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Degree: MFoodTech Dept/Institute/School: IFNHH

Thesis Title: Development of an acceptable, stable and safe nitrate-rich vegetable juice beverage.

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Development of an acceptable, stable and safe nitrate-rich vegetable juice beverage

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Food Technology

at Massey University, Albany, New Zealand.

Tejal Nikhil Kolte

2014

ABSTRACT

Ingestion of nitrates from a vegetable juice beverage has been reported to improve exercise performance. The research was therefore conducted to produce a vegetable juice beverage with stable nitrate content that could potentially enhance sports activity. In this study, a placebo drink was also produced with low nitrate content and to match the taste and quality parameters of the high nitrate juice beverage.

Juice was extracted from beetroot, pasteurised at 90±1°C for 15 s and blended with other ingredients and further tested for pH, titratable acidity, total soluble solids, nitrate and nitrite content and microbial counts. A sensory evaluation trial was conducted on four finalised juice blends along with the commercial product on the market. *Orange flavour low acid* beetroot juice beverage (1572±5 mg nitrate/L) was preferred formulation than the commercial juice beverage, BEET IT.

A shelf life trial, using a full factorial experimental design, was used to determine the effect of temperature (4±1°C and 20±1°C) and storage conditions (light or dark storage) on *orange flavour low acid* beetroot juice beverage. From the storage trial, the *orange flavour low acid* beetroot juice beverage containing more than 1500 mg nitrate/L, can be stored in transparent bottles and safely consumed after eight weeks storage if stored at 4±1°C.

The sensory results obtained from performing the triangle test on the *orange flavour low acid* formulation (standard beverage) and placebo drink suggested that only 28 % of the population could identify a difference between the two products. The placebo drink contained 181±4 mg nitrate/L which was nine times less than the nitrate concentration in the standard beverage.

In conclusion, an acceptable high nitrate juice beverage was formulated with a corresponding low nitrate drink placebo drink which could not be differentiated by consumers after sensory testing. It is recommended to develop a commercial manufacturing procedure to produce the nitrate juice beverage from beetroot, beet leaves and celery juices from which larger batches of samples can then be tested for exercise performance.

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LIST OF ABBREVIATIONS

°B/A	°Brix/Acid
ADI	Acceptable Daily Intake
H_a	Alternative Hypothesis
BW	Body weight
Ca^{2+}	Calcium
CIP	Cleaning In Place
CFU	Colony-Forming Unit
D	Decimal reduction time
DF	Degree of Freedom
EC	European Commission
EU	European Union
E.G.	Example
FAO	Food and Agriculture Organisation
FSANZ	Food Safety Australia New Zealand
GDP	Gross Domestic Product
HDPE	High Density Polyethylene
HPLC	High Pressure Liquid Chromatography
HTST	High Temperature Short Time
JEFCA	Joint Expert Committee of the Food and Agriculture
L	Lethal rate
F	Lethality
VO2	Maximal Oxygen Uptake
Wmax	Maximal power
min(s)	Minutes
MAP	Modified Atmosphere Packaging
NF	Nano Filtration
NZ	New Zealand
NO_3	Nitrate
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NO_2	Nitrite
N/A	Not applicable
H_o	Null Hypothesis
%	Percentage
PET	Polyethylene Terephthalate
PCA	Principal component analysis
PEF	Pulsed Electric Field
RCF	Relative Centrifugal Force
RO	Reverse Osmosis
SCF	Scientific Committee On Food
TPC	Total Plate Count
TTE	Transthoracic Echocardiogram

UHT	Ultra High Temperature
UK	United Kingdom
WHO	World Health Organisation
Y & M	Yeasts And Mould

CHAPTER 1
INTRODUCTION

Sport and recreation is highly valued in New Zealand (NZ) and individuals and communities invest considerable amounts of time and money in these activities. The sports and recreation sector contributed $5.2 billion to Gross Domestic Product (GDP) in 2008/09 or 2.8% of GDP (Sport and Recreation, 2011). This puts the sports and recreation sector on par with the dairy industry's contribution ($5.0 billion) to the New Zealand economy (New Zealand Institute of Economic Research, 2010). With growth of this sector, the demand for sports nutrition products in New Zealand is also steadily increasing. New Zealanders spend $1.2 billion per year on non-alcoholic cold beverages including sports drink consuming 640 million litres of soft drinks, fruit juice, bottled water, sports and energy drinks, and flavoured milk (New Zealand Juice and Beverage Association, 2014). Sport drinks continue to be in high demand as more and more people become involved in sports participation (New Zealand Institute of Economic Research, 2010). Consumer-focussed companies are capitalizing on this interest in sports performance by continually developing new products and improving the old ones that are designed to help athletes recover faster, play and practise harder and simply perform better overall. Among these popular products are energy bars and gels, fitness and sport drinks (Athletes, 2014). However, due to shifting market trends and declining life-cycle of the products, customers demand new sport products which can be consumed before, during and/or following the sports event and provide nutrients to enhance performance (Desbrow & Leveritt, 2005). The products are also expected to offer satiety feelings without causing stomach discomfort with minimal or no after taste (Priest et al., 2008a).

As it is often difficult to eat anything during or immediately after athletic participation, sport drinks can solve this problem by being a quick and easy way to prevent dehydration and muscle fatigue. The major reason for drinking beverages during sporting events is to reduce the fluid deficit incurred through the loss of sweat. Other considerations for consuming fluids during sporting events lasting longer than 45

minutes includes ingestion of common drink ingredients known to enhance performance such as carbohydrates (Burke & Deakin, 2010), electrolytes (Shirreffs & Sawka, 2011) and caffeine (Burke, 2008). Cool (Lee & Shirreffs, 2007) or icy (Ross et al., 2011) fluids may also help maintain thermal comfort and aid thermoregulation during exercise. This not only helps aid performance but also supports better recovery for subsequent activities especially important for well-trained and/or elite athletes who take part in more regular exercise.

For the past three years, endurance athletes have been consuming beetroot juice before their races to charge on its naturally occurring nitrate, which helps muscles use oxygen more efficiently (Athletes, 2014). Consuming dietary nitrate increases the natural nitric oxide production in the body, which relaxes and widens blood vessels, thereby reducing blood pressure and increasing blood flow to organs and tissues. As a result, nitric oxide can reduce the oxygen cost of exercise (Weitzberg et al., 2008).

A few manufacturers (firstly in United Kingdom and now in Australia) have emerged, offering bottled beetroot juice with a known nitrate content (McCubbin, 2013). These bottled beetroot juices are sold in selected health food stores. Most of the sports-based-nitrate-rich drinks on the market such as BEET IT, UPBEET, SUNRAYSIA and GO BEET are made by blending beetroot juice and apple juice. The sports-based-nitrate-rich drinks on the market are manufactured in UK and Australia and exported to New Zealand. The decision was to investigate different nitrate-rich vegetables in New Zealand besides beetroot due to the nutritional and functional benefits and also for the potential to utilise the waste stream (such as beet leaves and celery leaves) to reduce raw material costs. Generally only the purple red root with a small portion of beet stalk, is harvested for consumer consumption whereas the majority of the beet leaves are discarded as waste. Some of the nitrate-rich vegetables in New Zealand include beetroot, spinach and celery (Thompson, 2004).

The aim of this research was to develop a stable, safe and organoleptically acceptable nitrate rich vegetable juice beverage for sports performance and health beverage markets with an acceptable flavour.

The performance of the vegetable juice beverage will need to be tested for future research projects to check if the nitrate in the juice beverage helps in exercise related performance. To ensure it is tested properly, there is a need to include a placebo version to match the vegetable juice.

The following objectives were investigated to produce a stable juice from nitrate rich vegetables in New Zealand:

1. To develop a vegetable juice that provides more than 1000 mg/L of nitrates in a stable form, has a good sensory acceptability and is safe for consumption.
2. To quantify nitrate and nitrite concentrations in the vegetable juice using high performance liquid chromatography (HPLC).
3. To monitor various characteristics of the nitrate rich vegetable juice such as pH, acidity, brix, shelf life trial and microbial counts to ensure it is acceptable and safe for consumption.
4. To develop a placebo vegetable juice containing no nitrates which tastes the same as the vegetable juice with nitrates.
5. To determine the consumer acceptability of the nitrate rich vegetable juice and to determine by sensory testing if the placebo vegetable juice could be detected as different by a consumer panel.

CHAPTER 2

LITERATURE REVIEW

This chapter gives a brief overview on how nitrate helps to boost exercise-related performance and emphasises certain dietary sources of nitrates and nitrites found in New Zealand. It also summarises the importance of nitrate within the sports and health sector and tabulates some of the studies undertaken in the past using nitrate-enriched vegetable juice. A review of methods employed by researchers to facilitate the detection, determination and monitoring of nitrate and/or nitrite through HPLC is also presented. Processing and safety of the juice, along with some of the factors affecting the stability of nitrates in juice, are also discussed. Finally, the chapter summarises some of the available nitrate rich juices globally.

2.0 Nitrate, nitrite and nitrous oxide

Nitrogen is absorbed by plants from the soil, mostly as nitrate, and partly converted to ammonia in order to make proteins and other nitrogenous compounds through the nitrogen cycle (Lefebvre, 1976). Nitrates are used in agriculture as a fertilizer to replace the traditional use of livestock manure and in food processing as an approved preservative (European Food Safety Authority, 2008). Nitrate is ubiquitous and is present in food, water and in humans as a metabolite.

Sources of nitrate in the body can be grouped into two classes: exogenous and endogenous. The external sources are from foods and drinking water. Nitrate is a natural constituent of plants, their leaves and roots, and supports plant growth and development. Green leafy vegetables such as lettuce and spinach and other vegetables such as carrots, celery, radishes and beetroot constitute a major source of nitrate and nitrite in the human diet (Ministry of Agriculture, Fisheries and Food, 1998). It has been estimated that vegetables contribute approximately 80-92 % of the total nitrate (Dich et al., 1996) and 16-43 % of total nitrite (Walker, 1990) in an average daily diet. Drinking

water usually provides only a minor portion of the external nitrate, about 2.0-2.5 % (Ministry of Agriculture, Fisheries and Food, 1998).

Nitric oxide (NO) is a gaseous physiological signalling molecule and is known to influence a wide array of processes including skeletal muscle glucose uptake, vasodilation, neurotransmission, sarcoplasmic reticulum's calcium (Ca^{2+}) handling, mitochondrial respiration and skeletal muscle fatigue (Van Faassen et al., 2009). Therefore nitrate supplementation is seen as a way of increasing NO and having downstream effects on exercise performance (Feelisch et al., 2008; Van Faassen et al., 2009).

Nitrate is rapidly absorbed and metabolised by colonic bacteria (Weitzberg et al., 2008). Two pathways produce nitric oxide (NO) from nitrates: the L-arginine pathway and the nitrate-nitrite-NO pathway (**Figure 2.1**; Cosby et al., 2003; Weitzberg et al., 2008). The ability of humans to produce NO from the L-arginine pathway is complex and requires undisturbed blood supply and oxygen delivery. The L-arginine pathway is no longer fully functional when blood flow is impaired by occlusion or narrowing of vessels. Thus NO synthase (NOS) independent mechanisms must exist to maintain NO homeostasis under hypoxic (reduced oxygen) conditions. The reduction of nitrite to NO through the nitrate-nitrite-NO pathway reflects a major mechanism by which NO homeostasis is maintained independent of NOS (Weitzberg et al., 2008).

The right branch shown in **Figure 2.1** represents the conventional L-arginine-NOS-NO pathway while the left branch of the schematic represents the nitrate-nitrite-NO pathway (Cosby et al., 2003). The NOS-NO pathway requires L-arginine, oxygen and NADPH as essential substrates as well as co-factors: flavine adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4), haem and calmodulin (Cosby et al., 2003; Weitzberg et al., 2008). A portion of the NO oxidation products, nitrate, produced through the reaction between NO and oxyhaemoglobin, and nitrite produced by the oxidation of NO by ceruloplasmin, can be recycled back to NO via the nitrate-nitrite-NO pathway, as indicated by the dashed arrows (Cosby et al., 2003).

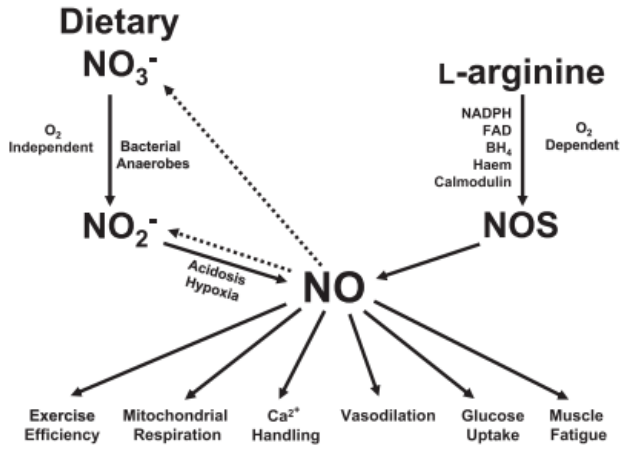

Figure 2.1: The pathways of NO generation in humans (Cosby et al., 2003)

The nitrate-nitrite-NO pathway is proposed as an alternative to the classical L-arginine-NOS-NO signalling pathway (Cosby et al., 2003). Ingested inorganic nitrate is rapidly absorbed from the gut and passes into systemic circulation with peak plasma nitrate observed 60 mins after nitrate ingestion. About 25% of nitrate consumed through food, passes into the enterosalivary circulation and is concentrated in the saliva (Zuckerbraun et al., 2011). The facultative anaerobic bacteria in the enterosalivary circulation reduces both plasma-extracted nitrate and dietary nitrate to form nitrite, resulting in salivary nitrite concentrations that are 1000 times higher than those found in human plasma (Cosby et al., 2003). Nitrite is protonated to form nitrous acid (HNO_2) when the nitrite-rich saliva meets the acidic gastric juice after swallowing (Weitzberg et al., 2008). This nitrous acid is then decomposed to NO and other reactive nitrogen intermediates within the acidic environment of the stomach, known as the acidic disproportionation process (Zuckerbraun et al., 2011). The gastric NO takes part in the human defence system against pathogens entering via the alimentary tract. However, some nitrite is absorbed from the stomach to increase circulating plasma nitrite and hence dietary nitrate supplementation represents a practical method to increase the circulating plasma nitrite (**Figure 2.2**). Bryan et al. (2011) showed that nitrate is excreted in human sweat and reduced to nitrite by skin bacteria. As normal skin is slightly acidic (pH ~ 5.5) this nitrite is reduced to NO. This NO is thought to inhibit skin pathogens, particularly fungi (Bryan et al., 2011) and when normal saliva is applied to healthy skin, high concentrations of nitrite considerably increase NO synthesis, to protect against infection and encourage wound healing (McKnight et al., 1997).

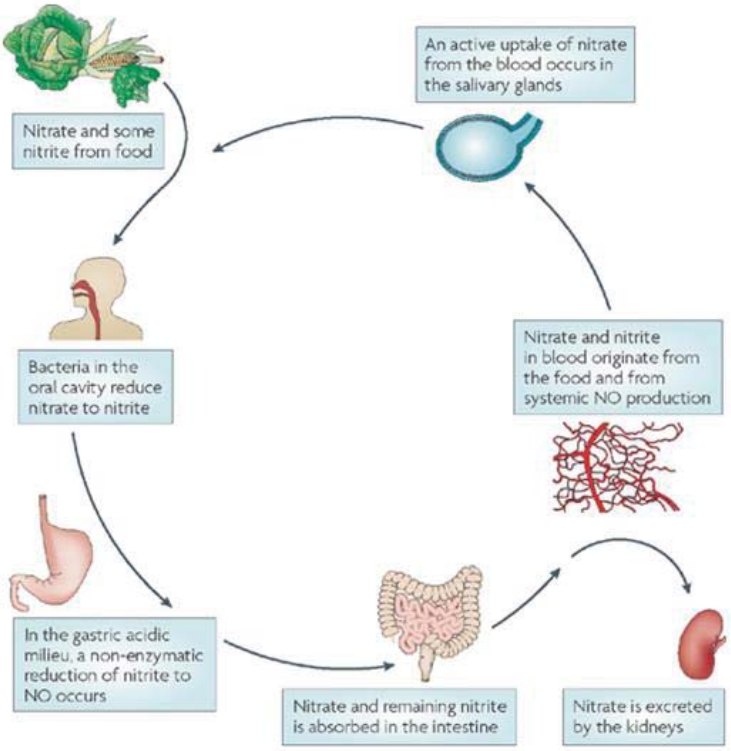

Figure 2.2: Formation of NO from nitrate-nitrite- NO pathway (Weitzberg et al., 2008).

It is important to note that the characteristic rise in plasma nitrate following an oral nitrate supplementation is largely abolished after antibacterial mouthwash or from spitting and not swallowing, indicating that the reduction of nitrate to nitrite in humans is dependent on the bacterial nitrate reductases (Weitzberg et al., 2008). Hence this alternative nitrate-nitrite-NO anaerobic pathway is important especially in hypoxic conditions such as exercise.

2.1 Dietary sources of nitrates and nitrites

Vegetables constitute the major dietary source of nitrate, generally providing from 300-940 mg nitrate/kg vegetable weight of the daily dietary intake (Bryan et al., 2011). Consumption of a typical western diet results in ingestion of approximately 1 to 2 mmol nitrate/day (62-124 mg nitrate/day) (Bryan et al., 2011). In contrast, the contribution of vegetables to nitrite intake is low, in fact lower than that from cured meat products (Santamaria, 2006). Nitrite is found in plant foodstuffs, typically 1 to 2 mg/kg of the fresh vegetable weight (Walker, 1996). Higher amounts of nitrite are found in contaminated food, food with broken vegetable tissues or in food stored for several days

at room temperature due to nitrate reductase activity from microbial contamination (Jones & Griffith, 1965; Santamaria, 1999).

Generally nitrate-accumulating vegetables come from the families of *Brassicaceae* (cabbage, cauliflower, broccoli, radish, rocket), *Chenopodiaceae* (beetroot, spinach, swiss chard), *Asteraceae* (lettuce) and *Apiaceae* (celery, parsley) (**Table 2.1)**. Nitrate content can also vary within species cultivars and even genotypes with different ploidy (Santamaria, 1999). These differences in the nitrate content could be correlated with the differing location of nitrate reductase activity and to a different degree of nitrate absorption and transfer in the plants (Maynard & Barker, 1979). Nitrate content differs in various parts of a plant which are listed by the decreasing nitrate content as follows: petiole> leaf> stem> root> inflorescence> tuber> bulb> fruit> seed (Santamaria, 1999). The nitrate is mainly located in the cell vacuoles and is transported by the xylem. The xylem carries water and nutrients from the roots to the leaves whereas the phloem carries the products of photosynthesis from the leaves to the growth points of the plant. This means that leaf crops, such as cabbage, lettuce and spinach have fairly large concentrations of nitrate whereas storage organs such as potato tubers, carrots, leeks and beans have relatively small concentrations (**Table 2.1**., European Food Safety Authority, 2008).

The two most significant contributors to both nitrate and nitrite exposure in the human diet were potatoes (32 %) and lettuce (29 %) (Thompson, 2004). However New Zealand lettuce and potato samples are not high in nitrate when compared with European and Asian data (Thompson, 2004). The EU has established different limits for nitrate concentrations in spinach and lettuce depending on the season of cultivation. Higher concentrations of nitrate are permitted for produce grown in the winter months than in the summer (European Commission, 1997). Muramoto (1999) explained the requirement of light energy for nitrate reductase activity and further described that the high nitrate in vegetables during fall/winter could be due to less nitrate reductase activity given the short daylight duration of time. On the other hand, in a recent study of vegetables grown in Korea, no significant variance in the nitrate reductase activity was found for most vegetables cultivated during the summer and winter (Chung et al.,

2003). Malmauret et al. (2002) reported higher median and maximum concentrations of nitrate in organically grown compared with conventionally grown lettuces.

