.*, 2015).

These authors reported on this for different guava leaf extracts, they attributed the effect to synergy between the different polyphenols (Favreau-farhadi *et al*., 2015).

Based on the afore-mentioned benefits, polyphenolic compounds have attracted the attention of the scientific community throughout the world. Moreover, there is a growing market globally for plant bioactive compounds with emphasis placed on finding more novel techniques that maximise extraction yield and exploit extractions from plant waste and by-products for potential use as nutraceuticals (Pinho *et al.*, 2014). There are many techniques to recover these antioxidants from plants, such as Soxhlet extraction coupled with maceration. However, extraction yield and antioxidant activity not only depend on the extraction method but also on the solvent used for extraction. Water is the safest solvent that may be used for extraction of bioactive compounds from plant material, however its capability is limited to polar compounds, and also results in lower extraction efficiency (Miller, 2016). Organic solvents, on the other hand, are frequently used for recovering polyphenols from plant matrices. The most suitable solvents are aqueous mixtures containing ethanol, methanol, acetone, or ethyl acetate. Although these solvents are effective in increasing the extraction yield, enormous volumes are used, and they are not environmentally friendly, hence, contributing to pollution. Hence "greener" extraction procedures are sought to reduce the environmental impact caused by these solvents (Cai *et al.*, 2018; Wang *et al.*, 2019).

Alternative green extraction processes which do not pose any danger to the environment, such as supercritical fluid and water extraction, ultrasound and pulsed electric field-assisted extraction are preferred over organic solvents due to concerns regarding their safety (Albahari *et al.*, 2018). However, the cost associated with procurement of these alternative technologies might prohibit their implementation. It is in the light of this information that the present study focused on exploring the combination of water and beta-cyclodextrin (β-CD).

Cyclodextrins (CDs) are cyclic oligosaccharides, typically containing six (α-CD), seven (β-CD), or eight (γ-CD) glucopyranose units with a truncated cone spacial geometry. These molecules are widely used in the food, pharmaceutical and chemical industries for their ability to form host-guest inclusion complexes with a wide range of bioactive compounds (Haidong *et al.*, 2011; Cai *et al.*, 2018). This results in modification of the physicochemical properties of the encapsulated compound leading to improved solubility, stability (Rajha *et al.*, 2015) and/or bioavailability, as well as their application in separation and purification operations.

Numerous studies have been conducted where the ability of CDs to improve the extraction of polyphenols from plant matrices was investigated. In these studies, extraction parameters, such as type of CD and concentration, temperature, and time are likely to influence the process, having possible interactions among the variables (Ratnasooriya & Rupasinghe, 2012; Aree & Jongrungruangchok, 2016; Cai *et al.*, 2018; Mourtzinos *et al.*, 2018). For instance, the extraction of some phenolic compounds from plants with different

aqueous CD solutions has been demonstrated to be an efficient and eco-friendly extraction process (Parmar *et al.*, 2015). A study by Ratnasooriya and Rupasinghe (2012) proved that amongst α-CD, γ-CD and β-CD, the latter was the most effective in recovering stilbenes, flavonols, and flavan-3-ols from grape pomace. In another study, Rajha *et al*. (2015) compared the efficacy of solvents, namely water, methanol and hydro-ethanol with that of a β-CD-water solution (37.7 mg.ml^{-1}) to extract polyphenols from vine shoots. Results showed that it took ten hours' extraction time to attain the same polyphenolic yield with pure water, compared to five hours using the β-CD solution. In addition, β-CD-assisted extraction also resulted in higher content of polyphenols than hydro-ethanol. The encapsulation role of β-CD extracted polyphenols played a role in increasing stability against degradation by oxidation compared to methanolic extracts. Diamanti *et al.* (2017) reported similar results where β-CD enhanced the total phenolic content and the radical scavenging activity of whole pomegranate extracts.

Cyclodextrins have been proven to effectively enhance extraction of polyphenolic compounds from plant material. Numerous authors who investigated application of β-CD to enhance the extraction and encapsulation of polyphenols from plant materials used response surface methodology (RSM) to select optimal extraction parameters that resulted in high yield and improved functional properties (Haidong *et al.*, 2011). Response surface methodology is a collection of statistics and mathematical-based techniques useful for development, improvement, and optimisation of products and processes (Miller, 2016; Favre *et al.*, 2018), in this case the yield of bioactive compounds. This technique is applied particularly when several input variables can potentially influence some performance measures or quality characteristics of the product/process. In several studies, some of the parameters that were optimised include β-CD concentration, solvent to plant material/polyphenol ratio (Koteswara *et al.*, 2020), extraction temperature and time (Rajha *et al.*, 2015; Mourtzinos *et al.*, 2018; Wang *et al.*, 2019). In instances where extraction was used in conjunction with a technology such as ultrasound, parameters associated with the specific technology were also optimised (Albahari *et al.*, 2018; Favre *et al.*, 2018).

Moreover, most authors who reported on the antioxidant activity of β-CD-assisted plant extracts, also reported on its protective effect against heat degradation, moisture uptake, oxidation as well as masking of off-flavours (Rakmai *et al.*, 2018; Dai *et al.*, 2019). These properties are crucial, bearing in mind the intended application of the green rooibos extracts in this study. Therefore, the current study was carried out with the aim of obtaining the β-CD concentration, time and temperature combination for the optimal recovery of polyphenols from green rooibos leaves. This involved comparing the extraction efficiency of β-CD to pure water, by quantifying the resultant polyphenol content and profile, as well as antioxidant activity of the extracts.

3.3 Materials and Methods

3.3.1 Green rooibos and reagents

The dry green rooibos leaves were obtained from the Oxidative Stress Research Centre at the Cape Peninsula University of Technology (Bellville, South Africa) sourced from (Rooibos Ltd., Clanwilliam, South Africa). Beta-cyclodextrin (β-CD) was purchased from Industrial Analytical (Kyalami, South Africa). Folin and Ciocalteu's phenol reagent, gallic acid monohydrate, and, 2,4,6-tri[2-pyridyl]-s-triazine, ascorbic acid, 2,2'-Azobis (2-methylpropionamidine) dihydrochloride, fluorescein disodium salt, Trolox, (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ethanol, polyphenolic standards quercetin and luteolin were purchased from Sigma-Aldrich (Kempton park, South Africa). Orientin, iso-orientin, vitexin, iso-vitexin, hyperoside and chrysoeriol were purchased from Extrasynthese (Genay, France). Aspalathin was a gift from Oxidative Stress Research Centre at the Cape Peninsula University of Technology (Bellville, South Africa). Ethylenediaminetetracetic acid (EDTA), glucose, potassium hydrogen phosphate, absolute ethanol, ascorbic acid, potassium ferricyanide, trichloroacetic acid, potassium persulfate, sodium chloride, acetic acid, ferrozine, ferric chloride, ferrous chloride acetonitrile and sodium carbonate, sodium acetate, hydrochloric acid, acetone, glacial acetic acid, perchloric acid, potassium-peroxodisulphate, citric acid and potassium metabisulphite were purchased from Merck (Modderfontein, South Africa). All chemicals used in this study were of analytical grade and chemical reagents were prepared according to standard analytical procedures. Prepared reagents were stored under conditions that prevented deterioration or contamination. The water used in this study was ultrapure water purified with a Milli-Q water purification system (Millipore, Microsep, Bellville, South Africa).

3.3.2 Solid liquid extraction of green rooibos plant

Green rooibos, as received, was coarsely milled (Fritsch, Germany) using a sieve with an aperture of 0.2 mm. The extraction of green rooibos was performed based on the method of Rajha *et al*. (2015) and Miller (2016) with slight modifications. Green rooibos leaves and 0 – 15 mM β-CD aqueous solutions in a 1:10 (w.v^{-1}) ratio in a 200 mL Schott bottle were homogenised using a polytron at 29 000 rpm for two min, followed by heating the mixture at 40 – 90°C on a temperature-controlled heating plate for 15 – 60 min with magnetic stirring at 1 500 rpm. The extracts were cooled immediately and centrifuged at 10 000 rpm for 15 min at 4°C. The supernatant was freeze-dried, and the resulting powder, termed crude green rooibos extract (CGRE), was stored in an air-tight container at – 20°C until further analysis.

3.4 Polyphenolic content

3.4.1 Total polyphenolic content (TPC) and quantification of major flavonoids

The TPC was determined following the Folin-Ciocalteu colorimetric assay described by Rajha *et al*. (2015) with slight modifications. A 1 mL aliquot of Folin-Ciocalteu reagent (10-fold diluted)

was mixed with 200 µL of CGRE in ethanol (0.5 mg.mL^{-1}). Then 800 µL of sodium carbonate solution (75 g.L^{-1}) was added. The mixture was incubated for 10 min at 60°C and then for 10 min at room temperature. The absorbance was measured at 750 nm using a spectrophotometer (Lambda 25, Perkin Elmer, Singapore). A calibration curve was drawn with gallic acid standards (10 – 100 µg.mL^{-1}). The total phenolic content was expressed as Gallic acid equivalent (mg GAE.g^{-1} of dry material).

Quantification of major flavonoids from (CGRE) was done following a modified HPLC method described by (Pantsi *et al.*, 2011). An Agilent Technologies 1200 Series HPLC (Santa Clara, CA, USA) system with a diode array detector and a 5 µM YMC-PackPro C18 (150 mm × 4.6 mm i.d.) column was used for the separation. Detection wavelengths were set at 280, 320 and 360 nm, and the mobile phases (A) was water containing 0.1% trifluoroacetic acid and (B) methanol containing 0.1% trifluoroacetic acid. The gradient elution started at 0 min 100 % (A) changing to 100 % (B) after 25 min. The flow rate was set at 1 mL.min^{-1}, the injection volume was 20 µL, and the column temperature was set at 21°C. Peaks were identified based on the retention time of the standards (orientin, iso-orientin, vitexin, iso-vitexin, hyperoside and chrysoeriol, quercetin, aspalathin and luteolin and confirmed by comparison of the wavelength scan spectra (set between 210 nm and 400 nm). The individual polyphenol content of the extracts was expressed as mg.g^{-1} of CGRE.

3.5 Antioxidant activity

3.5.1 Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power of CGRE samples was determined according to the method of Favre *et al*. (2020) with slight modifications. The FRAP reagent was prepared by mixing 2.5 mL of 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl with 2.5 mL of 20 mM FeCl$_3$·6H2O solution and 25 mL of 300 mM acetate buffer (pH 3.6). A 840 µL aliquot of freshly prepared FRAP reagent was mixed with 60 µL of each CGRE (0.5 mg.mL^{-1}). The solutions were kept for 30 min at 37°C followed by measuring the absorbance at 595 nm. The FRAP was expressed as ascorbic acid equivalent (µmol AAE.g^{-1} of dry material) using a standard curve.

3.5.2 Metal chelation (MTC)

The ferrous ion chelating effect of CGRE was determined following the method of Oh *et al*. (2013) with slight modifications. A 1 mL aliquot of each extract (10 mg.mL^{-1} in each respective solution of water or β-CD) was reacted with 100 µL of 2 mM ferrous chloride for 10 min, followed by addition of 100 µL of 5 mM ferrozine. After 10 min reaction time, 3 mL of the sample solution was added to the reaction mixture and allowed to react for a further 10 min. The absorbance of the mixture was measured at 562 nm using a spectrophotometer (Lambda 25, Perkin Elmer, Singapore). The percentage of chelating activity was calculated as follows:

$$\% \ Chelating \ activity = \left(1 - \left(\frac{A_s \ 562 \ nm}{A_c \ 562 \ nm}\right)\right) \times 100$$

Where: A_s is the absorbance of the sample at 562 nm, and A_c is the absorbance of the control at 562 nm.

3.5.3 Radical scavenging *via* 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS).

The Trolox equivalent antioxidant capacity was also applied based on the method of Damiani *et al*. (2019) with slight modifications. An ABTS stock solution of 7 mM was mixed with a 2.45 mM solution of a potassium persulfate in (1:1 v.v^{-1}) ratio. The mixture was left to react for 16 h until the reaction was complete. The resulting mixture was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm. The assay was initiated by mixing 2.7 mL of ABTS potassium persulfate solution with 0.3 mL of CGRE (0.5 mg.mL^{-1}). The absorbance at 734 nm (Lambda 25, Perkin Elmer, Singapore) was taken immediately after standing for 15 min. The ABTS was expressed as Trolox equivalent (µmol TE.g^{-1} of dry material) using a Trolox standard curve.

3.5.4 Oxygen Radical Absorbance Capacity (ORAC) assay.

Oxygen Radical Absorbance Capacity (ORAC) assay was employed following the method described by Ou *et al*. (2001) as modified by Del Castillo *et al*. (2005). Briefly, 2.225 mL of 48 nM fluorescein was premixed with sample (0.5 mg.mL^{-1} in ethanol), 375 µL Trolox (standard) or phosphate buffer and incubated for 30 s. This reading was the fluorescence at time zero. The assay was initiated by adding 375 µL of 143 mM 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH). Mixtures were kept in a waterbath at 37°C for 30 min. Fluorescence readings (λex = 493 nm and λem = 515 nm) were taken every 5 min after AAPH addition. The fluorescence decay curve was plotted and the area under the curve calculated. Blanks were prepared by replacing sample with phosphate buffer. Sample fluorescence values were corrected for the blank value. Scavenging activity of CGRE was expressed as Trolox equivalent (µmol TE.g^{-1} of dry material).

3.6 Experimental design and data analysis.

A Taguchi L9 orthogonal array experimental design was applied to investigate the optimal extraction conditions for maximum total polyphenolic content and antioxidant activity. As shown in Table 3.1, experiments were carried out with three factors at three levels resulting in 9 runs with independent variables (X) β-CD concentration (0 – 15 mM), reaction temperature (40 – 90 °C) and time (15 – 60 min) coded −1, 0, +1. The dependent variables (Y) were TPC, MT, ABTS, FRAP and ORAC. Optimisation to obtain the best combination that yields maximum

TPC and antioxidant activity of the β-CD-assisted extracts of green rooibos using response surface methodology (RSM). Statistical analysis was performed using SPSS 27.0 for Windows® and Design-Expert V8.0.6 software Microsoft Office 2007. Descriptive statistical analyses determined the mean and standard deviation of triplicates (n=3). Analysis of variance (ANOVA) established significant differences of the models and correlation coefficients. The level of confidence required for significance was selected at 95%.

3.7 Results and discussion

3.7.1 Fitting the model

Table 3.1 depicts the combinations of the coded input variables (β-CD concentration, reaction temperature and extraction time) and the resultant responses (TPC, MTC, ABTS, FRAP and ORAC). Response surface methodology applied revealed combinations that yielded the highest total phenolic content and antioxidant activity of crude green rooibos extracts. Experiment #3 (15 mM β-CD, temperature 40°C and time 60 min) was the optimal reaction conditions resulting in the highest ABTS (1689.70 µmol TE.g^{-1}) and FRAP (2097.53 µmol AAE.g^{-1}) values, meanwhile, for TPC, MTC and ORAC the distinction between 7.5 and 15 mM β-CD was not clear ($p > 0.05$). Nevertheless, overall extraction with β-CD proved to increase ($p < 0.05$) the polyphenolic content and antioxidant activity more than the water-based extracts, i.e. 0 mM β-CD.

The analysis of variance (ANOVA) as shown in Table 3.2 reveals that the experimental values of all response variables could be fitted on a model ($p < 0.0001$), thus proving that the significance of the model was higher than the aimed 95% confidence level. The insignificant lack of fit ($p > 0.05$) and the value of pure error prove the reproducibility of the experimental runs. The effect of each input variables on the response variables was determined. Varying the β-CD concentration had a significant effect ($p < 0.0001$) on all response variables. Reaction temperature (X_2) was significant ($p < 0.05$) for MTC, ABTS and ORAC only, and reaction time (X_3) was significant ($p < 0.05$) for ABTS, FRAP, and ORAC. In addition, interactions between β-CD concentration and temperature (X_1X_2) and temperature and time (X_2X_3) were only significant for ORAC. The coefficients of determination (R^2) for all models ranged between 0.8163 – 0.9537 %, which indicated that on average 88% changes in the response variable were as a result of the input variables, as well as an excellent fit of data on the mathematical model (Table 3.2).

Moreover, strong significant positive correlations between response variables were observed, with emphasis placed on the effect of TPC on all antioxidant assays (Figure 3.1).

Table 3.1: Coded independent and response variables for response surface design of crude green rooibos extracts.

Run	Extraction conditions			Response variables				
	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4	Y_5
	β-CD concentrations	Extraction temperature	Treatment time	Total polyphenolic content (TPC)	Iron chelation (MTC)	Radical scavenging (ABTS)	Reducing power (FRAP)	Total antioxidant activity (ORAC)
	(mM)	(°C)	(min)	(mg GAE.g^{-1})	%	(µmol TE.g^{-1})	(µmol AAE.g^{-1})	(µmol TE.g^{-1})
1	(+1)15	(1)90	(-1)15	374.73 ± 30.28[bcd]	70.99 ± 2.51[de]	1425.31 ± 69.58[bc]	1886.42 ± 138.72[c]	11791.39 ± 422.51[d]
2	(-1)0	(0)65	(1)60	299.5 ± 23.17[a]	44.00 ± 8.54[ab]	1092.0 ± 29.80[a]	157.50± 3.08[a]	7574.93 ± 119.89[a]
3	(+1)15	(-1)40	(1)60	398.25 ± 15.97[d]	92.95 ± 17.87[f]	1689.70 ± 23.88[d]	2097.53 ± 22.33[d]	11162.82 ± 104.32[cd]
4	(-1)0	(1)90	(0)30	281.7 ± 2.36[a]	38.00 ± 5.57[a]	1056.86 ± 62.84[a]	1472.78 ± 39.48[a]	6948.02 ± 391.44[a]
5	(0)7.5	(0)90	(1)60	361.84 ± 23.80[bc]	52.82 ± 3.20[abc]	1351.52 ± 14.79[b]	1830.63 ± 63.83[bc]	10813.97 ± 123.11[c]
6	(+1)15	(0)65	(0)30	387.81 ± 14.26[cd]	84.57 ± 13.01[ef]	1476.82 ± 89.61[c]	1927.37 ± 46.28[c]	11334.79 ± 270.66[cd]
7	(0)7.5	(0)65	(-1)15	351.62 ± 20.62[b]	59.09 ± 2.62[bcd]	134.96 ± 58.07[b]	1863.14 ± 140.69[bc]	9521.48 ± 356.62[b]
8	(0)7.5	(-1)40	(0)30	344.00 ± 2.48[b]	61.03 ± 2.93[cd]	1351.61 ± 28.58[b]	1732.12 ± 11.09[b]	8903.31 ± 279.79[b]
9	(-1)0	(-1)40	(-1)15	305.5 ± 21.00[a]	41.00 ± 9.64[a]	1050.70 ± 61.64[a]	1574.80 ± 69.01[a]	7234.37 ± 14.59[a]

Data presented as TPC – total polyphenolic content mg GAE.g^{-1} milligram gallic acid equivalent per gram, MTC* - metal chelation, ABTS**- 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) µmol TE.g^{-1} micromole trolox equivalent per gram, FRAP**- ferric reducing *antioxidant* power µmol AAE.g^{-1} – micromole ascorbic acid equivalent per gram and ORAC**- oxygen radical absorbance capacity of green rooibos crude plant extracts expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed. [abc] Means with different letter superscripts on the same row denotes significant differences ($p < 0.05$).

Table 3.2: Regression coefficients obtained by ANOVA to model the effects of variables (β-CD concentration, temperature and time) on the total phenolic content and antioxidant activity of crude green rooibos extracts.

Total polyphenolic content $R^2 = 0.8163$				Adjusted $R^2_{adj} = 0.7924$	
Source	SS	DF	MS	f-value	p-value
Model	38588.59	3	12862.86	34.07	< 0.0001
β-CD	37567.44	1	37567.44	99.51	< 0.0001
Temperature	434.58	1	434.58	1.15	0.2944
Time	586.57	1	586.57	1.55	0.2251
Lack of fit	1970.62	5	394.12	1.06	0.4159
Pure error	6712.69	18	372.93		

Metal chelation $R^2 = 0.8826$				$R^2_{adj} = 0.8658$	
Source	SS	DF	MS	f-value	p-value
Model	5155.77	3	1718.59	52.62	< 0.0001
β-CD	5046.53	1	5046.53	154.51	< 0.0001
Temperature	237.19	1	237.19	7.26	0.0136
Time	27.06	1	27.06	0.8286	0.3730
Lack of fit	86.70	5	17.34	0.46	0.7980
Pure error	599.19	16	37.45		

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) $R^2 = 0.9106$				$R^2_{adj} = 0.8989$	
Source	SS	DF	MS	f-value	p-value
Model	1.05×10^6	3	3.51×10^5	78.09	< 0.0001
β-CD	9.69×10^5	1	9.9×10^5	215.14	< 0.0001
Temperature	33354.30	1	33354.30	7.40	0.0122
Time	52806.68	1	52806.68	11.72	0.0023
Lack of fit	50643.50	5	10128.70	3.44	0.0234
Pure error	52975.45	18	2943.08		

Ferric reducing power $R^2 = 0.8757$					$R^2_{adj} = 0.8263$
Source	SS	DF	MS	*f*-value	p-value
Model	9.01×10^5	3	3.0×10^5	67.62	< 0.0001
β-CD	8.29×10^5	1	8.27×10^5	259.96	< 0.0001
Temperature	23025.92	1	23025.92	9.08	0.0592
Time	49683.13	1	49683.13	2.90	0.0079
Lack of fit	42846.9	5	8569.34	2.59	0.1859
Pure error	85123.63	17	5007.27		

Oxygen radical scavenging activity $R^2 = 0.9537$					$R^2_{adj} = 0.9427$
Source	SS	DF	MS	*f*-value	p-value
Model	8.54×10^7	5	1.71×10^7	86.61	< 0.0001
β-CD	6.06×10^7	1	6.05×10^7	307.02	< 0.0001
Temperature	3.02×10^6	1	3.02×10^6	15.32	0.0008
Time	1.82×10^6	1	1.82×10^6	9.23	0.0062
β-CD vs temperature	8.90×10^5	1	8.90×10^5	4.51	0.0457
Temperature vs time	9.37×10^5	1	9.37×10^5	4.75	0.0408
Lack of fit	1.357×10^5	3	3.44×10^5	1.99	0.1513
Pure error	1.302×10^6	18	1.73×10^5		

SS – sum of squares, MS – mean square, DF – degree of freedom. β-CD – beta-cyclodextrin concentration. Significance level = $p \leq 0.05$.
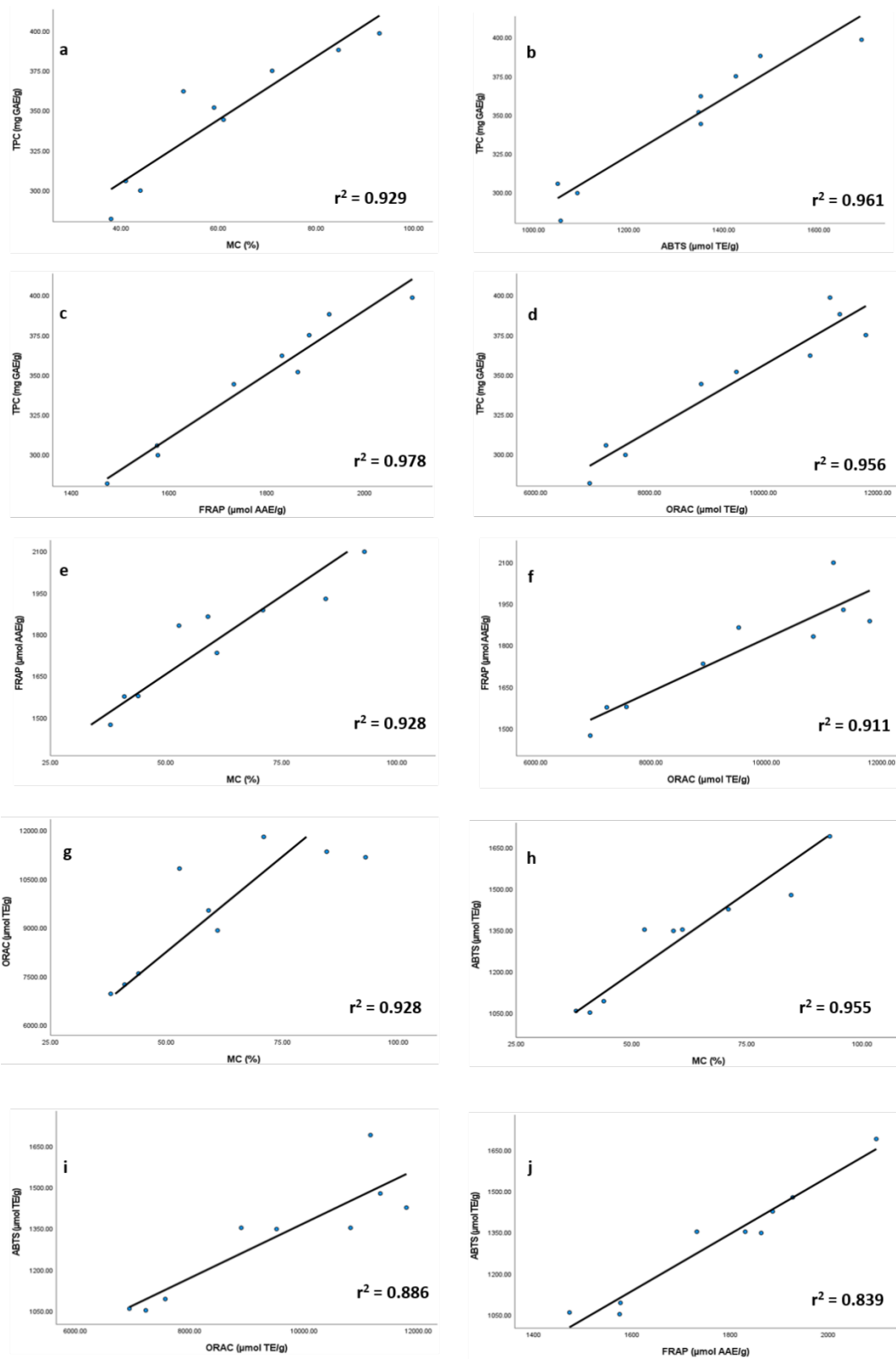
Figure 3.1: a – j Pearson correlation coefficient between total phenolic content and antioxidant activity.

3.7.2 Total polyphenolic content (TPC)

According to multiple linear regression analysis the model proposed for TPC was significant (p = 0.0001) and thus making it suitable to explain the results as displayed in Table 3.2. The coefficient of determination (R^2) and the adjusted (R^2_{adj}) were 0.8163 and 0.7924, respectively, with a lack of fit (p = 0.4159). This was a clear indication that the model was capable of explaining that 80% of the variability in TPC was as a result of the input variables. The following regression equation (Eq. (1)) was generated to determine the effect of each of the variables on the total polyphenolic content of green rooibos extracts, insignificant terms were excluded.

$$Y_1 \text{ (TPC, mg GAE/mL)} = 303.36 + 6.09X_1 - 0.20X_2 + 0.25X_3 \qquad (1)$$

As shown in table 3.1, the TPC content ranged from 281.7 to 398.24 mg GAE.g^{-1} of CGRE, with the highest TPC value observed at β-CD concentration of 15 mM, lowest extraction temperature (40°C) and longest heating time (60 min). In terms of single factors, only the effect of β-CD concentration (X_1) was significant (p < 0.0001), as the concentration increased there was an increase in TPC (figure 3.1). The other input variables, heating temperature X_2 and reaction time X_3 did not pose a significant (p > 0.05) effect.

A   B

Figure 3.2: Response surface plots for independent variables for total polyphenolic content of crude green rooibos extracts. (A) Effect of Beta-cyclodextrin and reaction temperature. (B) Effect of Beta-cyclodextrin and reaction time.

Water extracts, i.e. 0 mM β-CD exhibited the lowest (p < 0.05) TPC values compared to all concentrations (Table 3.1). Moreover, no significant (p > 0.05) differences were observed between all water extracts, although the temperature and time range was broad. Miller (2016) and Santos *et al*. (2016) reported on the importance of temperature-time effect on the extraction of phenolic compounds from green and red rooibos, respectively. They observed an

increase in TPC as the temperature and time increased ($p < 0.05$). Based on their result, in the current study, it would have been expected for the 0 mM: 90°C: 30 min extract to exhibit the highest TPC values compared to 40 and 65 °C. It has been proven numerous times that an increase in extraction temperature increases polyphenol content, due to their increased solubility and diffusion out of plant cells. We therefore speculate that the temperature of 90°C was too high, and this could have resulted in degradation of some polyphenols, resulting in reduced TPC value, hence, no significant differences were observed between the highest temperature with longer time exposure, compared to low temperature of 40°C for a shorter time of 15 min. Similar findings were observed by Mourtzinos *et al*. (2018) when they studied polyphenols from hemp oil by-products. Many studies that investigated the TPC of green rooibos extracts would have done so using either water or ethanol as solvents. Comparison with results reported in other studies investigating green rooibos reveals that our study reported both higher and lower TPC. For instance Lawal *et al*. (2019) and Coetzee *et al.* (2013)(Coetzee *et al*., 2013) reported lower TPC values of 250 and 243.7 mg GAE.g^{-1} for extracts obtained at a content of 1.33% at 100°C for 2 min and 10% at 100°C for 30 min, respectively. On the other hand, Damiani *et al.* (2019) reported higher TPC of 1019, 614.1 and 508.7 mg GAE.g^{-1} for microwaved, cold and regular brews, respectively. The variability of the results of the afore-mentioned studies are due to the differences in extraction conditions such as plant to solvent ratio, reaction temperature and exposure time. (Miller, 2016) also alluded to the effect of other aspects such as environmental conditions of the production area, production season, batch and processing methods on the TPF of green rooibos. However, it is worth to note that extraction parameters employed by Coetzee *et al*. (2013) closely resembled those employed in our current study.

With regards to β-CD-assisted extracts exhibited higher ($p < 0.05$) TPC than their water counterparts, this is in agreement with results of Diamanti *et al.* (2017) who extracted polyphenols from pomegranate using water, β-CD and hydroxypropyl beta-cyclodextrin (HP-β-CD). The polyphenol content increased significantly from 41, 59 to 71 mg GAE.g^{-1} of dry weight, respectively. To further corroborate this, Dai *et al.* (2019) observed a TPC of 236.6 for water and 252.5 g.kg^{-1} for 0.05% (w.v^{-1}) of β-CD based extracts. The superiority of β-CD solutions over water in our study and fellow researchers is attributed to the mechanism via which CDs function, as previously reported by Diamanti *et al*. (2017) and Mourtzinos *et al*. (2018). They credited the enhanced recovery of polyphenols from the aqueous matrix to encapsulation. As polyphenols diffuse out of the aqueous matrix into solution, they are incorporated/hosted into CD cavities, shielding them away from exposure to harsh extraction conditions that may lead to degradation.

With regards to β-CD assisted extracts, no significant differences were observed amongst extracts within and across concentrations, with a few exceptions, where 15 mM β-CD extracts produced at 40 and 60°C exhibited higher ($p < 0.05$) TPC than those at 7.5 mM.

various conclusions can be made with reference to the application β-CD in the current study. The use of β-CD allowed for the effective application of lower extraction temperatures coupled with longer exposure time for maximum recovery of polyphenols, a phenomenon also observed by Rajha *et al.* (2015) who investigated β-CD-assisted extraction of polyphenols from vine shoot cultivars, proving that longer extraction times were more effective in facilitating higher polyphenol content, as opposed to higher temperatures at the same extraction time. On the other hand, high extraction temperatures of 90°C which were considered detrimental in water-based extracts yielded higher TPC when β-CD was used; this can only mean that the protective effect *via* complexation came to play, where the same high temperature (90°C) in the presence of β-CD yielded increased TPC, even with longer exposure time. Similar inferences were made by Favre *et al*. (2018). The TPC is merely a sum of individual phenolic constituents.

With reference to major flavonoids, the order in terms of content decreased from aspalathin > hyperoside > orientin > iso-orientin > vitexin > isoviten > quercetin > luteolin > chrysoeriol (Table 3.3). Similar findings were also reported by Pantsi *et al.* (2011) who found aspalathin, hyperoside and iso-orientin as major flavonoids, with quercetin, luteolin, and chrysoeriol also detected in trace quantities in both green and red rooibos. Numerous studies have been conducted where aspalathin was not just the major flavonoid, but the major polyphenol amongst all classes (Joubert & DeBeer, 2011a; Gelderblom *et al.*, 2017; Damiani *et al.*, 2019). A clear distinction ($p < 0.05$) was observed between the aspalathin content of aqueous and β-CD assisted extracts, with the latter exhibiting the highest content as represented by 15 Mm: 40°C: 60 min extracts. A study done by Parmar *et al.* (2015) found that cyclodextrin-assisted extraction of apple pomace significantly ($p < 0.05$) increased the flavanols, dihydrochalcones and flavan-3-ols compared to aqueous extraction. However, CDs did not surpass methanol extracts. No significant ($p > 0.05$) differences were observed between aqueous and β-CD-assisted extracts of green rooibos for all other the other selected polyphenols (Table 3.3).