Table 2.1: Classification of vegetables in Italy according to nitrate content (mg/kg) (Santamaria, 2006)

Very low (<200)	Low (200–500)	Middle (500–1000)	High (1000–2500)	Very high (>2500)
Artichoke	Broccoli	Cabbage	Celeriac	Celery
Asparagus	Carrot	'Cima di rapa' (broccoli rab)	Chinese cabbage	Chervil
Broad bean	Cauliflower	Dill	Endive	Cress
Brussels sprouts Eggplant	Cucumber	'Radicchio'	Escarola	Lamb's lettuce
Garlic	Pumpkin	Savoy cabbage	Fennel	Lettuce
Onion	'Puntarelle' chicory	Turnip	Kohlrabi	Radish
Green bean			Leaf chicory	Red beetroot
Melon			Leek	Rocket
Mushroom			Parsley	Spinach
Pea				Swiss chard
Pepper				
Potato				
Summer squash				
Sweet potato				
Tomato				
Watermelon				

Based on published results, and knowledge of New Zealanders' vegetable consumption preferences, the following were identified as the most likely contributors to dietary intake of nitrate in Bari, Italy: cabbage, lettuce, silverbeet, celery, broccoli and perhaps watercress and courgette (Santamaria, 2006). Other vegetables of significance of nitrate content include beetroot, potatoes, carrot and pumpkin (Santamaria, 2006). New Zealand water supplies are low in nitrate with 85% of water samples collected between 1983 and 1989 contained no nitrate compared with a guideline maximum value of 44 mg/L (Thompson, 2004).

Table 2.2 shows the mean and range of nitrate and nitrite concentrations found in different vegetables in New Zealand (NZ) as part of the 'Total Diet survey' (Thompson, 2004).The highest nitrate concentration was found in watercress, celery and lettuce. Canned beetroot, spinach and silverbeet had lower amounts of nitrate than lettuce, celery and watercress but higher amounts than cabbage, broccoli, pumpkin, potato and carrot. Nitrite was not detected in any of the vegetable samples above the limit of detection with the exception of broccoli at 6 mg/kg (Thompson, 2004; **Table 2.2).**

Table 2.2: Concentration of nitrate and nitrite in different vegetables from the 'Total Diet survey' conducted in New Zealand (mg/kg fresh weight of sample; as sodium salt) (Thompson, 2004)

FOOD	NITRATE MEAN mg/kg AS $NaNO_3$	RANGE	NITRITE MEAN mg/kg AS $NaNO_2$	RANGE
GREEN VEGETABLES				
Cabbage	331	120-690	2.5	<5
Lettuce	1590	83-3420	2.5	<5
Silverbeet	740	190-1690	2.5	<5
Watercress	1640	870-2790	2.5	<5
Celery	1610	880-2320	2.5	<5
Broccoli	133	51-280	6	<5-27
Spinach	990	100-1560	2.5	<5
Beetroot, canned	763	260-2220	2.5	<5
Potato	48-240	48-240	2.5	<5
Carrot	<5-290	<5-290	2.5	<5
Pumpkin	<5-350	<5-350	2.5	<5

<less than

Table 2.3 shows the concentration of nitrate (mg/kg fresh weight basis) for different vegetables in New Zealand and compares them against international data. The results for vegetables from the 'NZ Total Diet survey' were lower than or comparable with nitrate results from overseas. Lower results for beetroot, broccoli, silverbeet and spinach relative to the overseas data can be explained by the difference in preparation and other factors (Thompson, 2004). In the 'NZ Total Diet survey', the nitrate concentrations reported in the 'NZ Total Diet survey' were from cooked vegetables whereas the overseas data represented nitrate concentrations in fresh vegetables (Thompson, 2004). Abo Bakr et al. (1986) have found that 14% to 79% of the nitrate contained in fresh vegetables is lost when they are cooked. Several other factors can affect the nitrate

uptake and accumulation in vegetable tissues e.g. genetic factors, environmental factors (atmospheric humidity, substrate water content, temperature, irradiance and photoperiod) and agricultural factors (nitrogen doses and chemical forms, availability of other nutrients, use of herbicides etc.) (Santamaria, 2001).

Table 2.3: Comparison of NZ nitrate concentrations in vegetables with the international data (mg/kg fr
Safety Authority, 2008; Food Standards Australia New Zealand, 2010; Thompson, 2004)

FOODS	NZ 1980	NZ 2004	NZ 2007	SAS 2010	UK 1994	UK 1999	Denmark 1999	Chi 200
Cabbage	542	275	331	346	860	338	-	153
Lettuce	450	823	1590	1144	3000	1051	2440	-
Silverbeet	1770	616	-	-	-	-	-	-
Watercress	-	1364	-	-	1300	-	-	-
Celery	4100	2339	1610	1527	1200	-	-	360
Broccoli	-	111	133	224	-	-	-	-
Spinach	-	824	990	2741	2100	1631	1783	-
Beetroot	2810	1935	763	2009	-	1211	1390	-
Potato	102	107	129	431.5	110	155	229	164
Carrot	-	48	-	7	210	97	-	-
Pumpkin	3	55	67	165.8	410	-	-	-

In the Symbio Alliance Survey (SAS), the nitrate concentration in fresh vegetable samples were determined in accordance with the accredited quality assurance procedures and the results were provided to Food Standards Australia & New Zealand (Food Standards Australia New Zealand, 2010) (**Table 2.3**). These results were consistent with a comprehensive survey of nitrate concentrations in vegetables in Europe which examined 41,969 analytical results (European Food Safety Authority, 2008). The results for nitrate are also consistent with those observed in a 2007 'NZ Total Diet survey' (Food Standards Australia New Zealand, 2010). Some variation in results between surveys is expected because it is known that nitrate concentrations are influenced in particular by the season, methods of production and sunlight available as mentioned earlier.

Comparatively fewer data are available for nitrite concentration in surveys of foods and beverages internationally. Unfavourable conditions such as high storage temperature and long storage periods have previously been shown to increase nitrite concentrations in vegetables (Chung et al., 2004). A survey conducted in Hong Kong found that nitrite concentrations in vegetables were generally low (around 1 mg/kg) but higher concentrations were reported in cabbage (3 mg/kg) and beetroot (8 mg/kg) (Food Standards Australia New Zealand, 2010).

2.2 Beetroot

Beetroot (*Beta vulgaris L.*) is a rich source of potent antioxidants and nutrients, including magnesium, sodium, potassium, vitamin A, B1, B2, B6, C and betalains (Singh et al., 2013). The green leafy parts of beetroots are also of nutritional value, containing beta-carotene and other carotenoids (Singh et al., 2013). Fresh beetroot are highly susceptible to spoilage due to their high moisture content and hence preservation methods such as juicing, canning, drying to produce beetroot chips are followed to ensure microbial safety of the products (Mathlouthi, 2001).

2.2.1 Beetroot types and cultivars

There are five types of beetroots namely processing beetroots, table beetroots, novelty beetroots, spinach beetroots and swiss chard.

2.2.1.1 Processing beetroots

The beetroot grown for processing are usually cylindrical in shape. Cylindrical beetroots offer greater uniformity and efficiency for sliced beetroot production, which is a major requirement for processors. Non-hybrid varieties include: Detroit Short Top, Ruby Ball and Scarlet Supreme. Hybrid variety: Red Ace F1 (Schrader & Mayberry, 2003).

2.2.1.2 Table beetroots

Table beetroots are known as fresh market beetroots which are red, round types, namely: Detroit stains and Ruby queen (Schrader & Mayberry, 2003).

2.2.1.3 Novelty beetroots

Novelty beetroots have unusual colours or shape. Red elongated varieties: Cylindra, Forono. Alternating red and white variety: Chiaggia yellow, Round variety: Burpee golden, Yellow elongated variety: Burpee Golden, White round varieties: Showhite, Albino. Most cylindrical varieties produce beetroots with an “earthy” taste (Schrader & Mayberry, 2003).

2.2.1.4 Spinach beetroots

Spinach beetroots are table beetroots grown for their succulent leaves which can be harvested over an extended period. The main variety grown is Burpee Red ball (Schrader & Mayberry, 2003).

2.2.1.5 Swiss Chard

Swiss chard is a beetroot grown for its edible leaves. It has large, well-developed petioles that may be green, red or multi-coloured. Green petiole variety: Lucullus. Red petiole varieties: Charlotte, Rhubarb Chard. Multi coloured petiole variety: Bright lights (red, yellow, white, orange, purple, pink) (Schrader & Mayberry, 2003).

Studies suggest that beetroot juice is a rich source of naturally occurring nitrates that have beneficial effects on health (Webb et al., 2008). A diet rich in beetroot juice may be a natural approach to help lower blood pressure and improve heart health (Moncada & Higgs, 1993). Beetroot juice studies have shown positive effects on the body during exercise and related performance benefits (Bailey et al., 2009; Jones et al., 2013; Vanhatalo et al., 2010).

2.3 Acceptable daily intake (ADI) of dietary nitrates and nitrites

The concept of Acceptable daily intake **(**ADI) is defined by the Joint Expert Committee of the Food and Agriculture (JEFCA) organization of the United Nations/ World Health Organisation (WHO) for substances intentionally added to food or for contaminants (pesticides, herbicides and fertilizers) (FAO, t2003 a,b). In light of the well-known benefits of vegetables and the lack of data on the possible effects of vegetable matrices on the bioavailability of nitrate, JEFCA considered it to be inappropriate to compare exposure to nitrate from vegetables with ADI or to derive limits for nitrate directly from vegetables. In 1990, JEFCA and the European Commission's Scientific Committee on Food (SCF) have set an ADI for added nitrate from food including, those added as preservatives at 0-3.7 mg/kg bodyweight or 260 mg per day for a 70 kg person (European Commission,1992). In 2002, the acceptable amount of added nitrite from the food daily diet set as 0.07 mg of nitrite per kg of body weight per day or 5 mg per day for a 70 kg person (FAO, 2003a,b).

Compared with the current ADIs, the ingestion of only 100 g of raw vegetables with a nitrate concentration of 2500 mg/kg will lead to an intake of 250 mg nitrate. Therefore consumption of 100 g of raw vegetables by a person whose body mass is 60 kg, would exceed the ADI for nitrate by 13%. Calculating the partial conversion of nitrate to nitrite (5%) after such consumption, the current SCF limit for nitrite (0.07 mg/kg bodyweight) would be exceeded by 247%. The SCF recommended the continuation of efforts to reduce exposure to nitrate via food and water since nitrate can be converted into nitrite and nitrosamines. The SCF suggested that good agricultural practices are adopted to ensure nitrate concentrations in food and water are as low as reasonably achievable (SCF, 1997).

Estimated daily intakes for New Zealand adults using UK and New Zealand analytical data have been calculated at 120 mg/day and 1.2 mg/day (equating to 1.61 and 0.016 mg/kg body weight/day for a 75 kg body weight) of nitrate and nitrite, respectively (Ministry for Primary Industries, 2013). An average adult New Zealander consumes about 0.01 mg of nitrite per kg of body weight per day or 14% of the ADI of nitrite and 0.7 mg per kg of body weight per day or 18% of the ADI of nitrate (Ministry for Primary Industries, 2013). About 10% of people with an average rate of conversion, and 50% of people with a high rate of conversion are estimated to exceed the ADI, when nitrate from the food is converted to nitrite in the body (Thompson, 2004).

People who eat large amounts of lettuce and those who have a high rate of conversion of nitrate to nitrite are potentially most at risk to health damage from nitrate, but to date there has been no evidence that this is a problem (Ministry for Primary Industries, 2013). It is known that conditions related to overexposure to nitrate intake are rare (Hill, 1991). For most people, fruit, which have low nitrate concentration (10 mg/kg), comprises up to half of the total recommended daily intake of 400 g of vegetables and fruit, actual nitrate intakes would be reduced to between 81-106 mg/day for the majority of the EU population (European Food Safety Authority, 2008). Further mitigation of nitrate intake may result from processing e.g. washing, peeling and/or cooking (Abo Bakr et al., 1986; Szponar et al., 1981)

Thompson & Subar (2013) reported that further studies should be conducted with an accurate method of assessment, such as total or duplicate diets and individual dietary records, as these give the best estimates of intakes. Also, foods should be analysed as ‘ready to consume’, thus accounting for losses of the chemicals during processing, food storage, preparation and cooking. This applies specifically to nitrite. Studies should be conducted during different seasons to account for natural variation in the concentrations in foods such as vegetables, particularly for nitrate (Thompson & Subar, 2013).

2.4 Role of nitrates in the sports performance and health markets

Apart from the dietary aspects of nitrate and nitrite, the interest in their biological role was sparked by the findings in the mid-1980s showing that these anions are generated

endogenously in our bodies (Lundberg et al., 2011). New discoveries in the field of nitrate and nitrite biology have provided mechanistic insights into the potential physiological roles of dietary nitrate and nitrite and their potential health benefits in sports and health sector (Bailey et al., 2009; Lundberg et al., 2011; Vanhatalo et al., 2010,). In addition to the sports performance benefits of nitrates, nitrate supplementation has also been shown to have various health benefits. The beneficial effects are actually produced by NO which has well known cardiovascular benefits (Webb et al., 2008). NO derived through nitrates is an incredibly important signalling molecule in the body and is vitally important for the cardiovascular system (Szabo, 2010). NO facilitates vasodilation in blood vessels, promoting increased blood flow and helps regulates blood pressure (Moncada & Higgs, 1993). Numerous studies have confirmed that nitrate rich diets have an antioxidant effect which offers significant protection against a wide range of degenerative diseases and high concentrations of nitrate may also be beneficial for cardiovascular health (Webb et al., 2008). Beetroot has been used in previous studies in a beverage form because it provides a very rich source of nitrate (Bailey et al., 2009; Cermak et al., 2012; Lansley et al., 2011; Vanhatalo et al., 2010). **Table 2.4** gives a brief overview of some of the sport and exercise-related benefits derived from consumption of naturally occurring nitrates in beetroot juice or sodium nitrate solutions. It appears that dietary supplementation with inorganic nitrate shows reduction in the oxygen cost of submaximal exercise (Lundberg et al., 2007). Submaximal exercise is a cardiorespiratory fitness exercise designed so that the intensity does not exceed 85 percentage of heart-rate reserve or maximal oxygen uptake (Larsen et al., 2011). Furthermore, increased dietary nitrate intake has the potential to enhance exercise tolerance during longer-term endurance exercise (Larsen et al., 2011).

As reported earlier, NO helps promote vasodilation in muscles enabling a better blood supply resulting in oxygen being able to diffuse deep into muscle tissue supplying larger number of contracting muscle cells (Steinberg et al., 1994). NO also has the ability to prevent blockages in the brain that potentially could lead to stroke and myocardial infarction (Bryan et al., 2011). Also, NO produced through the nitrate-nitrite- NO pathway might help prevent muscle cells, next to capillaries supplying blood, from

taking a major share of the oxygen allowing more distant muscle cells to achieve better oxygenation and therefore a more even oxygen distribution in the working muscles (Szabo, 2010). NO produced from nitrates also stimulates the production of muscle mitochondria (Steinberg et al., 1994). The mitochondrion is considered as the energy store in muscle cells and a greater number of mitochondria would enable more efficient oxidative phosphorylation (P/O ratio). The improved mitochondrial P/O ratio correlated to the reduction in oxygen cost during exercise (Larsen et al., 2011). Jones et al. (2013) also explains the dose-response effects of three different amounts of beetroot juice on several health and exercise outcomes.

Table 2.4: Studies of nitrate supplementation in trained and untrained populations

SUBJECTS AND STUDY DESIGN	NITRATE DOSE AND FORM	EXERCISE PROTOCOL	ENHANCED PERFORMANCE	COMMENTS
Cyclists (n=12 Males) Randomised double-blind placebo-controlled crossover design	6 days chronic supplementation 2 x 70 ml/day concentrated beetroot juice (Beet it Shot) Placebo: blackcurrant cordial	Cycling 30 min @ 45 % Wmax[1] + 30min @ 65 % Wmax + 10 km time trial	Perhaps	• Increase in mean power in time trial with beetroot juice compared with Placebo and faster time • VO_2[2]at 65 % Wma and 45 % Wmax was reduced with beetroot juice • No difference in blood pressure

[1] Maximal Power
[2] Maximal Oxygen Uptake

Trained cyclists (n = 9 Males) Randomised double-blind placebo-controlled crossover design	Acute dose 2.5 hr pre-exercise 6.2 mmol nitrate as 500 ml beetroot juice (Beet it) Placebo: nitrate depleted beetroot juice No dietary restriction of nitrate, but restriction of antibacterial mouthwash and chewing gum use.	Cycling 4 km time trial 16. km time trial	Yes Yes	• 2.8 % improvemer in 4 km time trial • 2.7 % improvemer in 16.1 km time tri • 7-11 % improvement in power output achieved with no increase in oxyger cost of exercise. • Resting Systolic blood pressure dropped in beetroo juice trial but no change in Diastoli blood pressure

Trained cyclists and triathletes (n = 9 Males). Randomised double-blind placebo-controlled crossover design	3 days chronic supplementation: Nitrate: 0.1 mmol sodium nitrate/kg/day Placebo: NaCl (equivalent to ~150-250 g nitrate rich vegetable) 3 doses per day with last dose 60 min pre-exercise. 3 day restricted dietary nitrate (avoidance of vegetables, cured meats,	Cycling 5 x 5 min incremental test (45, 60, 70, 80 and 85 % oxygen peak) + oxygen peak	Yes at submaximal intensities: enhanced economy No at maximal intensities	• No change in oxygen peak or ma power • Reduced oxygen cost at submaxima workloads with nitrate without change in lactate • Mean gross efficiency significantly increased from ~19.7 %. (Placebo to 21.1 % (Nitrate) • Resting Systolic and Diastolic BP both reduced with nitrate as compare

	strawberries, grapes and tea)			to placebo
Healthy, physically active subjects (n = 14 Males)	3 day chronic supplementation: Nitrate: 0.1 mmol sodium nitrate/kg/d day Placebo: NaCl 3 doses per day with last dose 90 min pre-exercise 3 day restricted dietary nitrate (avoidance of vegetables, cured	Cycling Day 4: cycling at 50 % VO_2	Not measured but enhanced economy	• 3 % reduction in oxygen cost of exercise. • Muscle biopsies collected to undertake analysis of mitochondrial properties. • Improvement in oxidative phosphorylation efficiency (P/O^3 ratio)

[3]Phosphate Oxygen ratio, the amount of ATP produced from the movement of two electrons through a defined electron transpo
oxygen atom

	meats, strawberries, grapes and tea)			
Healthy, physically active subjects (n = 9 Males) Double blind, placebo controlled, crossover study	6 day chronic supplementation: Nitrate: 6.2 mmol/day as 0.5 L/d beetroot juice (Beet It) Placebo: 0.5 L/day nitrate-depleted beetroot juice Dose consumed slowly 3 hr pre-	Running / Walking Day 4, 5, 6 Day 4: 2 x 6 min @ moderate intensity + 10 min walk @ 4 km/h + TTE[4] @ 'severe' intensity	Yes Enhanced efficiency	• Decreased oxygen cost of walk (12-1 %), moderate-intensity run and severe intensity ru • 6 % decrease in energy cost to run 1km. • Plasma nitrite increased 105 % with beetroot juice but no change in

[4]Transthoracic Echocardiogram (cardiac ultrasound)

	test. No dietary nitrate restriction	Day 5: 2 x 6 min @ moderate intensity + 1 x 6 min @ 'severe' intensity		placebo trial. • Systolic blood pressure reduced b 4 % in beetroot juice- no change in placebo. • Additional test on day 6 (incremental single legged knee extension in MRS scanner) showed n change in mitochondrial oxidative capacity
Healthy physically active subjects (n=9, 5Males, 3	15 day chronic supplementation Nitrate: 5.2 mmol/d as 0.5	Cycling Day 0, 1, 5, 15 2 x 5 min @ moderate	Yes	• Oxygen cost of moderate exercise was reduced by ~ % by acute beetroo

Females) Double blinded placebo controlled, crossover study	L/daybeetroot juice ('Beet It'). Placebo: 0.5 L/d low-joule black current cordial Dose consumed 2.5-5 hr prior to start of each test No dietary nitrate restriction	intensity + ramp incremental time trial exercise (10 min recovery).		juice at day 5 and day 15. • Plasma nitrite elevated by beetro juice at 2.5 hrs, da 5 and day 15 by 2 50 %. • Blood pressure lowered by beetro juice by ~4 %.
Healthy subjects (n=8 Males) Double blinded placebo	6 day chronic supplementation Nitrate: 5.5 mmol/day as 0.5 L/day beetroot juice ('Beet It').	Cycling Day 4, 5, 6 Day 4: 6 min @ moderate intensity + 25 min rest + 6	Enhanced efficiency	• Time trial exercise at severe intensity exercise increased by beetroot juice. • Plasma nitrite was increased by 96 %

controlled, crossover study	Placebo: 0.5 L/day low-joule black current cordial Dose sipped at regular intervals throughout the day Provided with a list of nitrate rich foods and asked to abstain from consuming these foods during the study.	min @ moderate intensity Day 5: 6 min @ moderate intensity + 25 min rest + 6 min @ high intensity Day 6: 6 min @ moderate intensity + 25 min rest + time trial exercise @ severe intensity		by beetroot juice. • Beetroot juice reduced Systolic blood pressure. • Reduced oxygen cost of moderate intensity exercise by 19 % by beetro juice. • Alteration in oxygen kinetics with beetroot juice amplitude of slow component reduce with severe exercise.

2.5 Nitrate health risk

Links between nitrate and health risk have been studied for more than 50 years, however none of the health claims against dietary nitrate have been substantiated (Addiscott, 2005; Dinkla, 1976; Shuval & Gruener, 1972).

Methaemoglobinaemia or blue baby syndrome is a condition for infants at risk from excessive nitrate consumption (Wright et al., 1999). In this condition, nitrite produced from nitrate blocks the ability of haemoglobin in the blood to carry oxygen, resulting in low tissue oxygen. However, recent studies have shown that methaemoglobinaemia is not caused by nitrate per se but by faecal bacteria that affects the infants and produce nitrite, followed by NO in their gut enabling the conversion of haemoglobin to methaemoglobin (McKnight et al., 1999). Studies have also shown that infants exposed to far higher concentrations of nitrate (up to 700 mg per day) do not develop methaemoglobinaemia (Sharma et al., 2013). Recent studies on both nitrate and nitrite in healthy adult and adolescent populations have failed to find any negative health impact with nitrate consumption (Addiscott, 2005; European Food Safety Authority, 2008; McKnight et al., 1999).

Other concern about nitrate and nitrite consumption relates to a reported increased risk of cancer (Correa et al., 1975; Hill, 1994; Moller, 1995; Speijers, 1996a; Speijers, 1996b; Yang et al., 1998). Epidemiological studies do not suggest that naturally occurring dietary nitrate intake is associated with increased cancer risk (Ward et al., 2010). There has been unclear evidence that high intake of sodium nitrite might be associated with increased pancreatic cancer risk. (Nothlings et al., 2005). Overall, the estimated exposures to nitrate from vegetables have not been reported to lead to likely health risks, while the benefits of consuming vegetables is well reported (McKnight et al., 1999). Nitrate in drinking water and food is readily converted to nitrites in the body (nitrites can also be ingested directly through processed meats). Nitrites can possibly react with amino acids to produce carcinogenic compounds known as nitrosamines (Addiscott, 2005). The potential risk does not apply to nitrates found in vegetables which do not contain amines (Bryan et al., 2011). World Health Organisation (1996) showed no evidence of increase cancer incidence when mice and rats were given sodium nitrite (130 mg/kg) in drinking water or through their feed for two years.

Epidemiological studies have not provided any evidence that there is an increased risk of cancer related to high nitrate intake from vegetables (Moller, 1995; National Academy of Sciences, 1981; World Health Organisation, 1996). In contrast though, Yang et al. (1998) found a positive correlation between nitrate exposure through drinking water and gastric cancer.

Ward et al. (2005) suggested that the intake of dietary nitrate is less likely to increase nitrosation (conversion of organic compounds to nitroso derivatives) because of the presence of nitrosation inhibitors in vegetables. High intakes of vegetables and fruits containing nitrate/nitrite decreases the risk of compounds produced from them due to high concentration of naturally occurring protective antioxidants present (Ames et al., 1993; Kahkonen et al., 1999; Ministry for Primary Industries, 2013). Other claims such as an increased risk of fetal mortality, genotoxicity, congenital malformation, tendency towards enlargement of the thyroid gland, incidence of childhood diabetes have been linked to nitrate intake from food and water (L'Hirondel, 2002). The situation facing the authorities in the 'nitrate and cancer' issue is thus complex and the authorities have concluded that exposure to the nitrate concentrations found in drinking water in the United States is unlikely to contribute to human cancer risk (American Water Works Association, 1999; European Food Safety Authority, 2008; L'Hirondel, 2002). Attempting to limit nitrate and nitrite exposure on the basis of carcinogenicity would impact on the diet and the consumption of vegetables in particular, as the primary source of risk for most of the U.S. population (American Water Works Association, 1999; European Food Safety Authority, 2008; World Health Organisation, 1996). But diets rich in vegetables have consistently been shown to reduce the cancer risk (European Food Safety Authority, 2008; World Health Organisation, 1996). Consumption of nitrate rich leafy green vegetables such as spinach, celery, lettuce and beet greens, in particular, could easily exceed the outdated recommended maximum for nitrate by following nutrition guidelines on vegetable intake (Bryan et al., 2011; European Food Safety Authority, 2008; Hutchinson, 2013; L'Hirondel, 2002; Ministry for Primary Industries, 2013; World Health Organisation, 1996). However, the theoretical cancer risk should be weighed against the benefits of eating vegetables due to their protective antioxidant properties (Addiscott, 2005; Bryan et al., 2011; European

Food Safety Authority, 2008; Shuval & Gruener, 1972; World Health Organisation, 1996).

2.6 Quantification methods to determine nitrates and nitrites

Few techniques possess sufficient generic applicability to enable detection of nitrates and nitrites amongst the huge number of potential interferences that can be encountered within environmental, food, industrial and physiological samples (Moorcroft et al., 2001), hence specific techniques are required for different systems. **Table 2.5** summarises some of the methods used in past studies to effectively quantify nitrates and nitrites. A large number of protocols including almost all major analytical methodologies have been developed to overcome the distinctiveness of the various conditions during quantification of compounds (Moorcroft et al., 2001; Tsikas, 2000). Nitrates and nitrites can be determined by various methodologies such as spectrophotometry and chromatographic methods such as High Pressure Liquid Chromatography (HPLC) with ultra violet (UV) detector, ion chromatography (IC) and gas chromatography (GC) (Butt et al., 2001; Cheng & Tsang, 1998; Chien-Chung et al., 2003; Hsu et al, 2009; Pinto et al., 2010; Tsikas, 2000; Yuegang et al., 2006). Simultaneous techniques include electrochemical and capillary electrophoresis, in which the analytes are detected independent of one another in a single measurement (Leone et al., 1994; Moorcroft et al., 2001; Trushina et al., 2005).

In the past decade, a number of IC and HPLC methods have been developed which are generally characterised by faster, more accurate and higher sensitivity than the spectrophotometric methods (Moorcroft et al., 2001). Measurements of nitrate and nitrite anions in various matrices are made with various difficulties especially due to the fact that nitrite is prone to fall beneath the detection limits of most methods used (Moorcroft et al., 2001; Pfaff, 1993; Tsikas, 2000; Wu et al., 2013). Nitrate and nitrite determination is very important because they are the main precursors of NO essential to improve exercise performance (Bryan et al., 2011; Wu et al., 2013).

A typical example of HPLC chromatogram with nitrate and nitrite peaks is shown in **Figure 2.3**. Chien-Chung et al. (2003) showed HPLC chromatograms of Chinese cabbage sample (B) and standard solution (A) containing 10 μg/ml of nitrate and nitrite

using HPLC analytical method under the condition for a mobile phase solution of 0.01 M octylammonium orthophosphate (**Figure 2.3**).

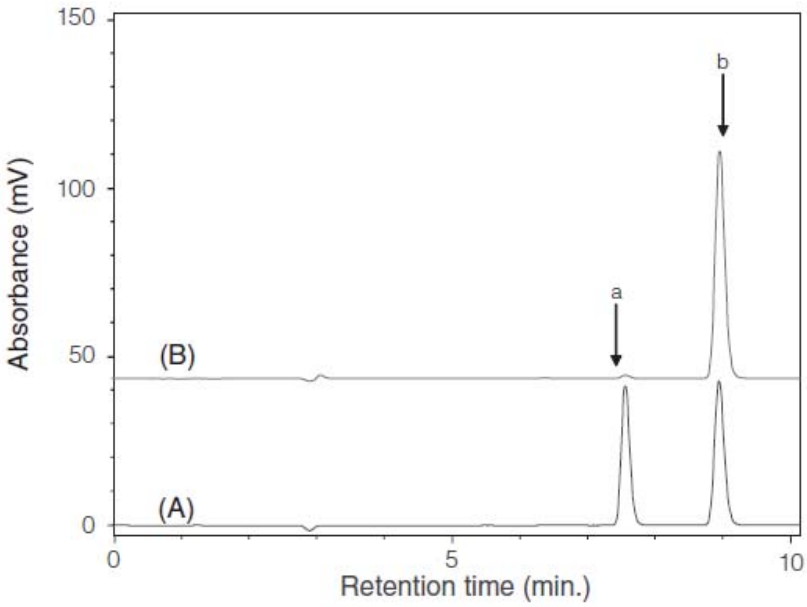

Figure 2.3: HPLC chromatogram with nitrate (b) and nitrite (a) peaks of organic non-heading Chinese cabbage sample (B) and standard solution (A) (Chien-Chung et al., 2003)

Table 2.5: High Pressure Liquid Chromatography (HPLC) methods for nitrate and nitrite determination

TYPE OF FOOD TESTED	WAVELENGTH	COLUMN	MOBILE PHASE	FLOW RATE	INJEC VOLU
Nitrate and nitrite	210-240 nm	Hypersil ODS 4.6-125 nm, 5 µm	PIC-A-Low UV (5 mM) in water/methanol (5 %), gradient	0.6 mL/min	50 µL
Canned vegetable juice	230 nm	Spherisorb C18 reversed phase (55µm, 250 × 4.6 mm i.d.)	0.01M octylammonium orthophosphate, isocratic	0.5 mL/min	20 µL
Spinach and lettuce	203-505 nm	Partisil PXS 10/25 (Currently available as Partisil 10 silica which is irregular in shape)	Tetraethylammonium, isocratic	0.6 mL/min	20 µL

Twelve marketed vegetables	213 nm	Phenomenex Luna C18 (5 µm, 250 × 4.6 mm i.d.)	30% methanol, pH 7, isocratic	0.8 mL/min	10 µL
Dew, rain, snow and lake water samples	205 nm	Phenomenex C 18 reversed phase column (150 mm x 2.00 mm i.d.,5 µm)	83% 3.0 mM TBA-OH titrant and 2.0 mM sodium phosphate buffer at pH 3.9 and 17 % acetonitrile organic solvent, isocratic	0.4 mL/min	20 µL
Ham	Not available	HyPurity RP C18 (5 µm, 150 x 3 mm)	0.01 M n-octylamine/5mM tetrabutylammonium hydrogenosulphate pH 6.5, isocratic	1 mL/min	20 µL

Cured meat and vegetables	214 nm	Phenomenex C18 110A Gemini column (250 mm x 4.6 mm x 5 µm)	Methanol:water (75:25) with 0.075M tetrabutylammonium phosphate, isocratic	1 mL/min	10 µL
Organic spinach and lettuce	220 nm	ACE C18, 5 µm	0.01M n-octylamine and 20% methanol, pH 6.6, isocratic	0.5 mL/min	Not av
Mammalian blood, urine and vegetal samples	222 nm and 520 nm	Lichrospher 100, Rp-18 (5 µm)	Tetrabutylammonium hydroxide 5 mM brought to pH 2.5 with sulphuric acid (a), acetonitrile (b) and methanol (c), gradient	Not available	100 µL

2.7 Factors affecting stability of nitrates in vegetables

Changes in nitrate and nitrite concentration in vegetables can be influenced due to a number of the processes including washing, peeling, boiling, and frozen storage.

2.7.1 Processing Factors

2.7.1.1 Washing

Nitrate is soluble in water and washing of leafy vegetables can reduce nitrate concentrations by 10-15 % (Dejonckheere et al., 1994). Mozolewski & Smoczynski (2004) showed that concentration of nitrate and nitrite in potatoes can also be decreased by 18 to 40 % and 25 to 75 %, respectively after preliminary processing methods (washing, peeling and rinsing).

2.7.1.2 Peeling

The nitrate content in two potato varieties (Innowator and Santana) before peeling were 258 and 349 mg/kg dry matter, respectively, and the level decreased significantly during French fries production (European Food Safety Authority, 2008). About 30 % of the nitrate was removed during peeling. Preheating and cutting reduced the nitrate content by a further 20 % and blanching by 30 %. After final frying only 5-6 % of the original nitrate content remained or 16-18 mg/kg dry matter (Rytel et al., 2005). Dejonckheere et al. (1994) showed that after peeling of potatoes, bananas and melons, the nitrate content decreased by 34 %, 62 % and 41 %, respectively. Czarniecka-Skubina et al. (2003) reported the reduction in nitrate and nitrite concentration in beetroots by 20 % and 6.6 %, respectively due to peeling. Szponar et al. (1981) observed higher decrease of nitrates after peeling (~39 %). Nitrate is not evenly distributed throughout the vegetable. For lettuce and spinach, elimination of the stem and midrib resulted in a decrease of the nitrate content of 30-40 % (Dejonckheere et al., 1994). Schuster & Lee (1987) reported that the ‘flesh’ makes up the bulk of the carrot has a significantly lower concentration of nitrate than the core tissue. The largest amount of nitrate in potatoes is found in and just under the skin; however nitrite is more evenly distributed throughout the potato (Marin et al., 1998).

2.7.1.3 Cooking

Different studies have shown reduction of nitrate concentration when vegetables are cooked in water. Peas, cabbage, beans, carrots, potatoes, spinach, endives and celery leaves lost between 16 to 79 % of the nitrate after cooking (Abo Bakr et al., 1986; Dejonckheere et al., 1994; Schuster & Lee, 1987). Varoquax et al. (1986) showed that the diffusion of nitrate from carrots depended on water temperature, surface area (thickness of the carrot slice) and ratio of carrot to water. Cooking vegetables tends to lower nitrate content since nitrate is soluble and readily leaches into cooking liquids. The loss upon cooking was more pronounced in leafy vegetables being 79.4 % for spinach, 62 % for jew's mallow (herb) and 31.5 % for cabbage (Abo Bakr et al., 1986). According to Lutsoya & Rooma (1971) about 85 % of the nitrates and nitrites present in vegetables passed into the cooking water. Hata & Ogata (1971) showed that the nitrate and nitrite content of potatoes were heat stable during cooking but losses occurred due to leaching from potato tissues into cooking water. Pickston et al. (1980) reported that the concentration of nitrate fell after cooking by an average of 24 % for potatoes. Huarte-Mendicoa et al. (1997) reported that boiling reduces nitrate content since nitrate is soluble and predisposed to readily leach into cooking liquids. The highest nitrate loss after boiling was found for celery (59.14 %), followed by Chinese cabbage (56.04%), lettuce (49.66 %) and English cabbage (46.69 %) (Huarte-Mendicoa et al., 1997).

Overall the losses of nitrite were greater than for nitrate when applying preliminary processing and heating methods.

2.7.1.4 Other methods of processing

Limited data are available on nitrate and nitrite concentration in canned vegetables. Jakszyn et al. (2004) reported that canned vegetables contained much higher amounts of nitrite (450 mg/kg) than those reported in the raw commodity, due to storage of the cans at ambient temperature. Bednar et al. (1991) analysed the nitrate and nitrite content of commercially processed and home-processed beetroots and spinach samples. The highest concentrations of nitrate were found in the home-frozen beetroots (41,250 ppm) and the home-canned beetroots (27,967 ppm). The mean concentrations of nitrate in the home processed beetroots were also higher than the commercially processed beetroots.

The higher concentrations of nitrate in the home-processed beetroots were due to the differences in processing methods (Bednar et al., 1991). In the home-processed method for canning, the beetroots were first precooked so that the skins could be removed. The cooking liquid was retained and used to cover the canned and frozen beetroots. The method used in commercial processing of beetroots is quite different. After the beetroots are precooked by steam, a high pressure water spray combined with agitation of the beetroots causes the skins to be removed. More water is added to the beetroots when they are canned. Since nitrate is a highly water soluble compound, much of it is removed during the commercial processing procedure (Bednar et al., 1991; Consalter et al., 1992). Bednar et al. (1991) showed that commercially processed beetroots contained considerably less nitrate than home processed beetroots. Significant decrease in the nitrate content in commercially processed beetroots may be attributed to leaching during the blanching operation because of nitrate's high water solubility (Bednar et al., 1991). Similar losses have been reported by other authors (Consalter et al., 1992; Forlani et al., 1997). In red beetroot, the nitrate was reduced by fermentation by up to 50 % and in white cabbage by up to 87 % (Preiss et al., 2002). For vegetables eaten raw, only handling and storage would impact the nitrate concentration. Prasad & Chetty (2008) also indicated that the nitrate values remain relatively constant after baking. Bednar et al. (1991) showed decreasing nitrate concentrations in home-processed canned beetroots with increasing storage time. Since no detectable concentrations of nitrite were found in the beetroots for this study, no conclusions regarding conversion of nitrate to nitrite during the storage period were made.

Overall, handling, storage, processing including washing, peeling and cooking can reduce the amount of nitrate in vegetables (Dejonckheere et al., 1994).

2.7.2 Storage time and temperature

Under certain storage conditions, nitrate can be converted to nitrite in vegetables. Nitrite tends to increase dramatically via microbiological reduction of nitrate in vegetables, and nitrate content decreases during a period of storage at ambient temperature (Ezeagu, 1996; Phillips, 1968). Wooton et al. (1985) explained a negligible amount of nitrite was found in fresh spinach during refrigerated storage. Phillips (1968) reported that under frozen storage of vegetables, nitrite accumulation was inhibited.

Chung et al. (2004) studied changes in the nitrate and nitrite content of four types of vegetables during storage at refrigerated (4±1°C) and ambient temperatures (20±1°C). Nitrate concentrations in spinach, crown daisy, organic Chinese spinach and organic non-heading Chinese cabbage were almost unaffected and remained high in the range 2830-5270 mg/kg at refrigeration temperature. In contrast, nitrite concentrations remained low (5 mg/kg) showing that nitrate and nitrite concentrations were scarcely affected during the refrigerated storage (Chung et al., 2004).