Table 3.3: Major selected flavonoids in crude green rooibos extract.

Run	β-CD (mM)	Temp (°C)	Time (min)	Aspalathin (mg.g^{-1})	Isorientin (mg.g^{-1})	Orientin (mg.g^{-1})	Isoviten (mg.g^{-1})	Vitexin (mg.g^{-1})	Hyperoside (mg.g^{-1})	Quercetin (mg.g^{-1})	Luteolin (mg.g^{-1})	Chrysoeriol (mg.g^{-1})
1	15	90	15	136.32 ± 11.64[b]	5.87 ± 0.33[b]	10.03 ± 1.78[a]	1.73 ± 0.63[ab]	2.34 ± 0.18[ab]	23.34 ± 4.87[a]	0.110 ± 0.01[c]	0.143 ± 0.04[c]	0.063 ± 0.01[c]
2	0	65	60	93.93 ±15.25[a]	5.94 ± 0.90[b]	8.88 ± 0.37[a]	1.68 ± 0.45[ab]	1.95 ± 0.16[bc]	21.34 ± 3.43[a]	0.090 ± 0.02[bc]	0.043 ± 0.01[a]	0.043 ± 0.02[ab]
3	15	40	60	172.25 ± 7.61[c]	7.93 ± 0.21[c]	8.86 ± 1.17[a]	2.31 ± 0.14[c]	2.53 ± 0.20[e]	29.27 ± 1.46[c]	0.137 ± 0.01[d]	0.070 ± 0.01[ab]	0.060 ± 0.00[c]
4	0	90	30	96.20 ± 4.22[a]	4.44 ± 0.56[a]	9.97 ± 1.16[a]	1.89 ± 0.07[abc]	1.76 ± 0.10[ab]	23.12 ± 0.56[ab]	0.080 ± 0.00[bc]	0.077 ± 0.01[b]	0.040 ± 0.00[ab]
5	7.5	90	60	107.70 ± 3.63[a]	4.14 ± 1.27[a]	10.17± 0.43[a]	1.55 ± 0.19[a]	1.69 ± 0.10[a]	19.61 ± 2.28[a]	0.070 ± 0.01[b]	0.057 ± 0.02[ab]	0.030 ± 0.01[a]
6	15	65	30	148.07± 11.10[b]	6.56 ± 0.23[b]	9.51 ± 0.99[a]	1.77 ± 0.38[abc]	2.49 ± 0.14[e]	23.55 ± 4.27[bc]	0.063 ± 0.02[b]	0.083± 0.01[b]	0.050 ± 0.00[bc]
7	7.5	65	15	134.48 ± 12.04[b]	5.51 ± 0.31[b]	10.60 ± 1.06[a]	1.37 ± 0.76[a]	2.30 ± 0.14[de]	21.26 ± 1.02[a]	0.040 ± 0.00[a]	0.063 ± 0.00[ab]	0.043 ± 0.00[ab]
8	7.5	40	30	143.23 ± 6.96[b]	4.05 ± 0.16[a]	13.46 ± 1.34[b]	2.24 ± 0.14[bc]	2.34 ± 0.17[de]	27.53 ± 1.15[bc]	0.107 ± 0.02[c]	0.083 ± 0.00[b]	0.050 ± 0.01[bc]
9	0	40	15	93.52 ± 3.58[a]	5.98 ± 0.18[b]	10.12 ± 0.36[a]	1.42 ± 0.04[a]	2.16 ± 0.08[cd]	18.75 ± 0.20[a]	0.083 ± 0.01[bc]	0.053 ± 0.01[bc]	0.043 ± 0.02[ab]

Data represented as polyphenols of green rooibos crude plant extracts expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed. [abcd] Means with different letter superscripts within and between columns denotes significant differences ($p < 0.05$).

3.7.3 Metal chelation

The metal chelation (MTC) assay is based on the ability of ferrozine to chelate ferrous ions (Fe^{2+}) and form a red coloured complex. Any compound that forms a coordinate complex with the metal ions exhibit chelating activity by competing with the ferrozine for ferrous ions (Oh *et al.*, 2013), which results in a decrease of the red colour of the ferrozine-Fe^{2+} complex. Iron has been found to accelerate the Maillard reaction. Therefore, we saw it fit to include MTC assay as an indirect indicator of the antioxidant activity of green rooibos plant extracts.

The model was significant for MTC of crude green rooibos extracts ($p < 0.0001$). The regression equation (Eq. (2)) below was generated to determine the effect of each of the variables on the metal chelation.

$$Y_2 \ (MTC, \%) = 48.39 + 2.39X_1 - 0.15X_2 + 0.055X_3 \tag{2}$$

The ANOVA results as presented in Table 3.2 indicate that the model possessed insignificant ($p > 0.05$) lack of fit, and the R^2 and R^2_{adj} value of the model was 0.8826 and 0.8658, respectively, therefore, the input variables were a true predictor of outcomes of metal chelation. The overall effects of the input variables on the responses was further analysed, and the results showed that β-CD X_1 and temperature X_2 significantly ($p < 0.05$) influenced the MTC. However, the effect of reaction time was insignificant ($p > 0.05$). Figure 3.2 A – B shows the interaction between the independent variables and their effects on the MTC.

A   B

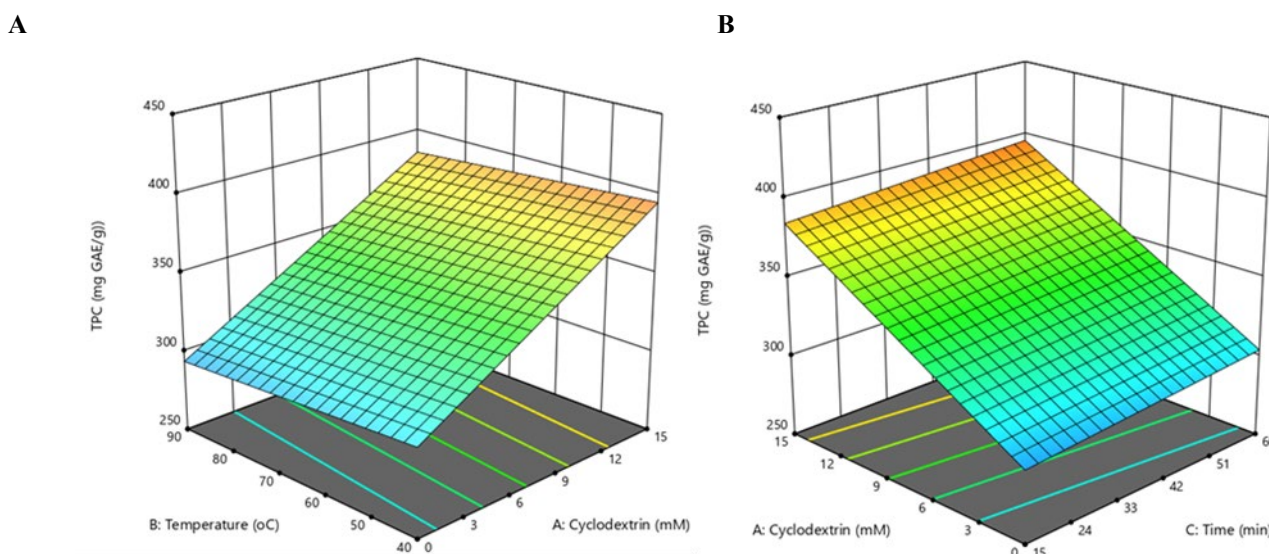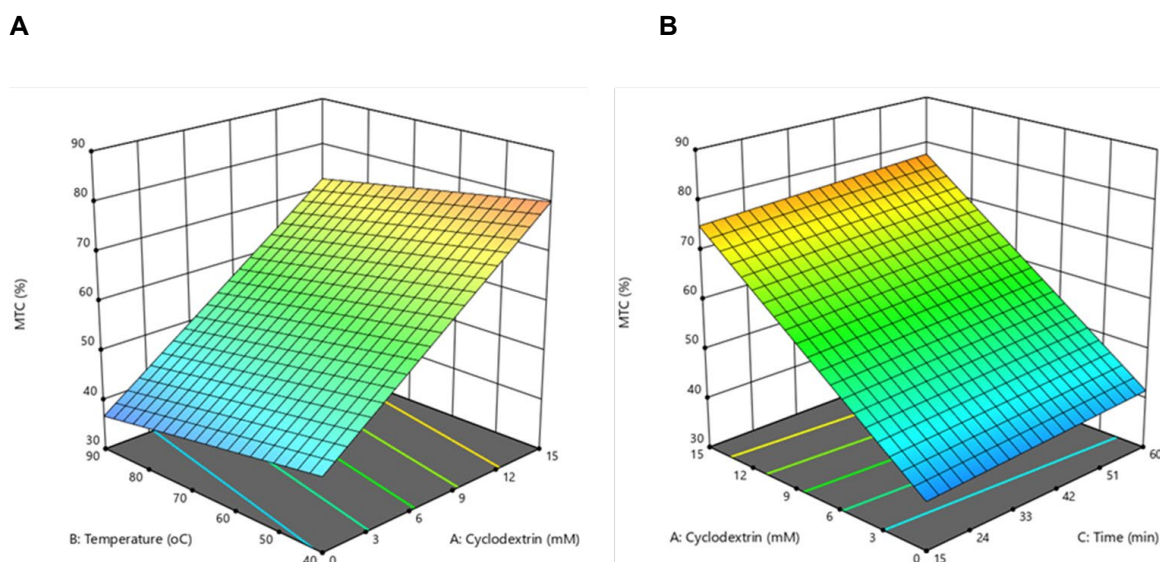**Figure 3.3:** Response surface plots for independent variables for metal chelation of crude green rooibos extracts. (A) Effect of Beta-cyclodextrin and reaction temperature. (B) Effect of Beta-cyclodextrin and reaction time.

The MTC of green rooibos plant extracts is depicted in Table 3.1. The highest value was reported at 93% for 15 mM β-CD: 40°C: 60 min extracts. Beta-cyclodextrin had a positive effect on MTC, causing an increase as the concentration increases, however, an inverse effect was observed for temperature (Fig. 3.2 A). Although not much research has been conducted on the metal chelation ability of green rooibos, Oh *et al*. (2013) reported on 66.54 % MTC of red rooibos extracts of 1:20 plant to water ratio obtained via steeping at 80°C for 10 min. The ability of CGRE in chelating metals such as iron and copper is crucial based on the expected function in inhibiting non-enzymatic browning in the next research chapter.

The main compounds known to possess metal chelation activity are flavonoids, and in green rooibos these are aspalathin, nothofagin, isorientin, orientin, isoviten, vitein, hyperoside, quercetin, luteolin and chrysoeriol which were found in varying quantities in our extracts (Table 3.3). Sinjman *et al.* (2009) studied the MTC of 13 common rooibos flavonoids based on the shift in the Fe^{2+}-induced absorption bands after the formation of a complex between the phenolic functional groups and Fe^{2+}. They found that chrysoeriol, vitexin, and isovitexin possessed no MTC properties, whereas, chelation of Fe^{2+} was observed for quercetin, rutin, isoquercitrin, and hyperoside, with quercetin exhibiting the strongest Fe^{2+} complex which proved to be irreversible when adding EDTA. Consequently, our current results show that the highest MTC was observed for 15 mM β-CD: 40°C: 60 min extracts, which happens to be the combination that also exhibited the highest quercetin at 0.137 mg.g^{-1} and 29.27 mg.g^{-1} hyperoside. Based on the MTC results, as well as the nine flavonoids identified in our study, there is no doubt that in green rooibos a synergistic effect between all flavonoids would result in heightened MTC activity.

The increase in MTC of β-CD-assisted extracts is linked to the TPC, where high concentrations led to high TPC, this further reiterates that the quantity of TPC correlates with MTC.

3.7.4 Radical scavenging 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).
The 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS•) cation radical scavenging method, also known as trolox equivalent antioxidant capacity (TEAC), measures the ability of a compound to donate hydrogen atoms similarly to DPPH, the difference is that the former is more suitable for both hydrophilic and lipophilic compounds (Oh *et al.*, 2013).

The input variables and responses of the ABTS radical scavenging activity of CGRE are shown in Table 3.1. Response surface methodology (RSM) was used to model the data and to obtain regression models that help understanding the effects of β-CD concentration

(X_1) temperature (X_2) and time (X_3) on the ABTS radical scavenging activity. For this purpose, regression equation was generated as follows (Eq. (3)):

$$Y_3 \ (ABTS, \mu mol \ TE. g) = 1112.85 + 30.94X_1 - 1.72X_2 + 2.36X_3 \tag{3}$$

As presented in Table 3.2, the model for the antioxidant activity as measured by the ABTS assay presented statistically significant differences ($p < 0.0001$), with values ranging from 1050.65 to 101689.70 μmol TE.g^{-1}. The R^2 (0.9106) and R^2_{adj} (0.8989) proving that the proposed model was able to explain that 90% variability in the response was due to the input variables. Moreover, the effect of each of the three input variables X_1, X_2 and X_3 was significant ($p < 0.05$) (Fig. 3.3). The highest ABTS value was recorded for 15 mM β-CD: 40°C: 60 min (Table 3.1).

A

B
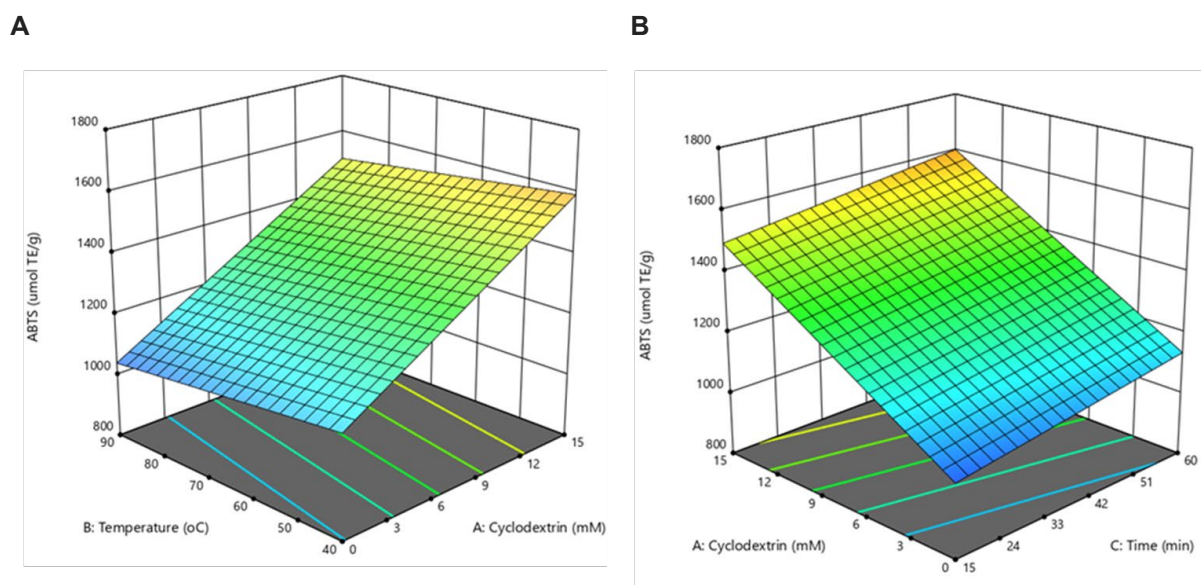
Figure 3.4: Response surface plot for the effect of (A) beta-cyclodextrin and reaction temperature (B) Beta-cyclodextrin and time on ABTS radical scavenging activity of green rooibos plant extracts.

Water-based extracts (0 mM) exhibited the lowest ABTS radical scavenging activity compared to the other concentrations ($p < 0.05$), and it followed a trend similar to what we observed with TPC, where no significant differences ($p > 0.05$) were noted between the water extracts. As the concentration of β-CD increased, an increase in ABTS radical scavenging was observed.

Comparing our results with other rooibos studies, Coetzee *et al*. (2014), and Lawal *et al*. (2019) reported ABTS values of 1408 and 1486 µmol TE.g^{-1} soluble solids of green rooibos,

respectively. These values are within the range obtained in the current study. It is still noteworthy to always bring in the differences in extraction conditions raised previously.

Rooibos flavonoids are considered to be the underlying basis for bioactivity of the plant. Flavonoids (C6 – C3 – C6) usually present a high antioxidant activity due to their high redox potential, which allow them to act n as reducing agents, hydrogen donors, oxygen suppressors, and transition ion metal chelators. When typical rooibos flavonoids were assayed, the reactivity of aspalathin against ABTS radical at an IC_{50} at 3.3 µM was equal to that of 3.4 µM EGCG, 3.6 µM quercetin and 4.4 µM nothofagin; higher than that of 11.37 µM trolox. Isovitexin and vitexin were the lowest radical scavengers at IC_{50} 1224 µM and 2313 µM, respectively (Sinjman *et al.*, 2009). The overall potency of the flavonoids in scavenging ABTS• in descending order was; aspalathin ≥ EGCG ≥ quercetin ≥ nothofagin > (+)-catechin >hyperoside > rutin ≥ luteolin ≥ iso-orientin ≥ trolox orientin ≥ isoquecitrin≥ chrysoeriol ≥ isovitexin ≥ vitexin (Sinjman *et al.*, 2009)

These flavonoids exhibited high antioxidant activity as single polyphenols, and when these are found in different quantities in green rooibos, they contribute to the increased antioxidant potency due to synergy. In the current study, aspalathin was the highest polyphenol at a range of 93.52 – 172.25 mg.g^{-1} (Table 3.3).

Concerning concentration, a similar trend observed for both TPC and MTC was observed, the increase in β-CD concentration had a direct relationship with ABTS radical scavenging activity. This observation was in agreement with results reported by numerous authors such as Favre *et al.* (2018; 2020) who reported on the improvement in antioxidant activity (DPPH radical scavenging) as a result of encapsulation. However, contradicting results were also reported. For instance, Albahari *et al.* (2018) observed no significant differences ($p > 0.05$) in ABTS radical scavenging activity of water-based olive pomace extracts and that extracted with 7mM β-CD was 31.5 and 32.48 mg TE.kg^{-1}, respectively.

On the other hand, Miguel *et al.* (2010) studied the effect of encapsulating thymol and carvacrol with β-CD. They reported an ABTS of 183.7 and 421.4 µmol TE.g^{-1} for thymol and β-CD- thymol complex, respectively. Meanwhile, carvacrol and carvacrol-β-CD complex values were insignificant ($p > 0.05$) at 460 and 490 µmol TE.g^{-1}, respectively. Kamimura *et al.*, 2014 found similar results, where free carvacrol showed a higher ($p < 0.05$) ABTS scavenging activity (7491 µmol TE.g^{-1}) capacity than complexed carvacrol (6752 µmol TE.g^{-1}), the authors attributed this to inclusion of carvacrol in HP-β-CD making the hydroxyl groups less available to react with the free radical. In conclusion, encapsulation does increase the phenolic content of CGRE, which in turn the antiradical potency.

3.7.5 Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) is a single electron transfer based assay, whereby the ability of a potential antioxidant to transfer an electron to reduce any ferric chloride to ferrous chloride is detected (Shahidi & Ambigaipalan, 2015). The FRAP of crude green rooibos extracts was measured using ascorbic acid as a standard, and the results are shown in Table 3.1.

The results of antioxidant activity measured by the FRAP assay presented statistically significant differences ($p < 0.0001$), ranging from 1472.78 to 2097.53 µmol AAE.g^{-1}. Experimental data was appropriately adjusted, presenting lack of fit ($p = 0.1859$), $R^2 = 0.8757$ and $R^2_{adj} = 0.8587$, proving that the proposed regression model was satisfactory to explain the variability in the data (Table 3.2). input variables β-CD concentration, extraction temperature and time employed in RSM to optimise the FRAP of green rooibos plant extracts are shown in Table 3.1

The ANOVA results shown in Table 3.2 indicate a relationship between the FRAP and the extraction conditions based on a regression coefficient ($R^2 = 0.8757$). The coded equation in taking in account on significant terms of FRAP of green rooibos plant extracts is as follows (Eq. (4)):

$$Y_4 \text{ (FRAP, µmol AAE/g)} = 1559.17 - 28.61X_1 - 1.43X_2 + 2.35X_3 \tag{4}$$

For FRAP, the independent variables X_1 (β-CD), and X_2 (temperature), as well as time X_3 were significant ($p < 0.05$), whereas, time X_3 and X_1X_2 interactions were insignificant. Figure 3.4 shows the RSM plots on the effect of temperature and β-CD, as well as time and β-CD on FRAP. The FRAP values ranged from 1472.78 – 2097.53 µmol AAE.g^{-1}. A positive relationship was observed between β-CD concentration and FRAP values. As the concentration of β-CD increased, there was an increase in FRAP. It was evident that, the highest FRAP value (average) was reported ($p < 0.05$) for 15 mM β-CD: 40°C:60 min, whilst water-based extracts (0 mM β-CD) were the lowest.
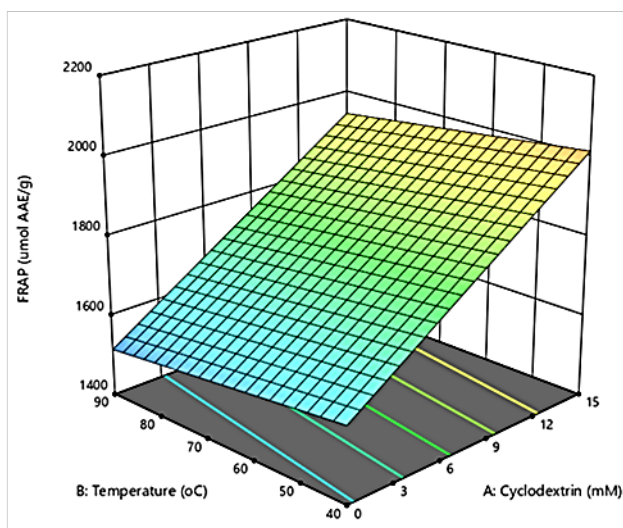
A   B
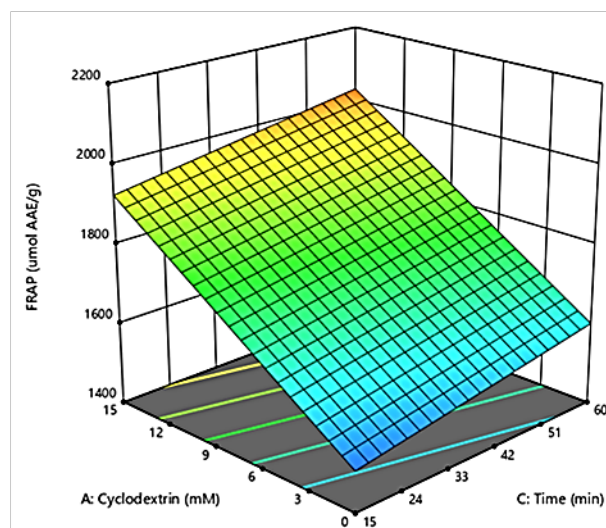
Figure 3.5: Response surface plots for independent variables for ferric reducing plasma of green rooibos plant extracts. (A) Effect of reaction temperature and Beta-cyclodextrin. (B) Effect of Beta-cyclodextrin and time.

However, values reported in this study were in the range of what was reported by Lawal *et al*. (2019) for green rooibos at 2012 µmol AAE.g^{-1}. Interestingly our values were comparable to that of red rooibos at 1638 µmol AAE.g^{-1}. Numerous authors have reported on the reduced antioxidant activity of red rooibos due oxidation of polyphenols during fermentation, thus the lower FRAP is expected. However, in our case lower values compared to that of Lawal *et al*. (2019) might be due to the dilution of the extract prior to analysis. They used 10 mg.ml^{-1}, which was 20 times our concentration at 0.5 mg.ml^{-1}. Moreover, various other factors such as the extraction parameters employed play a role, factors that we cannot account for since theirs was a commercial product.

The FRAP of β-CD-green tea complex at higher concentration of green tea was higher than that of a lower concentration. However, these authors did not make comparisons between the β-CD-green tea complex with the aqueous green tea extract (Koteswara *et al.*, 2020). When applying β-CD-assisted extraction of thyme and green pepper grains at similar concentrations applied in this study, Favre *et al*. (2018) found that β-CD at 15mM resulted in the highest FRAP than 7.5 and 0mM, with the latter exhibiting the lowest antioxidant activity.

3.7.6 Oxygen radical absorbance capacity

The oxygen radical absorbance capacity (ORAC) assay evaluates the potential of a test compound as a chain-breaking antioxidant. The ANOVA results showed that model proposed

for ORAC was significant (p = 0.0001). The variation in response around the fitted model was insignificant, thus the model fits the data well (lack of fit p = 0.1513). The relationship between radical scavenging and extraction parameters could be fitted to a regression model. Therefore, this proves that 94% changes in ORAC values was due to the effect of changes in input variables (Table 3.2). Evident to this is an R^2_{adj} (0.9427) close to the R^2 (0.9427), which denotes a satisfactory adjustment of the model to the experimental data. According to Parmar *et al.* (2015), an R^2 greater than 80% signifies a good fit for the model, which was true for our current study. Furthermore, the effect of each input variable on the ORAC of crude green rooibos extracts was evaluated from the regression equation in coded level (Eq. (5)).

$$Y_5 \text{ (ORAC, } \mu\text{mol TE/g)} = 8072.71 + 174.46X_1 - 22.21X_2 - 25.18X_3 + 1.93X_1X_2 + 0.65X_2X_3 \tag{5}$$

The influence of each input variable on the response value was significant, with β-CD concentration (p = 0.0001), reaction temperature (p = 0.0008) and time (0.0062). Furthermore, the interaction (X_1X_2 and X_2X_3) between independent variables were also significant, see Fig 3.2 for actual p-values. The three-dimensional response surface plots depicted in Fig 3.5 can be used to illuminate the influence of interaction on the response value.
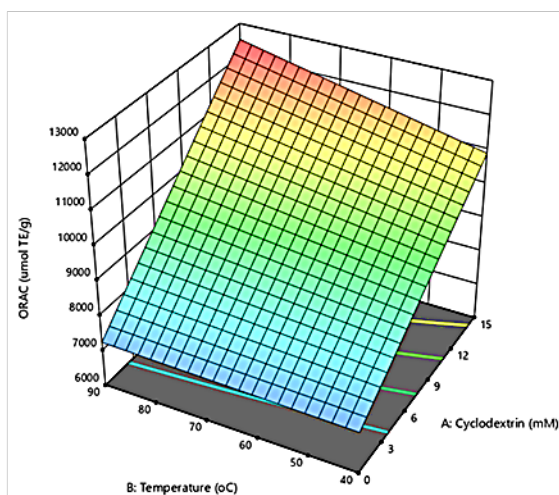
A   **B**
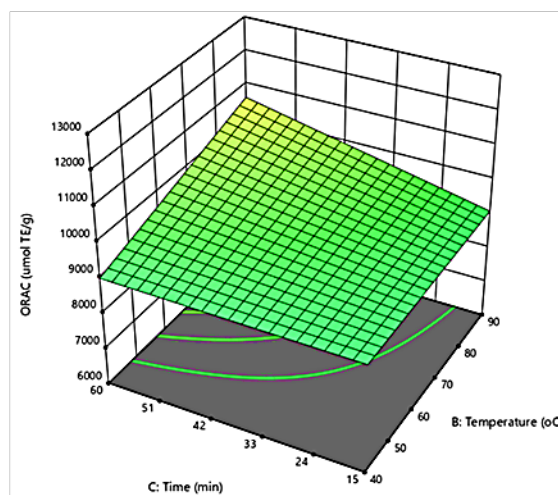
Figure 3.6: Response surface plots for independent variables on the oxygen radical absorbance capacity of crude green rooibos extracts. Effect of reaction temperature and Beta-cyclodextrin (A) and effect of reaction temperature and time (B).

The ORAC values of CGRE are shown in Table 3.1 and ranged between 6948.03 – 11791.39 μmol TE.g^{-1}. The lowest values were reported for all water-based extracts, consequently β-CD extracts exhibited the highest values, although there was little distinction between 7.5 and 15 mM.

In comparison to other rooibos research, Simpson *et al.* (2013) reported an ORAC value of 1 840 μmol GAE.L^{-1} for green rooibos infusions at 1.33% (w.v^{-1}) brewed for 5 min. When Lawal *et al*. (2019) reconstituted lyophilised green tea to a concentration of 1% (w.v^{-1}), they reported an ORAC of 4087 μmol TE.g^{-1}. On the other hand, Damiani *et al*. (2019) evaluated the impact of different brewing parameters and reported 11 700, 7 100 and 6 300 μmol TE.L^{-1} for 1.25% (w.v^{-1}) cold, regular and hot brews of green rooibos herbal teas, respectively. All these studies yielded varying results due to the differences in extraction parameters. Moreover, our values were significantly higher, and this could have been as a result of a lower (1:10 w.v^{-1}) plant to water ratio that resulted in extraction of more polyphenolic compounds. Considering that it is well-documented that the antioxidant activity of rooibos is due to its polyphenolic composition. Damiani *et al*. (2019) found strong significant ($p < 0.05$) correlation between increase in polyphenolic content with an increase in ORAC values. We also found a positive correlation between TPC and ORAC values ($R^2 = 0.878$). To illustrate this point further, Simpson *et al.* (2013) analysed common polyphenols found in rooibos. They found that quercetin was the highest followed by aspalathin > nothofagin > orientin > vitexin = iso-orientin = rutin > isoquercitrin > hyperoside. In our study, HPLC analysis of individual polyphenolic compounds of CGRE revealed aspalathin (93.52.01 – 172.25 mg.g^{-1}) as the most abundant, with quercetin amongst the lowest (0.040 – 0.137 mg.g^{-1}) reported polyphenols. In the study of Simpson *et al*. (2013), they proved that excellent peroxyl radical scavenging of quercetin was achieved at very low concentrations of 0.07 and 0.5 μM using fluorescein and pyrogallol red, respectively. Therefore, we also speculate that based on current TPC and individual polyphenols, that these had an influence on the ORAC values of green rooibos plant extracts. Evident to this is the correlation (0.878) as shown in Table 3.4

Concerning β-CD complexed plants extracts and compounds. Miguel *et al*. (2010) reported a significantly ($p < 0.05$) lower ORAC value for carvarcol-β-CD (28 μmole TE.mg^{-1}) compared to free carvarcol (33 μmole TE.mg^{-1}); on the other hand, no significant differences were observed between free thymol and the complexed counterpart exhibiting a similar ORAC value of 47 μmole TE.mg^{-1}. Although the latter results showed no differences, the reduction in antioxidant of carvarcol-β-CD was attributed to the blockage of some active functional groups (OH) that are responsible for donating the hydrogen atom. A similar phenomenon has been reported by Rakmai *et al.* (2018) on guava leaf oil.

3.8 Conclusions.

The use of β-CD in improving the extraction of bioactive compounds in green rooibos was assessed, with a view of influencing the physicochemical properties thereof. The optimum extraction conditions of CGRE functional compounds were evaluated through RSM. Run #3 with combination of 15 mM β-CD heated at 40°C for 60 min was identified as the optimum extraction conditions with a desirability of 0.985. Under these parameters, the CGRE presented the highest TPC, ABTS radical scavenging and FRAP values. With reference to MTC and ORAC assays the optimum conditions (15 mM β-CD heated at 40°C for 60 min) had no significant ($p > 0.05$) difference from other runs extracted with β-CD at 7.5 and 15 mM, but significantly higher ($p < 0.05$) compared to aqueous extracts (0 mm β-CD).

Based on the reported results, an increase in β-CD concentration proved to increase the extraction yield of polyphenols, thus positively contributing to the antioxidant activity, as confirmed by the correlation results. Rooibos polyphenols proved to be less sensitive to high temperatures than those from herbs or spices as reported by other researchers, since the decay at the higher temperatures was not evident. While thyme, and green, black and white pepper extracts showed a clear maximum in the TPC, antiradical and reducing power of the extracts as a function of temperature, these indices of antioxidant activities showed much lower temperature dependence in the rooibos extracts (Favre *et al.*, 2018, 2020).

Moreover, β-CD-based extractions can be applied in conjunction with alternative technologies such as ultrasound and pulsed electric fields in order to increase the antioxidant capacities and adequately modulate the polyphenol profile of the extracts. The oxidation status of the rooibos plant material also has an influence, besides the extraction conditions and have to be considered.

The obtained results provide the basis for selecting optimum conditions to prepare extracts with the maximum potential health-promoting properties, with potential technological applications also. The local communities and the development of family agriculture can benefit by enhanced perceived value of their crops under sustainable production and by the preservation of ancestral varieties for the manufacture of differentiated products with added value. These considerations are under the global recommendations of international organizations like WHO and UN for healthy populations and sustainable development. Moreover, of outmost importance is the main objective of this study, where green rooibos extracts were applied in canned apples to inhibit NEB reactions.

3.9 References

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CHAPTER 4: PHYSICOCHEMICAL PROPERTIES OF BETA-CYCLODEXTRIN-ASSISSTED EXTRACTS OF GREEN ROOIBOS (*ASPALATHUS LINEARIS*).