Yaneva et al. (1996) found that cold temperature could strongly reduce the activity of nitrate reductase in leaves of green vegetables by disturbing the internal electron transport of nitrate reductase. Although the nitrite concentration was not elevated during the refrigerated storage, it should nevertheless be of concern in terms of health risk and that the extremely high nitrate amounts (mean 5210 mg/kg) of fresh spinach significantly exceeded the maximum concentration set by the European commission (European Community, 2001). Organic Chinese spinach also contained a high nitrate concentration of 3450 mg/kg which is above the suggested hazardous level (>3100 mg/kg) for China (Zhou et al., 2000).

Figure 2.4 shows the contents of nitrate and nitrite in A) spinach B) crown daisy C) organic Chinese spinach and D) organic non-heading Chinese cabbage during storage at an ambient temperature (22±1°C) over 1 week. Nitrate and nitrite concentrations varied significantly during the ambient temperature storage during this period. Nitrate concentration of spinach, organic Chinese spinach and organic non-heading Chinese cabbage remained high in the range 2960-3960 mg/kg before the third stored day and then dropped significantly on the fourth day with a mean reduction of 87.4 % compared with the initial value (Chung et al., 2004).

In contrast, nitrite was not found in these vegetables during the first three days, while nitrite concentrations of organic Chinese spinach and organic non-heading Chinese cabbage increased dramatically in the range 1857-3617 mg/kg on the fourth day and declined for the sequential days. Nitrite concentration of spinach increased on the fourth to fifth days and reached a peak on the fifth day (4430 mg/kg) then reduced slightly for the rest of the storage period. The nitrite concentrations of the crown daisy were almost

negligible and only increased slightly on the fifth day but the increases were not significant.

The total concentrations of nitrate and nitrite decreased from the third day during storage at ambient temperature; however, the total concentrations remained high and essentially unaffected during storage when refrigerated. This suggests that the nitrate reductases in vegetables were considerably activated during the third to fifth days owing to ambient temperature storage (Chung et al., 2004). This contributed to the significant microbial reduction of nitrate, which led to the accumulation of high nitrite concentration. During refrigerated storage, the nitrite concentrations were essentially negligible. Comparison with the literature showed a similar result for nitrate's microbiological reduction to nitrite in foods when stored at room temperature (Jones & Griffith, 2006).

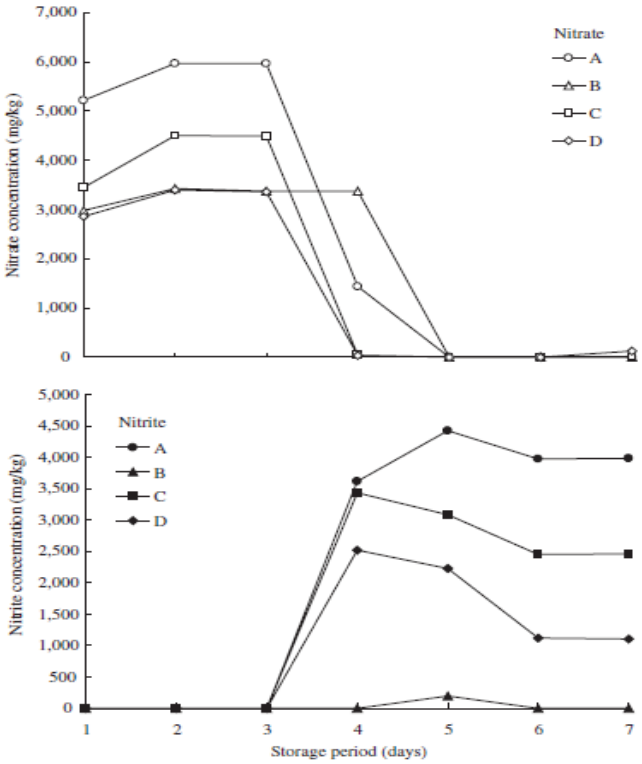

Figure 2.4: Effects of ambient (22±1°C) storage on nitrate (upper) and nitrite (lower) concentrations of four species of vegetables over 1 week. A, spinach; B, crown daisy; C, organic Chinese spinach; D, organic non-heading Chinese cabbage (Chung et al., 2004).

Hunt & Turner (1994) showed that nitrite concentrations in fresh, uninjured well-stored vegetable tissues are extremely low. It seems that the activity rate of nitrite reductase maintains in equilibrium with one of the nitrate reductase enzymes under proper storage conditions. The case of poor storage such as that beyond the normal 'use by' dates or at ambient temperatures may result in bacterial growth which could contribute to the increasing accumulation of high nitrite concentrations (Peterson & Stoltze, 1999). The variations are dependent on the differences between species-specific nitrate-reductase activities and the influence of levels of bacterial contamination (Chung et al., 2004). This reaction of microbiological reduction (the effect of nitrate reductase) during storage at room temperature (22±1°C) caused a significant elevation in nitrite concentration in vegetables (Chung et al., 2004). Chung et al. (2004) suggested that consumers store fresh vegetables purchased from the market immediately in a refrigerator and consume within three days of storage. Improper storage conditions at ambient temperature present a risk in terms of increased accumulation of nitrite. Proper storage of nitrate-containing vegetables at refrigerated temperatures was suggested as the appropriate way to prevent bacterial nitrite formation and thus improved safety during vegetable consumption (Chung et al., 2004). Schuster & Lee (1987) found no significant changes in nitrate or nitrite content of spinach, beet, carrot, parsley-root, celery or potatoes during frozen storage for up to 12 weeks since nitrate and nitrite accumulation is inhibited under frozen storage (Phillips, 1968).

Abo Bakr et al. (1986) also studied the effects of storage time on nitrate and nitrite content. Storage of frozen spinach for up to six months caused a reduction in the nitrate content. No nitrite was found in frozen spinach for the first four months of storage but it was detected after storage for six months. Phillips (1968) reported that there was no significant increase in the nitrite content of frozen spinach with period of storage up to 5 months. Walker (1975) showed that frozen spinach did not accumulate large amounts of nitrite (less than 4ppm) but on thawing nitrite concentration rose rapidly. Prasad & Chetty (2008) suggested that microbial reduction of nitrate to nitrite would not be expected to proceed at low freezing temperatures. A similar trend was observed when frozen cabbages were stored; the concentration of nitrate decreased and no nitrite was detected during the first period of storage, then it accumulated to a high concentration after three months' storage.

Cantliffe (1973) also identified nitrogen fertilisation and light intensity as major factors that influence nitrate concentration in vegetables. Gangolli et al. (1994) showed that vegetables grown in heated glasshouses have higher nitrate contents than those grown outdoors potentially because of lower light intensity and high nitrogen mineralization

2.8 Extraction of juice from vegetables

Juices are produced using combinations of physical destruction and enzyme-assisted reactions to expel the juice from fruit and vegetables, in some cases leaving large amounts of insoluble waste (Varnam & Sutherland, 1994). The juice can take a variety of forms such as clear clarified juices, light cloudy and heavy cloudy juices containing cellular material in suspension, pulpy juices and nectars made by pulping whole fruits or vegetables (Markowski et al., 2009). Different methods of juicing may affect the flavour and odour development of fruit and vegetable juices (Ahmed et al., 1978; Farnworth et al., 2001; Kotseridis & Baumes, 2000).

Single strength juices are unmodified after extraction; they typically have a short shelf life and may have only minimal further processing (Graumlich et al., 1986). Commercially available single-strength juices need preservation for distribution and storage, but typically have a shelf life of days or weeks, rather than months (Varnam & Sutherland, 1994). It has been suggested that fresh vegetable juices (unpasteurised) should generally be consumed within 24 hrs due to their potential for microbial growth (Hyun-Pa et al., 2007).

Nelson & Tressler (1980) grouped vegetable juice into six classes:

1. Juices prepared from acidic products (tomato and rhubarb) that can be processed at relatively low temperature.
2. Vegetable or blends that are acidified with highly acid products such as lemon juice concentrate, inorganic acids etc. (citrus, pineapple, tomato and rhubarb).
3. Vegetable juices acidified with organic acids to allow processing at relatively low temperatures.

4. Juices freshly extracted from non-acid vegetables (spinach, lettuce, beetroot) immediately before consumption that are not heat treated or acidified.
5. The excess juice obtained from fermented vegetables e.g. sauerkraut juice.
6. Vegetable juices or blends when not acidified that must be processed at relatively high temperatures to kill spores of spore forming microorganisms.

2.8.1 Stages of commercial juice production

Vegetable-juice processing is similar to fruit-juice processing and involves a common basic process that varies according to the type and structure of vegetable being juiced (Varnam & Sutherland, 1994). The five elements associated with juice production are harvesting, transport and storage, pre-treatments, juicing and post treatments (Bates et al., 2001; Varnam & Sutherland, 1994).

2.8.1.1 Harvest, transport and storage

The vegetable is harvested in a manner to minimise physical damage. Likewise with storage and transport, the produce should be handled with care and stored at a temperature appropriate for the post-harvest storage.

2.8.1.2 Pre treatments

Vegetables can be prepared for juice extraction using several different processes. Pre-treatments are designed to maintain the quality of the raw ingredients, to reduce surface microbial contamination and reduce particle size to optimise juice extraction (He et al., 2005). Once received at the factory the produce is graded and washed to ensure the produce is acceptable and free from gross damage and contamination and also to remove any foreign objects (Varnam & Sutherland, 1994). Washing can be in water flumes or spray washing depending on the product. The water used for washing vegetables or fruits can be hot or cold and/or contain a sanitiser such as chlorine or pass through a UV light tunnel (Varnam & Sutherland, 1994). Some produce with short growing seasons can be stored frozen and defrosted when required. This can improve

the juice yield as freezing damages the plant cell walls resulting in juice being more easily released (Varnam & Sutherland, 1994).

Thermal treatment of carrots prior to juice extraction has been found to be an important step in producing cloudy stable juices. Acidification is also deemed to be essential when using blanched carrots. Clarification can only be prevented by acidifying the mash before juice extraction. Acidification after juice extraction will result in poor cloud stability (Reiter et al., 2003).

Blanching is used as a pre-treatment to inactivate enzymes that will adversely affect the desired juice. European Food Safety Authority (2013) reported nitrate loses in vegetables such as lettuce, spinach, potato, green bean, carrot, red beet, white cabbage, Chinese cabbage and courgette after blanching due to its highly soluble nature. Some vegetables require physical size reduction prior to juice extraction (milling). Hammer or fixed knife mills are widely used as they give a high juice yield (Varnam & Sutherland, 1994). For stubborn material, pre-treatment with a macerating enzyme with or without heating to about 60 °C and holding up to 40 mins can greatly increase yield and subsequent pressing/clarification steps (Bouzrara & Vorobiev, 2000).

2.8.1.3 Juicing stage

In fruit and vegetables, tissue cells are surrounded with elastic membranes and rigid walls, which limit efficiency of the processing extraction (Praporscic et al., 2007). Juice is extracted from the produce using several different methods depending on the starting raw material and the type of juice required. Juice is either pressed from the raw material or centrifuged or a combination of both (Bouzrara & Vorobiev, 2000). The efficiency of these methods is improved by the use of other pre-treatment processes to reduce the particle size and/ or cause damage to the plant cell, or by addition of processing aids such as enzymes (Landbo & Meyer, 2001). Juice can be extracted by pressing using either batch or continuous methods depending on the scale of the juice to be extracted (Varnam & Sutherland, 1994). Different techniques, including thermal, electrothermal, pulsed electric fields (PEF), chemical and enzymatic pre-treatment are suggested to enhance pressure assisted juice extraction (Praporscic et al., 2007).

The rack and frame press is one of the oldest methods of juice extraction and often used by small processors. The pulp of soft produce is placed into a sterile heavy nylon or cotton bag in a frame on a wood or metal rack. Multiple bags are stacked up, the frame removed and pressure is applied to the stack with a hydraulic ram to express the juice. Although very labour intensive, this process produces high quality juice (Varnam & Sutherland, 1994). For larger volumes automated hydraulic presses are used but require press aids to provide firmness to the mash and to form channels for the juice to exit. Press aids reduce cloudiness and require long fibres for efficient action such as ground wood pulp or sterilised rice hulls (Varnam & Sutherland, 1994).

A screw press is a heavy graduated pitch screw fitting closely within a cylindrical screen (Bouzrara & Vorobiev, 2000). In a two stage process the easily removed juice is drained off, then the pulp passes through the screw where it is compressed. The action of the screw is aided by the interaction with compressor bars incorporated into the press. Screw presses require press aids and efficiently produce cloudy juice (Varnam & Sutherland, 1994). Belt presses are used effectively with firm fruit. Pulp blended with press aid is pressed between two mesh belts which pass through a series of rollers and the expelled juice is collected in a channel below (Varnam & Sutherland, 1994).). Subsequent phase separation with the decanter enables yields of 90 % or more to be achieved. The use of highly developed decanter technology results in a rapid, continuous and low-oxidation juicing resulting in high quality juices with high yields under hygienic conditions. A new process consisting of a combined pressing and pulsed electric field (PEF) treatment is proposed to increase the efficiency of juice extraction from beetroot pulp. The treatment causes pore formation and destruction of the semipermeable barrier of the cell membrane. Mechanical pressing associated with a PEF treatment allows the juice yield to be increased threefold with an energy consumption of about 2.92 x 10-2 kWh per kg of extracted juice. This process would provide a good alternative to the standard thermal and mechanical techniques for juice extraction (Bouzrara & Vorobiev, 2000).

Direct pressure of pressing has been the traditional juice processing method, however, the use of centrifugal separation of juice is becoming more common. The decanter centrifuge can be used in conjunction with a pressing system as a preliminary step to

increase efficiency (Moller et al., 2002). The centrifuge in conjunction with the pressing system provides a complete separation as a coarse primary stage and the second as a final clarification stage. The decanter is a horizontal scroll centrifuge with a cylindrical-conical solid-wall bowl for the continuous separation of solids out of suspensions (Ashurst, 2004).

Compared to pressing, centrifugation produces single strength cloudy juices with a high percentage (60 %) of small particles (1µM or smaller) in suspension compared to only 20 % in pressed juice (Ashurst, 2004). A major factor in the production of "naturally cloudy" juices is the rate of processing. To ensure stability, the juicing stage should be followed immediately by pasteurisation in order to inactive the enzymes naturally present in the fruit that cause deteriorative reactions (Ashurst, 2004). Decanters are frequently used in conjunction with disk-stack-type centrifuges in the pre-preparation of clear juices and juice concentrates, where the initial decanter treatment results in a partially clarified juice with a low level of suspended solids. This is followed by a clarification stage using a disk centrifuge whereby the solids are thrown outwards from the through-flow juice stream into a solids holding space and automatically discharged from there, as and when an optimum level of solids is reached (Ashurst, 2004).

2.8.1.4 Post treatments

Once juice is extracted from the vegetable any further processing is determined by or defined by its end use and desired shelf life (Varnam & Sutherland, 1994). Juice can undergo any or a combination of processes including clarification, heat treatments, non-thermal processing, mixing and homogenisation, concentration and packaging.

Clarification is normally a combination of enzyme treatment, fining, centrifugation and filtration. Treatment with pectolytic enzymes for juice clarification is useful but not essential (Varnam & Sutherland, 1994). Enzyme treatment in a holding tank for eight hrs at 15-20 °C or one hr at 45 °C is recommended for apples but will vary according to produce and enzyme but temperature between 20 °C and 40 °C should be avoided to minimise yeast growth (Varnam & Sutherland, 1994). Enzymes are used to help extract, clarify and modify juices from many crops including berries, stone and citrus fruits, grapes, apples, pears and even vegetables (Ashurst, 2004).

Centrifugation is used as a preliminary treatment to remove high or low density material. It is also applied after fining with bentonite, gelatine or water soluble chitosan (Varnam & Sutherland, 1994). Bentonite forms flocs with proteins and gelatine creates an insoluble floc with the phenolics and proteins. The insoluble flocs are removed by decanting, centrifuging or filtration.

Pasteurisation, a relatively mild heat treatment with less impact on product quality, is applied to inactivate enzymes and pathogenic microorganisms. Endospores of *Clostridium botulinum* are distributed in soil (Gillian et al., 2001) and lower incidence of contamination by *Clostridium botulinum* has been reported in potatoes post pasteurisation (Lund et al., 2008). Since the process is not severe enough to kill *Clostridium botulinum*, the pasteurised foods require refrigeration immediately after processing (Gillian et al., 2001; Sun, 2012). Thus pasteurised products have a limited shelf life in the distribution chain.

2.8.2 Methodologies for preservation of juice

The food industry is driven by the consumer need for high quality, minimally processed, additive free, shelf stable, convenient and safe food products (van Loey et al., 1998). To meet these needs existing thermal preservation processes are being improved (e.g. better process control) or adjusted (e.g. aseptic processing, ohmic and microwave heating), or new physical food preservation methods are being introduced such as the PEF and high hydrostatic pressure processing (HPP) (van Loey et al., 1998).

2.8.2.1 Pasteurisation-High Temperature Short Time (HTST) processing

Liquid products are relatively easy to pasteurise. The flow properties permit fast heat transfer by turbulent mixing using convection and conduction. The severity and duration of heat treatment depends on the nature of the product, pH, initial microbial load, type of heat processing and the type of microorganisms (Sun, 2012). Studies have shown that thermal death rates of bacteria generally proceed much faster with increased temperatures (Sun, 2012). It is therefore possible to apply the principles of pasteurisation and aseptic packaging processes for better quality retention. (Weng, 2012). High temperature short time (HTST) is a type of pasteurisation that is carried out using a plate heat exchanger and a separate holding tube (Ashurst, 1999).

Modern plate pasteurisers have four main sections: a generative section, a heating section, a holding section and a cooling section (Ashurst, 1999). A generative section consists of hot, already pasteurised juice which is fed back through one side to preheat the incoming cold juice and to be cooled back to a lower temperature in the process, thus saving energy. The heating section is supplied with hot water or steam to boost the juice to its pasteurising temperature. A holding section is accommodated within the heat exchanger or possibly is a length of piping insulated to prevent heat loss which could lead to inadequate pasteurisation. A cooling section uses chilled water as the cooling medium (Ashurst, 1999).

HTST is the most common method of heating juice products, it is used for heat sensitive products and can help to maintain the colour and flavour of the final product (Weng, 2012). Ultra High temperature (UHT) is another method of aseptic filling by heating a product for extremely short periods (1-2 s), at a temperature exceeding 135 °C (275 °F), which is the temperature required to kill spores in milk (Gedam et al., 2007; Weng, 2012).

For fruit juices, in traditional practise, the juices are heated up to 60-70 °C for 30 min, then filled at that temperature (Lewis & Heppel, 2000). After this treatment, the products are cooled back to room temperature. HTST pasteurisation is conducted at higher temperatures (>90 °C) for shorter times. This can for example, be carried out at 95-98 °C for about 15-30 s for apple juice (Wilbey, 2003a). Pasteurisation of citrus juice at 90 °C for 10 s denatures pectinase, thus preventing cloudiness breakdown in fresh juice (Wilbey, 2003a). Pasteurisation of apple juice at 89 °C for 90 s destroys potential spoilage organisms and denatures polyphenol oxidase, the enzyme that cause browning (Wibley, 2003a). Tomato juice is processed at 115 °C for 15 s (Wilbey, 2003a, 2003b). UHT-sterilised food products are stored packaged in consumer containers (laminated cartons of various sizes) or in institutional and commercial size packs. The packaged products can be stored at ambient temperatures for several months, sometimes up to 2 years.

How a juice is packaged impacts on its shelf life, whether it is hot or cold filled, aseptically or not, with or without gas flushing, depending on the type of gas used and

the packaging material. Protection from the environment, reduced exposure to light and oxygen can all maintain the quality of a juice.