4.1 Abstract

Beta-cyclodextrin (β-CD)-assisted extracts of green rooibos were proven to exhibited higher total polyphenolic content (TPC) and antioxidant activity (AA) compared to aqueous extracts. Based on these properties, these extracts are an ideal natural substitute to synthetic food additives in preventing non-enzymatic browning during processing and storage. The purpose of this study was to characterise the physicochemical properties of green rooibos extracted with water and 15 mM β-CD at 40°C for 60 min. The moisture content (MC), water activity (a_w) and colour (L*a*b*) of powders termed GRE and β-GRE were analysed. While thermogravimetric analysis (TGA) and Fourier-transform infrared spectroscopy (FT-IR) were applied to confirm formation of green rooibos and β-CD inclusion complexes.

No significant differences ($p > 0.05$) were reported between the MC of GRE and β-GRE at 3.29 and 2.45%, respectively. However, the a_w of β-GRE was significantly ($p < 0.05$) lower at 0.11 than that of GRE at 0.18, proving the effect of β-CD on formation of hydrogen bonds with displaced water. Samples extracted with β-CD resulted in increased lightness (L*), reduced redness (a*) ($p < 0.05$), with no significant differences ($p > 0.05$) on the yellowness (b*) of green rooibos.

Thermograms of β-CD, GRE and β-GRE revealed that the thermal degradation process took place in stages. Evaporation of water absorbed on the surface and cavity of β-CD was observed between 100 – 120°C resulting in 11.0% in weight loss. The second stage, associated with degradation of β-CD, occurred in the range 340 – 375°C. With reference to GRE, an initial weight loss of 2% was observed from 40 – 120°C, while the main degradation resulting in 40% loss in weight was observed from 180°C. The thermogram of β-GRE, on the other hand, was a superposition of both GRE and β-CD, thus confirming formation of inclusion complexes. As a result of complexation, the main degradation stage of β-GRE took place at a higher temperature of 260°C, compared to 180°C of GRE. Furthermore, complexation was confirmed via FTIR spectra.

Absorption spectra of β-CD and β-GRE samples overlapped at specific regions and showed specific spectral differences in comparison to the aqueous extract (GRE), while similarities between GRE and β-GRE were observed at 578, 1025, 1154 cm^{-1}. When the β-GRE inclusion complex formed, most characteristic peaks of GRE and β-CD disappeared or shifted in the newly formed complex. The 1255 cm^{-1} band depicting C – O stretching of carboxylic acid disappeared due to formation of hydrogen bonds with β-CD. The GRE 1030 –

1070, 1606 and 2939 cm^{-1} bands shifted to 1025, 1615 and 2939 cm^{-1} in the β-GRE complex, respectively.

Keywords: Green rooibos extract; β-cyclodextrin; Inclusion complex; Heat stability.

4.2 Introduction

Recent trends in alleviating toxic compounds generated in foods during processing and storage focus on utilisation of plant extracts. This fulfils the current trend of using natural ingredients as an alternative to synthetic additives (Oral *et al.*, 2014). Moreover, these polyphenolic-rich plant extracts are selected as food ingredient due to known health-promoting properties. *Aspalathus linearis* infusions have received great attention in recent years due to their bio-functional properties such as: antioxidant activity (Vhangani *et al.*, 2022), anti-inflammatory (Ku *et al.*, 2014), anti-carcinogenic (Marnewick *et al.*, 2005), antimicrobial, anti-obesity and hypoglycaemic (Beltrán-Debón *et al.*, 2011) activities with the action of polyphenols responsible for the above-mentioned properties (Joubert & DeBeer, 2011). Green rooibos is of interest in the present study since it contains individual polyphenols which have been proven to inhibit non-enzymatic browning reactions in food and model systems.

For instance, quercetin and catechin at 50 μg.ml^{-1} exhibited inhibitory capacity of 40 and 36% against glycation of glucose-bovine serum albumin compared to 17% of aminoguanidine (Wu *et al.*, 2009). Chlorogenic acid and epicatechin reduced acrylamide in an asparagine-fructose model system and biscuit. In addition, 0.01 M chlorogenic acid and epicatechin reduced the formation of furans in a glucose-glycine model system (Oral *et al.*, 2014). These polyphenols were found to exert their inhibitory capacity *via* forming adducts with NEB reactants or their intermediate products. Similar observations were made by authors who studied the anti-glycative effect of plant extracts, proving that these plant extracts exhibited considerably better anti-glycation than the identified individual polyphenols contained in them, attributing the superior inhibitory effect to the synergy between the different polyphenols (Favreau-farhadi *et al.*, 2015). Therefore, plant extracts, and in particular green rooibos, would be ideal as functional ingredients in food production. However, instability of polyphenols during heat processing and storage may pose a challenge of using native plant extracts (Albahari *et al.*, 2018). Consequently, development of encapsulation methods and delivery systems containing specific compounds can improve some physicochemical properties of these extracts in food products. Encapsulation of sensitive compounds provides improved stability during processing, and in the final product by preventing reaction with other components in food products, such as oxygen or water (Nesterenko *et al.*, 2013). The release of microparticle content at controlled rates can be triggered by shearing, solubilization, heating, pH or enzyme activity of these agents naturally present in certain plants, ensuring

their stability. Common encapsulation material includes carbohydrates, protein and lipid polymers such as maltodextrin, inulin (Human *et al.*, 2020), soy protein isolates, sodium alginate and cyclodextrins, to mention a few. The choice of polymer is crucial as it affects how the active compound is released. For instance, Hidalgo *et al.* (2018) reported on elevated furosine levels in soy protein isolates applied to encapsulate beetroot pomace due to thermal treatments. Similarly, maltodextrin reacts with glycine to form browning compounds (DaSilva, 2018). Grape skin phenolics encapsulated in sodium alginate beads interacted with alginate resulting in reduced bioactivity. Therefore, the encapsulating polymer should not participate in any chemical reaction during processing or storage. Cyclodextrins (CDs) were the obvious choice since they were proven not to participate in an NEB reactions (DaSilva, 2018).

Cyclodextrins are made up of cyclic oligosaccharides consisting of a number of α (1→4) linked D-glucose subunits. The most common forms named alpha (α), beta (β) and lambda (γ-CDs), are composed of six, seven and eight glucose units, respectively (Cai *et al.*, 2018). These molecules are widely used in the food, pharmaceutical and chemical industries for their ability to form host-guest inclusion complexes with a wide range of bioactive compounds (Haidong *et al.*, 2011; Cai *et al.*, 2018). This results in modification of the physicochemical properties of the encapsulated compound leading to improved rheological and structural properties such gelling, viscosity, solubility, stability, as reviewed by Navarro-orcajada *et al.* (2020). Maraulo *et al.* (2021) proved that β-CD enhanced the physical properties of olive pomace extracts via improved heat stability and reduced hygroscopicity, in addition to increased antioxidant activity.

In the present study, β-CD-assisted extracts of green rooibos, in particular those obtained following the conditions of 15 mM: 40°C: 60 min were found to possess the highest TPC and antioxidant activity. These extracts were selected to be applied in canned apples to inhibit NEB reactions. This was based on studies where β-CD-assisted extracts of thyme (Favre *et al.*, 2018) and green pepper (Favre *et al.*, 2020) reduced browning and HMF development in BSA-glucose model systems. Therefore, the aim of this study was to determine the impact of β-CD on the physicochemical properties of green rooibos extracts via determination of moisture content (MC), water activity (a_w), colour (L*a*b*), thermogravimetry analysis (TGA) and Fourier-transform infrared spectroscopy (FT-IR).

4.3 Materials and Methods
4.3.1 Chemicals
The dry green rooibos plant was obtained from Rooibos Limited (Clanwilliam, South Africa). Beta-cyclodextrin was purchased from Industrial Analytical (Kyalami, South Africa). The water

used in this study was ultrapure water purified with a Milli-Q water purification system (Millipore, Microsep, Bellville, South Africa).

4.3.2 Solid liquid extraction of green rooibos plant.

Green rooibos as received from Clanwilliam was coarsely milled (Fritsch, Germany) using a sieve with a porosity of 0.2 mm. The extraction of green rooibos plant was performed based on the method of Rajha *et al.* (2015) and Miller (2006) with slight modifications. Green rooibos plant and 0 and 15 mM β-CD aqueous solutions in a 1:10 (w.v^{-1}) ratio were blended in a Waring blender for two minutes at low power, followed by heating the mixture at 40°C for 60 min. The extracts were cooled immediately and centrifuged at 5 000 rpm for 15 min. The supernatant was transferred into pre-weighed aluminium dishes and mass taken, followed by freeze-drying. The powders resulting from β-CD assisted (β-GRE) and native (GRE) extracts were weighed to calculate the soluble constituent yield, followed by storage in an air-tight container at –20 °until further analysis.

4.4 Moisture content (MC) and water activity (a$_w$).

The MC and a$_w$ was determined using the gravimetric method described by Human *et al.* (2020). A 2 g sample of green rooibos extract (β-GRE and GRE) was heated at 100 °C for 60 min using an Infrared Moisture Analyser (Denver Instrument, Colorado, USA). The a$_w$ was measured at 25 °C, using a Novasina LabMaster-a$_w$ meter (Lachen, Switzerland).

4.5 Colour measurement.

The colour was determined following a modified method of Maraulo *et al.* (2021) by measuring CIELab parameters L* (brightness, 100 = white, 0 = black), a* (+ red; - green) and b* (+ yellow; - blue) parameters by means of a spectrophotometer (CM-5, Konika Minolta, Japan); measuring the colour spectra using a D65 day light source, large viewing area and the observer at 10° angle.

4.6 Thermogravimetric analysis (TGA).

Thermogravimetric analysis (TGA) of samples were performed using a Shimadzu DTG-60 instrument following the procedure described by Miller *et al.* (2018), with slight modifications. Freeze-dried GRE and β-GRE (3 – 5 mg) were accurately weighed into open aluminium cells and heated from 25 to 800 °C at a heating rate of 20°C.min^{-1}, and a nitrogen gas purge rate of 35 mL.min^{-1}. The mass loss and heat flow in the sample were recorded as a function of temperature with reference to an empty pan.

4.7 Fourier-transform infrared (FT-IR)

Fourier-transform infrared spectroscopy analysis was performed to investigate possible β-CD-green rooibos interactions based on the modified method of Tutunchi *et al.* (2019). Analyses were performed using a Perkin Elmer Fourier-transform infrared spectroscope (FT-IR) equipped with a universal attenuated total reflectance (UATR) polarisation accessory for spectra. Prior to sample analysis, a background spectrum was collected, and finely ground (mortar and pestle) GRE and β-GRE were placed directly covering the surface of the ATR crystal. All spectra were acquired by co-addition of 32 scans at a resolution of 4 cm^{-1} in the range of 400 – 4000 cm^{-1}. The UATR crystal was carefully cleaned with acetone to remove any residual sample prior to analysis.

4.8 Data analysis

Statistical analysis was performed using SPSS 27.0 for Windows®. Descriptive statistical analyses determined the mean and standard deviation of triplicates (n = 3). The independent sample T-test was performed to determine statistical differences between the two sample groups. The level of confidence required for significance was selected at 95%.

4.9 Results and discussions

4.9.1 Moisture content (MC), water activity (a_w) and colour (L*a*b*).

The MC and a_w of green rooibos extracts (GRE and β-GRE) are shown in Table 4.1. The MC of 3.29% was reported for GRE and was not significantly different (p > 0.05) from the 2.45% of β-GRE. The MC of β-GRE was in the range reported for aqueous extracts of green rooibos of 2.14% by Human *et al.* (2020) and 2.4% by Miller *et al.* (2018). Moreover, Miller *et al.* (2018) also found no significant (p > 0.05) differences between the MC of green rooibos vs 1:1 green rooibos-inulin and green rooibos-maltodextrin encapsulates. Meanwhile, Human *et al.* (2020) reported significant differences between those encapsulates. Beta-cyclodextrin-encapsulated extracts are known to inhibit moisture uptake, which in the case of our results was not demonstrated. Therefore, we assume that β-CD's inhibition of moisture uptake would be more clearly demonstrated via moisture sorption isotherms than a single point measurement of MC. Evident to the above statement, Maraulo *et al.* (2021) found that the MC of olive pomace and β-CD-olive pomace was similar until a_w of 0.33, however, as the a_w increased to 0.8, the water adsorption was lower for the extracts obtained with β-CD.

Table 4.1: Physicochemical properties and beta-cyclodextrin encapsulated green rooibos extracts.

Sample	a_w	MC	L*	a*	b*
GRE	0.181 ± 0.00^b	3.29 ± 0.18^a	47.95 ± 1.43^a	16.12 ± 0.51^b	23.01 ± 0.59^a
β-GRE	0.111 ± 0.01^a	2.45 ± 0.12^a	53.70 ± 0.80^b	15.96 ± 0.51^a	24.46 ± 1.01^a

Data presented as mean ± standard deviation (n = 3) of a_w water activity, MC moisture content, L* lightness (0-100), a* +red/-green, b* +yellow/-blue of GRE – Gren rooibos extracts and β-GRE – beta-cyclodextrin encapsulated green rooibos extract. ANOVA and T-test were performed. [ab] Means with different letter superscripts on the same column denotes significant differences ($p < 0.05$).

The a_w of β-GRE of 0.11, on the other hand, was significantly lower ($p < 0.05$) than that of GRE at 0.181. Our results are in accordance to those reported by Miller *et al.* (2018), but opposes Human *et al.* (2020)'s findings, where maltodextrin and chitosan encapsulates exhibited higher a_w compared to aqueous green rooibos powdered extract. In solution, the nonpolar β-CD cavity is occupied water molecules, but due to energetically unfavoured interactions, when another guest molecule is present, it readily substitutes the water as a guest molecule. Lower a_w exhibited by β-GRE is attributed to the interaction and binding of water by the by the hydrophilic sites of β-CD, resulting in reduced free water (dos Santos Ferreira *et al.*, 2022). This contributes to good storage stability of these powders when stored under dry conditions. An important factor considering the instability of aspalathin during storage (Miller *et al.*, 2018; Human *et al.*, 2020).

The colour of GRE and β-GRE powders presented as L*a*and b* values are shown in Table 4.1. The β-GRE powder was lighter and less red ($p < 0.05$) compared to that of GRE, while the yellowness was similar ($p > 0.05$). The effect of encapsulation on the colour of green rooibos extracts was investigated by Miller *et al.* (2018). They reported an increase in L* and decrease in a* and b* of inulin and maltodextrin encapsulates, with the increase more pronounced in the latter encapsulant. Regarding application of β-CD as an encapsulant, various authors reported different outcomes in terms of the colour of extracts. Tutunchi *et al.* (2019) found that 1 and 5% w.v^{-1} of aqueous β-CD had no significant effect on the lightness of red beet extract. However, using ethanol as a solvent at 1% β-CD increased L* value, while no significant difference ($p > 0.05$) resulted when the concentration of β-CD in ethanol was increased to 5%. The latter concentration was too high, resulting in decreased solubility of β-CD. Maraulo *et al.* (2021) also reported higher L*values of β-CD-assisted extracts of olive pomace compared to aqueous extracts. Moreover, they also reported the effect of varying a_w on the colour of extracts. They found that darkening of aqueous extracts was increased as the a_w increased, and that β-CD-assisted extracts maintained the lightness regardless of changes in a_w. This further proves the impact of β-CD encapsulation on plant extracts colour stability during storage. Plant extracts are known to impart natural pigments into food they are added

into. The colour of plant extracts is important as it determines the suitability of its use as food additive. The colour of green rooibos extract is important, in particular regarding its intended use in this study. The main aim of this study was to apply β-GRE to inhibit browning in canned apples. The deep red colour of the extract may have an effect in contributing to visual browning, therefore the lighter the colour of the extract with increased antioxidant activity, the better. Moreover, at high temperatures applied in processing, in this case canning, GRE polyphenols might oxidise, resulting in browning (Favreau-farhadi *et al.*, 2015; Qi *et al.*, 2018). Therefore, β-CD encapsulated extracts are protected from direct exposure to heat. It is worth reiterating that in the present study (Vhangani *et al.*, 2022) and that of Maraulo *et al.* (2021), β-CD encapsulated extracts exhibited the highest antioxidant activity (AA) compared to their aqueous counterpart. The lighter colour, as well as increased AA renders them suitable for application in inhibiting browning in canned apples.

4.9.2 Thermogravimetric analysis (TGA).

Thermogravimetric analysis (TGA) was performed to confirm complex formation. This was achieved via determining thermal stabilities based on mass reduction as a function of increased temperature. Thermograms of GRE, β-GRE and β-CD are illustrated in Figure 4.1. The thermal degradation process of pure β-CD underwent two major stages, with peaks appearing at approximately between 100 – 120°C, and 340 – 375°C. The weight loss of 11.0% occurred in the first stage, a phenomenon associated with evaporation of water absorbed on the surface of β-CD, as well as the water contained in the cavities. Li *et al.* (2020) and Koteswara *et al.* (2020) reported initial β-CD weight losses of 13% in the same region of 112 and 100°C, respectively. Both these authors ascribed the weight loss to water evaporation from the β-CD cavities, Koteswara *et al.* (2020) also mentioned evaporation of water adsorbed on the surface of the β-CD molecule. In the second stage, further weight loss in β-CD of 67% was observed from 340 to 375°C, and this was due to the main thermal decomposition of β-CD. Once more the main degradation of β-CD resulting in 76% wight loss was observed at 305 and 400°C by Koteswara *et al.* (2020) and Li *et al.* (2020). The result of this study lies within the ranges observed by these two authors.

With reference to GRE samples, the initial sample weight was slightly higher, and this can be linked to the hygroscopicity of the sample (based on visual observation). An initial weight loss of 2%, although not significant was observed from regions as low as 40 – 120°C. Miller *et al.*, (2018) reported similar results on aqueous extracts of green rooibos as loss as surface moisture. The main degradation of GRE resulted in 40% loss in weight and was seen from 180°C, followed by a gradual decrease in weight as the temperature increased to 400°C. This degradation pattern is synonymous with plant extract polyphenols, in particular green rooibos. Li *et al.* (2020) reported degradation of Mulberry polyphenols at 138 – 600°C resulting

in 59% mass loss. The second region of weight loss for green tea occurred at around 130°C with 10–14% weight loss, which continued up to 900°C.
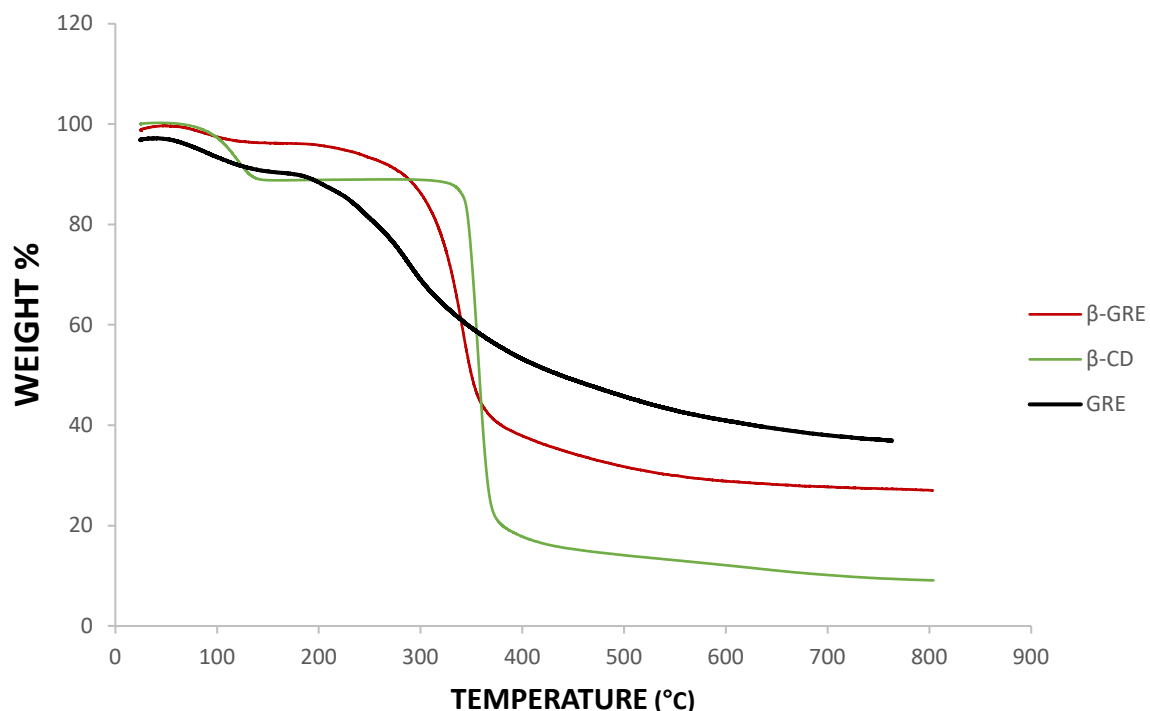
Figure 4.1: Thermogravimetric analysis (TGA) thermograms of beta-cyclodextrin (β-CD), aqueous.

The broad temperature range reported for crude plant extracts degradation may be due to the versatile mixture of compounds, decomposition of resistant aromatic structures requires higher temperatures. The thermogram of β-GRE, possessed characteristics of both GRE and β-CD, Li *et al.* (2020) referred to this phenomenon as superposition. For instance the initial phases resembled that of β-CD, where approximately 4% weight loss is seen at 60 – 100°C, albeit lower for β-GRE, a phenomenon once reported by Koteswara *et al.* (2020). This structural differences between β-GRE and β-CD, lie with the fact that the latter's truncad cavity was not occupied, therefore allowing more binding of water. Second stage degradation for β-GRE was observed at 260°C and resulted in a weight loss of 46%. Miller *et al*. (2018) reported that green rooibos encapsulated with inulin and maltodextrin started to degrade at 190 and 220°C, respectively. These results show that the thermal stability of GRE was improved by interaction with β-CD, thus, confirmation that complexation took place. Moreover, the thermal stability was greater than that reported by other authors involved in green rooibos encapsulation studies.

4.9.3 Fourier-transform infrared (FT-IR)
FT-IR analysis was performed to confirm encapsulation *via* vibrational deviations formed when

the host and guest molecules interact. Absorption spectra of β-CD, GRE and β-GRE in the 400 – 4000 cm^{-1} regions are depicted in Fig. 4.2. The GRE and β-CD spectra resemble those reported by Akinfenwa *et al.* (2021) and Li *et al.* (2020). All samples displayed peaks located at 1024 – 1 070 cm^{-1}, associated with the phenolic C – O bond, 1606 – 1646 cm^{-1} C=C stretching of aromatic rings, 2929 – 2939 cm^{-1} (C – H stretching) and the broad absorption band at 3267 – 3300 cm^{-1} represented the stretching vibration mode of O – H bond associated with the poly hydroxy groups of β-CD and polyphenols in GRE, in particular the flavonoids being the most abundant polyphenols known to exhibit a broad absorption band between 3,200 to 3,600 cm^{-1}. Pure GRE showed specific bands at 1 030 – 1 070, 1 255, 1 606, 2 939 and 3267 cm^{-1}. Meanwhile, β-CD spectra showed strong bands at 578, 1 024, 1 078, 1 153, 1 646, 2 946 and 3 274 cm^{-1}. The spectrum of β-CD and β-GRE samples overlapped at certain regions and showed certain spectral differences in comparison to the aqueous extract (GRE). Similarities between GRE and β-GRE were observed at 578, 1 025, 1 154 cm^{-1}. When the β-GRE inclusion complex formed, most characteristic peaks of GRE and β-CD disappeared, reappeared, or shifting of bands took place in the newly formed complex. For instance, the band 1 255 cm^{-1} depicting C – O stretching of carboxylic acid disappeared due to formation of hydrogen bonds with β-CD. The GRE 1 030 – 1 070, 1 606 and 2 939 cm^{-1} bands shifted to 1 025, 1 615 and 2 939 cm^{-1} in the β-GRE complex, respectively. Paczkowska *et al.* (2015) observed similar structural changes when forming rutin-β-CD complex. The above results indicate that successful encapsulation took place, and a new compound with new characteristics was formed.
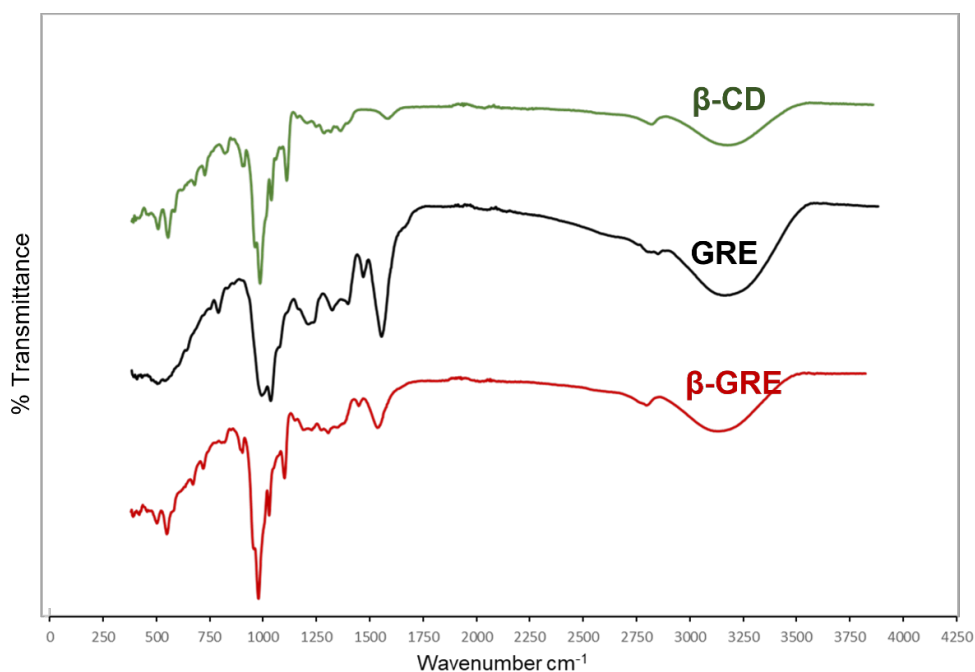
Figure 4.2: Fourier-transform infrared (FT-IR) spectra of beta-cyclodextrin (β-CD), aqueous green rooibos extracts (GRE) and beta-cyclodextrin assisted extracts of green rooibos (β-GRE).

4.10 Conclusions.

In this study the physicochemical properties of β-CD-assisted extracts of green rooibos were characterised in order to ascertain formation of inclusion complexes. The TGA thermogram and FT-IR spectrum revealed that green rooibos β-CD complexes were formed, this was based on the improvement in heat stability, as well as formation of a new molecule which was a superposition of β-CD and green rooibos. For future studies we recommend evaluation of sorption isotherm studies in order to determine the behaviour of the encapsulated extracts under various a_w as opposed to single value a_w and MC. In addition to sorption isotherm, the changes in colour as a result of storage at various a_w.

4.11 References

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CHAPTER 5: NON-ENZYMATIC BROWNING IN APPLES CANNED IN FRUIT JUICE DURING STORAGE.

5.1 Abstract

Non-enzymatic browning (NEB) of ascorbic acid (AA) added canned apples was investigated at 5, 23, 37 and 60°C for 24 weeks. Brix (°B), pH, browning indices (A_{294} and A_{420} nm, lightness (L*value) and colour difference (ΔE*)), reactant consumption (reducing sugars (RS) and AA) and intermediate NEB reaction products (furfural and hydroxymethylfurfural) were monitored. Kinetic modelling was applied to determine the reaction rate constant (k_0 and k_1), the kinetic order, and regression coefficient (R^2). Moreover, the temperature dependence of the NEB indicators was modelled via the Arrhenius equation to determine the apparent activation energy of the reaction.

The initial total sugar content comprised of 66% fructose, 22% glucose and 12% sucrose. The Brix (°B) ranged between 19.58 – 27.00 °B and pH 3.37 – 3.72. Significant changes ($p < 0.05$) in °Brix and pH were observed for each temperature at different storage times. Overall, an increase in °Brix was observed as the storage temperature and time increased, meanwhile, a decrease in pH was observed. As a result, the highest 27°B and lowest pH (3.37) was reported for samples at 60°C after 12 weeks of storage. However, no significant differences ($p > 0.05$) were reported between the pH and Brix of ascorbic acid added (+AA) samples and those without added ascorbic acid (-AA).

Concerning browning indices, the development of intermediate NEB reaction products as measured by A_{294} nm, increased from 4.65 – 281.96, while advanced products at A_{420} nm increased from 0.19 – 9.93 as the temperature and storage time increased. Colour development followed suit and was further demonstrated by the reduction in L* value from 6.64 – 5.98 observed visually as darkening of the samples, as a result, the ΔE* values (3.84 – 61.88) reported were categorised as perceivable browning compared to the reference sample (week zero), apart from +AA at 5°C stored for 4 weeks. In most cases, +AA exhibited faster progression in terms of colour formation compared to -AA. Testament to this was significantly higher ($p < 0.05$) reaction rate constants (k_0 and k_1) reported for +AA. It was evident that +AA samples at 60°C, after 12 weeks storage exhibited the highest A_{294} nm (281.96), A_{420} nm (9.93) ΔE* (61.88) and lowest L*-value (6.64). Absorbance at A_{294} and A_{420} nm followed first-order kinetics at 5 – 37°C and changed to zero-order at 60°C. The opposite was observed for L*-value, meanwhile ΔE* was adequately described as zero-order for all temperatures. An increase in reaction rate constants (k_0 and k_1) was observed as the storage temperature increased for all browning indices, and this was a clear indication that NEB

reactions are favoured by higher temperatures. Samples (+AA) exhibited higher Ea for browning indices compared to -AA, except for A_{294} nm.

No significant changes (p > 0.05) in RS were observed for all sample types. This might be attributed to hydrolysis of sucrose and complex carbohydrates, as well as progression of the MR and sugar degradation taking place simultaneously, that way the production and consumption reducing sugars was in equilibrium. The -AA content of unprocessed canned apples was 10 ± 0.68 mg.L^{-1}, while +AA was 997 mg.L^{-1} ± 1.87. After retorting, no AA was detected in the non-supplemented sample, meanwhile +AA was reduced to 290 mg.L^{-1}. The reduction in AA was insignificant at 5°C, as the temperature increased from 23 – 60 °C, AA decreased significantly (p < 0.05) with time. After 4 weeks of storage, approximately 1, 12, 26 and 92% of AA was lost at 5, 23, 37 and 60 °C, respectively. As a result of this occurrence, complete loss of AA was reported at week 20 and 10 for samples stored at 37°C and 60°C, the degradation of AA was described as zero-order kinetic model.

The HMF and furfural content of canned apples during storage at 5 – 60°C ranged between 0.4 – 26.19 and 1.02 – 64.31 mg.100g^{-1}. The furfural content was higher than HMF, and this was due to the high content of fructose in the sample. No significant increase (p > 0.05) in HMF was observed in all samples stored at 5°C, while for furfural this observation was made for 5 and 23°C. Similar to what was reported for colour development, at temperatures where significance in HMF and furfural were reported, it was observed at different storage times. Overall, the HMF of +AA was significantly higher (p < 0.05) than that of -AA, and that was attributed to the precedence of oxidative degradation of AA which produced reactive species, that in turn, served as reactants in the MR simultaneous to sugar degradation.

The reaction rate constants (k_0 and k_1) increased significantly (p < 0.05) with storage temperature for all samples. The variation in HMF and furfural formation was well fitted to the zero-order model. Moreover, the Ea of +AA was significantly higher than that of -AA for both determinations. Hence, we postulate that AA degradation was responsible for formation of Furfural, and HMF was formed because of mainly sugar dehydration and the MR.

5.2 Introduction

Thermal processing is aimed at converting raw material into end products with longer shelf-life. Not only does this enhance the palatability of these raw materials (Rufian-Henares & Pastoriza, 2016), both spoilage and pathogenic microorganisms are reduced or inactivated. During heat processing there are numerous chemical reactions, and these are usually enhanced by the heat applied, as well as determined by the variety of food components contained in the raw material. These chemical reactions can be desirable or undesirable, depending on the product type. Amongst those, non-enzymatic browning (NEB) is the most commonly occurring of them all. It is desirable due to the brown colour and aromas associated

with baked and fried goods, however, in some instances the brown colour can be perceived as spoilage, such as in UHT milk and powdered milk during storage (Rufian-Henares & Pastoriza, 2016). In this context, only the undesirable effects of NEB will be discussed. The deleterious effects of NEB reactions are as a result of the reaction proceeding via multiple mechanisms.

The aforementioned NEB reactions are of great significance considering that food safety is a major concern for food manufacturers, consumers and regulating authorities. In recent years, various organizations such as the International Agency for Research on Cancer (IARC), Food and Drug Administration (FDA) and the Joint Food Agricultural Organisation/World Health Organisation (FAO/WHO), identified some compounds that are generated during NEB reactions, especially during thermal processing as potentially toxic, mutagenic or even carcinogenic. Such compounds are formed through multiple pathways in which mainly lipids, carbohydrates, amino acids or ascorbic acid are thermally degraded (Nie *et al.*, 2013a; Bharate & Bharate, 2014). Some of these undesirable compounds include furfural, acrylamide and other similar heterocyclic compounds, whose concentration depends on the severity of the heat treatment applied as well as storage time (Nie *et al.*, 2013a; Anese *et al.*, 2014). These compounds have been identified in a wide range of processed foods such as fruits, honey, fruit juices, milk, extruded cereals and bread (Kowalski *et al.*, 2013). Amongst these, furans are known particularly in foods subjected to retorting in hermetically sealed containers which prevents its losses by volatilisation, thus permitting its accumulation (Nie *et al.*, 2013b). For this reason, these compounds are the main focus in this study.

Various studies have reported that the highest levels of furans are formed from the degradation of ascorbic acid. However, since ascorbic acid levels in foods are relatively low, as compared to sugars, amino acids, and unsaturated fatty acids, it can be expected that the latter food constituents are more important in the production of furans in foods than ascorbic acid. Therefore, it is crucial to identify compounds that are furan precursors, and these are namely: furfural, furosine, dimethyl hydroxyl furanone (DMHF) and 5-hydroxymethylfurfural (5-(hydroxymethyl) furan-2-carbaldehyde) (HMF). These thermally-induced toxicants are formed via one or more of the following three pathways: the Maillard reaction; caramelisation or ascorbic acid degradation.

Therefore, it is crucial to elucidate the exact chemical reaction/s taking place or sequence thereof. The first step in analysis is to take in consideration both product intrinsic and extrinsic factors. Important intrinsic factors include ingredients/raw materials composition, especially those known as major and minor reactants for each chemical reaction, pH and oxygen concentrations. Extrinsic factors include the processing parameters, in this instance temperature, product packaging and storage conditions. Monitoring of NEB reactions in food and model systems involves following kinetics of reactant consumption and product formation.

The rate at which certain reactants are consumed aid in mapping the pathway, thus following which chemical reaction proceeds first. In the same light, commonly known markers of NEB are also monitored. This can be done via monitoring the product during its real-time or accelerated shelf-life.

Numerous authors studied NEB reactions by evaluating common reactants and markers in food, especially fruits and their derivatives (juice, concentrates, puree, dried, preserves) during thermal processing and the course of the shelf-life (Aktağ & Gökmen, 2020; Aktag & Gokmen, 2021a; Buvé *et al.*, 2021; Agcam, 2022). Some of the investigations include studying NEB kinetics of peach puree (Garza *et al.*, 1999), colour stability of Cape Gooseberry juice (Olivares-tenorio *et al.*, 2017) and thermal stability of red pitaya juice during heating (Liao *et al.*, 2020b) at single or various temperatures. Some of these authors also went a step further to investigate the role of NEB in apple juice and concentrates (Burdurlu & Karadeniz, 2003; Paravisini & Peterson, 2018; Liao *et al.*, 2020b), as well as quality changes of orange juice during storage (Wibowo *et al.*, 2015c; Paravisini & Peterson, 2019). The afore-mentioned authors collectively studied common factors linked to NEB, these include acids (pH, titratable acidity and organic acid profile), sugars (total sugar content and sugar profile), amino acids, oxygen (headspace and dissolved) content, vitamin C (ascorbic and dehydroascorbic acid) content, markers (browning intensity, colour and fluorescence changes) as well as intermediate end-products (reactive carbonyl species, furfural and HMF). Using the factors, they applied kinetic modelling to track the rate of consumption or depletion of key reactants and formation of end-products. By doing so, specific NEB reactions could be elucidated (Burdurlu & Karadeniz, 2003; Liao *et al.*, 2020b).

Findings of these studies collectively concluded that an increase in processing/heating temperatures increased the depletion of oxygen, ascorbic acid (Olivares-tenorio *et al.*, 2017), sugars (fructose and glucose), and that in cases of high acidity, sucrose was hydrolysed to its monomers, thus heightening the reaction (Burdurlu & Karadeniz, 2003; Wibowo *et al.*, 2015c). In most cases, this consumption coincided with the change in colour and increase in HMF and furfural levels. Moreover, in studies involving storage, the HMF and furfural content depleted, showing its evolution to other NEB by-products (Wibowo *et al.*, 2015c; Liao *et al.*, 2020b).

The food industry has shifted to using fruit juice as a medium for canned fruit. Moreover, these canned products also come in new improved easy to open packaging with different oxygen transmission rate than the traditional can. Therefore, NEB kinetics for the new improved products are unknown. Moreover, most authors studied NEB kinetics of fruits and their derivative products, but none for canned fruit as a whole. Therefore, the aim of this study was to apply kinetic modelling in elucidating NEB reactions in apples canned in apple juice. This was done by monitoring depletion rates of key reactants, namely reducing sugars and ascorbic acid, formation of common NEB indices such as pH, Brix, browning intensity at 294

and 420 nm, colour difference, and the intermediate products furfural and HMF, during storage.

5.3 Materials and methods

5.3.1 Chemicals

'Golden Delicious' apples were purchased from a local supermarket (Bellville, South Africa). Apple concentrate (70°B) was purchased from Associated Fruit Processors (Paarl, South Africa). Sodium tetraborate, sodium dodecyl sulfate, 2-mercaptoethanol, dinitrosalicylic acid, sodium hydroxide, hydroxymethyl furfural, ascorbic acid, Absolute ethanol, thiobarbituric acid acetonitrile, metaphosphoric acid, Carrez I and II, trichloroacetic acid, fructose, glucose, sucrose, methanol and sulphuric acid were purchased at Merck (Modderfontein, South Africa). Sodium potassium tartrate, furfural and o-phthaldialdehyde was purchased at Sigma-Aldrich (Kempton Park, South Africa). Chemicals used in this study were of analytical grade and chemical reagents were prepared according to standard analytical procedures. Prepared reagents were stored under conditions that prevented deterioration or contamination. The water used was purified with the Milli-Q water purification system (Millipore, Microsep, Bellville, SA).

5.3.2 Apple canning process

The process of canning apples was conducted following the guidelines stipulated by the South African Agricultural Product Standards Act (APSA) 119 of 1990. Apple juice concentrate and slice width were prepared according to the specifications stipulated by the APSA Act 119 of 1990. Apple concentrate of 70 °Brix (°B) was diluted with ice cold water to a concentration of 30 °B, followed by the addition of 2 500 mg.kg^{-1} ascorbic acid. Apples were peeled, cored and dipped in ice cold 2% solution of calcium chloride (Ghoul, 2013) after which they were sliced longitudinally from the calyx to the stem into segments of not less than 40 mm in height with thickness ranging between 16 – 20 mm (APSA, 2015). Apple slices (70 g) were weighed into retortable laminated PET PE EVOH (160 mm x 100 mm) self-standing pouches. Ice cold juice concentrate (30 °B) was filled to a final mass of 120 g and the pouches sealed immediately with a pedal heat sealer. The sealed pouches were heat-processed in a horizontal retort at 100°C for 20 min. Temperature profiles in the retort, and at the coldest point of the of the sample was recorded using type T-thermocouples (Ellab, Hillerod, Denmark). Canned apples were stored in temperature-controlled incubators at 5, 23, 37 and 60 °C. Initial sampling was done prior to storage (i.e., immediately after retorting), thereafter sampling was done every second week (60°C) or monthly (5, 23, 37°C) as set out in Tables 5.1 and 5.2. At each sampling period, canned pouches were taken randomly from the incubators.

Table 5.1: Canned apple parameters.

Storage time	6 months
Sampling time	Weekly/monthly
Storage conditions	5°C, 23°C, 37°C and 60°C
No of samples per temperature	50
Net weight	120 g
Drained weight	65 g
Final °Brix	14 – 18

Table 5.2: Canned apple sampling schedule.

Week	Without Ascorbic acid (AA-)				With Ascorbic acid (AA+)			
0	X				X			
	5°C	23°C	37°C	60°C	5°C	23°C	37°C	60°C
2				X				X
4	X	X	X	X	X	X	X	X
6				X				X
8	X	X	X	X	X	X	X	X
10				X				X
12	X	X	X	X	X	X	X	X
14								
16	X	X	X		X	X	X	
18								
20	X	X	X		X	X	X	
22								
24	X	X	X		X	X	X	

5.3.2.1 Sample preparation

During specific sampling days, the canned products were collected from their respective storage, and placed in ice cold water to equilibrate the temperatures. Samples were then homogenised into a puree using a stick blender (Mellerware, SA). The pureed sample was used as it is for measurement of °B, pH and colour. All the other compositional analyses were done using water extracts of the puree. Two grams of apple puree was diluted with 4 mL Milli-Q water, vortexed for 1 min, and then centrifuged (Beckman Coulter, USA) at 12 000 g for 10 min. The resulting supernatant, termed Supernatant-A, was used for measurement of browning index and degree of glycation (Aktag & Gokmen, 2021a). Samples for HMF, furfural,

reducing sugars and sugar profile were further clarified using Carrez I and II solutions. For clarification, 1 mL of the Supernatant-A was mixed with 50 µL of Carrez I and 50 µL of Carrez II solutions, allowed to stand for 30 min, followed by centrifugation at 10,000 rpm for 5 min resulting in Supernatant-B (Aktag & Gokmen, 2021a).

5.4 Measurement of pH and °Brix

The pH of the pureed apple samples was measured using an 827 Lab pH meter (Metrohm, Switzerland) calibrated with buffer solutions of pH 4.0 and 7.0, respectively. The °Brix of the apple puree was determined using a handheld refractometer.

5.5 Determination of browning index

Browning index was conducted following the procedure described by Nie *et al.* (2017) and Wang *et al.* (2013). UV absorbance and browning of appropriately diluted Supernatant – A were determined at 294 and 420 nm, respectively, using a spectrophotometer (Lambda 25, Perkin Elmer, Singapore).

5.6 Colour measurement

The colour of the pureed apple samples was evaluated by measuring CIELab parameters L* (brightness, 100 = white, 0 = black), a* (+ red; - green) and b* (+ yellow; - blue) parameters by means of a spectrophotometer (CM-5, Konika Minolta, Japan), measuring the colour spectra using a D65 day-light source, large viewing area and the observer at 10°. The colour difference was calculated using the following formula:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5}$$

5.7 Determination of reducing sugar content

The reducing sugar content was determined following the dinitrosalicyclic acid (DNS) method described by Somjai *et al.* (2021).One mL of extract and 4 mL of DNS reagent were mixed and vortexed in a screw cap reaction tube. The mixture was heated in boiling water bath for 5 min. The reaction tube contents were cooled down, followed by addition of 10 mL of Milli-Q water. The absorbance of the sample solution was measured at 540 nm against a Milli-Q water blank using a UV-vis spectrophotometer (Lambda 25, Perkin Elmer, Singapore). A calibration curve was drawn with glucose standards (0.2 – 1.0 mg.mL^{-1}).

5.8 Quantification of vitamin C

Extraction and determination of vitamin C was carried out based on the modified HPLC procedure described by Agcam (2022). A 10 g sample of pureed apple was centrifuged

(Beckman Coulter, USA) at 10 000 g for 10 min at 4°C. The resulting supernatant was filtered through a 0.22 μm nylon filter (Millipore, Bedford, MA, USA). Twenty microlitres of the above sample was injected into an Agilent Technologies 1200 Series HPLC (Santa Clara, CA, USA) system with a diode array detector (DAD), while a Novapak C18 (250 mm × 4.6 mm i.d. 4 μm) column thermostated at 25°C was used for the separation. The mobile phase was 25 mM KH_2PO_4 (adjusted to pH 2.55 with phosphoric acid) with a flow rate of 1 mL.min^{-1}. Eluate was monitored by UV detection at 245 nm. Chromatograms were recorded and processed with EZStart Chromatography Software V.7.2. 1. Results were reported as mg.100 mL^{-1} of sample.

5.9 Determination of 5-hydroxymethyl furfural and furfural

Content of furfural and 5-hydroxymethyl furfural was determined according to the spectrophotometric method described by Bharate and Bharate (2014) and Liao *et al.* (2020), with slight modifications. Each 2 mL aliquot of sample was mixed with 2 mL of 12% trichloroacetic acid and 2 mL of 0.025 M thiobarbituric acid, reacted at 40 ± 0.5°C for 50 min, and cooled to room temperature after reaction. The absorbance was measured at 436 nm for furfural and 443 nm for HMF. The HMF and furfural concentrations were calculated through a calibration standard curve capturing the range from 0 to 6.6 mg.L^{-1}.

5.10 Data analysis

Statistical analysis was performed using SPSS 27.0 for Windows®. Descriptive statistical analyses determined the mean and standard deviation of triplicates (n=3). Analysis of variance (ANOVA) established significant differences ($p < 0.05$) of the means. The level of confidence required for significance was selected at 95%. To evaluate the effect of storage temperature and time on the formation of nonenzymatic browning products in apples canned in fruit juice, reactant consumption (reducing sugar and ascorbic acid degradation), evolution of brown products (absorbance at 294 and 420 nm, Lightness (L*), Colour difference (ΔE*)) and formation of intermediates and degradation products (HMF and furfural) were modelled using Excel regression analysis. Different kinetic models were applied and the best fit that describes the changes was selected, and these were: zero-order Eq. (1), first-order Eq. (2), second-order kinetic modelling Eq. (3). The reaction rate constants (k) and regression coefficient (R^2) were obtained for each dependent variable in relation to temperature and time. The best fitting model was decided based on the highest R^2 value. In the second step, the effect of temperature on *k* was calculated from the Arrhenius equation (Eq. (4)) to determine the activation energy (Ea) (Eq. (5)).

$$C = C_0 + k_0 t \qquad\qquad Eq.(1)$$
$$C = C_0 \, exp(k_1 t) \qquad\qquad Eq.(2)$$

$$c = \frac{c_0}{1 + c_0 kt} \qquad\qquad Eq.\,(3)$$

$$\ln k = \ln k_0 - \frac{Ea}{RT} \qquad\qquad Eq.\,(4)$$

$$E_a = -RT \cdot ln\left(k/A\right) \qquad\qquad Eq.\,(5)$$

Where C is concentration at time t, C_0 concentration at time zero, k_0 zero-order rate constant and t storage time, k_1 first-order rate constant, T is absolute temperature in (°K), k is the reaction rate constant at reference temperature in °K and R is the universal gas constant ($8.3145\ J.mol^{-1}.K^{-1}$).

5.11 Results and discussions

5.11.1 °Brix and pH

Temperature, time, pH and concentration of initial reactants (sugars, amino acid, ascorbic acid) are well established factors affecting occurrence of NEB reactions in foods during processing and storage (Pham *et al.*, 2020; Aktag & Gokmen, 2021a). In Food Science literature, the concept of sugar concentration is commonly referred to as total sugar content, total soluble solids, or total dissolved solids expressed as °Brix (°B) (Wibowo *et al.*, 2015c; Pham *et al.*, 2019). These terms will be used interchangeably in this chapter. Tables 5.3 and 5.4 show the evolution of total soluble solid content expressed as °B as well as the pH of apples canned in fruit juice stored at 5, 23 and 37°C for six months and samples stored at 60°C for three months. The initial °Brix prior to storage (week zero) ranged between 19.58 – 19.67 °B for samples with (+AA) and without added ascorbic acid (-AA), respectively. At the end of storage, it varied between 22 and 27°B depending on the storage temperature. It is worthy to mention that the final °Brix values were still within the cut-out Brix set by the APSA ACT 119 of 1990.

In general, no significant (p > 0.05) differences were observed in °Brix for all samples throughout storage (Table 5.3). An exception to this observation was when comparisons were made between respective initial °Brix (week zero) and weeks 2 – 24. At storage temperatures of 5 and 23°C significant increases (p < 0.05) in °Brix were observed at week 24 for samples with added ascorbic acid (+AA). Meanwhile, for samples without added ascorbic acid (-AA), significant increases (p < 0.05) were observed from weeks 16 (5°C) and 12 (23°C) relative to week 0. Similarly, at 37°C and 60 °C increases (p < 0.05) in °Brix were observed from week 12 and 2, respectively, for both +AA and -AA samples. It is worth to note that increases in °Brix observed at staggered storage weeks also reveal the progressive nature of NEB reactions as a function of temperature and time. Whereas, as the temperature increased, the significant increases (p < 0.05) in °Brix relative to week 0 were observed at earlier storage weeks (Table 5.3).

The increase in °Brix reported in the present study was similar to the findings of Wibowo *et al.* (2015) who also reported a slight increase in °Brix of reconstituted pasteurised orange juice stored at 20 and 28°C for 32 weeks. As the temperature increased to 35 and 42°C, the increase was observed at weeks 12 and 8, respectively. These authors linked the increase in °Brix to hydrolysis of fibres present in the orange pulp. As a result, we also speculate that in our study hydrolysis of sucrose, pectin and hemicellulose found in apples was initiated at temperature and time combinations applied during retorting, which then continued throughout storage, and this was more pronounced at higher temperatures (Table 5.3). This was also evident via palpable loss in firmness. However, contrary to our results, Pham *et al.* (2019) did not observe any significant changes ($p > 0.05$) in total soluble solids of 11.3 °B at pH 3.9 in orange juice stored at 42°C for 15 weeks. It is noteworthy to mention that both Wibowo *et al.* (2015) and Pham *et al.* (2019) used orange juice samples that were in the same range in terms of °Brix and pH, as well as processing and storage conditions. In addition, both studies also reported on the decrease in sucrose, and an increase in glucose and fructose content due to hydrolysis. The question then arises as to why they reported opposing results in terms of °Brix. A plausible explanation to this may be due to the complex nature of NEB reactions during processing and storage of fruit-based products as a response to reactant availability and concentration. For instance, hydrolysis of complex carbohydrates (such as pectin, hemicellulose) increases the concentration of simple sugars, fructose and glucose (in solution), which affects both the °Brix and reducing sugar content. However, hydrolysis of sucrose on the other hand does not contribute to an increase in °B, even though the individual fructose and glucose increase the reducing sugar content. This explains why Pham *et al.* (2019) did not observe an increase in °Brix.

In another study, where Vural Gokmen (2021) investigated formation of dicarbonyl compounds and HMF in pasteurised (85°C; 10 min) pomegranate and apple juice concentrates (30 – 70° B) during storage at 37°C for 20 weeks, and reported an insignificant increase ($p > 0.05$) in °B. However, their sample type was a concentrate, different to a fruit juice matrix used in Pham *et al.* (2019) as well as our present study's fruit in juice matrix.

Table 5.3: Brix (°B) of apples canned in apple juice.

	Temp °C	Storage weeks 0	2	4	6	8	10	12	16	20	24
	5	19.58 ± 0.63[ad]	-	19.5 ± 0.87[ad]	-	18.83 ± 0.29[a]	-	20.67 ± 1.44[bi]	20.17 ± 0.29[af]	21.00 ± 0.00[dk]	21.50 ±0.00[em]
	23	19.58 ± 0.63[ad]	-	20.00 ± 0.87[ae]	-	20.83 ± 0.29[cj]	-	20.33 ± 0.58[ag]	20.17 ± 0.29[af]	20.83 ± 0.29[cj]	21.67 ± 0.58[fm]
+AA	37	19.58 ± 0.63[ad]	-	20.67 ± 1.15[bi]	-	21.00 ± 1.00[dk]	-	21.67± 0.29[fm]	22.17 ± 0.76[in]	22.00 ± 0.00[hn]	22.33 ± 0.58[in]
	60	19.58 ± 0.63[ad]	21.83 ± 0.29[gn]	21.67 ± 1.15[fm]	22.17 ± 0.76[in]	22.67 ± 2.02[ln]	26.00 ± 1.00[q]	26.83 ± 1.04[q]			
	5	19.67 ± 1.15[ad]	-	19.33 ± 0.58[ac]		20.33 ± 1.15[ag]		21.00 ± 0.00[dk]	21.33 ± 0.29[el]	21.83 ± 0.29[gm]	22.17 ± 0.64[in]
-AA	23	19.67 ± 1.15[ad]	-	19.17 ± 2.02[ab]	-	20.67 ± 1.15[bi]	-	21.67± 0.29[fm]	22.00 ± 0.00[hn]	21.83 ± 0.49[gm]	22.17 ± 1.00[in]
	37	19.67 ± 1.15[ad]	-	20.5 ± 0.87[bh]	-	21.00 ± 0.00[dk]	-	21.67 ± 0.29[fm]	22.00 ± 0.00[hn]	22.50 ± 0.82[kn]	23.50 ± 0.00[no]
	60	19.67 ± 1.15[ad]	22.00 ± 1.32[hn]	22.33 ± 0.58[jn]	23.00 ± 0.87[mn]	24.50 ± 0.87[op]	25.67 ± 1.15[pq]	27.00 ± 0.00[q]		-	-

Data presented as Brix (°B) of canned apples stored for 24 weeks at various storage temperatures (5, 23, 37 and 60 °C) expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed.

[abc] Means with different letter superscripts on the same row and column denotes significant differences ($p < 0.05$). AA[+] – with added ascorbic acid, AA[-] without added ascorbic acid.

Therefore, while hydrolysis of complex carbohydrates very likely occurred in the present study and that of Pham *et al.* (2019), only sucrose hydrolysis occurred in the fruit concentrates in Aktag and Gokmen (2021a), resulting in an increase in fructose and glucose. The hydrolysis of either complex or simple carbohydrates also puts a different spin on NEB reaction pathways, especially when these reactions occur simultaneously in a product. In both cases, fructose and glucose are increased; depending on which sugar is dominant and its concentration, temperature, time, pH and presence of ascorbic acid or amine group, will then determine which pathway the NEB reaction might follow, and what products are formed (Aktag & Gokmen, 2021b; Agcam, 2022).

As mentioned previously, in the present study the sample is canned apples which, and in most cases examples of studies conducted on canned fruits are limited, therefore we then base our argument or comparison to fruit juices, concentrates and purees, with the latter closely resembling our sample type. Pham *et al.* (2020) conducted a study on the effect of orange fruit juice fractions (liquid, pulp and cloud) on NEB. They found that in a whole fruit juice, browning took place throughout the sample, however, upon separating each fraction, only the liquid serum showed browning. This further concludes that components responsible for NEB were dissolved in the serum, and that through hydrolysis to simple monomers, some of the polymers migrate into the serum.

The pH was initially recorded at 3.69 and 3.72 for samples with (+AA) and without added ascorbic acid (-AA), and at the end of storage the lowest pH was recorded at 3.37 for +AA samples stored at 60°C after 12 weeks. As the storage temperature and time increased, no significant differences ($p > 0.05$) in pH were observed for most samples. Moreover, hydrolysis could also be accelerated by the acidic pH of the samples (Table 5.4).

Therefore, a similar approach to the discussion on °B was adopted, where we reported significance changes in pH of week zero samples compared to weeks 2 – 24, significant decreases ($p < 0.05$) in pH were only observed for +AA samples stored at 23 and 37°C at 24 weeks, and 60°C at 6 weeks. With reference to -AA samples, a significant reduction ($p < 0.05$) in pH was only observed for samples stored at 60°C at week 10. Consequently, at week 12 differences in pH values were observed between +AA and -AA samples.

Potential hydrogen (pH) and °Brix are interdependent factors which are one of the key determinants of the other chemical reactions and the type of NEB pathway that may take place during processing and storage of fruit-based products. High acidic nature of fruits and fruit-based products (pH < 4), will accelerate hydrolysis of carbohydrates, freeing up simple/reducing sugars that may participate in initiating NEB reactions. In terms of NEB reaction types, the high acidic pH (3.37 – 3.72) Table 4.4 does not necessarily promote the MR, but is suitable for caramelisation and ascorbic acid degradation.

Table 5.4: pH of apples canned in apple juice during storage.

	Temp	Storage weeks									
	°C	0	2	4	6	8	10	12	16	20	24
	5	3.69 ± 0.02^{hp}		3.77 ± 0.14^{ms}		3.73 ± 0.06^{ip}	-	3.65 ± 0.04^{em}	3.66 ± 0.03^{em}	3.63 ± 0.02^{el}	3.64 ± 0.07^{em}
	23	3.69 ± 0.02^{hp}		3.74 ± 0.05^{iq}		3.74 ± 0.05^{iq}		3.64 ± 0.02^{em}	3.65 ± 0.05^{em}	3.65 ± 0.00^{em}	3.55 ± 0.01^{ce}
AA+	37	3.69 ± 0.02^{hp}	-	3.62 ± 0.01^{ej}		3.62 ± 0.01^{ej}	-	3.64 ± 0.10^{em}	3.60 ± 0.00^{ch}	3.60 ± 0.02^{ch}	3.50 ± 0.07^{bd}
	60	3.69 ± 0.02^{hp}	3.63 ± 0.00^{el}	3.61 ± 0.24^{di}	3.56 ± 0.01^{cf}	3.55 ± 0.09^{ce}	3.49+ 0.00^{bc}	3.37 ± 0.08^{a}		--	-
	5	3.72 ± 0.04^{hp}	-	3.73 ± 0.10^{ip}	-	3.87 ± 0.00^{rs}	-	3.86 ± 0.16^{qs}	3.75 ± ±0.01^{jr}	3.75 ± 0.03^{jr}	3.68 ± 0.06^{fp}
AA-	23	3.72 ± 0.04^{hp}	-	3.76 ± 0.09^{ls}	-	3.87 ± 0.01^{rs}	-	3.73 ± 0.00^{ip}	3.74 ± 0.03^{iq}	3.79± 0.08^{ns}	3.66 ± 0.06^{en}
	37	3.72 ± 0.04^{hp}	-	3.80 ± 0.00^{ps}	-	3.73 ± 0.07^{ip}	-	3.63 ± 0.01^{el}	3.72 ± 0.01^{hp}	3.62 ± 0.03^{ej}	3.64 ± 0.08^{em}
	60	3.72 ± 0.04^{hp}	3.73 ± 0.00^{ip}	3.68 ± 0.13^{fp}	3.62 ± 0.07^{ej}	3.67 ± 0.01^{eo}	3.55 ±0.08^{ce}	3.49 ± 0.14^{bc}			

Data presented as pH of canned apples stored for 24 weeks at various storage temperatures (5, 23, 37 and 60 °C) expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed.

[abc] Means with different letter superscripts on the same row and column denotes significant differences ($p < 0.05$). AA+ – with added ascorbic acid, AA- without added ascorbic acid.

Moreover, pH has been proven to have a direct impact on the formation and degradation of NEB intermediates such as furfural and HMF. The acidic pH values recorded in the present study (3.37 - 3.72) could enhance ascorbic acid degradation and decomposition of reducing sugars to form reactive carbonyl compounds (RCC) (HMF, methylglyoxal, glyoxal). Maillard-associated browning reactions involving the reaction between amino acids and formed RCC, could occur in conjunction with continued hydrolysis of carbohydrates (simple and complex) throughout storage (Pham *et al.*, 2020; Buvé *et al.*, 2021).

However, the synergistic relationship between °Brix and pH on NEB reactions has been studied and proven to be significant by several authors (Wibowo *et al.*, 2015a; Pham *et al.*, 2019). High temperatures (> 80° C) applied during processing of fruit and fruit-based products, coupled with inherent high carbohydrate content and acidic pH (< 4) are responsible for hydrolysis of simple (sucrose) and complex (pectin, lignin, cellulose and hemicellulose) carbohydrates, resulting in increased reducing sugars, and total sugar/soluble solids content (Pham *et al.*, 2019). In each case, the total dissolved solids/sugar content and pH determines the type of NEB reaction that is likely to take place in a product. In the current study we reported that significant changes in pH and °Brix were observed at higher temperatures of 37 and 60°C and longer storage time, mostly for +AA samples. This was attributed to hydrolysis of sucrose and other complex carbohydrates, followed by the MR involving the resulting reducing sugars and amino acids, hence the reduction in pH due formation of acids.

However, since significant differences in °Brix and pH were observed only in a few cases, we could not apply kinetics to evaluate the rate constant and regression coefficient as to determine the effect of these two variables in browning of canned apples.

5.11.2 Browning indices
5.11.2.1 Absorbance at 294 and 420 nm

The formation of intermediate colourless NEB reaction products is measured at 294 nm. Intermediate products are deemed precursors of the browning products formed in the final phase of browning reactions, and these are monitored by measuring the absorbance at 420 nm (Yu *et al.*, 2018). The absorbance at 294 nm is shown in Table 5.5. The initial absorbance was recorded at 4.65 for +AA and 5.60 for -AA samples. In general, as the storage temperature and time increased, an increase in browning was observed, with a few exceptions. These include +AA samples stored at 5 and 23°C which only exhibited a significant increase (p < 0.05) from the initial absorbance (week 0) starting at week 16 and 8, respectively. On the other hand, +AA samples stored at 37 and 60°C exhibited an increase from the onset of storage, this further proves the effect of storage temperature on browning evolution as reported by (Buvé *et al.*, 2021).

Table 5.5: Browning index ($A_{294\ nm}$) of apples canned in apple juice during storage.

AA	Temp °C	Storage weeks 0	2	4	6	8	10	12	16	20	24
+	5	4.65 ± 0.05^a	-	5.48 ± 0.13^{ab}	-	5.47 ± 0.06^{ab}	-	5.55 ± 0.01^{ab}	10.93 ± 0.01^g	18.05 ± 0.22^j	18.67 ± 0.57^{jk}
	23	4.65 ± 0.05^a	-	6.68 ± 0.50^{ae}	-	7.80 ± 0.34^{cf}	-	8.78 ± 0.17^{ef}	14.25 ± 0.06^{hi}	20.21 ± 0.39^k	22.70 ± 0.14^l
	37	4.65 ± 0.05^a	-	7.23 ± 0.14^{be}	-	8.16 ± 0.09^{df}	-	14.45 ± 0.19^{hi}	22.37 ± 0.29^l	46.45 ± 0.95^p	55.22 ± 1.00^q
	60	4.65 ± 0.05^a	36.16 ± 0.31^n	82.68 ± 0.55^r	116.64 ± 1.10^u	176.52 ± 0.83^x	227.33 ± 4.62^y	281.96 ± 2.60^{aa}			
-	5	5.60 ± 0.12^{ab}	-	5.82 ± 0.13^{ac}	-	5.92 ± 0.12^{ac}	-	5.80 ± 0.10^{ac}	10.90 ± 0.16^g	15.74 ± 0.42^{hi}	16.16 ± 0.25^i
	23	5.60 ± 0.12^{ab}	-	6.20 ± 0.37^{ad}	-	7.89 ± 0.18^{cf}	-	8.18 ± 0.10^{df}	13.82 ± 0.31^h	18.91 ± 0.17^{jk}	19.20 ± 0.21^{jk}
	37	5.60 ± 0.12^{ab}	-	7.25 ± 0.04^{be}	-	8.25 ± 0.08^{df}	-	9.82 ± 0.12^{fg}	14.51 ± 0.02^{hi}	33.79 ± 0.51^m	40.75 ± 0.97^o
	60	5.60 ± 0.12^{ab}	35.78 ± 0.81^n	91.83 ± 2.02^s	104.27 ± 1.26^t	145.38 ± 2.89^v	173.00 ± 3.46^w	231.87 ± 0.98^z			

Data presented as absorbance at 294 nm of canned apples stored for 24 weeks at various storage temperatures (5, 23, 37 and 60 °C) expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed. [abc] Means with different letter superscripts on the same row and column denotes significant differences ($p < 0.05$). AA – ascorbic acid

As a result, the highest absorbance was recorded at 281.96 for +AA samples stored at 60 °C after 12-week storage (Table 5.5). Moreover, as opposed to results reported for pH and °Brix, significant differences ($p < 0.05$) in absorbance between storage temperatures per week were also recorded, these observations were made from week 12 (+AA) and 20 (-AA).

Regarding absorbance at 420 nm, an increase in browning was observed with an increase in storage temperature and time, with a few exceptions (Table 5.6). At 5°C storage, no significant change ($p > 0.05$) in browning was observed for the first 8 and 12 weeks of storage for +AA and -AA samples, respectively. At 23°C, a significant increase1 ($p < 0.05$) from day zero was observed, however, thereafter browning remained constant from weeks 4 to 8, for +AA samples and weeks 4 - 12 for -AA. However, at 37°C significant increases were observed throughout storage, apart from samples without ascorbic acid at week 8 and 12. Our findings are in agreement with results reported by Paravisini and Peterson (2018; 2019) when they studied NEB development during storage of juice, respectively. In both studies they found that browning intensity (420 nm) of apple and orange juice stored at 4 and 35°C for 8 – 10 weeks exhibited a similar trend, whereby at refrigerated temperatures of 4°C no significant increase in browning was observed for orange juice throughout an eight-week storage, meanwhile, with apple juice, significant differences ($p < 0.05$) were observed after 10 weeks of storage. The discrepancy above might be attributed to the shorter storage period of eight weeks for orange, compared to ten weeks for apple juice. Another plausible explanation would be the difference in sugar profile of orange compared to apple juice. Apple juice contains higher quantities of fructose compared to orange juice (Wibowo *et al.*, 2015c; Pham *et al.*, 2020; Aktag & Gokmen, 2021a). In fruits and fruit-based products, fructose was found to possess a higher potential than glucose in inducing browning during processing and storage (Agcam, 2022). Consequently, at 35°C, an increase in absorbance was observed after four weeks of storage for both apple and orange juice. In both these instances, temperature and time parameters applied by Paravisini and Peterson (2018; 2019) were similar to those applied in the current study.