2.9 Juice Safety

Microbial growth in a food product can be prevented or minimised using a variety of different factors relating to the product and the environment it is processed and stored in. Many of these factors work in synergy such as pH and temperature. A pH reduction or increase (from the microorganism's optimum) can allow the product to be heat treated at lower temperatures while still resulting in the same level of microbial destruction (Koutsoumanis et al., 2006).

2.9.1 Fruits and Vegetables

Raw fruit and vegetables are sources of microorganisms mainly derived from soil, water and air including saprophytes such as coryneforms, lactic acid bacteria, spore-formers, coliforms, micrococci and pseudomonas (Wiles & Walker, 1951). Bacteria are the predominant microorganism in vegetables with significant number of yeasts and moulds and a smaller number of fungi (Bari et al., 2005). The bacteria include many potential food borne pathogens such as *Listeria monocytogenes, Salmonella spp, Staphylococcus botulinum, Shigella spp, Bacillus cereus* and *Staphylococcus aureus* (Bari et al., 2005). Contamination of fruits and vegetable products with enteric pathogens (*Escherichia coli 0157:H7, Listeria monocytogenes, Salmonella spp*) are of most concern as they have a low infectious dose or have the potential for growth in food prior to consumption even when stored under refrigerated conditions (Ukuku et al., 2005).

Microbial contamination of raw vegetables usually occurs on exposed surfaces, while internal tissues remain essentially free of microorganisms (Bari et al., 2005). The post-harvest growth of fungi in subsurface tissue can alter the pH of the plant tissues allowing the pathogenic bacteria to grow where it actually would not in healthy vegetables or fruit (Bari et al., 2005). The presence of microorganisms in a vegetable product reflects the effectiveness of anti-microbial treatments at any step from planting, processing through to consumption (Jay, 1996; Ukuku et al., 2005).

2.9.2 Microbial Control Factors

Microbial growth can be influenced by factors that are characteristic to the product (intrinsic) and/ or from factors in the surrounding environment (extrinsic). These factors are detailed in **Table 2.6**. Extrinsic factors affect both the product and the microorganisms (Bari et al., 2005; Jay, 1996).

Table 2.6: Intrinsic and Extrinsic microbial growth factors (Bari et al., 2005; Jay, 1996)

Intrinsic factors	Extrinsic factors
• Moisture content and water activity (aw)	• Temperature of storage
• pH and acidity	• Relative humidity of the environment
• Nutrients (water, energy source, nitrogen, vitamins and minerals)	• Presence and concentration of gases: carbon dioxide (CO_2), ozone (O_3) and oxygen (O_2) are toxic to certain microorganisms.
• Biological structure: structure of the microorganism and physical barriers of the plant e.g. skin	• Modified atmosphere packaging (MAP) : the use of CO_2, Nitrogen (N_2) and ethanol to change the environment in a package
• Oxidation-Reduction (Redox) potential ; a measure of the ease by which a substance gains or losses electrons	• The presence and activities of other microorganisms.
• Naturally-occurring antimicrobials	
• Competitive microflora	

The ability of microorganisms, including pathogens, to grow depends greatly on the combinations of intrinsic and extrinsic factors that are naturally present or introduced at any stage of the life of a plant product from growing, harvesting, production, processing, distribution and preparation at the site of consumption (Bari et al., 2005; Jay, 1996). Hurdle technology uses combinations of multiple intrinsic and extrinsic factors as 'barriers' against the growth of microorganisms in a product (Jay, 1996). Many of the factors have synergistic interactions. For example the pH of the product significantly affects the lethality of heat treatment. As the pH is reduced away from that required for optimum growth of microorganisms (generally pH 7), less heat is required to inactivate the microorganisms. Therefore a product can be pasteurised first followed by a reduction in pH to <4.6 with acids and/or lemon concentrate under aseptic conditions to prevent microbial growth (Bates et al., 2001).

2.9.2.1 Heat treatments for microorganisms

The use of heat treatment processes to preserve food is based on the destructive effect heat has on microorganisms. Food products can be either sterilised or pasteurised. Sterilisation is the destruction of all viable organisms in a food product. Pasteurisation by heat implies the destruction of all disease-producing organisms or the destruction of spoilage organisms in other food products (Jay, 1996; Wilbey, 2003a).

The most common pasteurisation time-temperature combination for milk is 72 °C for 15 s. Alternative temperature-time combinations for milk pasteurisation includes 89 °C /1.0 s, 90 °C/0.5 s, 94 °C/0.1 s or 100 °C /0.01 s (Jay, 1996). Heat treatments to eliminate yeasts and lactobacilli are more severe than for elimination of vegetative pathogens.

In citrus juices, heat treatment at 70 °C for 60 s or 85 °C for 30 s is used to eliminate yeasts (Wilbey, 2003a). Fruit juices generally have a pH <4.5, so growth of pathogenic bacteria will generally not be supported (Wilbey, 2003a). Carrot juice is processed at 105 °C for 30 s to eliminate microbes (Chen et al., 1995). In commercial practise, juices are usually pasteurised using HTST process of 90-95 °C for 10-20 s (Loong & Goh, 2004). Mixed vegetable juice acidified to a pH below 4 was subjected to pasteurisation regimes between 80 to 100 °C (Loong & Goh, 2004). Microbial counts in cucumber

juice drinks were completely inactivated by heat treatment at 85 °C for 15 s in combination with addition of a preservative (Zhao et al., 2013).

2.9.2.2 Effect of freezing on microorganisms

For multiple reasons, freezing cannot be solely relied upon as a method to destroy food borne microorganisms for food preservation. Destruction of microbial growth through freezing depends on the type, state and strain of microorganism; the type of freezing used the nature and composition of food, the length of time of freezer storage, and the freezing temperature (Jay, 1996). More microorganisms are destroyed at -4 °C than at -15 °C, with temperatures below -24 °C having no additional deteriorative effect on microorganisms (Jay, 1996). Furthermore Jay (1996) suggests that it is the time-temperature pattern characteristic of thawing that is potentially more detrimental than freezing. During thawing, the temperature rises rapidly to near the melting point and remains there throughout the long thawing period. This provides considerable opportunity for chemical reactions, re-crystallisation and even microbial growth if thawing is extremely slow (Jay, 1996).

2.10 Juice Flavour Perception

The flavour of a product is an important quality attribute that is linked to whether or not that product is accepted or rejected by a consumer (Cadwallader, 2005). Flavour is the integrated perception of aroma (odour) and taste and to a lesser extent pain or nerve response (e.g. heat of capsaicin), texture and mouth feel and overall appearance (Cadwallader, 2005).

Fruit and vegetable flavour are composed of a wide range of chemical compounds from non-volatile taste-active (including both inorganic and organic compounds) to volatile aroma-active organic molecules. Most often it is the aroma components that are the predominant contributors to the distinct flavour of fruit or vegetables (Cadwallader, 2005).

Some non-volatile compounds such as sugars and organic acids /lemon juice impart sweet and sour tastes, respectively. The percentage of soluble solids (°Brix) and °Brix to acid ratio are often used as indices of ripeness and flavour quality (Cadwallader, 2005).

Consumption of beetroot juice is not as popular as other fruit and vegetable juices such as tomato, carrot, apple or mango due to its perceived issues of taste, texture and urinary colouration (Manoharan et al., 2012). However, beetroot juice has a relatively pleasant taste in comparison with other vegetable juices due to its relatively high sugar content (Thakur & Das Gupta, 2006). Since earthy flavours are associated with beetroot juice, its compatibility with other food flavours is important (Thakur & Das Gupta, 2006).

The major contributor to the earthy flavour in beetroots is geosmin (*trans* 1,10-dimethyl-trans-9-decalol) (Acree et al., 1977). Acree et al. (1977) identified geosmin and potato-like odour which produced an odour characteristic of the beet. Raw beetroot showed low scores of aroma attributes but when the roots were boiled or baked, they developed aromas of raw beetroot, berry juice and baked potato. As beetroot stores its excess energy as sugar and not as carbohydrate polymers, a simpler relationship between sugar content and sensory evaluated sweetness was proposed by McBurney & Bartoshuk (1973). The perceived sweetness of beetroot may be influenced by bitter-tasting compounds in the root, by mixture interactions (McBurney & Bartoshuk, 1973). In general bitter-tasting compounds are well known for their ability to suppress the perceived sweetness in mixtures (Lawless, 1979). The sensory attributes bitterness and astringency were identified in beetroot but the chemical background for these compounds was not investigated by researchers (Acree et al., 1977; McBurney & Bartoshuk, 1973).

Sensory evaluation comprises of a set of techniques for accurate measurement of human responses to food and minimises the potentially biasing effects of brand identity and other information that influences consumer perception (Lawless & Heymann, 1988). Sensory evaluation consists of evoking, measuring, analysing and interpreting responses perceived of foods using all human senses without biasing the consumer's perception (Lawless & Heymann, 1988). The consumer sensory techniques used in this study aimed to cover all four terms (evoking, measuring, analysis and interpreting), in order to provide an accurate representation of the sensory perceptions associated with different flavoured vegetable juices. No instrument can replicate or replace the human response thereby making sensory evaluation an essential component in food product development (Lawless & Heymann, 1988).

Consumer testing is an effective method of testing that is employed in order to gauge consumer preference and/or acceptance to a product or product idea (Lawless & Heymann, 1988; Meilgaard et al., 1999). Consumer testing helps to gain information on consumer opinion about their product preference (Meilgaard et al., 1999).

2.11 Nitrate-rich beverages for sports performance on market

There are several nitrate-rich beverages on the market that claim to boost endurance and improve exercise performance. **Table 2.7** shows an overview of the current nitrate rich drinks on the market in the United Kingdom and Australia. **Table 2.7** emphasises the quantity of juice retailed in the supermarket, ingredient listing, amount of nitrate and the cost per 100 ml. Almost all the formulations are a combination of beetroot and apple juice and none are made in New Zealand. Many studies have been conducted in the past using BEET IT for exercise related performance studies. Beetroot juice (BEET IT) reduces muscle metabolic perturbation during hypoxic exercise and restores exercise tolerance and oxidative function. Consumption of organic beetroot juice (BEET IT, containing ~300-500 mg of nitrate) in acute (2.5 hr prior to exercise) or chronic (daily for up to 6 days) doses may result in a reduced oxygen cost of low-, moderate- and high-intensity exercise (Bailey et al., 2009; Lansley et al., 2011). Acute loading doses of organic beetroot juice (BEET IT) have been shown to significantly enhance the performance of a 4 km and a 16.1 km cycling time-trial effort (by 2.7-2.8 %, respectively), via a greater power output per L of oxygen consumed (Lansley et al., 2011). Lee (2013) has shown a 4.2 % improvements in intermittent, team sport running tasks when beetroot juice supplements (BEET IT) were consumed. These are important findings not only relevant for exercise physiology but also for patients with limited oxygen delivery to the working muscle (Vanhatalo et al., 2010). Kenjale et al. (2011) found that beetroot juice (Biotta Beet juice containing 1.5 g/L nitrate) improved exercise performance in patients with peripheral artery disease. In a free-living environment, people consuming an unrestricted diet and a single dose of 500 g of beetroot and apple juice (SUNRAYSIA beetroot and apple juice), a significant trend to lower blood pressure by 4–5 mmHg at 6-hr was observed only in men (Coles & Clifton, 2012).

Table 2.7: Competitive nitrate rich beverages

PRODUCT NAME	COUNTRY OF MANUFACTURE	PRICE PER 100ml	QUANTITY RETAILED	INGREDIENT LISTING	NITRATE CLAIMED
BEET IT juice James White Drinks (2014)	United Kingdom	NZ $ 1.38	• 1L carton • 750 ml glass bottle • 250 ml polyethylene terephthalate (PET)	Beetroot juice (90 %), apple juice (10 %).	1g nitrate/L
BEET IT shot (2 types) -organic -sports James White Drinks (2014)	United Kingdom	NZ $ 8.40	• 70 ml PET bottle	Organic beetroot juice (98 %), organic lemon (2 %)	Organic shot (0.3 g nitrate/ 70 ml) Sports shot (0.4 g nitrate/70 ml)

SUNRAYSIA Sunraysia (2013)	Australia		• 750 ml glass bottle and carton	Beetroot juice (72 %), Apple juice (28 %).	1.2 g/L Coles & Clifton (2012)
UPBEAT NitrateMax USD (2014)	Western Australia		• 250 ml PET bottle	Beetroot juice (85 %), Apple (15 %)	1.6 g nitrate/L

My t juice My t juice, 2014	Australia		• 1L and 250 ml PET bottle	Combination of beetroot and apple juice (percentages not specified)	N/A
Go Beet Go beet (2010)	Australia		• 200 ml PET bottle	Combination of beetroot and apple juice (percentages not specified)	1.3 g nitrate/L

2.12 Conclusions

Vegetables such as celery, lettuce, spinach, beetroot, radish, swiss chard constitute a major source of nitrate, generally providing more than 2500 mg/kg of nitrates. The acceptable daily intake for added nitrate and nitrite in food products are 260 mg and 5 mg per day for a 70 kg person, respectively (European Commission, 1992). Juices extracted from high nitrate vegetables especially beetroot have been used in the studies for sports and exercise performance. Excessive intake of nitrate and nitrite has been reported to increase the risk of cancer but none of the studies have been linked to the nitrates and nitrites from vegetables. Processing factors such as washing and cooking have been reported to cause nitrate loses in vegetables due to nitrate's water soluble property. Cold storage conditions (refrigeration and frozen storage) tend to inhibit the nitrate reductase activity in vegetables thereby inhibiting the conversion of nitrate to nitrite and preventing microbial growth. In order to receive the exercise related benefits from nitrate rich beverages, a minimum of 1 g nitrate per L is recommended in the final juice.

CHAPTER 3
MATERIALS AND METHOD

3.1 Resources used for juice extraction and testing

All vegetable juices were produced in the Institute of Food, Nutrition and Human Health (IFNHH) pilot plant and were tested in the chemical, microbiology and food technology laboratories at Massey University, Albany. The details of the equipment used in the production processes and for testing purposes can be found in **Table 3.1** and **3.2,** respectively.

Table 3.1: Resources used for juice extraction and storage

RESOURCE	MODEL AND MANUFACTURER	CITY, COUNTRY
Chiller (4 °C)	Cold Master Products Ltd	Auckland, New Zealand
Food Processor	Sunbeam Multipurpose	Auckland, New Zealand
Freezer (-18 °C)	Cold Master Products Ltd	Auckland, New Zealand
Freezer (-80 °C)	Thermo Electron Corporation, Thermo Fischer Scientific Inc.	Albany, New Zealand
Lab scale juicer	N-1000, Avanti, Food equipment distributors	Australia/New Zealand
Pasteuriser	Alpha Laval	Hamilton, New Zealand
plastic bottles	300 ml sterilised transparent plastic bottles with yellow cap by Arthur Holmes Ltd	Wellington, New Zealand
Translucent high density polyethylene (HDPE) bottle	1 L HDPE blow moulder natural bottle and tamper cap by Arthur Holmes Ltd	Wellington, New Zealand

Table 3.2: Resources used for juice testing

RESOURCE	MODEL AND MANUFACTURER	CITY, COUNTRY
99 % Ethanol	Alpha Tech	Auckland, New Zealand
Centrifuge (swing out rotor)	HeraeusLabofuge 400R, Thermo Scientific Ltd	Waltham, MA, USA
Corning centrifuge tubes (15 ml, 30 ml)	Thermo Fisher Scientific Inc.	Albany, New Zealand
Digital balance weighing to 4 decimal place	Sartorius CP8201, Thermo Fisher Scientific Inc.	Albany, Auckland
Digital refractometer	Atago PR-101, Global science Ltd	Albany, New Zealand
Glass vials (2 ml, 32 × 11.6 mm	Thermo Fisher Scientific Inc.	Albany, New Zealand
Handheld refractometer	BLS45-07, Thermo Fisher Scientific Inc.	Albany, New Zealand
HPLC Column (5µm, 120A, 4.6 × 250mm i.d.).	Gracesmart	Deerfield IL, USA
Microwave	Thermo Fisher Scientific Inc.	Albany, New Zealand
Milli-Q water	Synergy Millipore	MA,USA
Nylon Filter (0.45 µm)	Raylab	Auckland, New Zealand
Pasteur Pipette	Raylab	Auckland, New Zealand
pH510 Cyber scan pH meter	Eutech, Thermo Fisher Scientific Inc.	Albany, New Zealand
Plastic syringe (3 ml)	BD Leur-Lok Tip, RayLab	Auckland, New Zealand
Stirrer/hotplate	KendroVariomag, Sigma-Aldrich Ltd	Auckland, New Zealand

3.2 Raw materials used

Vegetables (beetroot, celery, lettuce and spinach) for preliminary laboratory work were sourced from local supermarkets (Auckland, New Zealand). For pilot scale juicing, beetroot and celery were sourced from Freshmax Ltd (Auckland, New Zealand) and Fresh Connection Ltd (Auckland, New Zealand), respectively. The commercial product (BEET IT) used for consumer sensory evaluation was purchased from a health store (Auckland, New Zealand) and had a best before of six months. Other ingredients used for blending purposes and placebo development with their respective suppliers are listed in **Table 3.3**. Beetroot juice concentrate 615 and Lemon juice concentrate 400 GPL was sourced from Germany and Argentina, respectively. Lemon juice concentrate 400 GPL had a best before of 2 years from the date of manufacture.

Table 3.3: Ingredients sourced for blending and placebo development

INGREDIENTS	°BRIX	SUPPLIER
Apple Flavour NAT 407540-DG	-	Sensient NZ Ltd
Beetroot juice concentrate 615	69	Zymus NZ Ltd
Fresh Up Crisp apple juice	10-11	Frucor Beverages Ltd
Lemon Flavour N326-DG	-	Sensient NZ Ltd
Lemon juice concentrate 400 GPL	41-46	Directus Ltd
Orange Flavour N264-DG	-	Sensient NZ Ltd
White Sugar	-	NZ sugar Ltd

3.3 Quantification of nitrates and nitrites in juice using high pressure liquid chromatography (HPLC)

The quantification of nitrates and nitrites in juice was based on the quantification methods described by Cheng & Tsang (1998) and Chien-Chung et al. (2003) (**Table 2.5, Chapter 2**).

3.3.1 Instruments and chemicals

A Shimadzu high performance liquid chromatography (HPLC) system, equipped with a RF-20A xs UV-VIS detector and LC-20AD chromatopac integrator was utilized in this

study (Shimadzu, Japan). Nitrates and nitrites present in the juice were separated by isocratic HPLC using 0.01M octylammonium orthophosphate (as mobile phase) performed on a Grace Smart RP18 column. The Shimadzu HPLC was fitted with a SIL-20 AC HT autosampler, CTO-20 AC column oven and DGU-20 A5 degasser. pH measurements were determined using a pH510 Cyberscan pH meter.

Octylamine and all other reagents (analytical grade) were purchased from Sigma Aldrich Co. (St Louis, MO, USA). Milli-Q water was prepared from a Synergy UV system (Millipore, MA, USA).

3.3.2 Preparation of mobile phase

The mobile phase consisted of 0.01 M octylammonium orthophosphate. The pH 3.5 of the mobile phase was adjusted with orthophosphoric acid (85 %) to pH 3.5. The mobile phase was freshly prepared every 2 weeks and stored at 4±1°C. Standard solutions of sodium nitrate and sodium nitrite powder were diluted to a series of concentrations of 0.1, 1, 10, 50 and 100 µg/mL in double deionized water and stored at 4±1°C for use. The solutions were freshly prepared every seven days. Each standard solution was injected in duplicate and a standard curve was generated.