Samples stored at 60°C exhibited the highest increase in browning, for instance after two weeks of storage the absorbance value for +AA samples was 2.36 which was even higher or equal to that of +AA samples at 5 and 23°C after 24 weeks of storage, and 37°C for -AA samples. Based on the results in Tables 5.5 and 5.6, it is evident that progression of brown compound formation was faster in +AA samples compared to -AA samples. Moreover, similar trends of browning were observed between absorbance at 294 and 420 nm, where +AA samples were superior. The concept of colourless intermediate correlating with brown compound formation was reported by Yu *et al.* (2018) when they investigated NEB in ascorbic acid/amino acid model system.

Table 5.6: Browning index (A $_{420\,nm}$) of apples canned in apple juice.

AA	Temp °C	Storage weeks									
		0	2	4	6	8	10	12	16	20	24
+	5	0.19 ± 0.01^a	-	0.25 ± 0.04^{ab}	-	0.28 ± 0.02^{ab}	-	0.50 ± 0.00^{df}	0.71 ± 0.01^{gi}	1.24 ± 0.04^{kl}	1.63 ± 0.12^n
	23	0.19 ± 0.01^a	-	0.44 ± 0.04^{ce}	-	0.48 ± 0.03^{df}	-	0.68 ± 0.03^{gi}	0.95 ± 0.01^j	1.39 ± 0.01^{lm}	2.30 ± 0.16^p
	37	0.19 ± 0.01^a	-	0.59 ± 0.02^{eh}	-	0.75 ± 0.04^{hi}	-	1.14 ± 0.01^k	1.78 ± 0.19^o	2.94 ± 0.09^r	3.80 ± 0.18^t
	60	0.19 ± 0.01^a	2.36 ± 0.19^{pq}	2.47 ± 0.13^q	3.81 ± 0.03^t	5.35 ± 0.06^u	7.94 ± 0.07^w	9.93 ± 0.11^x	-	-	-
-	5	0.28 ± 0.02^{ac}	-	0.26 ± 0.02^{ab}	-	0.27 ± 0.03^{ab}	-	0.37 ± 0.03^{bd}	0.67 ± 0.05^{gi}	1.16 ± 0.04^k	1.30 ± 0.02^{kl}
	23	0.28 ± 0.02^{ac}	-	0.47 ± 0.05^{df}	-	0.54 ± 0.00^{eg}	-	0.61 ± 0.01^{eh}	0.83 ± 0.01^{ij}	1.51 ± 0.17^{mn}	1.88 ± 0.04^o
	37	0.28 ± 0.02^{ac}	-	0.64 ± 0.02^{fh}	-	0.91 ± 0.09^j	-	0.93 ± 0.01^j	1.17 ± 0.04^k	2.51 ± 0.10^q	2.98 ± 0.06^r
	60	0.28 ± 0.02^{ac}	1.61 ± 0.01^n	1.87 ± 0.12^o	2.87 ± 0.22^r	3.43 ± 0.17^s	3.94 ± 0.0^t	6.09 ± 0.04^v	-	-	-

Data presented as absorbance at 420 nm of canned apples stored for 24 weeks at various storage temperatures (5, 23, 37 and 60 °C) expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed. [abc] Means with different letter superscripts on the same row and column denotes significant differences ($p < 0.05$). AA – ascorbic acid

Another important variable in terms of browning development is the presence of ascorbic acid (+AA). It is evident in our study that supplementation with ascorbic acid influenced both intermediate (A_{294nm}) products and browning ($A4_{20nm}$) development, especially at higher temperatures of 37 and 60°C for longer storage times (Tables 5.5 and 5.6). Ascorbic acid is known to participate in the NEB directly as a dicarbonyl or via its aerobic/anaerobic degradation products in Maillard-associated reactions. Pham *et al.* (2020) concluded that ascorbic acid was essential in inducing browning in orange juice model systems. Therefore, we conclude that storage temperature and time, as well as addition of ascorbic acid influenced the browning intensity of canned apples.

5.11.3 Colour

5.11.3.1 Lightness

An increase in storage temperature and time caused darkening of canned apple samples, this is reflected in the decrease in lightness (L*) as shown in Table 5.7. The L* value decreased as the storage temperature and time increased, with the lowest values reported for all samples stored at 60°C for weeks 10 – 12, and visually these were the darkest samples. With reference to +AA samples stored at 5°C, no significant differences ($p > 0.05$) were observed between week 0 and week 4, however, as storage temperature increased to 23, 37 and 60°C, differences ($p < 0.05$) between week 0 and subsequent weeks were observed, however, it is worthy to note that at temperatures of 5 and 23°C, significant differences ($p < 0.05$) between subsequent weeks were observed between the last two storage weeks, 20 and 24 only. Samples stored at 37°C exhibited a significant decrease ($p < 0.05$) in L* value between storage weeks throughout storage, meanwhile at 60°C a similar trend was observed, however, at week 10 and 12 at 60°C, change in browning was not significant ($p > 0.05$). Similar occurrences were observed by Burdurlu and Karadeniz, (2003) when investigating the effect of storage on NEB in apple juice concentrates.

On the other hand, -AA samples at week 0 had a significantly lower ($p < 0.05$) L* value compared to their counterpart (+AA). This might have been attributed to the oxidising potential of AA that resulted in bleaching of the substrates (juice and apple slices) as reported by Luo *et al.* (2011). A significant decrease ($p < 0.05$) in lightness were observed between week zero samples, and subsequent weeks of storage for all temperatures. Some trends observed with +AA samples were also observed in -AA samples. For instance, significant differences between storage weeks for 5 and 23° was also observed for -AA samples in the last two weeks. Differences in L* values were observed throughout storage weeks for 37°C, and the L* value of samples at 60°C remained constant from week 10 to 12 (Tables 5.7).

Table 5.7: Browning index (Lightness L*) of apples canned in apple juice.

AA	Temp (°C)	Storage weeks									
		0	2	4	6	8	10	12	16	20	24
	5	65.98 ± 0.52^{aa}		63.84 ± 0.58^{zaa}		63.52 ± 0.84^{z}		61.94 ± 0.29^{yz}	62.70 ± 0.51^{z}	54.47 ± 0.75^{sv}	50.59 ± 0.61^{pr}
	23	65.98 ± 0.52^{aa}		58.76 ± 0.41^{wx}		56.48 ± 0.53^{vw}		55.89 ± 0.20^{v}	55.34 ± 1.13^{uv}	53.06 ± 0.58^{ru}	46.18 ± 2.34^{mn}
	37	65.98 ± 0.52^{aa}		52.24 ± 0.20^{rs}		48.04 ± 0.31^{np}		41.83 ± 0.25^{k}	34.80 ± 0.33^{hi}	27.98 ± 0.00^{fg}	23.97 ± 2.08^{e}
+	60	65.98 ± 0.52^{aa}	51.27 ± 0.67^{qr}	29.43 ± 0.75^{g}	18.82 ± 0.32^{d}	11.69 ± 0.35^{c}	8.96 ± 0.18^{ab}	6.64 ± 0.42^{a}			
	5	60.20 ± 0.57^{xy}		56.39 ± 0.20^{vw}		56.60 ± 0.54^{vw}		55.81 ± 0.33^{v}	55.08 ± 0.23^{tv}	48.48 ± 0.61^{np}	42.11 ± 7.64^{k}
	23	60.20 ± 0.57^{xy}		56.08 ± 0.47^{v}		52.71 ± 0.46^{rt}		52.17 ± 0.22^{rs}	49.62 ± 0.68^{oq}	47.64 ± 3.88^{no}	37.96 ± 2.44^{j}
	37	60.20 ± 0.57^{xy}		52.52 ± 0.83^{rt}		48.77 ± 0.34^{oq}		43.09 ± 0.21^{kl}	36.82 ± 0.67^{ij}	33.28 ± 0.00^{h}	25.69 ± 1.20^{ef}
-	60		44.98 ± 0.64^{lm}	27.93 ± 0.50^{fg}	19.83 ± 0.31^{d}	12.92 ± 0.17^{c}	11.05 ± 0.28^{bc}	8.61 ± 0.31^{ab}			
		60.20 ± 0.57^{xy}									

Data presented as lightness of canned apples stored for 24 weeks at various storage temperatures (5, 23, 37 and 60 °C) expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed. [abc] Means with different letter superscripts on the same row and column denotes significant differences ($p < 0.05$). AA – ascorbic acid

Overall, the reduction in L* value was greater in samples without ascorbic acid. This was evident in lower storage temperatures of 5 and 23°C, with few exceptions at 37 and 60°C. A possible explanation could be linked with the time that lapsed between peeling, addition of juice and heat processing. As previously mentioned, some unavoidable EB might have been initiated. Samples with ascorbic acid could have been protected by the natural reduction role of ascorbic acid, a protective effect not present in the -AA samples, therefore, a slight onset in enzymatic browning that resulted in pre-heating darkening might have occurred. The decrease in L* value is in accordance with the increase in browning at 420 nm. In both cases, brown coloured compounds are generated, and they intensify as the storage temperatures and time increase throughout storage.

5.11.3.2 Colour difference

Table 5.8 depicts the colour difference (ΔE) of apples canned in apple juice during storage. Colour difference remains the best parameter used to describe colour variations since it is calculated from the L*, a* and b* value coordinates (Ibarz *et al.*, 1999). It depicts the change in visual colour in comparison to a given reference, in this case week zero samples. Colour difference is a more accurate predictor of colour formation than the L*a*b* coordinates, since it tells us about noticeable changes that can be perceived visually. According to literature, ΔE of 1 is the threshold at which a trained observer would notice the difference between two objects, whereas ΔE between 4 and 8 is deemed acceptable, and above 8 is deemed unacceptable, and likely to be rejected by consumers (Mokrzycki & Tatol, 2011). The colour difference of apples canned in fruit juice ranged from 3.84 to 61.88 as depicted in Table 5.8. Most values reported in this study, with the exception of samples stored at 5°C for four weeks, were all categorised as perceivable browning compared to the reference sample (week zero).

The effect of storage time and temperature on colour difference followed a similar trend to what was observed with aforementioned indices (abs 294, 420 nm and L* value). At 5°C the increase in colour difference of +AA and -AA samples were observed from week 4 to 8 ($p < 0.05$), then it remained constant from week 12 to 16 ($p > 0.05$), followed by a significant increase to week 20 ($p < 0.05$), and thereafter remined constant till the end of storage (24 weeks) ($p > 0.05$). Samples at 23°C exhibited an increase from week 4 to 8 ($p < 0.05$)and then remained constant until week 20 ($p > 0.05$), and then an increase at week 24 ($p < 0.05$). At 37 and 60°C, increases ($p < 0.05$) in ΔE were observed throughout storage, with an exception of -AA samples stored at 37°C, where no increase was observed between week 12 and 16.

Table 5.8: Browning index (ΔE colour difference) of apples canned in apple juice.

AA	Temp	Storage weeks								
	(°C)	2	4	6	8	10	12	16	20	24
+	5	-	3.84 ± 0.20^a	-	7.36 ± 0.15^c	-	10.84 ± 0.38^d	12.67 ± 0.37^{de}	17.15 ± 2.56^{gj}	18.96 ± 0.56^{il}
	23	-	12.44 ± 0.11^{de}	-	15.51 ± 0.21^{fg}	-	18.24 ± 0.56^{gk}	18.50 ± 0.32^{hk}	19.39 ± 0.72^{jl}	23.91 ± 3.67^{mn}
	37	-	16.51 ± 0.39^{fi}	-	20.51 ± 0.34^{kl}	-	25.58 ± 0.33^{no}	33.89 ± 0.27^q	40.83 ± 0.47^{tu}	43.63 ± 2.46^v
	60	19.06 ± 0.12^{il}	38.83 ± 0.05^{st}	48.49 ± 0.25^w	55.97 ± 0.19^x	59.11 ± 0.34^y	61.88 ± 0.05^z	-	-	-
-	5	-	4.6 ± 0.01^{ab}	-	11.55 ± 0.47^{de}	-	11.99 ± 0.29^{de}	12.21 ± 0.15^{de}	17.50 ± 0.06^{gj}	21.57 ± 7.31^{lm}
	23	-	6.56 ± 0.67^{bc}	-	11.75 ± 0.10^{de}	-	15.8 ± 0.51^{fh}	17.2 ± 0.09^{gj}	17.49 ± 3.57^{gj}	24.37 ± 1.92^n
	37	-	10.34 ± 0.19^d	-	13.95 ± 0.24^{ef}	-	25.50 ± 0.35^{no}	26.99 ± 0.06^o	30.52 ± 0.44^p	36.70 ± 1.30^{rs}
	60	19.00 ± 0.07^{il}	34.55 ± 0.12^{qr}	41.69 ± 0.27^{uv}	48.77 ± 0.41^w	50.63 ± 0.29^w	54.10 ± 0.25^x	-	-	-

Data presented as lightness of canned apples stored for 24 weeks at various storage temperatures (5, 23, 37 and 60 °C) expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed. [abc] Means with different letter superscripts on the same row and column denotes significant differences ($p < 0.05$). AA – ascorbic acid

In most cases, +AA samples exhibited higher ΔE compared to -AA, especially at 37 and 60°C. Hence, it was possible that ascorbic acid was directly involved in inducing NEB taking place in these samples, this might be *via* its direct participation as a reactant during aerobic/anaerobic degradation to form reactive carbonyl compounds (HMF, furfural, glyoxal) or which then participate in the so-called Maillard-associated reactions, whereby the formed reactive carbonyl compounds react with amino acids to form brown polymers.

5.11.3.3 Kinetic modelling for browning indices

Based on the above reported results for all browning indices (abs 294 and 420 nm, lightness, and colour difference), it is evident that in most cases, +AA samples exhibited significantly higher ($p < 0.05$) browning indices compared to -AA. Therefore, it was essential to establish kinetics pertaining to the evolution of the brown colour; this aided in characterising the type of NEB reactions that occurred in the samples. Kinetics determine the rate at which the reaction progressed at each temperature; this was achieved *via* the reaction rate constant. Moreover, the temperature dependence of the NEB reactions was modelled with the Arrhenius equation to be able to determine the apparent activation energy required to drive the reaction (VanBoekel, 2021).

Experimental data collected for absorbance at 294 and 420 nm were fitted to kinetic models regressed against storage time. Tables 5.9 and 5.10 show the rate of intermediate and advanced brown compounds formation for +AA and -AA, in response to storage temperature and time as zero- and first-order reaction kinetics. Evolution of colour was adequately described by both zero- and first-order kinetics, however, the one which exhibits the best fit was selected. The selection was based on the highest coefficient of determination (R^2) value as described by Chutintrasri & Noomhorm (2007).

An increase in reaction rate constants (k_0 and k_1) was observed as the storage temperature increased ($p < 0.05$) (Tables 5.9 and 5.10), and this was a clear indication that NEB reactions are favoured by higher temperatures (Chutintrasri & Noomhorm, 2007; Lyu *et al.*, 2018). For instance, the k_0 for A_{294} nm for +AA samples at 5, 23, 37 and 60 were 0.6488, 0.7826, 2.1817 and 23.3590, respectively. Similar findings were reported for k_1 (Table 5.9). With regards to supplementation, +AA samples exhibited higher ($p < 0.05$) reaction rate constants (k_0 and k_1) in both models compared to -AA for A_{294} and A_{420} nm. This further indicates that colourless NEB intermediates at 294 nm progressed faster to brown coloured compounds as measured at 420 nm in +AA samples. At storage temperatures of 5, 23 and 37°C, the reaction was classified as first-order (green highlight). However, as the temperature increased to 60°C, a zero-order model had a better fit (blue highlight). This was based on the zero-order model determination coefficients (R^2) of 0.9916 and 0.9838 for +AA and -AA sample, compared to 0.8234 and 0.7933 obtained for fist-order models (Tables 5.9 and 5.10).

Table 5.9: Kinetic parameters for absorbance (A_{294} nm) of canned apples.

Kinetic model	T (°C)	+ Ascorbic acid			- Ascorbic acid		
		k	Ea (KJ.mol^{-1})	R^2	k	Ea (KJ.mol^{-1})	R^2
$n = 0$	5	0.6488[aB]		0.8196	0.5043[aA]		0.8118
	23	0.7826[bB]		0.9234	0.6443[bA]		0.9003
	37	2.1817[cB]		0.8647	1.4714[cA]		0.8043
	60	23.3590[dB]		0.9916	17.9790[dA]		0.9838
$n = 1$	5	0.0647[1B]		0.8598	0.0516[1A]		0.8237
	23	0.0676[1B]	10.90	0.9730	0.0579[1A]	10.89	0.9419
	37	0.1085[2B]		0.9765	0.0857[2A]		0.9226
	60	0.2991[3B]		0.8234	0.2640[3A]		0.7933

$n = 0$ Zero order kinetics, $n = 1$ First order kinetics, T temperature, k rate constant, Ea activation energy, R^2 regression coefficient. Lowercase ([abcd]) and number ([1234]) superscripts in the same column denote statistical difference ($p < 0.05$). Uppercase ([AB]) superscripts across rows denote statistical difference ($p < 0.05$).

Table 5.10: Kinetic parameters for absorbance (A420 nm) of canned apples.

Kinetic model	T (°C)	+ Ascorbic acid			- Ascorbic acid		
		k	Ea (KJ.mol^{-1})	R^2	k	Ea (KJ.mol^{-1})	R^2
$n = 0$	5	0.0600[aB]		0.8794	0.0471[aA]		0.8348
	23	0.0774[bB]		0.8605	0.0641[bA]		0.8678
	37	0.1477[cB]		0.9227	0.1079[cA]		0.8641
	60	0.7723[dB]		0.9581	0.4224[dA]		0.9459
$n = 1$	5	0.0928[1B]	4.72	0.9735	0.0760[1A]	3.58	0.8827
	23	0.0937[1B]		0.9707	0.0763[1A]		0.9589
	37	0.1162[2B]		0.9617	0.0902[2A]		0.9334
	60	0.2679[3B]		0.7760	0.2082[3A]		0.8031

$n = 0$ Zero order kinetics, $n = 1$ First order kinetics, T temperature, k rate constant, Ea activation energy, R^2 regression coefficient. Lowercase ([abcd]) and number ([1234]) superscripts in the same column denote statistical difference ($p < 0.05$). Uppercase ([AB]) superscripts across rows denote statistical difference ($p < 0.05$).

Testimony to the adequacy of fit of either zero- or first-order in terms of browning is the activation energy (Ea) for each model. The Ea values are depicted in Tables 5.9 and 5.10. The Ea was calculated based on recommendations of van Boekel (2021), where the Arrhenius equation is applied only for temperatures which adhered to the same order, in this case 5 to 37°C. Van Boekel (2021) alluded that inclusion of other temperatures outside of the said model may introduce temperature dependence creeps in the Arrhenius equation, whereas the reaction only accounts for temperature dependence of the rate constant (VanBoekel, 2021). The Ea for absorbance at 294 nm was calculated using the reaction rate constants (k_1) for 5 – 37°C. No significant differences ($p > 0.05$) were observed between the Ea of +AA at 10.89 and -AA samples at 10.90 kJ.mol^{-1}. Meanwhile for A_{420} nm, a significant difference ($p < 0.05$) in Ea of +AA and -AA were observed at 4.71 and 3.58 kJ.mol^{-1}, respectively. This implies that browning at A_{420} nm in -AA canned apples was less temperature dependent than in +AA samples. Higher Ea signifies greater temperature sensitivity of colour change due to browning during thermal processing.

Comparing our results with those of other authors, similar conclusions were made by Garza *et al.* (1999) and Ibarz *et al.* (1999) when studying NEB kinetics of peach and apple puree, respectively. These authors also observed the temperature dependency of reaction rate constants. However, they could not assign which model was superior due to insignificant differences ($p > 0.05$) between the R^2 of the models. On the other hand, Lyu *et al.* (2018) reported similar results where both zero- and first-order models fitted the change in browning (A_{420}) when studying kinetic modelling of NEB in peach juice during storage. They characterised the A_{420} as best fitted by a combined kinetic model.

The progressive increase in R^2 with treatment temperature, reaching maximum values for the samples treated at 60°C for first order (0.9836 – 0.9888) indicates that this model fits better the higher the storage temperature, and, in consequence, the more developed the non-enzymatic browning reactions are (Garza *et al.*, 1999).

In addition, our assumption is also based on results obtained by Yu *et al.* (2018) on browning kinetics of L-ascorbic acid-basic amino acid model systems. They concluded that L-ascorbic acid degradation led to formation of colourless intermediates, which then reacted with lysine, histidine, or arginine to form brown polymers *via* the MR. A plausible explanation for our results could be, that under these conditions of acidic pH (3.37 – 3.72), initial retort and storage temperatures, and presence of common NEB reactants (ascorbic acid, sugars, amino acids); the aerobic ascorbic acid degradation pathway took precedence over the MR or sugar dehydration. At temperatures of 5 - 37°C, ascorbic acid related browning took place, hence the fit was described as first-order. Then at higher temperatures of 60°C, we postulate that the Maillard-associated reactions took place and was due to the reactivity of either the ascorbic acid degradation products and amino acids, or inherent sugars. Although it is a known that the

MR is not favoured by acidic environment (pH < 4), however, elevated storage temperature (60°C), as well as increased concentration/supply of reactants overcome the effect of pH. It is also worthy to mention that zero-order kinetic models are synonymous with the Maillard reaction type of browning (Garza *et al.*, 2000). The complexity of NEB during processing and storage of fruit and fruit-based products makes it difficult to establish a single kinetic model that adequately describes the entire reaction mechanism (Lyu *et al.*, 2018)

Kinetic parameters for lightness (L* value) of canned apple slices are shown in Table (5.11). ANOVA Duncan's multiple range test revealed that significant increase ($p < 0.05$) in reaction rate constants were observed as the storage temperature increased for both models, except for k_1 at 5 and 23°C for -AA samples. This is evident that NEB reactions are favoured by higher temperatures. However, an anomaly was observed, where k_0 for 5 and 23°C was higher ($p < 0.05$) for -AA compared to +AA samples. This might be attributed to the bleaching effect in AA, where the L* value was higher for +AA at week zero. Therefore, at lower temperatures the browning of +AA lagged, resulting in -AA samples browning faster. As the temperature increased to 37 and 60°C, the reaction rate constants for +AA was higher than that of -AA. However, the about-turn in terms of higher reaction rates is linked to the effect of higher temperatures on the faster progression of AA degradation during storage.

Moreover, significant differences ($p < 0.05$) between regression coefficients (R^2) of samples across models were also observed. As per rule, the model with the highest R^2 was deemed the best fit for explaining the evolution of lightness. As a result, the reduction in L-value of samples stored at 5 – 37°C, was best characterised as zero-order, and as the temperature increased to 60°C, first-order model became the best fit. This observation is an inverse of what was observed for A_{294} and A_{420} nm in terms of model adequacy. This could be linked to the fact that at A_{294} and A_{420} nm specific NEB markers are tested. Meanwhile the L* value, is not specific to any NEB marker, and can also be linked to darkening due to other chemical reactions such as carotenoid polymerisation as reported by Chutintrasri and Noomhorm (2007).

Another plausible explanation may be linked to a phenomenon pointed out by Garza *et al.* (1999) during heating of peach puree. They stated that NEB reactions cannot be adequately described by simple/single models based on its two-stage reaction. The first stage characterised by coloured compound formation follows zero-order, meanwhile the second stage comprised of decomposition of coloured polymers into non-coloured *via* first-order. They then proposed that the colour formation during NEB of peach should be expressed as combined kinetics. This was corroborated by Lyu *et al.* (2018).

Table 5.11: Kinetic parameters for lightness (L*) of canned apples.

Kinetic model	T (°C)	Ascorbic acid				No ascorbic acid		
		k	Ea (KJ.mol⁻¹)	R²		*k*	Ea (KJ.mol⁻¹)	R²
n = 0	5	-0.5868^{aA}		0.8044		-0.6394^{aB}		0.8120
	23	-0.6424^{bA}		0.8680		-0.7741^{bB}		0.9009
	37	-1.6766^{cB}	22.14	0.9896		-1.3748^{cA}	16.47	0.9936
	60	-5.0072^{dB}		0.8903		-4.2439^{dA}		0.8897
n = 1	5	-0.0101^{1A}		0.7897		-0.0125^{1B}		0.7861
	23	-0.0116^{2A}		0.8644		-0.0158^{2B}		0.8613
	37	-0.0411^{3B}		0.9765		-0.0335^{3A}		0.9756
	60	-0.2018^{4B}		0.9888		-0.1681^{4A}		0.9836

$n = 0$ Zero order kinetics, $n = 1$ First order kinetics, T temperature, k rate constant, Ea activation energy, R² regression coefficient. Lowercase ([abcd]) and number ([1234]) superscripts in the same column denote statistical difference ($p < 0.05$). Uppercase ([AB]) superscripts across rows denote statistical difference ($p < 0.05$).

The Ea of L* value is depicted in Table 5.11. The Ea value of +AA samples was significantly higher ($p < 0.05$) at 22.14 kJ.mol⁻¹ compared to 16.47 kJ.mol⁻¹ of -AA.

The kinetic models for total colour difference (ΔE*) of canned apples are shown in Table 5.12. The reaction rate constants (k_0 and k_1) increased significantly ($p < 0.05$) as the storage temperature increased, a phenomenon associated with NEB reactions and has been reported by several authors (Wibowo *et al.*, 2015a; Pham *et al.*, 2019). The reaction rate constant for +AA samples stored at 5 and 23°C were significantly lower ($p < 0.05$) than -AA; however, this occurrence was reversed as storage temperatures increased to 37 and 60°C. Similar observations were made for the L* value. The R² values for zero-order models ranged between 0.8780 to 0.9532 and 0.8958 to 0.9057 for +AA and -AA samples, respectively. These were significantly higher ($p < 0.05$) than those obtained for first-order models; as a result, zero-order kinetic models were deemed to adequately explain the changes in ΔE* (green highlights). Chutintrasri and Noomhorm (2007) found that colour difference of peach puree fitted zero-order kinetics. Meanwhile, Garza *et al.* (1999) described colour difference in peach puree during heating at 80 – 98°C as mixed order kinetics characterised by both zero- and first order models.

The effect of temperature variation on ΔE* during storage was established *via* the Arrhenius as depicted in Table 5.12. In the present study, the Ea was calculated for zero-order as described by van Boekel (2021). The Ea of +AA was significantly higher ($p < 0.05$) at 27.49

kJ.mol^{-1} compared to -AA samples at 22.75 kJ.mol^{-1}. This implies that colour change in +AA samples was more sensitive to a change in temperature compared -AA samples.

Table 5.12: Kinetic parameters for colour difference (ΔE) of canned apples.

Kinetic model	T (°C)	Ascorbic acid			No ascorbic acid		
		k	Ea (KJ.mol^{-1})	R^2	*k*	Ea (KJ.mol^{-1})	R^2
n = 0	5	0.5889^{aA}	27.49	0.9532	0.6568^{aB}	22.75	0.9057
	23	0.7687^{bA}		0.9875	0.8327^{bB}		0.9020
	37	1.4625^{cB}		0.9834	1.3047^{cB}		0.9516
	60	4.0348^{dB}		0.8780	3.2972^{dB}		0.8958
n = 1	5	0.0330^{1A}		0.9043	0.0512^{1B}		0.8908
	23	0.0515^{2A}		0.9772	0.0624^{2B}		0.784
	37	0.0763^{3B}		0.9319	0.0685^{3A}		0.9011
	60	0.1042^{4B}		0.7702	0.0934^{4A}		0.8015

n = 0 Zero order kinetics, *n = 1* First order kinetics, T temperature, k rate constant, Ea activation energy, R^2 regression coefficient. Lowercase (^{abcd}) and number (^{1234}) superscripts in the same column denote statistical difference ($p < 0.05$). Uppercase (^{AB}) superscripts across rows denote statistical difference ($p < 0.05$).

Comparing all the browning indices, ΔE* was the only parameter where one model could adequately fit to explain the change at all temperature range. This could be attributed to the fact that when calculating ΔE*, parameters L*, a* and b* values are considered. Garza *et al.* (2000) described ΔE* as a holistic view for describing colour variation. To corroborate their statement further, in their study they found that total colour difference was the most sensitive parameter for measurement of colour. Although ΔE* is preferred, Buvé *et al.* (2021) cautioned that it may not be used as an NEB indicator, but as a general measure of colour changes.

5.11.4 Reducing sugar content

Numerous authors who studied NEB browning of fruits and fruit-based products monitor the change in reducing sugar (RS) (Somjai *et al.*, 2021; Agcam, 2022), total soluble solids (Wibowo *et al.*, 2015c), individual sugar composition (Wibowo *et al.*, 2015c; Valero, 2017; Pham *et al.*, 2019; Aktag & Gokmen, 2021a) or a combination thereof. In terms of composition, Garza *et al.* (2000) found that in apple puree important sugars were hexoses (glucose, fructose) and sucrose. Similarly, Aktag and Gokmen. (2021) in their study of dicarbonyl compound and HMF formation in a range of apple juice products found they contained an average of 60% fructose, 19.5% glucose and 21% sucrose of the total sugar. In this study we focused on the change in RS as a measure of progression of NEB reactions, in addition, the initial composition of major simple sugars was analysed. We found that fructose constituted the highest content at 62%, followed by glucose (26%) and sucrose (12%) of the total sugar. This excludes other complex carbohydrates such as fibres. Although, our sample matrix was different from those studied by (Aktag & Gokmen, 2021a), the base ingredient being apples are similar. Therefore, reference will be made to such products.

The RS content of apples canned in fruit juice ranged from 2.61 to 4.24 g.L^{-1} (Table 5.13). No significant ($p > 0.05$) changes in RS were observed for most samples, except for +AA samples stored at 60°C for 12 weeks, where a significant decrease ($p < 0.05$) in RS was observed in relation to the control/week zero sample. However, looking carefully at the pattern of the results displayed in Table 5.13, the RS of samples stored at 23, 37 and 60°C increased and then decreased as the storage time elapsed, albeit this was not significant ($p > 0.05$). This pattern is indicative of a common occurrence during NEB reactions in fruit and fruit-related products. The initial increase is synonymous with hydrolysis of sucrose and other complex sugars to increase reducing sugars, followed by consumption of the resultant sugars *via* Maillard, or sugar dehydration (Aktag & Gokmen, 2021b).

For instance the study of Wibowo *et al.* (2015) reported at least 8% increase in reducing sugar of orange juice after 8 weeks of storage at 20 °C due to hydrolysis of sucrose. This was attributed to acidic pH (3.68 – 3.74), temperature, as well as a sample comprising of at least four times sucrose compared to the current study at 46%, 31% fructose and 23% glucose. Based on the above values, sucrose hydrolysis was inevitable. However, similar to our findings, a study by Paravisini and Peterson. (2019) investigating NEB browning mechanisms in orange juice, highlighted that reducing sugars such as glucose and fructose do not always reduce in concentration during storage, as they can be simultaneously used up *via* the MR and generated from sucrose hydrolysis. Wang *et al.* (2006) and Valero. (2017) on the other hand, reported an increase in reducing sugars of carrot juice concentrate and canned peaches during storage, respectively.

Table 5.13: Reducing sugar (RS) content of apples canned in apple juice during storage.

AA	Temp °C	Storage weeks									
		0	2	4	6	8	10	12	16	20	24
+	5	3.39 ± 0.12^{bd}		3.56 ± 0.26^{bd}		3.63 ± 0.31^{bd}		3.47 ± 0.18^{bd}	3.59 ± 0.30^{bd}	3.66 ± 0.33^{bd}	3.67 ± 0.65^{bd}
	23	3.39 ± 0.12^{bd}		3.95 ± 0.77^{cd}		3.57 ± 0.43^{bd}		3.63 ± 0.42^{bd}	3.76 ± 0.12^{cd}	3.50 ± 0.39^{bd}	3.63 ± 0.30^{bd}
	37	3.39 ± 0.12^{bd}		4.06 ± 0.41^{d}		3.86 ± 0.39^{cd}		3.81 ± 0.32^{cd}	3.72 ± 0.34^{bd}	3.60 ± 0.36^{bd}	3.51 ± 0.57^{bd}
	60	3.39 ± 0.12^{bd}	4.01 ± 0.11^{cd}	4.24 ± 0.14^{d}	4.18 ± 0.04^{d}	4.00 ± 0.09^{cd}	3.21 ± 0.28^{ac}	2.61 ± 1.16^{a}			
-	5	3.50 ± 0.13^{bd}		3.56 ± 0.09^{bd}		3.58 ± 0.10^{bd}		3.63 ± 0.20^{bd}	3.67 ± 0.23^{bd}	3.66 ± 0.21^{bd}	3.63 ± 0.17^{bd}
	23	3.50 ± 0.13^{bd}		3.81 ± 0.86^{cd}		3.85 ± 0.25^{cd}		3.72 ± 0.42^{cd}	3.78 ± 0.55^{cd}	3.60 ± 0.44^{bd}	3.48 ± 0.50^{bd}
	37	3.50 ± 0.13^{bd}		3.93 ± 0.15^{cd}		3.93 ± 0.28^{cd}		3.74 ± 0.24^{cd}	3.62 ± 0.25^{bd}	3.63 ± 0.08^{bd}	3.44 ± 0.45^{bd}
	60	3.50 ± 0.13^{bd}	4.12 ± 0.07^{d}	4.19 ± 0.10^{d}	4.15 ± 0.07^{d}	4.10 ± 0.02^{cd}	3.20 ± 0.59^{ac}	2.90 ± 1.07^{ab}			

Data presented as reducing sugar content ($g.L^{-1}$) of canned apples stored for 24 weeks at various storage temperatures (5, 23, 37 and 60 °C) expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed. [abc] Means with different letter superscripts on the same row and column denotes significant differences ($p < 0.05$). AA – ascorbic acid

These authors credited this unconformity to the higher reaction rate of sucrose inversion, compared to progression of the Maillard reaction. It should be noted that both authors made this conclusion based on sucrose being at the highest concentration, thus contributes to a substantial amount of individual glucose and fructose.