The injection volume was 10 µL and a flow rate of 0.8 mL/min was used for the mobile phase. Nitrates and nitrites were determined at 213 nm and 193 nm, respectively. Peaks were integrated using Millennium®$_{32}$ Chromatography Manager Software (Waters Corporation). When the injections of the standard solution gave reproducible retention times and a peak area, each sample solution was then injected for analysis. The peaks of the sample were identified by comparison to the peaks of the standards. The amounts of nitrate and nitrite in the test solution were calculated from the peak areas by using linear regression equations of nitrate and nitrite standard curves.

3.3.3 Sample Analysis

Each juice sample was left to thaw on the bench at room temperature (20±1°C) and was centrifuged for 10 minutes at 2054 g (g force) at 20 °C. The supernatant was collected and filtered through a 0.45 µm nylon filter into 2 ml glass vials prior to analysis for nitrate and nitrite content. Samples were diluted with double deionized water to ensure

the concentrations fell within the standard curve range. All samples were analysed in triplicate within 1 hr of sample preparation. **Figure 3.1** shows a typical HPLC chromatogram of beetroot juice and standard solution with nitrate and nitrite peaks.

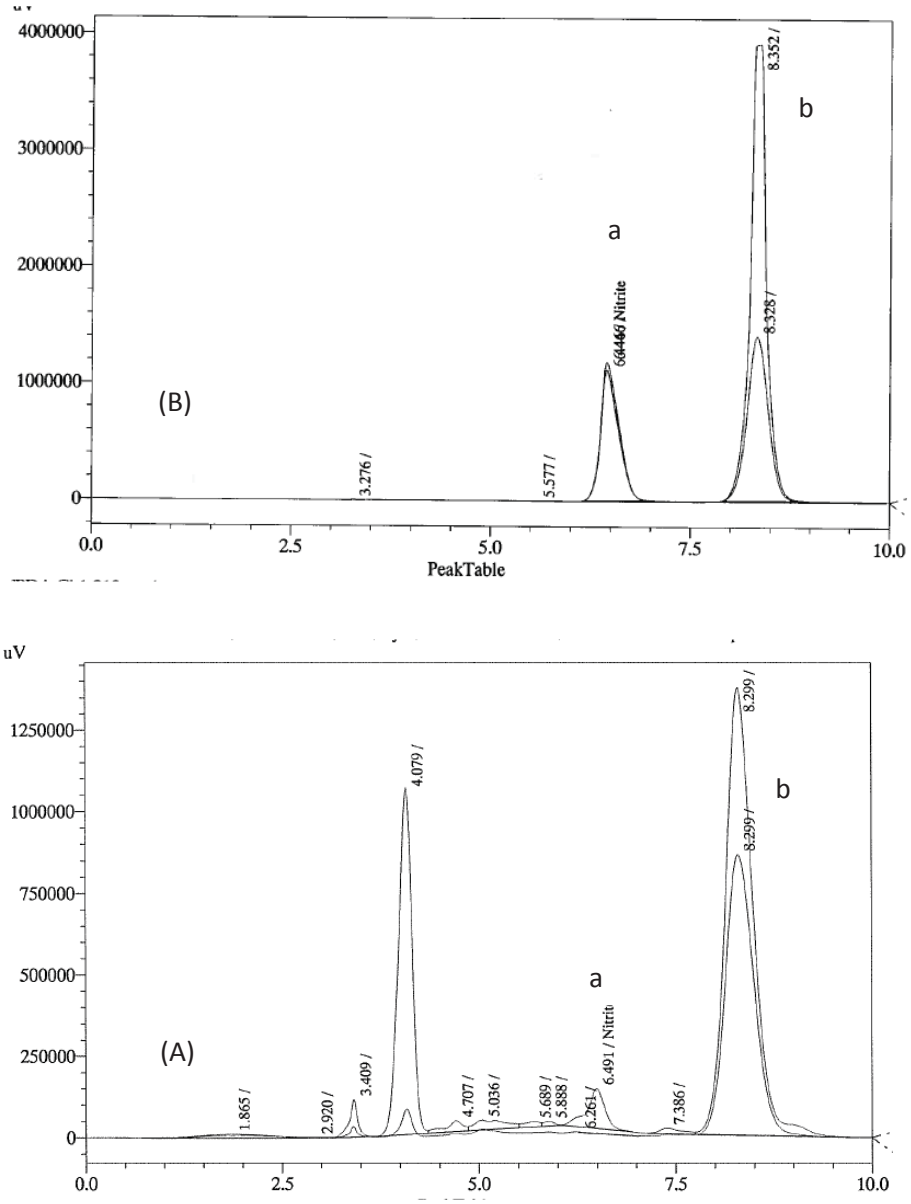

Figure 3.1: HPLC chromatograms of raw beetroot juice sample (B) and standard solution (A) containing 500 µg/ml of sodium nitrate and sodium nitrite using the HPLC analytical method under condition for a mobile phase solution of 0.01 M octylammonium orthophosphate and adjusted to a pH value of 3.5. The individual nitrite and nitrate peaks of the sample and the standard solution were shown as symbols a and b as indicated, respectively.

The injection volume of 10 μL and a flow rate of 0.8 mL/min were applied. The nitrite and nitrate peaks of the sample and standard solution were shown as 'a' and 'b' as indicated in **Figure 3.1**, respectively.

3.4 Juice analysis- Quality parameters

Each frozen juice sample was thawed to room temperature (20±1°C) for pH analysis, titratable acidity and total soluble solids (°Brix). Tests were conducted in triplicate. The equipment used in the biochemical analyses are detailed in the **Table 3.2.**

3.4.1 pH

Juice pH was determined using a pH meter with temperature adjustment and refillable combination electrode and stirred during pH measurement. For each juice treatment the final pH values were the average of three separate juice samples, and the temperature recorded.

3.4.2 Total titratable acidity

3.4.2.1 Preparation of reagents

Phenolphthalein (1 %) in 99 % ethanol: 1g indicator powder was dissolved into 100 ml absolute alcohol using a volumetric flask. It was ensured that the powder was fully dissolved prior to making to volume and mixed thoroughly. The final solution was stored in brown bottle with tightly secured lid.

Potassium hydrogen phthalate- as standardising solution: A small quantity of potassium hydrogen phthalate was dried for 2 hrs at 120 °C in an oven-proof small dish and cooled in an air-tight dessicator with fresh dessicant for at least 60 mins. The powder was stored in an air-tight bottle until used. Potassium hydrogen phthalate (0.2 g) was accurately weighed into a 150 ml conical flask. Distilled water (30-50 ml) was added and potassium hydrogen phthalate crystals were dissolved by heating carefully on a hotplate (set to 50±1 °C). The solution was allowed to cool to room temperature.

NaOH solution (0.1M): 4 g NaOH pellets were dissolved in 1 L CO_2- free distilled Milli-Q water in a 1 L volumetric flask. Once the solution was cooled, it was inverted carefully for thorough mixing.

Standardisation of NaOH solution: NaOH solution was standardised each time before titration of juice beverage samples.

3.4.2.2 Determination of Titratable acidity

Initial volume of the NaOH solution in the burette was recorded (v_i). The solution was titrated against NaOH solution until the first sign of a persistent, faint pink colour was observed. The final volume (v_f) of the NaOH in the burette was recorded and the volume of the titre ($v_a = v_f - v_i$) was calculated.

The concentration (molarity) of NaOH solution was calculated as follows:

$$\text{NaOH Molarity}(\text{mol/L}) = \frac{\text{mass of potassium hydrogen phthalate (g)}}{\text{NaOH titre volum } (v_a) \times 0.204229} \quad \text{…….. (Eq 1)}$$

The test was repeated until at least quadruplicate results were concordant (i.e. NaOH molarity > 0.0010 mol/L).

Titratable acidity of juice samples was determined using the method described by Friedrich (2001) with modifications. Juice (10 ml) was diluted with Milli-Q distilled water to 20 ml and the sample was titrated against 0.1 M NaOH to an end point of pH 8.2. The titratable acidity was calculated as g/100 ml (as citric acid) juice and was the average three separate juice samples (triplicate samples).

The titratable acidity (TA) in terms of standard acid was calculated using the equation below (Friedrich, 2001):

$$Titratableacidity(g/100ml) = \frac{V \times N \times meq\ weight \times 100}{1000 \times v} \quad \text{…….(Eq 2)}$$

V= Volume of NaOH solution used for titration (ml)

N= Normality of NaOH solution

Meq.weight. = milli-equivalent weight of the main acid (citric acid, 64., malic acid, 67)

v=sample volume (ml)

3.4.3 Total soluble solids

The total soluble solids of the juice samples were measured using a digital hand held refractometer with automatic temperature adjustment. The refractometer was standardised to zero using Milli-Q distilled water. The total soluble solids were expressed as °Brix, and the results were the average of three separate juice samples (triplicate samples).

NB: The total soluble solids for the preliminary experiments were determined using a hand held refractometer.

3.5 Microbiological analysis of juice samples

Juice samples were tested at the Institute of Food, Nutrition and Human Health (IFNHH) Microbiological Laboratory (Massey University, Albany). The samples were collected aseptically into sterile plastic tubes (25 ml) for analysis. Total aerobic plate count (cfu/ml) and yeasts and moulds (cfu/ml) were tested following the 141.393 Food Microbiology and safety Lab Instruction Manual (Mutukumira & Liu, 2011). Serial dilutions were carried out with sterile peptone water and then plated out with the appropriate media. The media used for total aerobic plate count and yeasts and moulds were Plate Count Agar (PCA) and Yeast Extract Glucose Chloramphenicol (YGC) agar, respectively. Peptone solutions were used to dilute beverage samples to concentrations suitable for enumeration by pour plating. The PCA plates for total aerobic plate counts were stored at 30±1°C for three days whereas the YGC plates for yeasts and moulds were stored at 25±1°C for five days.

To check if the pasteurisation step eliminated the bacterial and fungal growth, water samples before and after passing through the pasteurisation unit were tested as a part of the preliminary experiments.

Based on the microbiological reference criteria for ready to eat foods and packaged waters including mineral waters, the maximum recommended limits (New Zealand Food Safety Authority, 1995) of the chosen microbial tests are:

- Aerobic Plate Count at 35 °C 10^5 cfu/ml
- Coliform 10^2 cfu/100ml

Yeasts and moulds are the spoilage organisms and do not pose a food safety concern (New Zealand Food Safety Authority, 1995), therefore no limits exist. Three juice samples were tested for APCs, yeast and moulds, though the NZFSA requires five replicates (New Zealand Food Safety Authority, 1995). However, three replicates were chosen because of the limited juice availability.

Eight (four before pasteurisation, four after pasteurisation) beetroot samples were randomised and sent to an external laboratory (Assure Quality, Auckland) for the Total coliforms test (**Appendix F**).

3.6 Juice Production

Two preliminary experiments were undertaken firstly to determine the optimal high nitrate vegetables in New Zealand and secondly to determine the optimal storage temperature for nitrate and nitrite stability in vegetable juice over two weeks.

All vegetable juices were extracted using a juicer in the Food Technology laboratory at Massey University (Albany). Vegetables were sourced from local supermarkets in Auckland city for all the preliminary experiments. All vegetables were washed to remove any dirt and soil residues. Non-edible parts of each sample were removed and discarded. Vegetables were cut into small pieces (4 cm × 1 cm) and further chopped in a food processor (2 mm × 2 mm). Vegetables were weighed before juicing to calculate the yield. The juicer was washed with water and clean dried between vegetables to eliminate any traces from the previous vegetables. Juice was extracted by the centrifugal mechanism of the flat cutting blades in the juicer wall followed by spinning the produce at a high speed to separate the juice from the pulp. The juice was collected in a beaker whereas the pulp was discarded. The juice was filtered through a 200 mm stainless steel sieve before pasteurisation.

Individual juices were pasteurised at 90 ± 1°C for 15 s. Juice samples were collected before and after pasteurisation and tested for nitrates, nitrites, microbial counts and quality parameters. The pasteurised juice was stored in translucent high density polyethylene (HDPE) bottles (1 L) at -20 ± 1°C until used for further blending.

3.6.1 Determination of nitrate rich vegetables in New Zealand

Vegetables reported to be rich in nitrate concentrations in New Zealand were selected to test for nitrate and nitrite content in extracted juices. Based on information in the literature and availability, four vegetables were selected for quantification of nitrates and nitrites; lettuce (*Lactuca Sativa*), spinach (*Spinacia Oleracea*)**,** beetroot (*Beta vulgaris L*) and celery (*Apiumgraveolens var. dulce*). Each were juiced using a lab scale juicer (**Section 3.6**) and then quantified for nitrates and nitrites using HPLC (**Section 3.3**).

Beetroot and celery leaves were separated from the stalks and also juiced for testing. Juices were produced from following vegetables:

- Beetroot
- Beetroot leaves
- Celery stalk
- Celery leaves
- Spinach
- Lettuce

Figure 3.2 gives a process overview of the lab scale juice extraction. Since the amount of juice from the preliminary experiment was lower than the minimum quantities needed for the pasteurisation unit, all the preliminary juice samples were pasteurised using a laboratory microwave at 800 Watts for 15 s. Individual juice(s) were heated to a temperature of 90±1°C followed by cooling to room temperature by resting samples on the bench. Unheated and heated samples (x12) were assessed for taste by informal sensory evaluation (**Section 4.1.1**) and quantified for nitrates and nitrites by HPLC.

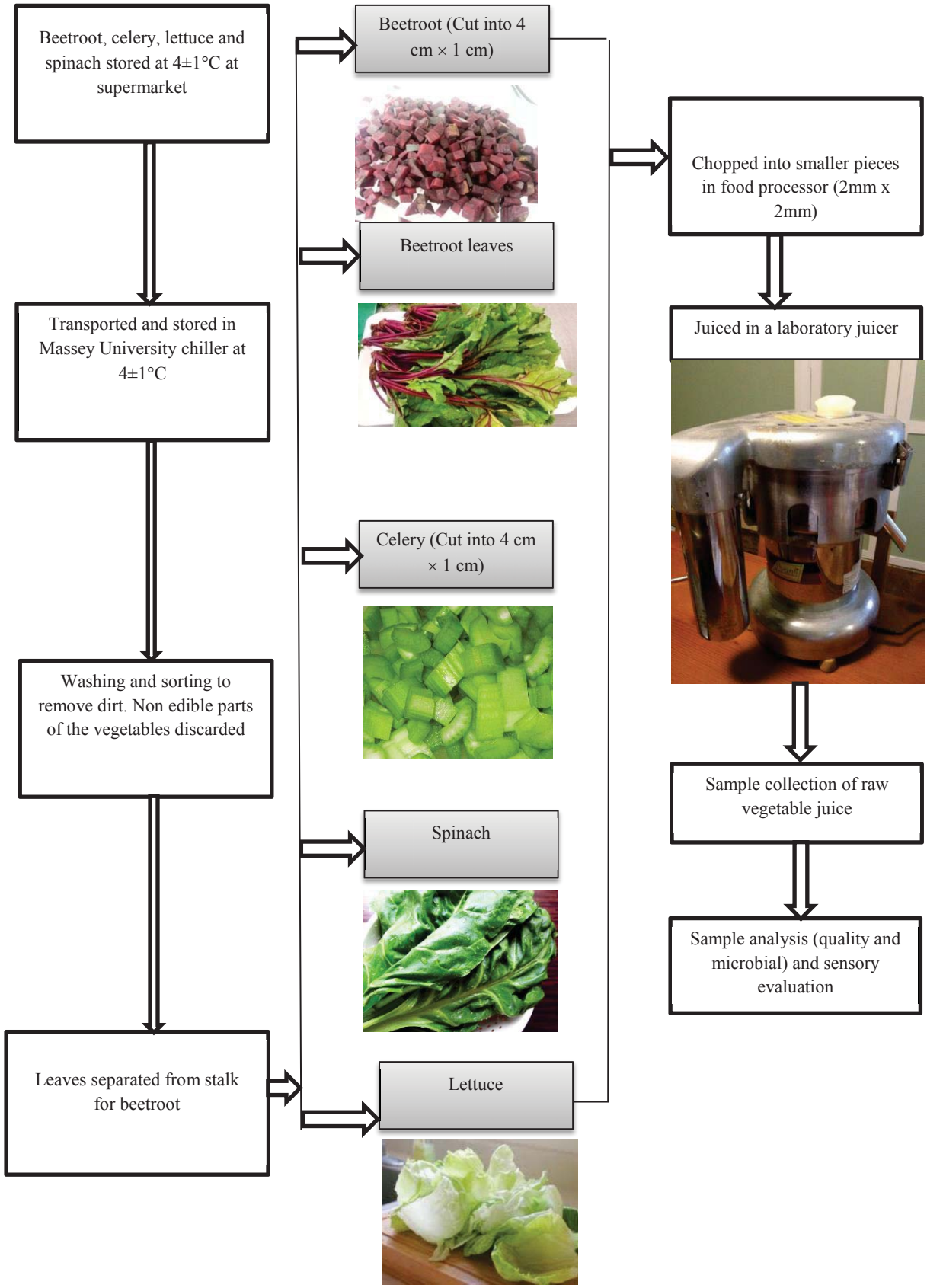

Figure 3.2: Process flow diagram for juice production

3.6.2 To determine appropriate temperature for nitrate stability for sample storage

The stability of nitrates and nitrites in beetroot juice was tested by monitoring concentrations over a period of two weeks (after 1, 2, 5, 7 and 14 days) after storage at three different temperatures (-80±1 °C, -20±1 °C and 4±1 °C). Beetroot juice was produced as described in **Section 3.6**. Quality testing such as pH, titratable acidity (g/100 ml (as citric acid) and soluble solids (°Brix) was tested (**Section 3.4**) and analysed by HPLC (**Section 3.3)**. The samples were filtered and pipetted into 30 ml Corning centrifuge tubes and stored at three different temperatures and thawed to the room temperature (20±1°C) before analysis.

3.7 Extraction of juice from pilot scale quantities

Beetroot with attached leaves (*Beta vulgaris*), 30 kg (3 × 10 kg batches) were supplied by Freshmax NZ Ltd (Mt Wellington, New Zealand) from one grower from the same planting. Celery (*Apiumgraveolens*), 10 kg was supplied by Fresh Connection Ltd (Mt Wellington, New Zealand). Juice from these vegetables was extracted as described in **Section 3.6**. Beetroot leaves were separated from the beetroots and juiced on the same day as celery (10 kg). The beetroot were divided into three equal batches (10 kg each) and juiced on three separate days. The juice was sieved through a 200 mm stainless steel sieve strainer before passing it through the pasteurisation unit (**Figure 3.3**) on the same day it was extracted and then stored at -20±1°C in HDPE bottles until further use.

Individual batches of juice was pasteurised at 90±1°C for 15 s as determined by the preliminary pasteurisation experiment and literature data (Jay, 1996; Loong & Goh, 2004; Wibley, 2003a). In order to achieve the correct holding time and temperature, the flow rate of the juice was set to 1L/min using water before processing. All of the beetroot, celery and beet leaf juices were pasteurised using the Massey University pilot plant pasteuriser and aseptically dispensed into each HDPE 1 L bottle in a clean workstation at ambient temperature. Each bottle was labelled appropriately and stored at -20±1°C until further analysis. Samples were collected before and after pasteurisation and tested for microbial counts, quality parameters and quantified for nitrates and nitrites.

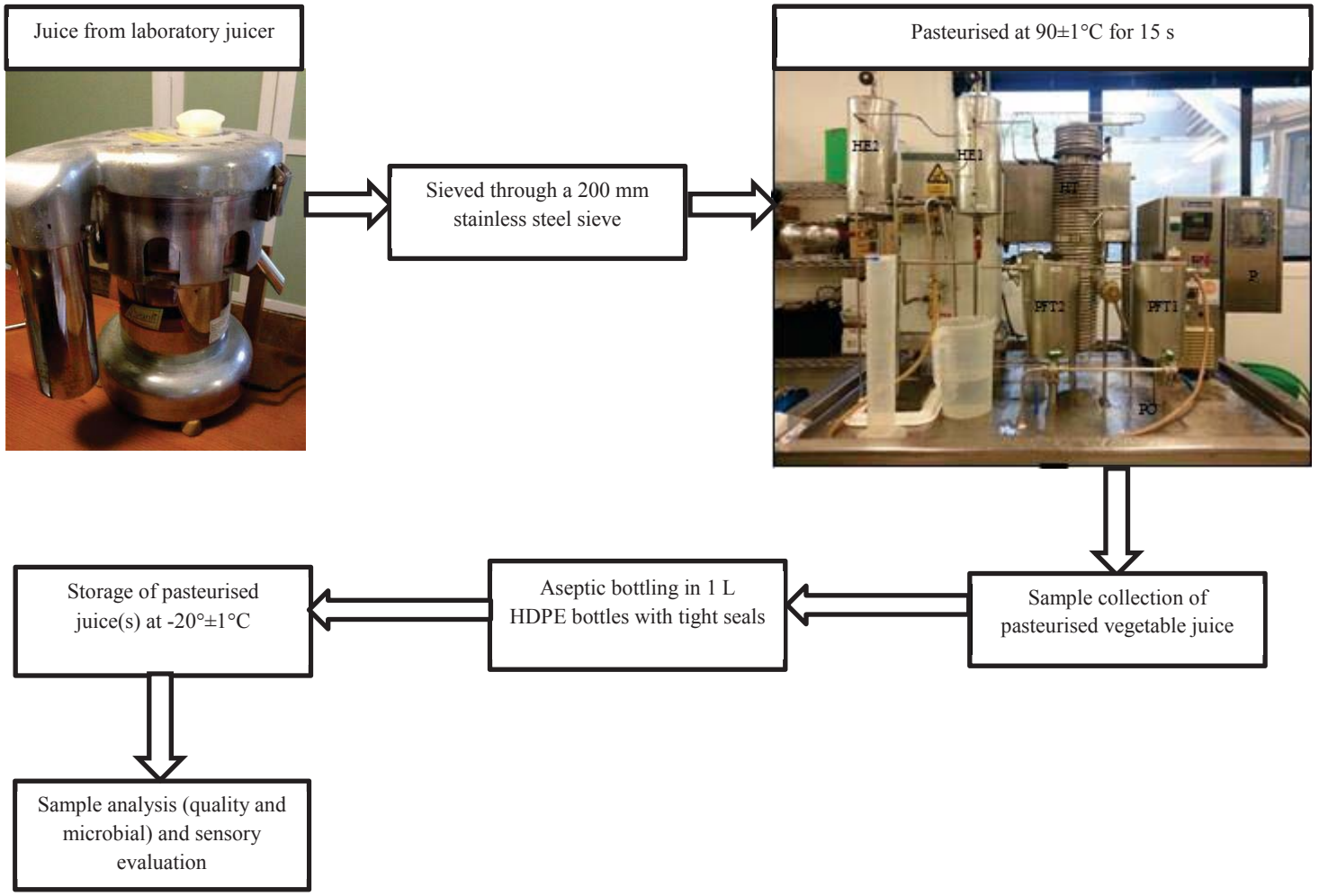

Figure 3.3: Process flow diagram post juice extraction (PFT1- Product feed tank 1, PFT2- Product feed tank 2, HE1- Heat exchanger 1, HE2-Heat exchanger 2, HT- Holding tube, PO- Product outlet, P- Power)

3.8 Juice blends

A blending plan was generated using MINITAB 16 Statistical software's mixture design. The mixture design was made based on component(s) such as ingredients and the percentage of each component in the mixture. The blends were randomly produced, generating a blending plan (formulations) in the form of a worksheet. Nitrate (g/L) concentration in the final formulation was calculated based on the different percentage of components in the blending formulations. Eight formulations were finalised for

analysis and taste testing. All juices were blended to a °Brix of around 10-11 and a nitrate content greater than 1000 mg/L.

Frozen juices were defrosted in cold water (8±1° C) for three hours on the morning of blending. The thawed juices were weighed according to the formulation (final volume=300 ml) and mixed in a plastic beaker. The final juice was stored in 1 L HDPE bottles and stored frozen at -20±1°C until further tasting and formulation development. Final formulation blends were tested for concentrations of nitrates and nitrites and microbial counts before being presented for informal sensory tasting (refer to results section). Ingredients used for blending formulations and placebo formulation with the company name are found in **Table 3.3**. The specifications of some of the ingredients in **Table 3.3** are attached in **Appendix A**.

3.8.1 Placebo formulation development

Frozen juices were thawed in cold water for three hours before placebo juice development. Placebo formulation was made with a combination of ingredients such as beetroot juice, apple juice, sugar, water, lemon juice concentrate and flavouring to produce a drink with low concentrations of nitrate. The titratable acidity g/100 ml (as citric acid) and total soluble solids of the placebo drink were matched with beverage blended to contain high nitrate content from Section 3.8.

Final formulations (300 ml each) were produced for informal sensory evaluation. Nitrate (mg/L) concentration was determined for all the placebo samples to ensure the levels were less than 200 mg/L. A sensory triangle test was also conducted on the placebo verses a nitrate rich formulation (standard beverage) to determine if the placebo beverage was significantly different ($P<0.05$). Placebo formulations developed for consumer triangle test evaluation were subjected to microbial testing prior to the consumer triangle test.

3.9 Sensory evaluation of juice blends

Nine blends (explained in Chapter 4) were narrowed down to four blends after initial screening. The four blends including BEET IT were subjected to sensory evaluation to investigate acceptability of flavour, acidity, sweetness and overall liking of the juice.

The data analysed from this evaluation provided a final formulation for this project and was used for storage trial. The samples were organoleptically assessed by consumers using sensory rating scales (**Appendix B**).

3.9.1 Sample preparation

All juices were made in the product development laboratory (Massey University, Albany) as explained in **Section 3.6**. Blending of different juices was carried out one day prior to sensory testing and samples were stored in 1 L HDPE at 4±1°C. In total, four juices (results section) were produced. Strict hygiene and sensory practices were carried out to ensure the safety of the beverages produced. The samples were also tested in the microbiological laboratory for total aerobic counts and yeasts and moulds and ensured they were within the acceptable limits before conducting the sensory evaluation. Each panellist received 10 ml juice samples served in 60 ml clear plastic sample cups (Galantai Plastics Group Limited, Auckland, New Zealand) coded with a three digit random number. The plastic cups were labelled a day before and sealed in a 5L air tight plastic container to prevent dust, dirt from entering the cups. Chilly bins and ice packs were used to maintain temperature below 4±1°C.

3.9.2 Testing location

Consumer testing was carried out in two locations- Auckland based gym (Club Physical, New Zealand) and Massey University sensory booths (Sensory laboratory, Massey, Albany) over two days. Two locations were chosen to ensure enough consumers were tested. Sensory evaluation was carried out just outside the gym but inside the building premises and an appropriate table was provided for the preparation area. Juices were maintained at 4±1°C by storing the juices in chill bins containing ice packs, hourly temperature checks were performed on the juice. The second testing of the juice assessments were held under temperature controlled condition (20±1°C) in the sensory booths at Massey University. The sensory booths were separate from preparation area to prevent the panellist from having physical or visual access to information that may bias their response. The juice was served between 3 to 5°C in the cups placed on white serving trays and appropriate questionnaires were provided.

3.9.3 Panels and participants

Sensory evaluation was completed by 70 consumers in total, 40 consumers at the gym and 30 consumers at Massey University tasting booths . Every panellist was given a participation information form and a consent form to fill out (**Appendix C**). An ethics approval was granted before commencing testing as attached in **Appendix C**. Sensory testing was carried out over two days, from 9:00 am to 2:00pm at Massey University.

3.9.4 Presentation of samples

Five cups (60 ml), labelled with 3-digit random numbers were placed on the white serving trays based on randomisation chart as attached in **Appendix D**. The juice samples were presented following a complete block Williams Latin squared design, balanced and randomised for carry-over effects, to avoid artefacts due to presentation order and sensory adaptation due to continuous exposure (Lawless & Heymann, 1998).

Juice was served at 4±1°C directly from fridge or chilly bin as this represented the beverage drinking temperature if purchased from a retail display unit. The consumer sensory evaluation forms (**Appendix B**) were arranged according to the consumer number and the randomisation chart. Appropriate stationary and serviettes were also presented to the consumers in the tray to give the test. A glass of tap water (20±1°C) was served with every tray and panellists were asked to rinse their mouths with water between samples. Five samples (*apple flavour high acid, orange flavour high acid, apple flavour low acid, orange flavour low acid* and *BEET IT*) per consumer were presented with the respective consumer sensory form under white lighting set up at the sensory booth at Massey and in daylight in the gym premises.

3.9.5 Sensory evaluation form structure

Consumer panellists were required to perform a simple test by using sensory scales commonly treated as interval scales. The scale was divided into intervals of equal size, labelled with descriptive term and numbers (Lawless & Heymann, 1998). Seven and nine point sensory scales were used in this study (Lawless & Heymann, 1998). Seven point scale was used to determine the liking for attributes such as sweetness, flavour and acidity liking whereas nine point scale was used to determine the overall product liking.

Nine point scale was used for overall product liking because consumer responses are repeated more consistently and also the test discriminated between the items at nine point scale as compared to seven point scale (Lawless & Heymann, 1998). On the sensory scale, the numbers on the left hand side indicated the least liked attributes, whereas those on the right were most liked. Randomisation of samples helped in ordering samples such that each unit had an equal chance of being chosen at each stage of the ordering process. Consumers were asked to fill out their gender and select an age group to study the demographics within the consumer trial. From the analysis of consumer forms, 36 males and 34 females attended the consumer tasting aged between 20 to 60 years old.

3.9.6 Discrimination triangle sensory test

The sensory evaluation was conducted in the sensory laboratory at Massey University, Albany. Three coded samples were presented to each panellist, and each panellist was asked to pick out which sample they felt was different to the other two (Meilgaard et al, 1999). All six possible combinations of presentation were randomly presented to the panellists (AAB, ABA, BAA, BBA, BAB, ABB). Twenty-five consumers participated in the triangle test as recommended by Lawless & Heymann, 1998. Placebo and high nitrate rich beverage were prepared and stored frozen (-20±1°C) for three days and analysed for microbial and fungal counts prior to tasting. Random three digit codes were used for samples. Three sample cups (two – same formulation, one-different formulation) were presented to every panellist in a randomised order (Table in **Appendix D**). Panellists were asked to place at tick for the odd tasting beverage. It was advised to taste the samples from left to right and to drink water in between samples and before the test. Statistics was performed to test for significance using MINITAB 16. More specific details about the consumer sensory evaluation are explained in **Section 3.8**.

3.9.7 Statistical analysis of sensory data

Microsoft Excel (2010) was used to calculate means, standard errors, standard deviations and graphically present the results. All the sensory data acquired from the consumer tasting evaluation was analysed statistically using MINITAB 16. The data

was analysed statistically to test the hypotheses and to find any significant differences between samples and if they depend upon other variables. All other statistical data was derived by conducting t tests on MINITAB 16 and chi test on EXCEL 2010.

The normality of the population was tested using a Ryan-Joiner test. For this analysis, the following null hypothesis was tested:

The data $\{x_1, x_2, \ldots, x_n\}$ are a random sample of size n from a normal distribution

Test statistic: RJ/r= the sample correction coefficient calculated from z percentile, observation) pairs where the z percentiles are for proportions (i-0.375/n+0.25) (Chantasorn, 2011).

In a Ryan Joiner test, if the data have a normal distribution, then the normal probability plot (plot of normal scored against the data) will be close to a straight line and the correlation r will be close to 1 (Chantasorn, 2011). If the data are sampled from a non-normal distribution then the plot may show a marked deviation from a straight line, resulting in a smaller correlation (r) (Kuo, 2001). Smaller values of r, are therefore regarded as stronger evidence against the null hypothesis (Kuo, 2001).

One way analysis of variance (ANOVA) was conducted on samples to determine whether there were any significant differences between the means of sample groups using p values. Any significance level (i.e. p value) of less than 0.05 indicated a significant difference between the mean of at least one pair of samples. As a result, the null hypothesis (H_o) was rejected and the alternative hypothesis (H_a) was accepted. The current hypothesis for the study is:

Null hypothesis (H_o): There is no significant difference among the samples

Alternative hypothesis (H_a): There is a significant difference among the samples

Analyses of Variance (ANOVA) for attributes were conducted using adjusted sum of squares (SS) for tests. Mean squares (MS) for the samples and error were calculated by dividing each SS by its representative degree of freedom (DF). The F values were compared to P value in order to determine whether there was any significant difference

among the means for the treatment. The F values were determined by dividing the MS values by MS error.

Finally, Principal Components Analysis was conducted on overall product liking for all the samples using the MINITAB software explained in more details in Chapter 5.

3.10 Storage trial conditions and experimental design

A storage trial was carried out to determine the shelf life of the drinks. Bottles of pasteurised juice (beetroot juice, beetroot leaves juice, apple juice) were removed from the freezer on the night before bottling for the storage trial and allowed to thaw at room temperature (20±1°C). *Orange flavour low acid*, finalised juice blend was made on the morning of the storage trial. Addition of the orange flavour was found to mask the beetroot earthy flavour notes.

Bottles (100ml) used for storage trial were cleaned and sterilised before filled with beverage. To ensure maintenance of product sterility, aseptic techniques were used at all times while handling the beverage ingredients, mixing, filling and storage. All equipment used for juice making were sanitised using food grade ethanol 70%. The working station used for production and filling of juice into bottles was also sanitised using ethanol 70% at the beginning of the blending process.

Each bottle was assigned to a storage temperature; 4±1°C or 20±1°C under light or dark storage according to the experimental design and sampling requirements. In total, 64 bottles including the duplicates were used in the storage trial (eight bottles per week, two per treatment). Each bottle was labelled with its designation and filled with juice in a randomised order.

3.10.1 Experimental design for storage trial

A full balanced factorial experimental design (**Figure 3.4**) was used to analyse the effects of temperature and light on the final juice product over an eight week period in simulated retail refrigerated storage conditions. Sixteen bottles were stored to allow for sampling of two bottles each treatment per week of testing.

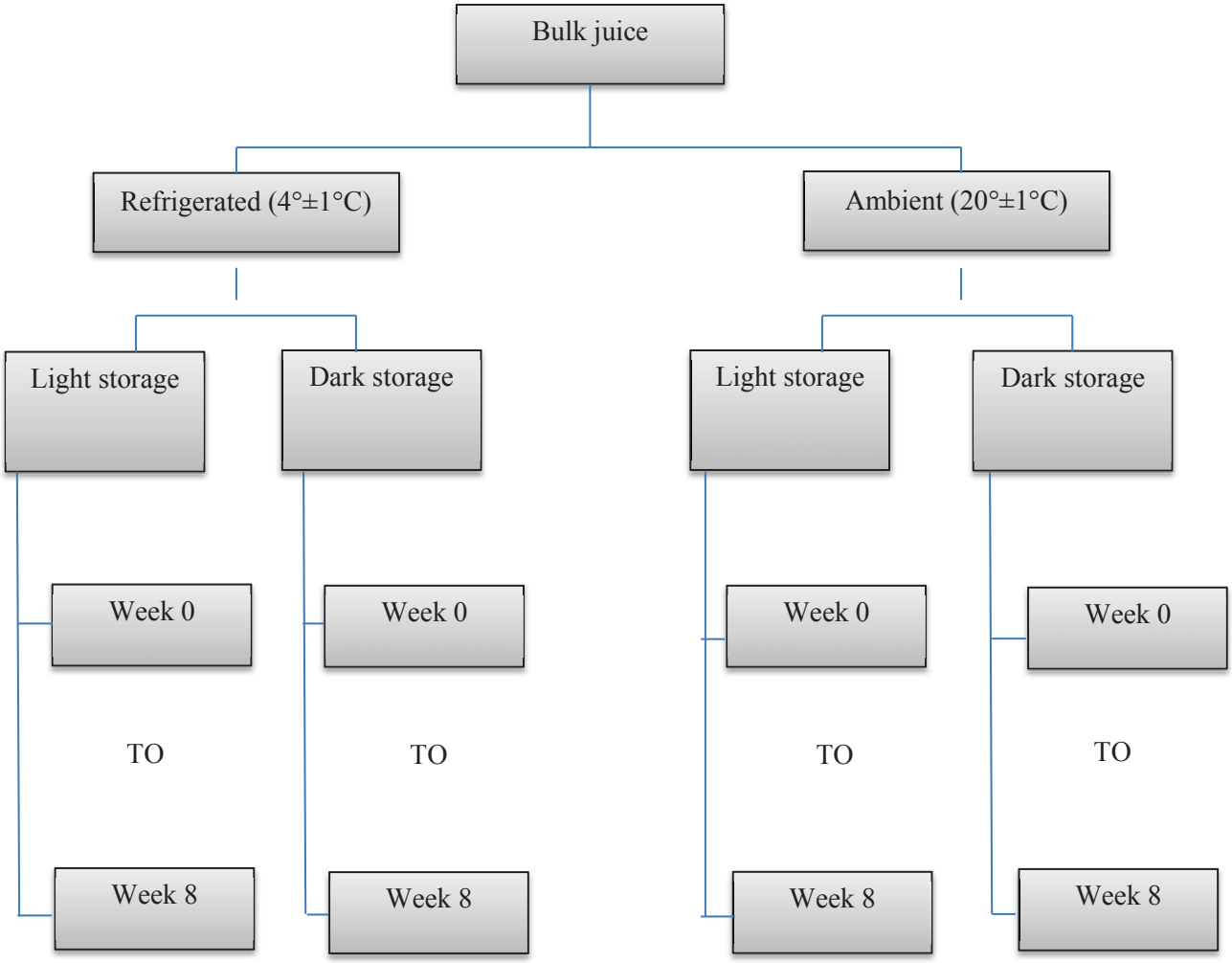

Figure 3.4: Storage trial full balanced factorial experimental design for refrigerated (4±1°C) and ambient (20±1°C) storage time in light and dark conditions.

3.10.2 Storage conditions

The juice blend for the storage trial was stored in 100 ml clear glass bottles with a plastic screw top. Bottles and tops were washed in a dishwasher followed by drying of the bottles in an oven at 120±1°C for 30 mins. The lids were soaked in 95 % ethanol prior to sealing the bottles.

A retail style refrigerated display unit (4±1°C) with clear doors was used for the storage trial. For light storage, the bottles were layered onto an open cardboard tray box and displayed at the front of the refrigerated retail unit to receive natural day light. For dark storage, the bottles were layered in an enclosed cardboard box and stored at the back

(away from daylight) in the same refrigerated unit. Additional black A4 paper was used to cover the storage trial bottles under dark storage to ensure no light would enter the box.

For the 20±1°C storage trial, the bottles were layered in a similar fashion for both light and dark storage in a temperature controlled room. All the bottles at both temperatures were stored at the same distance from each other. A temperature log was maintained and routinely checked (twice a week) for storage temperatures. Bottles were removed from the refrigerated and ambient storage on a weekly basis for sub-sampling and /or immediate analysis.

3.10.3 Storage time

Samples for quality checks such as pH, acidity and °Brix were assessed at 0, 1, 2, 4, 6, 8 weeks of storage, microbiological testing were assessed at 0, 1, 2, 3, 5, 7, 8 weeks of storage and quantification of nitrates and nitrites were assessed every week at 0, 1, 2, 3, 4, 5, 6, 7, 8. Quality testing and microbial testing was not carried out on the same weeks due to time limitations on obtaining microbiological test results prior to sensory assessments to assure product safety.