We postulate that in the current study, hydrolysis took place although sucrose was the lowest at 12% of the total sugar, ideally its invert sugars wouldn't necessarily contribute to a massive increase in RS compared to above-mentioned studies. We therefore, further speculate that the RS was due to other complex carbohydrates present as apple fibers (pectin hemicellulose) undergoing hydrolysis as reported by Pham *et al.* (2019). This can also be corroborated by visual observation of softened apple slices during sampling.

The decrease in RS at 60 °C at 12 weeks storage could be due to the higher reaction rate of the formed free sugars (fructose and glucose) with available amino acids *via* the MR as reported by Pham *et al* (2019; 2020).

5.11.5 Ascorbic acid degradation (AA).

The -AA concentration in canned apples before heat processing was 10 ± 0.68 mg.L^{-1}, and this value was close to what Louarme and Billaud (2012) reported for raw 'Golden Delicious' apples at 1.0 ± 0.2 mg.100 g^{-1} for puree preparation. The +AA content before retorting was 997 mg.L^{-1} $\pm$ 1.87. Table 5.14 shows the evolution of +AA during storage of canned apples at 5, 23, 37 and 60°C for 24 weeks. The +AA content prior to storage (week zero) was 290 mg.L^{-1}. Meanwhile, nothing was detected for -AA after heat processing. Reduction in AA observed above might be due to loss during preparation and heat processing. Rawson *et al.* (2011) described AA as a heat sensitive nutrient, and that its loss is further accelerated during storage.

The AA of samples stored at 23 – 60 °C decreased significantly ($p < 0.05$) with an increase in storage temperature and time (Table 5.14). With reference to samples stored at 5°C, a decrease was observed from week 8 to 12 ($p < 0.05$), from which it remained constant ($p > 0.05$) to week 24. After 4 weeks of storage, approximately 1, 12, 26 and 92% of ascorbic acid was lost at 5, 23, 37 and 60 °C, respectively. As a result of this occurrence, complete loss of AA was reported at week 20 and 10 for samples stored at 37°C and 60°C, respectively. Losses in ascorbic acid during storage of fruit and fruit-based products is a common occurrence. Several authors have reported on this phenomenon (Wibowo *et al.*, 2015c,b; Herbig & Renard, 2017). Wibowo *et al*. (2015) reported on the degradation of ascorbic acid in orange juice packaged in PET bottles as the storage temperature increased from 20°C to 42°C for 8 weeks.

Table 5.14: Ascorbic acid content (mg.L^{-1}) of apples canned in apple juice during storage.

Storage parameters				
Time	**Temperature (°C)**			
(W)	**5**	**23**	**37**	**60**
0	290.39 ± 3.93^n	290.39 ± 3.93^n	290.39 ± 3.93^n	290.39 ± 3.93^n
2	nd	nd	nd	34.93 ± 0.65^c
4	287.10 ± 5.00^n	253.70 ± 6.12^{kl}	215.33 ± 5.51^j	22.64 ± 11.55^b
6	nd	nd	nd	4.13 ± 0.12^a
8	275.50 ± 4.00^m	217.30 ± 3.25^j	132.79 ± 9.25^g	0.00 ± 0.00^a
10	nd	nd	nd	0.00 ± 0.00^a
12	259.63 ± 4.15^l	171.15 ± 7.04^i	65.61 ± 1.48^e	0.00 ± 0.00^a
16	258.33 ± 8.33^l	144.67± 5.51^h	31.00 ± 6.25^{bc}	nd
20	254.13 ± 4.51^{kl}	104.02 ± 1.91^f	0.00 ± 0.00^a	nd
24	245.95 ± 2.01^k	54.67 ± 0.14^d	0.00 ± 0.00^a	nd

Data presented as ascorbic acid content (mg.L^{-1}) of canned apples stored for 24 weeks at various storage temperatures (5, 23, 37 and 60 °C) expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed. [abc] Means with different letter superscripts on the same row and column denotes significant differences (p < 0.05). ND not determined.

They attributed the loss to presence of dissolved and headspace oxygen, as well as oxygen permeation through the packaging during storage. Testament to this was a sharp decrease in headspace and dissolved oxygen in pasteurised orange juice during first four weeks of storage. They attributed this loss to oxidation.

Under acidic conditions, such as those reported in this study (Table 5.4), oxygen oxidises AA to dehydroascorbic acid (DHA). DHA is highly unstable and further hydrolyse to 2,3-diketogulonic acid (DKG). DKG is subsequently decarboxylated to form xylosone, which is later converted to reductones and furanic intermediate browning compounds (Rufian-Henares & Pastoriza, 2016).

It should be noted that in the previous sections we reported on the effect of supplementation with AA on NEB indices. It was reported that although in most cases, no significant differences (p > 0.05) in browning indices (absorbance at 294 and 420nm, L*-value and ΔE*) were observed between most +AA and -AA samples stored at 5°C and 23°C. At 37 and 60°C, +AA samples exhibited higher browning indices compared to -AA (Tables 5.5 – 5.8). Therefore, this highlights the direct involvement of ascorbic acid in the progression of NEB reactions. Our results are in accordance with those observed by Pham *et al*. (2021) when they concluded that ascorbic acid was responsible for inducing NEB in an orange juice model system. Meanwhile, Pham *et al*. (2020) observed that orange juice model systems

supplemented with AA resulted in higher browning intensity and higher quantities of furfural on samples stored at 42°C after 4 weeks. Similarly, Wibowo *et al*. (2015) showed that higher concentrations of AA resulted in faster formation of furfural in mango juice during storage.

5.11.5.1 Kinetic modelling of ascorbic acid

The kinetic parameters for AA degradation are depicted in Table 5.15. The reaction rate constant of ascorbic acid increased significantly ($p < 0.05$) with storage temperature. The degradation of ascorbic acid could be explained by both the zero- and first-order kinetic models. Similar findings were reported by (Agcam, 2022) , however, in the current study the zero-order was selected as a better fit. This is true even though the determination coefficient R^2 for samples stored at 60°C was the lowest for zero order models. This might be influenced by the complete destruction of ascorbic acid detected from storage week 8 onwards, thus depicted as 0.00 mg.L^{-1}. This explains why the predicted lines did not fit well with actual measured values. In instances where both zero- and first-order models can describe evolution of a determinant, a combined kinetics can be employed. Lyu *et al.* (2018) reported on the degradation of ascorbic acid in peach juice during storage as best described by a combined kinetic model. Burdurlu *et al.* (2006) on the other hand described the loss of AA in citrus juice (orange, grapefruit, tangerine, and lemon) concentrates at all temperatures as a first-order reaction. Meanwhile Wibowo *et al.* (2015c) reported the degradation of AA in orange juice as best described by a first-order kinetic model.

The estimated apparent activation energy of 43 kJ.mol^{-1} was close to that described by Burdurlu *et al.* (2006) and Wibowo *et al.*, (2015c) on the degradation of AA in fruit juices at 45 kJ.mol^{-1}. In this case, -AA samples were not analysed for AA, therefore there is no comparisons to be made.

Table 5.15: Kinetic parameters for ascorbic content (mg.L^{-1}) of canned apples.

Kinetic model	T (°C)	k	Ea (KJ.mol^{-1})	R^2
n = 0	5	-1.9324[a]		0.9500
	23	-9.6353[b]		0.9966
	37	12.5323[c]	43.23	0.9210
	60	17.2084[d]		0.4851
n = 1	5	-0.0072[A]		0.9540
	23	-0.0643[B]		0.9209
	37	-0.2608[C]		0.8710
	60	-0.4864[D]		0.8991

n = 0 - Zero order kinetics, n = *1* - First order kinetics, T- temperature, k - Rate constant, Ea - Activation energy, R^2 - Regression coefficient. Lowercase ([abcd]) and uppercase ([ABCD]) superscripts across rows denote statistical difference ($p < 0.05$).

5.11.6 Hydroxymethyl furfural (HMF) and furfural.

The accumulation of HMF during storage of apples canned in fruit juice at 5, 23, 37 and 60°C is shown in Table 5.16. The HMF content ranged from 0.40 – 26.19 mg.100g^{-1}. At temperatures of 5°C no significant increase ($p > 0.05$) in HMF content was observed throughout storage for all sample types. However, as the temperature increased to 23, an increase ($p < 0.05$) in HMF in relation to week zero/control was observed from week 16 for +AA and 20 for -AA. Meanwhile, at 37°C the increase was observed from week 12 and 8 for +AA and -AA, respectively. At the highest temperature of 60°C, significant increases ($p < 0.05$) in HMF were observed from the onset of storage. As a result, the highest HMF content of 26.19 mg.100g^{-1} was reported at 60°C. The no observed changes in HMF at 5°C corresponds to the results of Selen Burdurlu and Karadeniz. (2003) when studying HMF accumulation in apple concentrates. They too did not observe any significant increase in HMF at 5°C throughout 4 months of storage. However, Wibowo *et al*. (2015) observed some significant increase ($p < 0.05$) in HMF for pasteurised orange juice stored at 20°C storage, albeit the increase was only observed from 20 of 32 storage weeks. These results further highlight the effect of temperature on progression of NEB reactions.

Table 5.16: Hydroxymethyl furfural (HMF) of apples canned in apple juice.

AA	Temp °C	Storage weeks 0	2	4	6	8	10	12	16	20	24
+	5	0.49 ± 0.03^{ab}	-	0.51 ± 0.02^{ab}	-	0.53 ± 0.03^{ab}	-	0.54 ± 0.03^{ab}	0.55 ± 0.02^{ab}	0.56 ± 0.00^{ab}	0.52 ± 0.02^{ab}
	23	0.49 ± 0.03^{ab}	-	0.73 ± 0.06^{ac}		1.57 ± 0.04^{ad}	-	1.75 ± 0.05^{ad}	2.00 ± 0.01^{ce}	2.26 ± 0.06^{df}	2.77 ± 0.04^{dg}
	37	0.49 ± 0.03^{ab}	-	1.48 ± 0.03^{ad}	-	1.89 ± 0.12^{bd}	-	2.62 ± 0.14^{dg}	3.29 ± 0.27^{eh}	4.92 ± 0.32^{ik}	5.84 ± 0.18^{k}
	60	0.49 ± 0.03^{ab}	3.56 ± 0.96^{fh}	6.16 ± 0.95^{k}	8.19 ± 1.89^{l}	12.75 ± 0.56^{n}	19.51 ± 1.79^{q}	26.19 ± 0.71^{r}	-	-	-
-	5	0.40 ± 0.05^{a}	-	0.39 ± 0.05^{a}	-	0.44 ± 0.01^{ab}	-	0.46 ± 0.01^{ab}	0.48 ± 0.01^{ab}	0.49 ± 0.01^{ab}	0.55 ± 0.10^{ab}
	23	0.40 ± 0.05^{a}	-	0.84 ± 0.11^{ac}		1.53 ± 0.10^{ad}	-	1.65 ± 0.09^{ad}	1.83 ± 0.08^{ad}	2.14 ± 0.06^{ce}	2.66 ± 0.15^{dg}
	37	0.40 ± 0.05^{a}	-	1.45 ± 0.15^{ad}	-	2.43 ± 0.26^{df}	-	2.84 ± 0.15^{dg}	3.52 ± 0.28^{fh}	4.51 ± 0.33^{hj}	5.67 ± 0.25^{jk}
	60	0.40 ± 0.05^{a}	3.91 ± 0.59^{gi}	7.64 ± 1.14^{l}	10.34 ± 1.70^{m}	12.47 ± 2.47^{n}	15.09 ± 2.00^{o}	17.77 ± 1.23^{p}	-	-	-

Data presented as hydroxymethyl furfural content (mg.100 g^{-1}) of canned apples stored for 24 weeks at various storage temperatures (5, 23, 37 and 60 °C) expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed. [abc] Means with different letter superscripts on the same row and column denotes significant differences ($p < 0.05$). AA – ascorbic acid

With reference to supplementation with AA, no significant differences ($p < 0.05$) in HMF were observed between +AA and -AA at temperatures of 5 to 37°C throughout storage. However, significant differences ($p < 0.05$) were observed in samples stored 60°C, albeit this was seen from week 4 to 6, where -AA exhibited higher ($p < 0.05$) HMF than +AA. At week 8, no significant differences ($p < 0.05$) were observed between +AA and -AA. However, from week 10 to 12 an increase ($p < 0.05$) was observed, but this time +AA samples exhibited higher HMF than AA-. The sudden change in pattern of HMF formation in terms of +AA and -AA samples might be attributed to composition. It has been reported by several authors that acidic pH, fructose and ascorbic acid are main instigators of HMF formation in fruit based-products (Louarme and Billaud, 2012; Wibowo *et al.*, 2015a; Thuy & Pham, 2020; Agcam, 2022). Hu *et al.* (2006) observed that formation of HMF in carrot juice concentrates at high storage temperatures of 37°C was due to a decrease in pH. In the present study, the lowest pH of 3.37 was reported for +AA samples stored at 60°C, which in turn exhibited the highest HMF (Tables 5.3 and 5.16).

Both fructose and glucose participate in NEB reactions to form HMF *via* the MR or sugar degradation. However, in the present study, the former reaction would initially have been limited by the acidic pH (Table 5.3), thus allowing the latter to take precedence. Testament to this are the findings of Gürsul Aktağ and Gökmen (2020) who reported that no MR took place in peach nectar, apple, and orange juice of pH (3.36 – 3.53) stored at 4, 27 and 37°C for 24 weeks. They attributed this to the low pH and high water activity of the samples. Furthermore, their findings were corroborated by no consumption of amino acids or reduction in pH throughout storage. They attributed browning to the degradation of sugars at low pH.

The reaction involving sugar degradation begins with enolisation of either fructose or glucose to form an 1,2-enediol intermediate, which then dehydrates in several steps to form HMF. The ketose position of the carbonyl group makes fructose more favourable to undergo enolisation compared to glucose, thus making it more reactive in the formation of HMF. To corroborate this, Paravisini and Peterson (2019) stated that formation of reactive carbonyl compounds such as glyoxal, methylglyoxal, and 3-deoxyglucosone in orange juice during storage was higher for fructose compared to glucose. This proved that fructose was the main precursor. HMF formation *via* the MR on the other hand takes place after enolisation of sugar from Amadori rearrangement products. To complete the process, the formed HMF condensates with other amino compounds to form brown polymeric compounds. In addition, a study conducted by Agcam (2022) proved the dependence of HMF formation on sugar type. In their study, fruit juice-sugar model systems heated at 90°C yielded HMF content ranging between 0.25 – 0.44 $\mu mol.L^{-1}$ for glucose and 0.46 – 1.74 $\mu mol.L^{-1}$ for fructose added samples. Furthermore, they found that the HMF increased as the temperature increased from 90, 105 and 120°C. In addition, Paravisini and Peterson (2018) found that under acidic conditions,

fructose was more reactive than glucose in forming reactive carbonyl compounds such as HMF, glyoxal, methylglyoxal, and 3-deoxyglucosone that further participate in Maillard-associated browning reactions. Regarding the effect of AA and acidic pH, the study of Wibowo *et al.* (2015a) found that addition of AA and citric acid (CA) in mango juices enhanced HMF formation, and that the rate was faster for CA-juice and CA-AA-Juice compared to AA-juice only. In this case, acidic conditions were more vital in NEB formation.

To corroborate this further, Gursul Aktag and Gokmen (2021) found that the concentration of HMF in pomegranate juice concentrate was higher than that of apple juice concentrate, despite the lower fructose levels in the former. They concluded that the initial pH of pomegranate was 3.2 compared to 3.7 of apple concentrate influenced greatly HMF formation. This can explain how high HMF content in the present study coincided with lower pH (Table 5.4), and this was observed at longer storage time.

With reference to furfural, the content ranged between 1.02 – 64.31 mg/100 g (Table 5.17), these values reported above were significantly ($p < 0.05$) higher than those reported for HMF content of 0.40 – 26.19 mg.100 g^{-1} (Table 5.16). Results of this study are different from what was reported by Pham *et al.* (2019) for orange juice, where HMF was higher than furfural at 25.91 and 2.21 mg.L^{-1}, respectively. This might be due to their sugar composition of sucrose: glucose: fructose of 50: 23: 27, respectively. No significant differences ($p > 0.05$) were observed in HMF of all samples at 5 and 23°C throughout storage. Our results are in agreement with those reported by Wibowo *et al.* (2015b) where furfural was only formed when orange juice was stored at temperatures of 28 °C and above. Meanwhile, at 37°C significant increases in relation to the week zero/control were observed from week 12 and 16 onwards for AA+ and AA- samples, respectively.

Samples stored at 60°C exhibited a significant increase ($p < 0.05$) in furfural content from the onset of storage, and +AA exhibited the higher furfural content than its -AA. Buve *et al.* (2018) indicated that AA is an important precursor for furfural formation in the juice-based medium. Similarly, Wibowo *et al.* (2015b) reported a faster formation of furfural during storage of mango juice when a higher AA concentration was added to the juice. This explains the high content of furfural in ascorbic-added samples at 60°C. Furfural formation in fruit and fruit-based products is attributed to ascorbic acid, oxygen, and acidic pH. In the presence of oxygen, AA is oxidised to dehydroascorbic acid, followed by hydrolysis to 2,3-diketogulonic acid (DKGA). Decarboxylation of DKGA leads to formation of to xylosone and 3-deoxypentaone, it is at this point of the reaction where common features of NEB reactions start to show.

Table 5.17: Furfural content of apples canned in apple juice.

AA	Temp °C	Storage weeks 0	2	4	6	8	10	12	16	20	24
+	5	1.02± 0.20^{ab}	-	1.00 ± 0.13^{a}	-	1.07±0.04^{ac}	-	1.09±0.03^{ac}	0.08 ± 0.03^{ac}	1.16±0.14^{ac}	1.14 ± 0.12^{ac}
	23	1.02± 0.20^{ab}	-	1.25± 0.17^{ac}	-	1.57 ± 0.36^{ac}		1.94± 0.06^{ac}	2.01 ± 0.10^{ac}	2.28±0.19^{ad}	3.37±0.01^{ad}
	37	1.02± 0.20^{ab}	-	1.72 ± 0.24^{ac}		2.65 ± 0.88^{ac}		5.45±2.10^{cf}	7.66±1.47^{hi}	12.44 ± 1.63^{kl}	17.96±6.70^{l}
	60	1.02± 0.20^{ab}	16.68 ± 3.26^{kl}	26.00 ± 5.10^{m}	32.00 ± 1.99^{n}	44.72 ±2.35^{o}	53.08 ±0.55^{p}	64.31 ±4.38^{q}	-	-	-
-	5	1.03 ± 0.18^{ab}	-	1.25 ± 0.00^{ac}	-	1.27±0.01^{ac}	-	1.26±0.01^{ac}	1.24 ± 0.01^{ac}	1.29 ± 0.01^{ac}	1.27 ± 0.01^{ac}
	23	1.03 ± 0.18^{ab}	-	1.25±0.02^{ac}		1.62±0.07^{ac}		1.66± 0.06^{ac}	1.95 ± 0.15^{ac}	2.47±0.16^{ac}	3.77±0.67^{ae}
	37	1.03 ± 0.18^{ab}	-	1.60±0.31^{ac}	-	2.86± 0.51^{ad}		5.44±2.31^{bf}	6.81±2.69^{dg}	10.53±2.56^{gi}	19.83 ± 7.83^{lm}
	60	1.03 ± 0.18^{ab}	8.53±1.78^{fh}	9.62 ±0.19^{gi}	11.53 ± 2.16^{hj}	14.55 ± 1.37^{jk}	24.44±4.33^{m}	30.40 ± 0.26^{n}	-	-	-

Data presented as furfural content (mg.100 g^{-1}) of canned apples stored for 24 weeks at various storage temperatures (5, 23, 37 and 60 °C) expressed as mean ± standard deviation (n = 3). ANOVA and Duncan's multiple range tests were performed. ^{abc} Means with different letter superscripts on the same row and column denotes significant differences ($p < 0.05$). AA – ascorbic acid

This explains the high content of furfural in AA samples at 60°C. Furfural formation in fruit and fruit-based products is attributed to ascorbic acid, oxygen, and acidic pH. In the presence of oxygen, AA is oxidised to dehydroascorbic acid, followed by hydrolysis to 2,3-diketogulonic acid (DKGA). Decarboxylation of DKGA leads to formation of to xylosone and 3-deoxypentaone, it is at this point of the reaction where common features of NEB reactions start to show (Valero, 2017). Xylosone is further degraded to form reductones and ethylglyoxal, while 3-deoxypentaone degrades to form furfural and 2-furan-carboxylic acid. These degradation products are highly reactive and continue NEB *via* the MR by reacting with amino acids or polymerising to form brown polymers (Bharate & Bharate, 2014).

As mentioned, formation of furfural was highly dependent on the pH, presence of oxygen as well as AA. However, the in -AA samples no trace was detected after heating, and this could only be due to loss during preparation and heat processing. However, since furfural is linked to both MR and AA degradation, we hypothesise that some of natural occurring AA had been converted to dehydroascorbic acid during analysis, and thus was not detected. Furthermore, only fructose, glucose and sucrose were analysed as sugars in the canned apple products. We also hypothesise that some pentose sugars might be present in the apple as well as the apple reconstituted concentrate. Pentose sugars are known to undergo formation of furfural during heating *via* the MR.

5.11.6.1 Kinetic modelling for Hydroxymethyl furfural (HMF) and furfural

Tables 5.18 and 5.19 depict the kinetic parameters for HMF and furfural content. The effect of storage temperature on the reaction rate agreed with results obtained for browning indices (Table 5.5 – 5.8). The reaction rate constants (k_0 and k_0) increased significantly ($p < 0.05$) with storage temperature for all samples. The variation in HMF formation was well fitted to the zero order as determined by regression coefficients significantly higher ($p < 0.05$) than those obtained for first-order model. Studies such as those conducted by Wibowo *et al.* (2015a;b) and Agcam (2022) have shown that evolution of HMF was described as zero order (green highlights). No significant differences ($p > 0.05$) in reaction rate constant were observed between +AA and -AA samples at 5, 23 and 37°C. However, at 60°C the significant difference ($p < 0.05$) was observed, with +AA higher HMF than -AA.

Regarding furfural, the reaction rate constants (k_0 and k_1) increased significantly ($p < 0.05$) as the storage temperature increased. Insignificant differences ($p > 0.05$) between reaction rate constant were observed between AA+ and AA- samples at 5°C and 23°C. Meanwhile, as the storage temperature increased to 37 and 60°C, significant differences ($p < 0.05$) were observed, with +AA samples exhibiting higher reaction rate constants. The linear increase in furfural content as a function of time was best modelled by a zero-order model (green highlights).

Table 5.18: Kinetic parameters outputs for hydroxymethyl furfural (HMF) of canned apples.

Kinetic model	T (°C)	Ascorbic acid			No ascorbic acid		
		k	Ea (KJ.mol^{-1})	R^2	k	Ea (KJ.mol^{-1})	R^2
$n = 0$	5	0.0020^{aA}		0.5337	0.0039^{aB}		0.9329
	23	0.0996^{bB}	86.06	0.9203	0.0647^{bA}	65.31	0.8793
	37	0.7042^{cB}		0.9189	0.1413^{cA}		0.9820
	60	5.0535^{dB}		0.9901	0.3123^{dB}		0.9914
$n = 1$	5	0.0059^{1A}		0.5394	0.01281^{1B}		0.9437
	23	0.0756^{2B}		0.8397	0.05932^{2B}		0.7005
	37	0.2164^{3B}		0.9572	0.10443^{3B}		0.8807
	60	1.4139^{4A}		0.7107	0.26014^{4B}		0.7945

$n = 0$ Zero order kinetics, $n = 1$ First order kinetics , T temperature, k rate constant, Ea activation energy, R^2 regression coefficient. Lowercase ([abcd]) and number ([1234]) superscripts in the same column denote statistical difference ($p < 0.05$). Uppercase ([AB]) superscripts across rows denote statistical difference ($p < 0.05$).

Table 5.19: Kinetic parameters outputs for furfural of canned apples.

Kinetic model	T (°C)	Ascorbic acid			No ascorbic acid		
		k	Ea (KJ.mol^{-1})	R^2	k	Ea (KJ.mol^{-1})	R^2
$n = 0$	5	0.0064^{aA}		0.8686	0.0070^{aA}		0.438581
	23	0.0853^{bA}		0.8981	0.0984^{bB}		0.844245
	37	0.6899^{cA}	89.30	0.9068	0.6982^{cB}	67.29	0.835867
	60	5.0535^{dB}		0.9911	2.2295^{dA}		0.926295
$n = 1$	5	0.0059^{1A}		0.8642	0.0060^{1A}		0.433133
	23	0.0449^{2A}		0.9620	0.0487^{2B}		0.947894
	37	0.1216^{3B}		0.9932	0.1206^{3A}		0.990709
	60	0.2730^{4B}		0.7497	0.2263^{4A}		0.75547

$n = 0$ Zero order kinetics, $n = 1$ First order kinetics, T temperature, k rate constant, Ea activation energy, R^2 regression coefficient. Lowercase ([abcd]) and number ([1234]) superscripts in the same column denote statistical difference ($p < 0.05$). Uppercase ([AB]) superscripts across rows denote statistical difference ($p < 0.05$).

Similarly Wibowo *et al.* (2015b) indicated that furfural formation was best fitted to zero-order model in pasteurised orange juice stored between 20 and 42°C. At storage temperatures of 5°C no furfural was detected, which explains why the predicted line did not fit well with

measured values, especially for -AA samples. Thus, we can conclude that the formation of furfural does not necessarily follow a zero-order model, it rather shows the best fit among models evaluated in this study.

In the second step of analysis, the Arrhenius equation was used to describe the temperature dependence of the reaction rate constant (k_0 and k_1) for all kinetic models to determine the apparent activation energy (Ea) of HMF and furfural. The Ea reported for HMF content for +AA was significantly higher ($p < 0.05$) at 86.06 kJ.mol^{-1} for +AA compared to 65.31 kJ.mol^{-1} for -AA samples. Meanwhile, for furfural it was 89.30 and 67.29 kJ.mol^{-1}, respectively. In this case, accumulation of HMF and furfural in +AA was more sensitive to temperature changes compared to -AA. Formation of HMF was dependent on sugar type, pH and temperature.

HMF and furfural formation during processing and storage of fruits and fruit-based products is mainly attributed to NEB pathways, namely caramelisation, ascorbic acid degradation, and the Maillard reaction. These pathways are dictated by product composition and preparation or storage conditions; at times they may take place simultaneously. Agcam (2022) attributed browning of their fruit juice-sugar model systems to the Maillard and caramelisation, and that was true owing to the employed temperatures. In the present study caramelisation is ruled out, we however speculate that ascorbic acid degradation was the first reaction to take place and gave rise to furfural, followed by a combination of sugar acid degradation resulting in HMF and the MR due to the reactivity of the degradation products with amino acids. The acidic nature of the product would have initially retarded the MR, however hydrolysis and degradation products accumulated and served as substrates, overcoming the pH hurdle.

5.12 Conclusions

This chapter aimed to evaluate the type of NEB reactions taking place in canned apples during storage. Overall, samples with added ascorbic acid resulted in higher browning as well as formation of HMF and furfural. Kinetics revealed that AA degradation preceded the MR and sugar degradation. In this study, the two important intermediate products evaluated was HMF and furfural. It is recommended that future studies should include reactive carbonyl species such as glucosone, 3-deoxyglucosone and fructofuranosyl. These will aid in identifying the NEB reactions, more closely it also recommended to include minor assays such as amino acid profile, dissolved and headspace oxygen.

5.13 References

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CHAPTER 6: INHIBITION OF NON-ENZYMATIC BROWNING REACTIONS IN APPLES USING BETA-CYCLODEXTRIN-ASSISTED EXTRACTS OF GREEN ROOIBOS (*ASPALATHUS LINEARIS*).

6.1 Abstract

Antioxidant activity associated with green rooibos (*Aspalathus linearis*) infusions is attributed to the activity of polyphenols, particularly aspalathin and nothofagin. Beta-cyclodextrin-assisted extracts of green rooibos (β-GRE) were optimally extracted *via* conditions of: 15 mM β-CD: 40°C: 60 min, yielding the highest total polyphenol content (TPC) 398.25 mg GAE.g^{-1}, metal chelation (MTC) 93%, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging of 1 689 µmol TE.g^{-1}, ferric reducing antioxidant power (FRAP) of 2 097 µmol AAE.g^{-1}, oxygen radical absorbance capacity (ORAC) of 11 163 TE.g^{-1} and aspalathin 172.25 mg.g^{-1}. The extracts were investigated as natural inhibitors of non-enzymatic browning (NEB) reactions in apples canned in fruit juice during storage. Comparisons were made between control (without added extracts) and aqueous extracts (GRE) of β-GRE of the same extraction conditions. The concentrations of inhibitors were varied between 0.25 – 0.5% to evaluate the highest level of inhibition, and possible pro-oxidant effect. The β-GRE and GRE samples were stored at 23 and 37°C for six months. Lightness L*value and ΔE* determined the effect of inhibitors against browning development in canned apples. Meanwhile, furfural and HMF formation determined the ability to inhibit intermediate NEB reaction products. The efficiency of β-GRE and GRE as inhibitors was demonstrated in terms of percentage inhibition in relation to the control over the duration of storage, as well as the reaction rate constants (k_0).

Storage temperatures played a significant ($p < 0.05$) role in the progression of NEB reactions in canned apples. The L*value decreased and ΔE* increased significantly as the storage time increased, where, during the first 8 weeks of storage, all samples stored at 23°C exhibited acceptable ΔE* values ranging between 1.89 – 4.87, with GRE 0.25 and β-GRE 0.5 exhibiting the lowest values. Meanwhile at 37°C, acceptable ranges were reported at week 4 for GRE and β-GRE only, i.e., the control. Therefore, temperature had an influence on the efficacy of extracts against NEB reactions.

Overall, β-GRE samples demonstrated superior inhibitory ($p < 0.05$) effect, compared to GRE. In most cases, higher inhibition was observed for samples stored at 23°C. For instance, β-GRE 0.25 and 0.5 exhibited the highest inhibition against browning development *via* L*value (40.93 – 46.67%), β-GRE 0.25 for ΔE* (46.67%) and β-GRE 0.25 and 0.5 for HMF (59.55 – 67.33%). In terms of furfural, no significant differences ($p > 0.05$) were reported

between all GRE and β-GRE samples, although inhibition of furfural was reported at a range between 62.69 – 72.29%. Inhibitory effect against NEB at the 23°C was further confirmed by higher (p < 0.05) rate constant (k_0) exhibited by the control in relation to GRE and β-GRE for L*value, ΔE* furfural and HMF, although no significant differences (p > 0.05) were observed amongst these extracts except for HMF. Increased storage temperature of 37°C reduced (p < 0.05) the inhibitory efficacy of all extract types, resulting in comparable abilities between GRE and β-GRE. In some cases, β-GRE 0.5 exhibited less inhibition (p < 0.05) than GRE, and even showing pro-oxidant activity, i.e., -17.17% for ΔE*. In addition, no significant differences (p > 0.05) in k_0 were observed for all browning indices at 37°C, with the exception of β-GRE 0.25 and 0.5 exhibiting the lowest k_0 for HMF at 0.1215 and 0.1143, respectively.

The dose-dependency of GRE and β-GRE against inhibition was more pronounced for GRE samples, where an increase in concentration from 0.25 to 0.5 resulted in increase in inhibition of ΔE*and HMF at 23°C, while those observed for 37°C was, L*value and furfural. Meanwhile, in most cases no significant (p > 0.05) effect of concentration on inhibition was observed for β-GRE except for ΔE* at 23°C and L*value at 37°C.

Keywords: Green rooibos extract; Encapsulation; β-cyclodextrin; Polyphenols; Hydroxymethyl furfural; Inhibition.

6.2 Introduction

During processing and storage of fruit and fruit-based products, non-enzymatic browning (NEB) reactions are responsible for the formation of undesirable brown colour and producing undesirable toxic compounds (Buvé *et al.*, 2021).

In the light of this information, prevention of NEB reactions is of outmost importance. Some mitigation strategies are possible, such as reduction in processing temperatures and time, substituting highly reactive ingredients with the less reactive to inhibit NEB reactions during processing and storage. However, these interventions are not applicable to all product types, and pose the risk production of products of inferior quality in terms of attributes such as taste, colour and flavour. Therefore, alternative inhibition methods must be pursued.

Chemical inhibitors are the most preferred choice due to low cost and high performance. Amongst them, sulphites have proven to be superior in inhibiting NEB reactions, especially blocking the initial condensation step of the MR *via* attaching to the carbonyl of sugars, or stabilising the intermediate HMF from reacting further (Bharate & Bharate, 2014). Another synthetic AGE inhibitor, aminoguanidine (AG), is known as an excellent trapper of MGO (Khan *et al.*, 2020). However, both these inhibitors have been implicated in terms of safety concerns (Vally *et al.*, 2009; Wang *et al.*, 2016; Khan *et al.*, 2020). Due to the critical role that glycation plays in various diseases, there is a need to find natural antiglycation

agents, which could be delivered through the diet. Therefore, the best alternatives are the utilisation of plant extracts (Yeh *et al.*, 2017).

Plant extracts are good candidates due to their versatile activities, having bioactive constituents, such as flavonoids, phenolic acids, curcumin, vitamin E and C. Crude plant extract bioactives have been assessed for their ability to inhibit NEB/glycation markers such as HMF, furfural, glyoxal (GO), methyl glyoxal (MGO) and development of AGEs (Rudnicki *et al.*, 2007; Wu *et al.*, 2009; Hafsa *et al.*, 2018; Khan *et al.*, 2020). Using model systems of bovine serum albumin (BSA) and glucose, Favre *et al.* (2020) proved that green pepper extract was effective in reducing the formation of HMF. A study by Wu *et al.* (2009) showed that guava leaf extracts exhibited a slight inhibitory effect against formation of Amadori products, and good inhibitory against dicarbonyl compounds and AGEs. Moreover, its activity was higher than that of AG. The inhibitory capacity was widely attributed to the presence of polyphenolic compounds (Wu *et al.*, 2009; Lavelli and Sri Harsha, 2019; Favre *et al.*, 2020). The anti-glycation properties of polyphenols have been linked to their antioxidant activity (Wang *et al.*, 2016; Hafsa *et al.*, 2018; Lin and Zhou, 2018)

Epigallocatechin gallate (EGCG) has been found to scavenge free radicals formed during oxidation of the Schiff base (Wang *et al.*, 2016). In addition, quercetin was found to inhibit AGE production through trapping and blocking MGO and GO, as well scavenging free radicals (Yeh *et al.*, 2017). Ferulic acid (FA) has been found to inhibit dicarbonyl compounds (Wu *et al.*, 2009; Yeh *et al.*, 2017), formation and prevent CML and CEL developments in model systems, however, its efficacy gravitates more toward prevention of late stage glycation (Srey *et al.*, 2010). In addition, the synergy between two or more polyphenols exhibits better anti-glycation functions than the use of a single compound. This applies to plant extracts that contain a variety of phenolic compounds. Wu *et al*. (2009) found that gallic acid, catechin, quercetin and ferulic acid were the main polyphenols contained in guava leaf extracts in descending order, where they reported that inhibition properties of guava leaf extracts was more effective than that of AG. Of interest in this current study, several authors reported on the phenolic content of green rooibos. The major phenolic compounds in green rooibos are the flavonoids and phenolic acids class (Joubert & DeBeer, 2011b; Miller, 2016; Damiani *et al.*, 2019; Lawal *et al.*, 2019). Based on results from studies and reviews on literature by the afore-mentioned authors, aspalathin and nothofagin are the main flavonoids with others such as quercetin present at lower concentrations. Moreover, Joubert and DeBeer (2014) further reported that aspalathin was a potent antioxidant which compared favourably with EGCG. Gallic and ferulic acid were amongst other phenolic acids in green rooibos. It has to be noted that the unfermented green rooibos plant contains more phenolic compounds than what have been mentioned above, however, the ones highlighted here are those implicated in exhibiting anti-glycation or anti-browning properties (Wu *et al.*, 2009; Wang *et al.*, 2016; Yeh *et al.*, 2017).

Although these plant extracts or polyphenols have been proven to exhibit excellent anti-browning activities in vitro, the final intended application is in food processing and storage. Crude plant extracts or purified individual polyphenols thereof have been reported to affect colour and flavour changes when incorporated in food. This is influenced by either natural pigments, flavours or reaction of polyphenols with other food components resulting in colour augmentation. These changes are also accelerated by high temperatures employed during heat processing leading to reduced bioactivities. For instance, 1 – 1.5% EGCG successfully reduced the browning rate of bread rolls, however at increased concentrations of 3% it exhibited pro-oxidative activity (Favreau-farhadi *et al.*, 2015). Qi *et al.* (2018) also reported the efficacy of 0.01 – 0.1 mg.L^{-1} flavan-3-ols against the MR, however at 0.3 – 1 mg.L^{-1} darkening occurred due to autoxidation at high temperatures. The afore mentioned instabilities limitations may pose as a challenge of using native plant extracts (Albahari *et al.*, 2018).

Consequently, development of encapsulation methods and delivery systems containing specific compounds can improve some physicochemical properties of these extracts in food products. Encapsulation of sensitive compounds provides improved stability during processing, mask off-flavours or odours, and in the final product by preventing reaction with other components in food products such as oxygen (Nesterenko *et al.*, 2013). Common encapsulation material includes carbohydrates, protein and lipid polymers such as maltodextrin, inulin (Human *et al.*, 2020), soy protein isolates, sodium alginate and cyclodextrins to mention a few. The choice of polymer is crucial as it affects how the active compound is released. For instance, Hidalgo *et al.* (2018) reported on elevated furosine levels in soy protein isolates applied to encapsulate beetroot pomace due to thermal treatments. Similarly, maltodextrin reacts with glycine to form browning compounds (DaSilva, 2018). Lavelli and Sri Harsha, (2019) reported that sodium alginate encapsulated grape skin extracts exhibited reduced potential to inhibit glycation of fructose-BSA and MGO-BSA model systems, this was due to some phenolics interacting with alginate, resulting in a decrease release. Therefore, cyclodextrin were the obvious choice since they were proven not to participate in any NEB reactions (DaSilva, 2018). Moreover, unlike other potential encapsulants, beta-cyclodextrin (β-CD) is also known to aid in extraction of these polyphenols from their sources, hence the term "β-CD assisted extracts" used interchangeably with β-CD encapsulation (Favre *et al.*, 2018; Maraulo *et al.*, 2021). Maraulo *et al.* (2021) proved that β-CD enhanced the physical properties of olive pomace extracts *via* improved heat stability and reduced hydroscopicity, in addition to increased antioxidant activity. Diamanti *et al.* (2017) reported similar results where β-CD enhanced the total phenolic content and the radical scavenging activity of whole pomegranate extracts.

In terms of encapsulation of plant extracts with β-CD encapsulated plant extracts, inhibition of NEB reaction products. Favre *et al.* (2018; 2020) proved that β-CD-assisted extracts of thyme and green pepper were effective in retarding browning development (A_{420nm}) and HMF formation in glucose-BSA model systems, respectively.

Therefore, the aim of this study was to apply β-CD-assisted green rooibos plant extracts in apples canned in fruit juice during processing and storage to mitigate common NEB markers such as browning development and HMF and furfural formation.

6.3 Materials

6.3.1 Chemicals

The dry green rooibos was obtained from a major local producer (Rooibos Ltd., Clanwilliam, South Africa). Beta-cyclodextrin (β-CD) was purchased from Industrial Analytical (Kyalami, South Africa). 'Golden Delicious' apples were purchased from a local supermarket (Bellville, South Africa). Apple concentrate (70°B) was purchased from Associated Fruit Processors (Paarl, South Africa). Hydroxymethyl furfural, ascorbic acid, thiobarbituric acid, Carrez I and II, trichloroacetic acid, fructose, glucose, sucrose, methanol and sulphuric acid were purchased from Merck (Modderfontein, South Africa). Furfural was purchased at Sigma-Aldrich (Kempton Park, South Africa). Chemicals used in this study were of analytical grade and chemical reagents were prepared according to standard analytical procedures. Prepared reagents were stored under conditions that prevented deterioration or contamination. The water used was purified with the Milli-Q water purification system (Millipore, Microsep, Bellville, SA).

6.4 Methods

6.4.1 Solid-Liquid Extraction of Green Rooibos extracts

Green rooibos, as received, was coarsely milled (Fritsch, Germany) using a sieve with an aperture of 0.2 mm. The extraction of green rooibos was performed based on the method of Rajha *et al.* (2015) and Cai *et al.* (2018) with slight modifications. Green rooibos of 10 g and a 100 mL of 0 and 15 mM β-CD (1.7%) aqueous solutions (1:10 (w/v)) ratio in a Schott bottle were homogenised using a Polytron homogeniser at 29,000 rpm for 2 min, followed by heating the mixture at 40°C on a temperature-controlled heating mantle for 60 min with magnetic stirring at 1,500 rpm. The extracts were cooled immediately and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was freeze-dried, and the resulting powder termed native green rooibos extracts (GRE) and β-CD-assisted extracts of green rooibos (β-GRE) were stored in air-tight containers at –20°C until further analysis.

6.4.2 The process of canning

The process of canning apples was conducted following the guidelines stipulated by the South African Agricultural Product Standards Act (APSA) 119 of 1990. Apple juice concentrate and slice width were prepared according to the specifications stipulated by the APSA (Act 119 of 1990). Apple concentrate of 70 °Brix (°B) was diluted with ice cold water to a concentration of 30°B, followed by the addition of 2,500 mg.kg^{-1} ascorbic acid, green rooibos extracts (15 mM β-CD: 40°C:60 min) as prepared in Chapter 4. To evaluate the effect of β-CD encapsulation, aqueous GRE (0 mM β-CD: 40°C: 60 min) at 0.25 and 0.5% were also prepared. Apples were peeled, cored and dipped in ice cold 2% solution of calcium chloride (Ghoul, 2013) after which they were sliced longitudinally from the calyx to the stem into segments of not less than 40 mm in height with thickness ranging between 16 – 20 mm (APSA, 2015). Apple slices (70 g) were weighed into retortable laminated PET PE EVOH (160 mm x 100 mm) self-standing pouches. Ice cold juice concentrate (30°B) was filled to a final mass of 120 g and the pouches sealed immediately with a pedal heat sealer. The sealed pouches were heat-processed in a horizontal retort at 100°C for 20 min. Temperature profiles in the retort, and at the coldest point of the of the sample was recorded using type T-thermocouples (Ellab, Hillerod, Denmark). Canned apples were stored in temperature-controlled incubators at 23 and 37°C. Initial sampling was done prior to storage (i.e., immediately after retorting), thereafter sampling was done monthly (23 and 37°C) as set out in Tables 6.1 and 6.2. At each sampling period, canned pouches were taken randomly from the incubators.

Table 6.1: Canned apples with added extracts parameters.

Storage time	6 months
Sampling time	Monthly
Storage conditions	23°C, and 37°C
No of samples per temperature	50
Net weight	120 g
Drained weight	65 g
Final °Brix	14 – 18

Table 6.2: Canned apples with added extracts sampling schedule.

Week	Green rooibos extracts (GRE)				β-CD green rooibos extracts (β-GRE)			
	23°C		37°C		23°C		37°C	
	Gre 0.25	Gre 0.5	Gre 0.25	Gre 0.5	β-Gre 0.25	β-Gre 0. 5	β-Gre 0.25	β-Gre 0. 5
0	X[1]	X[1]	X[1]	X[1]	X[1]	X[1]	X[1]	X[1]
4	X	X	X	X	X	X	X	X
8	X	X	X	X	X	X	X	X
12	X	X	X	X	X	X	X	X
16	X	X	X	X	X	X	X	X
20	X	X	X	X	X	X	X	X
24	X	X	X	X	X	X	X	X

[1]The storage temperatures of 23 and 37°C did not apply to control/ week zero

6.4.3 Sample preparation

During specific sampling days, the canned products were collected from their respective storage, and placed in ice cold water to equilibrate the temperatures. Samples were then homogenised into a puree using a stick blender (Mellerware, SA). The pureed sample was used as it is for measurement of °B, pH and colour. All the other compositional analyses were done using water extracts of the puree. Two grams of apple puree was diluted with 4 mL Milli-Q water, vortexed for 1 min, and then centrifuged (Beckman Coulter, USA) at 12,000 g for 10 min. The resulting supernatant termed Supernatant-A was used for measurement of browning index and degree of glycation (Aktag & Gokmen, 2021a). Samples for HMF, furfural, were further clarified using Carrez I and II solutions. For clarification, 1 mL of the Supernatant-A was mixed with 50 µL of Carrez I and 50 µL of Carrez II solutions, allowed to stand for 30 min, followed by centrifugation at 10,000 rpm for 5 min resulting in Supernatant-B (Aktag & Gokmen, 2021a).

6.4.4 Measurement of pH and °Brix

The pH of the pureed apple samples was measured using an 827 Lab pH meter (Metrohm, Switzerland) calibrated with buffer solutions of pH 4.0 and 7.0, respectively. The °Brix of the apple puree was determined using a handheld refractometer.

6.4.5 Colour measurement

The colour of the pureed apple samples was evaluated by measuring CIELab parameters L* (brightness, 100 = white, 0 = black), a* (+ red; - green) and b* (+ yellow; - blue) parameters by means of a spectrophotometer (CM-5, Konika Minolta, Japan), measuring the colour spectra using a D65 day-light source, large viewing area and the observer at 10°. The colour difference was calculated using the following formula:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \qquad Eq.\ (1)$$

Where:

ΔL^* is the difference in lightness/darkness value.

Δa^* is the difference on red/green.

Δb^* is the difference on yellow/blue.

The percentage inhibition of browning was calculated following the formula described by Bharate and Bharate (2014).

$$\% \ Browning \ inhibition \ L = \frac{\Delta L^* \ control - \Delta L^* \ sample}{\Delta L^* \ control} \qquad Eq.\ (2)$$

Where:

$\Delta L^*_{control}$ is the difference between the measured L* value for the control at week 24 and the corresponding value at week 0.

ΔL^*_{sample} is the difference between the measured L* value for the sample with added extract at week 24 and the corresponding value at week 0.

$$\% \ Browning \ inhibition \ \Delta E = \frac{\Delta \Delta E^* \ control - \Delta \Delta E^* \ sample}{\Delta \Delta E^* \ control} \qquad Eq.\ (3)$$

Where:

$\Delta DE^*_{control}$ is the difference between the calculated ΔE^* for the control at week 24 and the corresponding value at week 4.

ΔDE^*_{sample} is the difference between the measured ΔE^* for the sample with added extract at week 24 and the corresponding value at week 4.

6.4.6 Determination of 5-hydroxymethyl furfural and furfural

Furfural and 5-hydroxymethyl furfural content were determined according to the spectrophotometric method described by Bharate and Bharate (2014) and Liao *et al.* (2020), with slight modifications. Each 2 mL aliquot of sample was mixed with 2 mL of 12%

trichloroacetic acid and 2 mL of 0.025 M thiobarbituric acid, reacted at 40 ± 0.5°C for 50 min, and cooled to room temperature after reaction. The absorbance was measured at 436 nm for furfural and 443 nm for HMF. The HMF and furfural concentrations were calculated through a calibration standard curve capturing the range from 0 to 6.6 mg.L^{-1}. The percentage of furfural and HMF inhibition was calculated following the formulae:

$$\% \, Furfural \; inhibition \; = \frac{\Delta Furfural \; control - \Delta Furfural \; sample}{\Delta Furfural \; control} \qquad Eq. \, (4)$$

Where:

Δ Furfural $_{control}$ is the difference between the furfural value for the control at week 24 and the corresponding value at week 0.

Δ Furfural $_{sample}$ is the difference between the furfural value for the sample with added extract at week 24 and the corresponding value at week 0.

$$\% \, HMF \; inhibition \; \Delta E = \frac{\Delta HMF \; control - \Delta HMF \; sample}{\Delta HMF \; control} \qquad Eq. \, (5)$$

Where:

$\Delta HMF_{control}$ is the difference between the HMF value for the control at week 24 and the corresponding value at week 0.

ΔHMF_{sample} is the difference between the HMF value for the sample with added extract at week 24 and the corresponding value at week 0.

6.5 Data analysis

The inhibitory effect of GRE and β-GRE in canned apples against NEB reactions was determined *via* percentage inhibition of (Lightness (L*), colour difference (ΔE*)), and formation of intermediates products (HMF and furfural) calculated following Eq. (2 – 5). In addition, zero-order reaction kinetics Eq. (6 – 8) for each index was applied to determine the effect of added extracts in reducing the reaction rate constant (k_0) as well as the regression coefficient (R^2) thereof. The effect of temperature on *k* was calculated from the Arrhenius equation Eq. (10) to determine the activation energy (Ea) Eq. (11).

Statistical analysis was performed using SPSS 27.0 for Windows®. Analysis of variance (ANOVA) established significance of each dependent factor. Descriptive statistical analyses determined the mean and standard deviation of triplicates (n = 3). Significant differences among means were determined by Duncan's multiple range tests. The level of confidence required for significance was selected at 95%.

$$L = L_0 + k_0 t \qquad\qquad\qquad\qquad\qquad\qquad Eq. \, (6)$$

$$\Delta E = \Delta E_0 + k_0 t \qquad\qquad\qquad Eq.\,(7)$$

$$HMF = HMF_0 + k_0 t \qquad\qquad\qquad Eq.\,(8)$$

$$Furfural = Furfural_0 + k_0 t \qquad\qquad\qquad Eq.\,(9)$$

$$\ln k = \ln k_0 - \frac{Ea}{RT} \qquad\qquad\qquad Eq.\,(10)$$

$$E_a = -RT \cdot ln\left(k/A\right) \qquad\qquad\qquad Eq.\,(11)$$

Where L, ΔE, HMF and Furfural is concentration at time t, L_0, ΔE_0, HMF_0 and $Furfural_0$ concentration at time zero, k_0 zero-order rate constant and t storage time. T is absolute temperature in (°K), Ea is the activation energy and R is the universal gas constant (8.3145 J.mol^{-1} K^{-1}).

6.6 Results and discussions

6.6.1 Inhibition of colour formation *via* L* value and Δ*E

Results of colour development *via* L*values and ΔE* of canned apples with added green rooibos extracts (GRE and β-GRE) stored at 23 and 37°C are shown in Figure 6.1, Tables 6.3 and 6.4. Initially all samples displayed varying levels of L*values. As expected, the control exhibited the highest ($p < 0.05$) L*value of 58.91 at week zero. Upon addition of green rooibos extracts (GRE and β-GRE), the red component (a*) increased (results not shown) and brightness (L*) decreased ($p < 0.05$) due to GRE and β-GRE imparting the natural red colour to the canned samples, albeit no significant differences ($p > 0.05$) were observed amongst the L*values of the samples initially (Table 6.3). This change was also visible to the naked eye and agrees with the observation that crude plant extracts or purified individual polyphenols affect colour changes when incorporated in food or model systems (Figure 6.1). This colour change can be ascribed to either natural pigmentation, or reaction of polyphenols with food components resulting in colour augmentation (Favreau-farhadi *et al.*, 2015; Hidalgo *et al.*, 2018; Qi *et al.*, 2018). For instance, Hidalgo *et al.* (2018) experienced colour change when they added native and encapsulated beetroot pomace extract (BPE) during water biscuit baking. They observed a sharp decrease ($p < 0.05$) in L* and increase in a* values due to the presence of betacyanins. In the present study, at the end of storage, the control and GRE reported the lowest L* value (34.58 – 37.42) at 23°C, while at 37°C it was observed for β-GRE

and GRE at 0.5%, similarly Hidalgo *et al.* (2018), also reported greater reduction in L*values as the concentration BPE increased from 5.7 – 14.19%.

Based on these results, it is evident that the decrease ($p < 0.05$) in L*value was more pronounced at 37°C compared to 23°C, and this is attributed to influence of temperature on browning development (Lyu *et al.*, 2018). Furthermore, brown colour development of samples was further explained *via* ΔE^*, where an increase ($p < 0.05$) was observed during storage (Table 6.4). Therefore, reporting on the changes in colour of each sample cannot be directly compared to the control, and more so since there are variations in the extract concentrations. Hence, it was worthy to determine the percentage inhibition (%IL*) of browning *via* colour formation for each concentration, as well as the reaction rate constant (k_0) and activation energy Ea, with the latter two being more accurate in determining the progression of the reaction and inhibitory effect of added extracts.

The %IL was used to describe the effectiveness of added GRE and β-GRE in lessening the decrease in lightness (L*) during storage, i.e., in inhibiting browning. Table 6.5 depicts the % inhibition of colour formation *via* L*value and ΔE^*. The %IL* value ranged between 0.20 – 46.67%, with the lowest reported for GRE 0.5 (0.2%) and β-GRE 0.5 (2.44) at 37°C, and the highest for β-GRE 0.5 (46.67%) at 23°C. The inhibitory effect of β-GRE increased as the extract concentration increased, on the other hand, no significant differences ($p > 0.05$) were observed as a function of concentration for GRE samples stored at 23°C. However, as the storage temperature increased to 37°C, the overall %IL*value was reduced, and worsened ($p < 0.05$) as the concentration increased to 0.5% for both extract types. Moreover, an opposite trend to what was observed at 23°C was reported, where GRE 0.25 samples exhibited higher % inhibition capacity than β-GRE 0.25.
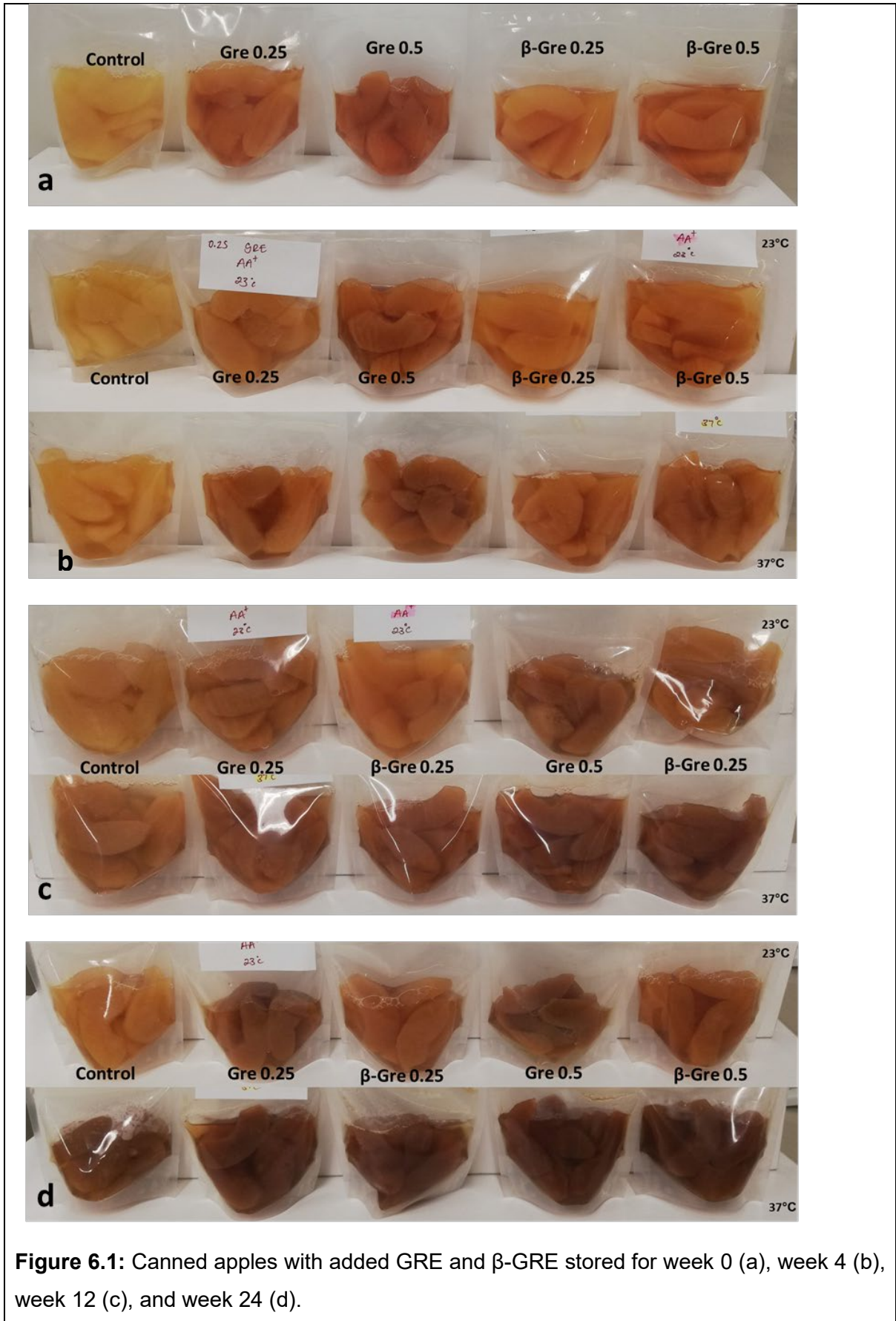
Figure 6.1: Canned apples with added GRE and β-GRE stored for week 0 (a), week 4 (b), week 12 (c), and week 24 (d).

Table 6.3: Lightness (L* value) of canned apples with added green rooibos extracts.

Storage temp	Storage time	Samples type and concentration				
		Control	Gre 0.25	Gre 0.5	β-Gre 0.25	β-Gre 0. 5
	0	58.91 ± 1.92^{k}	53.80 ± 1.75^{ij}	52.39 ± 1.87^{hij}	54.15 ± 0.99^{j}	52.55 ± 0.61^{hij}
	4	57.6 ± 1.50^{k}	52.15 ± 0.45^{hij}	50.72 ± 0.81^{hi}	53.69 ± 0.53^{hij}	50.73 ± 0.80^{hi}
	8	57.45 ± 2.14^{k}	50.85 ± 1.72^{hij}	50.38 ± 2.37^{h}	52.91 ± 0.58^{hij}	51.06 ± 2.09^{hij}
23°C	12	46.55 ± 2.05^{g}	43.71 ± 1.20^{defg}	44.22 ± 2.08^{efg}	40.04 ± 1.03^{bc}	44.35 ± 1.56^{efg}
	16	45.89 ± 2.10^{fg}	42.94 ± 1.17^{cdef}	41.30 ± 0.89^{cde}	40.75 ± 2.40^{cd}	44.3 ± 0.88^{efg}
	20	41.48 ± 0.88^{cde}	40.17 ± 1.53^{bc}	35.59 ± 1.57^{a}	41.53 ± 1.91^{cde}	41.48 ± 1.89^{cde}
	24	37.42 ± 1.11^{ab}	36.29 ± 1.70^{a}	34.58 ± 1.31^{a}	41.54 ± 4.87^{cde}	41.09 ± 0.66^{cde}
	0	58.91 ± 1.92^{R}	53.8 ± 1.75^{PQ}	52.39 ± 1.87^{PQ}	54.15 ± 0.99^{Q}	52.55 ± 0.61^{PQ}
	4	53.45 ± 2.05^{PQ}	50.86 ± 2.56^{OP}	43.75 ± 2.66^{KL}	48.51 ± 0.82^{NO}	51.06 ± 1.04^{OPQ}
	8	48.05 ± 0.96^{NO}	46.13 ± 0.91^{MN}	41.84 ± 3.87^{IJK}	39.9 ± 1.16^{HIJK}	42.93 ± 0.64^{KL}
37°C	12	41.54 ± 0.79^{IJK}	42.14 ± 0.97^{JK}L	39.22 ± 1.53^{GHI}	39.6 ± 1.87^{GHIJ}	37.81 ± 0.70^{FGH}
	16	38.2 ± 1.12^{FGH}	38.71 ± 3.13^{GHI}	36.33 ± 2.25^{EFG}	35.31 ± 1.20^{DEF}	36.59 ± 0.77^{EFG}
	20	34.69 ± 0.74^{DE}	34.03 ± 1.79^{CDE}	32.66 ± 0.87^{CD}	33.79 ± 0.89^{CDE}	33.92 ± 0.36^{CDE}
	24	33.14 ± 1.69^{CD}	33.16 ± 2.95^{CD}	26.67 ± 3.88^{A}	31.11 ± 0.19^{BC}	27.41 ± 0.50^{A}

Data presented as Lightness (L* value) of canned apples with added green rooibos extracts stored at 23 and 37°C for 24 weeks expressed as mean ± standard deviation (n = 3). Gre 0.25 – green rooibos native extract at 0.25%, β-GRE 0.25 – beta-cyclodextrin encapsulated green rooibos extract.

Table 6.4: Colour difference (ΔE*) of canned apples with added crude green rooibos extracts.

Storage Temp	Storage time	Samples type and concentration				
		Control	Gre 0.25	Gre 0.5	β-Gre 0.25	β-Gre 0. 5
23°C	4	3.72 ± 1.10[ab]	1.66 ±1.13[a]	4.01 ± 1.24[ab]	3.06 ± 0.56[ab]	1.89 ± 0.91[a]
	8	4.25 ± 1.03[ab]	4.31 ± 0.05[ab]	4.87 ± 1.53[b]	4.36 ± 1.03[ab]	1.67 ± 1.07[a]
	12	13.46 ± 2.40[fi]	11.27 ± 1.57[df]	10.30 ± 1.76[ce]	15.39 ± 2.56[hi]	8.40 ± 0.94[c]
	16	14.15 ± 1.02[gi]	12.94 ± 2.63[eh]	12.82 ± 1.14[eh]	14.76 ± 1.01[gi]	9.22 ± 1.26[cd]
	20	19.07 ± 1.81[k]	15.99 ± 1.36[ij]	18.30 ± 1.28[jk]	14.56 ± 1.08[gi]	12.04 ± 1.18[eh]
	24	23.27 ± 2.69[l]	19.46 ± 1.36[k]	19.41 ± 1.36[k]	15.52 ± 1.64[hi]	12.61 ± 0.18[eg]
37°C	4	10.00 ± 1.79[BD]	2.93 ±1.28[A]	6.85 ±1.66[B]	6.99 ± 1.20[B]	2.15 ±1.03[A]
	8	15.19 ± 2.04[FG]	7.34 ± 1.68[B]	9.01 ± 1.48[BC]	14.88 ± 1.80[FG]	8.92 ± 1.01[BC]
	12	21.02 ± 2.68[IK]	11.49 ± 1.37[CE]	12.59 ± 1.72[DF]	15.65 ± 1.37[FG]	14.35 ± 1.82[EG]
	16	24.61 ± 0.76[KL]	15.90 ± 1.69[F]	15.83 ± 1.45[G]	20.35 ± 2.02[IJ]	16.29 ± 1.13[GH]
	20	29.28 ± 2.50[M]	20.65 ± 2.95[IJ]	19.55 ± 0.99I	22.87 ± 1.59[JL]	19.08 ± 1.57[H]
	24	31.02 ± 1.69[M]	21.94 ± 2.64[IK]	25.83 ± 1.14[L]	25.49 ± 2.41[L]	26.78 ± 1.73[L]

Data presented as colour difference (ΔE*) of canned apples with added green rooibos extracts stored at 23 and 37°C for 24 weeks expressed as mean ± standard deviation (n = 3). [abc] Means with different letter superscripts of the same upperase or lowercase on the same row denotes significant differences (p < 0.05)Gre 0.25 – green rooibos native extract at 0.25%, β-GRE 0.25 – beta-cyclodextrin encapsulated green rooibos extract.

Percentage (%) inhibition is a good indicator of inhibitory effect of extracts; however, it has to be noted that it only considers the initial and final L*values, without reflecting the rest of storage weeks. Therefore, the determination of rate constant (k) becomes a suitable method to supplement the % inhibition. Hence, to further confirm the effect of addition of green rooibos extracts against browning of canned apples, kinetics was applied to establish reaction rates constant (k_0) for each temperature for the duration of storage. Table 6.5 depicts the rate constant (k_0) of zero-order reactions (as the determined order in Chapter 5) for samples with added extracts. At all storage temperatures GRE and β-GRE markedly decreased ($p < 0.05$) the browning rate relative to the control, expectedly, the highest ($p < 0.05$) k_0 values were reported for the control at 0.9786 and 1.1130 compared to GRE and β-GRE. Favreau-farhadi *et al*. (2015) studied the inhibition kinetics of epigallocatechin gallate (EGCG) and rosmarinic acid (RA) against browning of applesauce and bread rolls. They too reported a higher rate constant (k_1) for control compared with 1 – 1.5% EGCG and 1% RA added samples. At 23°C the k_0 decreased ($p < 0.05$) in the order from Control > GRE 0.5 = GRE 0.25 = β-GRE 0.25 > β-GRE 0.5. However, at 37°C, the order of decrease in k_0 was Control > β-GRE 0.5 > GRE 0.25 = GRE 0.55 = β-GRE 0.25. This implies that amongst the samples with added green rooibos extract, the rate of colour formation at 37°C progressed faster in β-GRE 0.5 compared to all samples. Numerous authors reported on the anti-browning properties of crude plant extracts or purified bioactives thereof, where polyphenols were linked to the antioxidant activity. In the present study, the antioxidant activity of GRE, in particular the superior activity of β-CD-assisted extracts (β-GRE) exhibited *via* metal chelation and radical scavenging was reported. Moreover, these were correlated to the total polyphenolic content, with aspalathin content being the highest amongst the determined flavonoids (Vhangani *et al*., 2022).

The results leading to colour development and reduction of the rate thereof was associated with the action of polyphenols in scavenging radicals produced during reducing sugar dehydration which leads to formation of dicarbonyl compounds, and eventually browning *via* polymerisation. However, the reduction in inhibitory capacity, in particular β-GRE 0.5 can be attributed to increased concentration and release of polyphenols which in the presence of heat results in autooxidation resulting in browning, and not necessarily Maillard reaction, AA, or sugar dehydration associated-browning. Another argument can be that the natural red pigment in green rooibos polymerised. Favreau-farhadi *et al*. (2015) added EGCG (3%) and RA (1.5%) in bread rolls dough and ready-made apple sauce. They found that rate of browning in bread rolls accelerated with increased concentration of EGCG (3%) and RA (1.5%) and was higher ($p < 0.05$) than that of the control. Unlike in the present study, these polyphenols did not initially impact any colour in the dough, but high temperatures employed during baking had an influence, since browning was not experienced in applesauce. It is

worthy to note that the applesauce was readymade applesauce and then stored at 37°C for 30 days. In the present study, GRE and β-GRE were incorporated in canned apples before retorting, followed by storage for 24 weeks at 37°C. Therefore, for applesauce the temperature of 37°C was low compared to temperatures employed during baking. Hence, autoxidation of polyphenols was not initiated in applesauce, but in bread rolls. Favreau-farhadi *et al*. (2015) reported that polyphenols have optimal concentrations and/or conditions as antioxidants, beyond which they promote oxidation, and this phenomenon is evident in the results of the present study, where high concentrations of polyphenols in β-GRE and high retorting temperatures resulted in increased browning in apples.

Colour difference describes colour variations by considering lightness (L*), a* (red/green) and b* (yellow/blue) coordinates (Ibarz *et al*., 1999). Therefore, ΔE* is a more accurate predictor of colour formation than the individual L*a*b* coordinates and serves as a direct reflection of noticeable changes that can be perceived visually. As mentioned in the previous chapter, ΔE* of 1 is the threshold at which a trained observer would notice the colour difference between two objects, whereas ΔE* between 4 and 8 is deemed acceptable, but above 8, ΔE* is deemed unacceptable, and likely to be rejected by consumers (Mokrzycki & Tatol, 2011). The colour difference of canned apples with added extracts ranged from 1.66 to 31.02 as depicted in Table 6.4. In the present study, samples stored at 23°C were still deemed acceptable at week 8, after which ΔE* drastically increased beyond unacceptable ranges. However, at 37°C only GRE 0.25 could reach 8 weeks within the acceptable range. All the other samples with added extracts maintained the acceptable threshold until 4 weeks of storage.

Percentage inhibition of ΔE* is displayed in Table 6.5. At 23°C an increase in concentration of extracts resulted in an increase ($p < 0.05$) in %IΔE*, and this increase was greater ($p < 0.05$) for β-GRE compared to GRE added samples. Meanwhile, at 37°C no significant differences ($p > 0.05$) in %IΔE* were observed between GRE 0.25, 0.5 and β-GRE 0.25 extracts, and a further drastic decrease was observed for β-GRE 0.5 at -17%. This correlates with results reported for L*value regarding loss of inhibitory effect by β-GRE 0.5. Although L*value for β-GRE 0.5 was comparable to that of GRE 0.5, in terms of %IΔE*, β-GRE 0.5 exhibited a negative result, which usually denotes prooxidative properties due to autooxidation. Qi *et al.* (2018) found that flavan-3-ols added at higher concentrations of 0.3 – 1 mg.L^{-1} in fried potatoes to mitigate NEB reactions also resulted in a significant increase ($p < 0.05$) in ΔE*, which was not experienced at 0.01 – 0.1%. It is worth mentioning that the highest %IΔE* was reported for β-GRE 0.5% at 23°C, which is similar to what was reported for %IL*value. This is expected since ΔE* is derived from L*value, while taking in consideration the contribution of the a* and b* coordinates.

Moreover, the k_0 for ΔE^*, as presented in Table 6.5 reported similar results to what was obtained for L*value. The progression of colour change at 23°C for the control was equal (p > 0.05) to that of GRE 0.25 and 0.5, with all β-GRE proving to significantly (p < 0.05) reduce the rate of colour change than the Control, and all GRE samples. Furthermore, at 37°C, the Control, β-GRE 0.5, and GRE 0.25 exhibited similar k_0, with only GRE 0.5 and β-GRE 0.25 showing inhibitory activity towards reducing the rate of colour change. Looking at colour development holistically, at the lower temperature of 23°C, β-GRE exhibited the highest %IL*value and %IΔE, but at a higher storage temperature of 37°C, the rate of browning intensified resulting in reduction in inhibitory capacity of β-GRE, with the lower capacity more pronounced in β-GRE 0.5 than β-GRE 0.25. Concerning GRE samples, at a concentration of 0.25%, these extracts showed no changes (p > 0.05) in inhibitory capacity for both L*value and ΔE*, whilst the inhibitory capacity of GRE 0.5 decreased. Both native and encapsulated green rooibos extracts were effective in reducing overall browning in canned apples at 23°C, which is an ambient temperature where canned food products are likely to kept throughout its shelf-life. This then proves that these may be suitable anti-browning agents. However, sensory evaluation studies have to be conducted in evaluating the effect of green rooibos addition in terms of taste, flavour and overall acceptability.

Table 6.5: Inhibitory capacity of green rooibos extracts against browning of canned apples during storage.

Sample type		Lightness value (L*)			Colour difference (ΔE*)		
Conc [ ]	Temp (°C)	% IL*	(k₀)	r²	% IΔE*	(k₀)	r²
Control	23	-	-0.9786 ± 0.38[d]	0.9286	-	1.0205 ± 0.69[b]	0.9377
Gre 0.25	23	18.45 ± 3.64[c]	-0.7536 ± 0.39[bc]	0.9575	8.94 ± 0.99[b]	0.8984 ± 1.09[b]	0.9582
Gre 0.5	23	17.07 ± 1.93[c]	-0.8283 ± 0.17[c]	0.9454	21.23 ± 1.20[c]	0.8555 ± 1.91[b]	0.9546
β-Gre 0.25	23	40.93 ± 2.71[d]	-0.6620 ± 0.55[b]	0.6918	36.22 ± 4.33[d]	0.6551 ± 1.32[a]	0.6803
β-Gre 0.5	23	46.67 ± 5.96[e]	-0.5325 ± 0.81[a]	0.8946	45.24 ± 3.06[e]	0.6106 ± 0.23[a]	0.8957
Control	37	-	-1.1130 ± 1.27[C]	0.9670	-	1.0785 ± 0.51[C]	0.9465
Gre 0.25	37	18.55 ± 2.44[c]	-0.9199 ± 1.42[A]	0.9730	9.68 ± 1.46[b]	0.9954 ± 1.00[BC]	0.9321
Gre 0.5	37	0.20 ± 0.98[a]	-0.9179 ± 1.83[A]	0.9705	9.54 ± 1.3[b]	0.9262 ± 2.04[AB]	0.9582
β-Gre 0.25	37	9.82 ± 1.68[b]	-0.9210 ± 0.43[A]	0.9208	11.96 ± 2.15[b]	0.8657 ± 1.42[A]	0.9060
β-Gre 0.5	37	2.83 ± 0.83[a]	-1.0361 ± 0.744[B]	0.9622	-17.17 ± 6.51[a]	1.0929 ± 0.47[C]	0.9454

Data presented as % inhibition of decrease in lightness (% IL*) and increase in colour difference (% IΔE*), reaction rate constant k_0 (week^{-1}) and regression coefficient (r^2) of colour change in canned apples with added green rooibos extracts stored at 23 and 37°C for 24 weeks expressed as mean ± standard deviation (n = 3). Gre 0.25 – green rooibos native extract at 0.25%, β-GRE 0.25 – beta-cyclodextrin encapsulated green rooibos extract. [abc] Means with different letter superscripts of the same case (low/upper) on the same column denotes significant differences ($p < 0.05$).

6.6.2 Inhibition of Furfural and HMF formation.

Table 6.6 depicts the furfural content of canned apples with added green rooibos extracts (GRE and β-GRE) stored at 23 and 37°C (Table 6.6) The furfural content ranged from 1.42 – 24.92 mg.100 g^{-1}, with no significant differences ($p > 0.05$) observed between the control and all samples at week zero. The highest furfural content was reported as 4.18 and 24.92 mg.100 g^{-1} for control samples at 23 and 37°C at week 24. No significant increases ($p > 0.05$) were observed in furfural content of GRE and β-GRE stored at 23°C. This implies the inhibitory effect of these extracts at 23°C. However, significant increases ($p < 0.05$) were observed at 37°C as the storage time increased, indicating the effect of increased temperature on accelerating furfural formation, as well as the inhibitory effect of each sample type.

As a means to evaluate the impact of added extracts on furfural formation during storage, the inhibition capacity was calculated based on the control in relation to each sample with added extract. The results depicted in Table 6.7 revealed that GRE and β-GRE exhibited excellent inhibition capacity at 23°C against furfural formation at 62.69 – 72.29%, even though no significant differences were observed between them. As stated previously, in the presence of oxygen, ascorbic acid becomes oxidised to dehydroascorbic acid. The possible inhibition mechanism of furfural formation was due to donation of a hydrogen to reduce dehydroascorbic acid back to ascorbic acid. That way ascorbic acid degradation, consequently, furfural formation is delayed. As the storage temperature increased to 37°C, a significant reduction in furfural inhibition was observed, with the lowest reported at 10.17% for GRE 0.25. At higher temperatures NEB reactions are favoured, such as sugar hydrolysis followed by dehydration to form highly unstable radicals. Thus, GRE and β-GRE's inhibitory effect diminishes since there are more reactions to combat. Therefore, greater inhibition in furfural formation was observed for samples stored at 23 *vs* 37°C.

Likewise, furfural formation, as described by reaction rate constants, showed that the control sample at 23°C exhibited a significantly higher k$_0$ ($p < 0.05$) compared to GRE and β-GRE treated samples. Moreover, no significant difference ($p > 0.05$) observed between all samples with added extracts is in accordance with what was reported in % furfural inhibition at the same temperature (Table 6.6). At 37°C the reaction rates were on average 12 times higher than those reported at 23°C, once again highlighting the effect of elevated temperature on increased reaction rates, as has been observed repeatedly.

Table 6.6: Table 6.6: Furfural content of canned apples with added crude green rooibos extracts.

Storage temp	Storage time	Samples type and concentration				
		Control	Gre 0.25	Gre 0.5	β-Gre 0.25	β-Gre 0. 5
23°C	0	1.75 ± 0.12^{ad}	1.62 ± 0.10^{ad}	2.06 ± 0.27^{af}	1.42 ± 0.36^{ab}	1.54 ± 0.30^{ac}
	4	1.39 ± 0.18^{a}	1.59 ± 0.18^{ad}	2.10 ± 0.48^{af}	1.24 ± 1.11^{a}	1.64 ± 0.14^{ad}
	8	1.28 ± 0.31^{a}	1.68 ± 0.13^{ad}	2.18 ± 0.29^{af}	1.70 ± 0.89^{ad}	1.78 ± 0.21^{ad}
	12	1.92 ± 0.44^{ae}	1.59 ± 0.54^{ad}	2.09 ± 0.78^{af}	1.39 ± 0.42^{a}	1.56 ± 0.42^{ad}
	16	3.47 ± 1.39^{gi}	1.71 ± 0.22^{ad}	2.40 ± 0.70^{cf}	1.77 ± 0.14^{ad}	1.73 ± 0.14^{ad}
	20	3.69 ± 1.24^{hi}	2.08 ± 0.15^{af}	2.76 ± 0.71^{eg}	1.96 ±1.16^{ae}	2.39 ± 0.16^{bf}
	24	4.18 ±0.64^{i}	2.52 ± 0.31^{df}	3.00 ± 0.78^{fh}	2.13 ± 1.26^{af}	2.42 ± 0.26^{cf}
37°C	0	1.75 ± 0.21^{A}	1.62 ± 0.13^{A}	2.06 ± 0.26^{A}	1.42 ± 0.30^{A}	1.54 ± 0.30^{A}
	4	2.81 ± 0.33^{AC}	2.31 ± 0.14^{A}	2.19 ± 0.17^{A}	2.40 ± 0.08^{AB}	2.57 ± 0.25^{AB}
	8	6.62 ± 0.28^{GJ}	4.88 ± 0.60^{DF}	4.10 ± 0.84^{CE}	4.80 ± 0.42^{DF}	3.84 ± 1.19^{BD}
	12	7.47 ± 0.77^{IK}	6.09 ± 0.69^{FI}	5.42 ± 0.45^{EG}	5.68 ± 1.75^{FG}	5.91 ± 0.49^{FH}
	16	12.04 ± 0.55^{M}	7.98 ± 0.48^{JK}	8.68 ± 1.19^{K}	6.43 ± 1.22^{GI}	7.36 ± 0.44^{HK}
	20	17.10 ± 1.26^{O}	14.06 ± 1.02^{N}	10.81 ± 1.20^{LM}	10.37 ± 0.54^{L}	10.60 ± 1.71^{L}
	24	24.92 ± 0.54^{Q}	22.39 ± 0.15^{P}	13.90 ± 1.91^{N}	16.38 ± 0.98^{O}	14.37 ± 0.62^{N}

Data presented as furfural content (mg.100g^{-1}) of canned apples with added green rooibos extracts stored at 23 and 37°C for 24 weeks expressed as mean ± standard deviation (n = 3). [abc] Means with different letter superscripts of the same upper or lowercase on the same row denotes significant differences (p < 0.05). Gre 0.25 – 0.5 green rooibos native extract at 0.25 and 0.5%, β-GRE 0.25 – 0.5 beta-cyclodextrin encapsulated green rooibos extract.

Table 6.7: Inhibitory capacity of green rooibos extracts against formation of furfural and HMF in canned apples during storage.

Sample type		Furfural			HMF		
Conc [ ]	Temp (°C)	%IF	(k_0)	r^2	%Inhibition	(k_0)	r^2
Control	23	-	0.1241 ± 0.55^b	0.7787	-	0.0865 ± 0.85^d	0.9511
Gre 0.25	23	62.69 ± 1.46^c	0.0320 ± 0.69^a	0.7081	12.75 ± 12.75^a	0.0746 ± 0.14^c	0.9297
Gre 0.5	23	64.55 ± 4.16^c	0.0379 ± 0.24^a	0.8948	50.20 ± 3.56^d	0.0459 ± 0.60^b	0.9567
β-Gre 0.25	23	72.29 ± 1.20^c	0.0322 ± 0.12^a	0.8688	59.55 ± 5.64^e	0.0273 ± 0.46^a	0.8002
β-Gre 0.5	23	63.54 ± 9.57^c	0.0376 ± 0.17^a	0.8676	67.33 ± 5.22^e	0.0301 ± 0.47^a	0.9121
Control	37	-	0.8657 ± 1.42^B	0.9388	-	0.2124 ± 0.08^D	0.9751
Gre 0.25	37	10.17 ± 1.34^a	0.7160 ± 1.6^{AB}	0.8802	11.51 ± 1.21^a	0.1774 ± 0.08^C	0.8998
Gre 0.5	37	47.10 ± 1.51^b	0.4892 ± 0.47^A	0.9414	24.03 ± 8.39^b	0.1573 ± 0.11^B	0.9274
β-Gre 0.25	37	35.04 ± 3.09^b	0.5275 ± 0.77^A	0.8763	36.56 ± 3.14^c	0.1215 ± 0.04^A	0.8797
β-Gre 0.5	37	43.87 ± 1.51^b	0.4877 ± 0.69^A	0.9485	45.77 ± 2.62^{cd}	0.1143 ± 0.61^A	0.9518

Data presented as % inhibition of furfural and hydroxymethyl furfural in canned apples with added green rooibos extracts stored at 23 and 37°C for 24 weeks expressed as mean ± standard deviation (n = 3). Gre 0.25 – green rooibos native extract at 0.25%, β-GRE 0.25 – beta-cyclodextrin encapsulated green rooibos extract. [abc] Means with different letter superscripts on the same column denotes significant differences ($p < 0.05$).

No differences ($p < 0.05$) were observed between the control sample and GRE 0.25%. Meanwhile, k_0 for GRE 0.5, β-GRE 0.25 and 0.5 were significantly lower ($p < 0.05$) than for the control sample, but not different from each other ($p > 0.05$). Inhibition of furfural formation by various polyphenols has been reported in literature, even though the focus was more in model systems than real food. Oral *et al.* (2014) found that polyphenols (tyrosol, epicatechin, punicalgin, chlorogenic, caffeic and ellagic acid) and extracts from olive mill waste, pomegranate peel and European cranberry juice were effective against inhibiting furfural formation in glycine-glucose model systems produced at higher temperatures of 140 and 180°C. The inhibitory effect of GRE and β-GRE is linked to their antioxidant activity, in particular the radical scavenging by donation of hydroxyl groups to trap reactive carbonyl species produced during ascorbic acid degradation, as well as glucose and fructose autoxidation. The radical scavenging activity of GRE and β-GRE was reported in our previous paper (Vhangani *et al.*, 2022).

In terms of HMF the content ranged between 0.61 – 5.67 mg.100 g^{-1} (Table 6.8). Similar to furfural, the highest HMF content was reported for the control sample after 24 weeks of storage at 37°C. Overall, the HMF content was lower compared to furfural, and this may be attributed to the sugar composition of the canned apples. As mentioned in the previous chapter, the canned apples comprised of 66% fructose, 22% glucose and 12% sucrose of the total sugar (results not shown). Based on the composition, it was speculated that furfural formation was achieved *via* oxidative degradation of ascorbic acid, sugar dehydration and the Maillard reaction, with fructose being the main precursor rather than glucose. Sucrose, on the other hand, was hydrolysed to yield its invert sugars (results not included). For this reason, the furfural content was higher than HMF

In terms of inhibitory effect of green rooibos extracts, the % HMF inhibition increased significantly ($p < 0.05$) as the concentration of GRE increased from 0.25 to 0.5%, meanwhile no significant differences ($p > 0.05$) were observed between β-GRE samples at both storage temperatures, although, the highest inhibitions were recorded for β-GRE at 59.55 to 67.33% (Table 6.8). At 37°C, a similar trend was observed where an increase in GRE concentration resulted in increased % inhibition of HMF, with no significant difference observed for β-GRE. However, it should be noted that an overall decrease in inhibition was reported for all extracts as a function of increased storage temperature. To confirm the inhibitory action mentioned in the aforementioned results, the k_0 values of the control sample was significantly higher ($p < 0.05$) than that of all samples with varying concentrations of extracts for both 23 and 37°C. The rate of HMF formation was reduced by addition of increasing concentrations of GRE. The lowest k_0 was reported for both β-GRE at 0.25 and 0.5 since no significant differences were observed between them. Similarly, Favre *et al*. (2020) also correlated total phenolic contents

and antioxidant activities, with the antiglycation properties of green, white, and black peppers *via* HMF inhibition.

The study of Qi *et al.*, (2018) mentioned earlier proved the link between anti-browning capacity and inhibitory activity against hydroxymethyl furfural formation. Flavan-3-ols at 50, 100 and 150 µg.mL^{-1} showed a dose-dependent inhibition against HMF formation in glucose-asparagine model systems. Meanwhile, in a real food system, the reduction of HMF formation in fried potato chips dipped in 0.01 – 0.1 mg.mL^{-1} of flavan-3-ols also increased with increased concentration, however concentrations higher than 0.1 mg.mL^{-1} caused a decline in inhibition. This phenomenon also coincided with the reduction in lightness and increase in ΔE. High concentrations of added phenols that resulted in darkening of the chips, also resulted in reduced HMF reduction. Therefore, as the polyphenols participate in autoxidation, its inhibitory effect against NEB is also reduced. In the present study we also reported the decline in inhibitory effect of GRE and β-GRE against HMF.

To finally conclude on the holistic inhibitory effect of GRE and β-GRE, the activation energy (Ea) of each extract for each dependent variable is shown in Table 6.9. Activation energy (Ea) is defined as the minimum energy required to start a chemical reaction, and in this instance the effect of temperature is combined for 23 and 37°C for each sample treatment over the storage period calculated from the Arrhenius equation (Lyu *et al.*, 2018; VanBoekel, 2021). In terms of browning development *via* L*value and ΔE*, the control and all GRE samples exhibited the lowest Ea ($p < 0.05$), revealing that GRE was a less effective inhibitor against browning development. Although the Ea of GRE 0.25 for L*value was not significantly different from β-GRE 0.25 ($p > 0.05$), β-GRE 0.5 showed the highest Ea of 36.33 kJ.mol^{-1} indicating greater inhibitory power *via* resisting initiation of browning.

Concerning intermediate NEB product formation, no significant differences were observed ($p > 0.05$) between the Ea of HMF for the control and GRE 0.25 samples at 49.12 and 47.23 kJ.mol^{-1}, respectively. The Ea of GRE 0.5 and β-GRE 0.5 was comparable, which was equal to that of β-GRE 0.25. In terms of furfural, the highest Ea of 167.13 kJ.mol^{-1} was reported for GRE 0.25 followed β-GRE 0.25 at 148.82 kJ.mol^{-1}, while no significant differences were observed between GRE 0.5 and β-GRE 0.5 ($p > 0.05$). Overall, samples treated with green rooibos extracts exhibited the highest Ea compared to the control. Therefore, furfural formation required less energy to form in the control samples and was restricted by the presence of GRE and β-GRE.

Table 6.8: Hydroxymethyl furfural content of canned apples with added crude green rooibos extracts.

Storage time	Storage time	Samples type and concentration				
		Control	Gre 0.25	Gre 0.5	β-Gre 0.25	β-Gre 0. 5
	0	0.65 ± 0.68^a	0.62 ± 0.48^a	0.71 ± 1.1^a	0.59 ± 1.88^a	0.61 ± 0.92^a
	4	0.78 ± 0.62^{ab}	0.64 ± 0.09^{ab}	0.72 ± 0.55^a	0.74 ± 0.96^{ab}	0.80 ± 0.19^{ab}
	8	1.63 ± 0.73^{jl}	1.44 ± 1.02^{hi}	1.14 ± 0.29^{cf}	1.09 ± 0.45^{ce}	0.94 ± 0.66^b
23°C	12	1.76 ± 0.58^{jl}	1.63 ± 0.55^{jl}	1.29 ± 0.44^{eh}	1.18 ± 0.77^{cf}	1.01 ± 0.17^{cd}
	16	1.95 ± 0.77^l	1.78 ± 0.78^{kl}	1.44 ± 0.84^{hi}	1.28 ± 1.14^{eh}	1.22 ± 0.82^{eg}
	20	2.33 ± 1.66^m	1.93 ± 1.07^l	1.56 ± 1.94^{ij}	1.19 ± 0.87^{df}	1.31 ± 1.36^{eh}
	24	2.79 ± 1.11^n	2.46 ± 1.62^m	1.76 ± 1.02^{jl}	1.31 ± 0.98^{eh}	1.36 ± 1.66^{gh}
	0	0.65 ± 1.08^{AB}	0.62 ± 0.46^A	0.71 ± 0.86^{AB}	0.59 ± 0.81^A	0.61 ± 0.92^A
	4	0.92 ± 0.78^B	0.76 ± 0.81^{AB}	0.85 ± 0.77^{AB}	0.79 ± 0.89^{AB}	0.84 ± 1.01^{AB}
	8	2.20 ± 0.59^{FG}	1.90 ± 1.14^{DE}	1.47 ± 1.92^C	1.88 ± 1.24^{DE}	1.76 ± 0.96^{DE}
37°C	12	2.91 ± 0.14^J	1.91 ± 0.98^{DE}	$1.71 \pm 1 .06^{CD}$	2.04 ± 0.78^{EG}	1.81 ± 0.94^{DE}
	16	3.36 ± 1.03^K	2.28 ± 0.1^{GH}	2.52 ± 1.12^{HI}	1.96 ± 1.40^{DF}	2.04 ± 1.15^{EG}
	20	4.46 ± 0.67^M	3.53 ± 0.89^K	3.43 ± 0.97^K	2.77 ± 2.58^{IJ}	2.79 ± 0.05^J
	24	5.67 ± 0.96^P	5.09 ± 1.09^O	4.76 ± 1.33^N	3.86 ± 1.07^L	3.48 ± 1.03^K

Data presented as hydroxymethyl furfural content ($mg.100g^{-1}$) of canned apples with added green rooibos extracts stored at 23 and 37°C for 24 weeks expressed as mean ± standard deviation (n = 3). Gre 0.25 – green rooibos native extract at 0.25%, β-GRE 0.25 – beta-cyclodextrin encapsulated green rooibos extract.

Table 6.9: Activation energy (KJ.mol^{-1}) of green rooibos extracts against browning of canned apples during storage.

Sample type	Browning development		Intermediate products	
	L*value	ΔE*	HMF	Furfural
Control	7.03 ± 1.79[a]	3.16 ± 3.33[a]	49.12 ± 7.12[a]	101.88 ± 3.53[a]
Gre 0.25	10.83 ± 2.15[ab]	5.87 ± 4.70[a]	47.23 ± 3.53[a]	167.13 ± 5.34[d]
Gre 0.5	5.55 ± 1.72[a]	4.42 ± 2.31[a]	67.42 ± 3.10[b]	140.06 ± 1.83[b]
β-Gre 0.25	18.84 ± 10.28[b]	15.54 ± 3.64[b]	81.92 ± 5.64[c]	148.82 ± 3.54[c]
β-Gre 0.5	36.33 ± 1.39[c]	32.06 ± 7.47[c]	73.12 ± 8.54[bc]	136.79 ± 3.63[b]

Data presented as activation energy KJ.mo^{l-1} for Lightness (L*-value), colour difference (ΔE*), hydroxymethyl furfural (HMF) and furfural of canned apples with added green rooibos extracts stored at 23 and 37°Cfor 24 weeks expressed as mean (n = 3). Gre 0.25 – green rooibos native extract at 0.25%, β-GRE 0.25 – beta-cyclodextrin encapsulated green rooibos extract. [abc] Means with different letter superscripts on the same column denotes significant differences ($p < 0.05$).

6.7 Conclusions

The results of this study revealed that green rooibos extracts were able to inhibit browning development with β-GRE 0.25 and 0.5 exhibiting higher ability to reduce browning development. However, as the temperature increased to 37, the inhibitory effect of green rooibos was reduced. Formation of HMF and furfural followed the same trend. When comparing the β-GRE 0.25 and 0.5 exhibited higher inhibition of HMF compared to GRE, although no significant differences ($p > 0.05$) were observed between furfural inhibition. It is worth to note that furfural inhibition ranged between 62 – 72%. These results were further proved *via* activation energy (Ea), where the control sample exhibited Ea comparable to that of GRE for brown development and HMF, while β-GRE exhibited a high Ea which indicated resistance to browning and furfural formation.

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CHAPTER 7: GENERAL SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

7.1 General Summary and Conclusions

The general aim of present PhD work was to determine the anti-browning/anti-glycative potential of β-CD-assisted extracts of green rooibos by first gaining insight into the antioxidant activity of encapsulated extracts, followed by application of the extracts in canned apples during storage. The following sections summarises the findings of each research chapter.

The first research chapter's objective (Chapter 3) was to optimise β-CD-assisted extraction of bioactives from green rooibos in order to obtain compounds with the highest TPC and antioxidant activity. Under a concentration of 15 mM β-CD heated at 40°C for 60 min, CGRE exhibited the highest TPC, ABTS radical scavenging and FRAP content ($p < 0.05$). Although no significant ($p > 0.05$) differences were observed between the MTC and ORAC content of 15 mM β-CD: 40°C: 60 min from other β-CD-assisted extracts, a significant difference ($p < 0.05$) was observed in comparison to all aqueous extracts. Furthermore, a positive strong correlation between TPC and antioxidant activity assays was established at R^2 (0.929–0.978).

The second research Chapter 4 was aimed at ascertaining formation of inclusion complexes between green rooibos and β-CD, as obtained in Chapter 3, in order to evaluate their suitability as browning inhibitors in canned apples. This was achieved *via* studying the physicochemical properties linked to storage and processing conditions that CGRE was to be exposed during processing. Physicochemical properties results proved that encapsulation posed no significant effect ($p > 0.05$) on the MC between GRE and β-GRE, however, encapsulation reduced the a_w of β-GRE by 39%. In addition, β-GRE samples were lighter (L*) and less red (a*) compared to their counterpart ($p < 0.05$). Therefore, this proved that β-GRE could be applied in canned apples and impart less intense red colour compared to GRE. Regarding suitability of application of green rooibos extracts during thermal processing, thermogram (TGA) and FT-IR spectrum proved that green rooibos β-CD complexes were successfully formed, resulting in formation of a new molecule which was a superposition of β-CD and green rooibos, which resulted in improvement in heat stability of β-GRE, compared to GRE.

The third research Chapter 5 aimed at applying reaction kinetics to evaluate NEB reactions taking place in AA-supplemented canned apples during storage. This was achieved via monitoring reaction rate constants (k_0 and k_1) and activation energy (Ea) required for key reactant consumption and intermediate product formation. The effect of non-specific browning indicators such as pH and Brix was no significant, thus kinetics was not applied for these indices. Reaction rates for browning development in terms of A_{294} and A_{420} nm, L* and ΔE*

were higher for ascorbic acid added samples (+AA) compared to those without (-AA), with the reaction order characterised mostly as zero-order from 5 to 37°C, and first-order for samples stored at 60°C. Changes in reducing sugar content throughout storage was insignificant ($p > 0.05$), and this was attributed to sugar hydrolysis and degradation, as well as the MR taking place simultaneously in canned apples. Ascorbic acid degradation kinetics was also temperature dependent, with samples stored between 5 – 37°C following zero-order and 60°C, first-order. The progression of Intermediate browning products, HMF and furfural, was faster for +AA than -AA samples. Furthermore, kinetics revealed that that AA degradation preceded the MR and sugar degradation in +AA samples.

The final research Chapter 6 was the main research chapter, where extracts with maximum TPC and antioxidants activity (15 mM β-CD: 40°C: 60), and proven to be stable against high temperatures were added in canned apples processed and stored at 23 and 37°C. These were compared to an aqueous counterpart (0 mM β-CD: 40°C: 60). The results obtained showed that overall, browning development as measured by L^* and ΔE^* was reduced by the addition of green rooibos extracts (GRE and β-GRE), with β-GRE 0.25 and 0.5 exhibiting the highest ability. The increase in storage temperature from 23 to 37°C resulted in reduction in inhibitory effect of green rooibos was reduced. In terms of intermediate product formation, β-GRE 0.25 and 0.5 exhibited higher inhibition capacity against of HMF compared to GRE. No significant differences ($p > 0.05$) were observed for furfural inhibition between GRE and β-GRE added samples, although overall furfural inhibition was in the range 62 – 72%. Furthermore, the effect of temperature on browning of canned apples was determined via the Ea. development and HMF and furfural formation was revealed via Ea. GRE added samples exhibited Ea comparable to that of the control sample for L^* and ΔE^* development and HMF, while β-GRE exhibited a high Ea which indicated resistance against browning and furfural formation.

Therefore, the following conclusions regarding the use of β-CD in the present study could be made based on the pre-formulated hypothesis:

Successful complexation between β-CD and green rooibos was confirmed by TGA and FT-IR

Beta-cyclodextrin-assisted extracts of green rooibos enhanced the TPC, thus positively contributing to enhanced antioxidant activity, and these increased as the β-CD concentration increased.

Beta-cyclodextrin-assisted extracts of green rooibos resulted in reduced a_w and improved the heat degradation temperature compared to aqueous.

Application of β-CD-assisted extracts of green rooibos reduced browning development, furfural and HMF formation in canned apples during storage.

7.2 Future Studies and Recommendations

In light of the antioxidant and TPC recovery, β-CD-assisted extraction can be applied in conjunction with alternative processing technologies such as ultrasound and pulsed electric fields, in order to increase the antioxidant capacities and adequately modulate the polyphenol profile of the extracts. More importantly, achieve the same content of TPC by applying shorter extraction time than 60 min. Moreover, it is advisable to evaluate the oxidation status of polyphenols during extraction.

With reference to characterisation of physicochemical properties of β-CD-assisted green rooibos extracts, future studies can also include evaluation of sorption isotherm studies to determine the behaviour of the encapsulates under various conditions of a_w, as opposed to one-time a_w and MC measurement. In addition to sorption isotherm, the changes in colour as a result of storage at various a_w can be monitored.

With reference to elucidating NEB reaction types and kinetics of canned apples, monitoring of initial concentration and consumption of oxygen (headspace and dissolved), ascorbic acid type (oxidised and reduced) and amino acid profile.

In the present study, only intermediate products HMF and furfural were evaluated. It is recommended that future studies should include reactive carbonyl species such as glucosone, 3-deoxyglucosone and fructofuranosyl. These will aid in confirming specific NEB reaction types.