At each time, bottles were removed from the refrigerated and ambient trial and treated as below

- Samples were tested at weeks 0, 1, 2, 4, 6 and 8 for soluble solids (**Section 3.4.3**), pH (**Section 3.4.1**) and titratable acidity (**Section 3.4.2**) measurements.
- Microbiological testing was conducted at weeks 0, 1, 2, 3, 5, 7 and 8 for total aerobic plate count and yeasts and moulds (**Section 3.5**).
- Nitrates and nitrites were quantified every week (0, 1, 2, 3, 4, 5, 6, 7 and 8) to study the effect of light and temperature on their stability (**Section 3.3**).

The results for each treatment are the average of two bottles. Depending on the analytical method, replication of the test method was also conducted.

CHAPTER 4
JUICE EXTRACTION AND TESTING

The results from the preliminary tests to determine the optimal high nitrate vegetables in New Zealand are presented and discussed in **Section 4.1**. The effects of storage temperature on nitrate and nitrite stability in vegetable juice samples were determined and the results are presented and discussed in **Section 4.2**. The results from juice extraction of different vegetables is presented and discussed in **Section 4.3**.

4.1 Extraction of juice from nitrate rich vegetables in New Zealand

Juices were extracted from selected vegetables using a lab scale juicer as described in **Section 3.6**. The weights of some selected nitrate rich vegetables of New Zealand, volume of retrieved juice and the juice yield (mL juice/g wet weight of vegetable) are presented in **Table 4.1**.

Table 4.1: Juice yield (mL juice/ g wet weight of vegetables) of nitrate rich vegetables from New Zealand

VEGETABLES	TOTAL WEIGHT (g)	JUICE (ml)	JUICE YIELD (mL juice/ g wet weight of vegetable)
Beetroot raw	1021	492	0.48
Beetroot leaves	259	103	0.40
Celery stalk	1061	522	0.49
Celery leaves	351.4	116.1	0.33
Lettuce (Iceberg)	1057	382	0.36
Spinach	1076	271	0.25

The juice yield values of the vegetables in **Table 4.1** were less than 0.50 mL juice/ g wet weight of vegetable since a laboratory bench top juicer was used for all the vegetables. The vegetables were chopped into smaller (2 mm × 2 mm) pieces in a food processor to reduce the particle size so that during juicing, increased juice yields were obtained. In industrial juicers, comminution maximises the juice yield by disrupting cellular structures and exposing a greater surface area for extraction.

The nitrate concentration in the extracted juices was determined by HPLC as described in **Section 3.3**. The nitrate (mg/L) and nitrite (mg/L) concentration in the extracted raw vegetable juices, prior to any heat treatment are shown in **Table 4.2**. Celery and beetroot had significantly higher nitrate and nitrite content than spinach and lettuce (P value= 0.002) (**Table 4.2**).

The content of nitrates and nitrites is determined mainly by the content of these compounds in the raw materials (Goulding, 2000). The difference in nitrate and nitrite concentrations in vegetables could be due to a number of factors. Nitrogen fertilisers are introduced in horticulture with the aim to increase the crop yields (Goulding, 2000). Sometimes, excess of nitrates from fertilisers in the soil induces their accumulation in crops leading to increased nitrate concentrations (Shaviv & Mikkelsen, 1993). Fertilising conditions, plant susceptibility, climate, soil and activity of soil and root micro flora are other factors that influence the content of nitrates and nitrites in field crops (Mills & Jones, 1979). The content of nitrates in stored vegetables is influenced mainly by storage temperature, ventilation, access to day light and microbiological quality of stored vegetables (Smiechowska, 2003). Nitrate content can also vary within species, cultivars and even genotypes with different ploidy (Blom-Zandstra, 1989). The differences between nitrate concentrations in different varieties have been most extensively studied in relation to lettuce where open leaf varieties generally have higher nitrate concentrations than tight-headed varieties such as iceberg (Scientific Committee on Food, 1997).

European Commission Regulation No. 563 (2002) reported that in some regions of Europe, nitrate concentrations in vegetables are frequently higher than those set in the Annex of Regulation (EC) No 466 (2001), although the general trend shows that the nitrate concentrations in lettuce are decreasing (Santamaria, 2006). The use of calcium

cyanamide as a nitrogen source consistently resulted in lower nitrate concentrations in the lettuce compared with calcium ammonium nitrate (Byrne et al, 2001). Lettuce and spinach had significantly lower concentrations of nitrate (lettuce p value = 0.001, spinach p value= 0.003) and nitrite (lettuce p value = 0.009, spinach p value= 0.01) than beetroot and celery (**Table 4.2**). These results are similar to the high nitrate concentrations (mg/kg) for beetroot and celery in comparison to spinach and lettuce in the year 1980 and 2004 New Zealand survey report (**Table 2.3**). Nitrate content in spinach was highly dependent on season and nitrite concentrations (mg/L) were high for beetroot and celery (Santamaria, 1999). Santamaria (1999) observed spinach was higher in nitrate in autumn-winter than in spring (2580 mg/kg compared to 1622 mg/kg) and inner petioles of celery accumulated less nitrate than the outer ones but the difference was not significant between petioles and leaflets.

In fresh, undamaged vegetables, the nitrite concentrations are low, since the conversion of nitrate to nitrite is minimal (Food Standards Australia New Zealand, 2010). However, under improper post-harvest storage conditions, the nitrite concentrations can increase in vegetables as a result of bacterial contamination and endogenous nitrate reductase action (European Food Safety Authority, 2008). It is also suspected that pureeing or juicing releases endogenous nitrate reductase thus causing excessive formation of nitrite particularly in vegetables with high nitrate content (Chan, 2011). It is thus recommended to immediately store vegetables and juice to prevent bacterial contamination and reduction of nitrate to nitrite (Centre of Food safety, 2010).

Results showed (**Table 4.2)** that no significant losses in nitrate and nitrite were observed (p values > 0.05) when raw vegetable juices were heated to 90±1°C for 10 sec in a microwave.

Table 4.2: Nitrate and nitrite concentrations in extracted vegetable (beetroot, beet leaves, celery stalk, cele spinach) raw and heated (90±1°C) juices with p values. Nitrate and nitrite values are mean ± SE (n=3).

VEGETABLES	RAW JUICE	HEATED JUICE	P VALUES (FOR DIFFERENCE BETWEEN RAW AND HEATED JUICES)	RAW JUICE	HE
	NITRATE (mg/L) STANDARDISED TO 10° BRIX	NITRATE (mg/L) STANDARDISED TO 10° BRIX		NITRITE (mg/L) STANDARDISED TO 10° BRIX	NITR (mg/ STAN TO 1
Beetroot	4390 ± 31	4401 ± 35	0.91	377 ± 2	
Beet leaves	4112 ± 29	4156 ± 31	0.63	352 ± 3	
Celery stalk	6534 ± 36	6560 ± 33	0.82	521 ±3	
Celery leaves	6880 ± 12	6978 ± 15	0.41	360 ± 4	
Lettuce (Iceberg)	849 ± 22	846 ± 23	0.94	93 ± 4	
Spinach	993 ± 22	990 ± 20	0.94	96 ± 3	

4.1.1 Informal sensory evaluation of juices

The extracted juice samples were tasted in an informal tasting. Beetroot juice was sweet whereas the beet leaves juice was astringent, had a bitter after taste and a grassy chlorophyll smell. Celery juice had a strong celery smell and taste. Both spinach and lettuce juices were found to be bitter.

Based on this first tasting, it was decided to continue with only beetroot and celery juices for further blending trials. Both had high nitrate concentrations and were not bitter or astringent. Beet leaves are higher in potassium, magnesium, vitamin C, thiamine, riboflavin and vitamin B6 than the root (Musembwa, 2010), and are especially rich in chlorophyll which helps in the cleansing of the lymphatic system (Musembwa, 2010). Therefore celery and beetroot along with their leaves (normally disposed of as waste material) were used to make future blends.

4.2 Impact of storage conditions on nitrate/nitrite concentrations in vegetable juices

The stability of nitrate and nitrite in beetroot juice was determined by storing juice samples at three different temperatures and monitoring nitrate/nitrite concentrations over two weeks**.**

The results in **Table 4.3** show that there was a significant increase in the nitrate concentrations at 4±1°C on the fifth day (4677 ± 11 mg/L), seventh day (4675 ± 16 mg/L) and 14th day (5057 ± 11 mg/L) from the nitrate concentration of 4449 ± 15 mg/L on day zero (p values are presented in **Appendix E**). Significant increases in the nitrate concentrations were also observed at -20±1°C on day seven (4589 ± 9 mg/L) and day 14 (4840 ± 8) from 4419 ±13 mg/L at day zero and at -80±1°C on day 14 (4711 ± 21 mg/L) from 4399 ± 10 mg/L on day zero ($P < 0.05$; **Appendix E**). No significant difference in the nitrate concentrations were observed at -80±1°C for the first seven days, at -20±1°C for the first five days and at 4±1°C over the first two days ($P > 0.05$). The effect of refrigeration (4±1°C) storage on nitrate and nitrite concentrations of spinach, crown daisy, organic Chinese spinach and organic Chinese cabbage has been studied by Chung et al. (2007). Nitrate concentrations in these vegetables were almost

unaffected and remained high in the range of 2830-5270 mg/kg. Nitrate concentrations could increase due to varying storage temperature and food processing methods (Ekart et al., 2013).

Table 4.3: Changes in nitrate (mg/L) concentration in beetroot juice stored at three different temperatures over time. Values are mean ± SE (n=3).

NITRATE (mg/L)			
STANDARDISED TO 10° BRIX			
	TEMPERATURE (±1°C)		
TIME (DAY)	**-80°C**	**-20°C**	**4°C**
0 day	4399 ± 10	4419 ± 13	4449 ± 15
1 day	4405 ± 14	4424 ± 16	4454 ± 15
2 days	4451 ± 9	4467 ± 12	4576 ± 16
5 days	4440 ± 23	4481± 27	4677 ± 11
7 days	4566 ± 5	4589 ± 9	4675 ± 16
14 days	4711 ± 21	4840 ± 8	5057 ± 11

The nitrite concentration (mg/L) of beetroot juice stored at three different temperatures tested over two weeks (**Table 4.4;** p values are presented in **Appendix E)**. Overall, no significant difference ($P > 0.05$) was observed in the nitrite concentration over a period of two weeks at all three storage temperatures (**Appendix E**). It was evident that nitrate in the beetroot juice was not reduced to nitrite during frozen and refrigerated storage. This implies that the activity of endogenous nitrate reductase in vegetables tends to be inactivated under cold storage conditions (Chung et al., 2007). Phillips (1968) reported that during frozen storage of vegetables, nitrite accumulation was inhibited. Cold temperature has been shown to strongly reduce the activity of nitrate reductase in leaves of green vegetables by disturbing the internal electron transport of nitrate reductase

(Yaneva et al., 1996). At higher temperatures, significant microbial reduction of nitrate occurs which leads to the accumulation of high nitrite concentrations (Jones & Griffith, 1965).

Table 4.4: Changes in nitrite (mg/L) concentration in beetroot juice at three different temperatures over time. Values are mean ± SE (n=3).

NITRITE (mg/L) STANDARDISED TO 10° BRIX			
	TEMPERATURE (±1°C)		
TIME (DAY)	-80°C	-20°C	4°C
0 day	110 ± 1	109 ± 2	105 ± 3
1 day	110 ±1	106 ± 2	103 ± 0
2 days	104 ± 3	103 ± 4	100 ± 0
5 days	103 ± 3	102 ± 5	98 ± 4
7 days	99 ± 3	100 ± 2	95 ± 1
14 days	95 ± 2	94 ± 1	92 ± 2

Nitrite concentrations in fresh, uninjured, well stored vegetable tissues have been shown to be extremely low (Ezeagu, 1996; Hunt & Turner, 1994). Chung et al. (2007) suggested that the activity rate of nitrate reductase maintains in equilibrium with one of the nitrate reductase enzymes under proper refrigerated storage conditions. Nitrite concentration remains constant over time under cold storage conditions (≤ 4±1°) due to inhibition of nitrate reductase activity, which converts nitrates to nitrites. Poor storage of vegetables such as that beyond the normal 'use by' dates or at ambient temperatures may result in bacterial growth contributing to high nitrite content due to nitrate reductase activity by microbes (Chung et al., 2007).

The beetroot juice was also tested for quality parameters: pH, titratable acidity and % soluble solids (°Brix) as shown in **Table 4.5**. There was no significant difference ($P > 0.05$) in the pH values over two week storage period (p values are presented in **Appendix E**). A slight pH drop was observed between day zero and day 14, however the difference was not significant. The pH drop could be due to any microbial growth. This correlates with the titratable acidity values, where a slight increase was observed on the 14th day compared to day zero, however the difference was not significant ($P > 0.05$). The total soluble solids remained constant throughout the two week storage period.

Table 4.5: The pH, titratable acidity and % soluble solids of raw beetroot juice over two weeks (Storage temperature = -20±1°C, Testing temperature = 25±1°C). Values are mean ± SE (n=3).

BEETROOT	**pH**	**TITRATABLE ACIDITY (g citric/100ml)**	**% SOLUBLE SOLIDS (°BRIX)**
0 day	6.9 ± 0.00	0.19 ± 0.03	11.1 ± 0.00
1day	6.89 ± 0.00	0.19 ± 0.03	11.1 ± 0.00
2 days	6.24 ± 0.1	0.20 ± 0.02	11.2 ± 0.1
5 days	6.24 ± 0.1	0.20 ± 0.03	10.9 ± 0.1
7 days	6.25 ± 0.00	0.20 ± 0.00	11.00 ± 0.00
14 days	6.19 ± 0.00	0.20 ± 0.00	11.1 ± 0.00

4.3 Pilot Plant production of beetroot, celery and beet leaves juices

For scale up production, 30 kg of beetroot, 5.36 kg beet leaves including stalks and 10 kg celery were juiced on four different days followed by pasteurisation and appropriate storage as explained in **Section 3.6**. **Table 4.6** shows the volume of juice (ml) recovered and the juice yield (mL juice/ g wet weight of vegetables). Beetroot juice yields were found to be lower in these trials compared to the preliminary trials (**Section 4.1**). This is likely due to the sourcing of beetroot and celery from different suppliers.

Table 4.6: Juice yield (mL juice/ g wet weight of vegetables) of beetroot, beet leaves and celery

VEGETABLES	TOTAL WEIGHT (g)	JUICE (ml)	JUICE YIELD (mL juice/ g wet weight of vegetables)
Beetroot lot 1	9821	2720	0.28
Beetroot lot 2	9670	2600	0.27
Beetroot lot 3	9624	2600	0. 27
Beet leaves	5358.5	2550	0.48
Celery	9589	5610	0.59

It was observed from informal tasting that the beetroots from Freshmax (**Figure 4.1-B**) were fresh, rich in colour, had a firm texture and long crisp leaves as compared to the beetroots purchased from a supermarket (**Figure 4.1-A**) which had soft texture and wilted leaves indicating the beetroots had been out of the ground for several days before they were purchased.

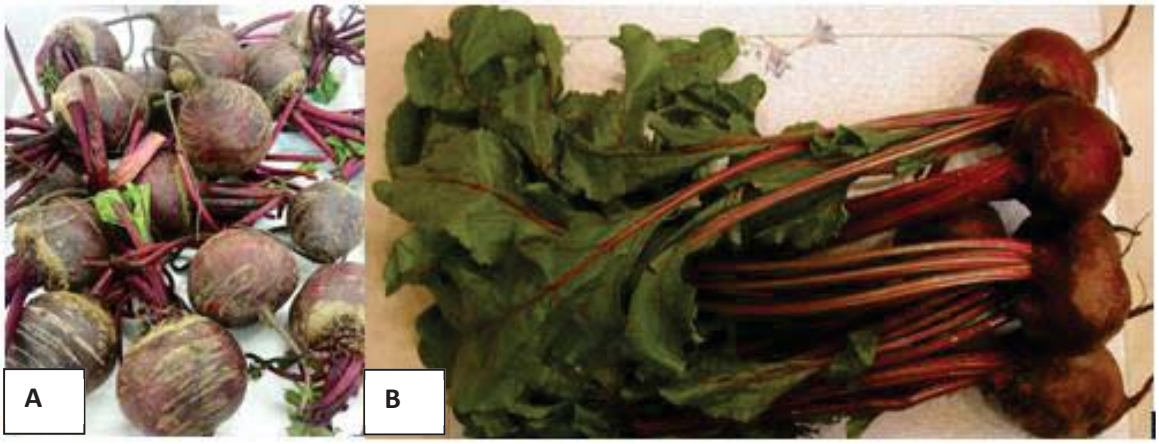

Figure 4.1 (A) Left photo : Beetroots purchased from supermarket **(B) Right photo:** Beetroots purchased from Freshmax NZ Ltd.

The softer texture of the beetroots from the supermarket was easier to chop in the food processor whereas the firmer beetroots resulted in a lower juice yield and greater pulp waste content from the juicer.

Beet leaves are removed from the beetroots before they are sold in the supermarket. Beet leaves have a higher water content compared to the beetroot itself and hence they had higher juice yield values (**Table 4.6**). Beet leaves were also easier and faster to juice as compared to the beetroot. The yield of beet leaves from Freshmax NZ Ltd (48 %) was higher than the yield of beet leaves from the supermarket (40 %) because the leaves supplied by Freshmax (**Figure 4.2 B**) were fresher, longer and crisper as compared to the beet leaves from supermarket (**Figure 4.2 A**).

Figure 4.2 (A) Left photo: Beet leaves/stalks purchased from supermarket **(B) Right photo:** Beet leaves purchased from Freshmax NZ Ltd

4.3.1 Pasteurisation of vegetable juices

Individual juices were pasteurised at 90±1 °C for 15 s. The flow rate of juice in the pasteuriser was maintained at 1 L/min. Samples were collected before and after pasteurisation and tested for pH, acidity, % soluble solids, nitrates and nitrites within 48 hrs of pasteurisation. Samples were stored at -20±1°C before analysis. All samples were tested on the day of pasteurisation for total bacteria plate counts and yeast and mould counts.

4.3.2 Microbiological evaluation of vegetable juices

Beetroot juice (lots: 1, 2 and 3), celery juice and beet leaves juice before and after pasteurisation were tested for total plate count (TPC) and yeasts and moulds (Y & M). The results of the microbiological evaluation of TPC and yeasts and moulds of beetroot, celery and beet leaves juice are presented in **Table 4.7**.

Table 4.7: Microbiological test results for beetroot, beet leaves and celery juices before and after pasteurisation. Values are mean (n=3)

BEETROOT JUICE	BEFORE PASTEURISATION		AFTER PASTEURISATION	
	TPC cfu/ml	Y &M cfu/ml	TPC cfu/ml	Y &M cfu/ml
LOT 1 (10kg)	5.0×10^6	38	<50	<10
LOT 2 (10 kg)	4.2×10^6	26	<50	<10
LOT 3 (10 kg)	5.9×10^6	41	<50	<10
Celery	8.9×10^3	99	<50	<10
Beet leaves	4.8×10^6	110	<50	<10

cfu/ml=colony forming units per ml of sample. TPC-Total Plate Count; Y & M- Yeasts and Mould

As expected a significant reduction in the microbial counts were observed post pasteurisation. The recommended maximum limit for aerobic plate counts according to the Microbiological Reference criteria for Food (New Zealand Food safety Authority, 1995) is 10^5 cfu/ml of sample at 35°C (**Section 3.5**). The results obtained for pasteurised juice were below the recommended maximum limit for beetroot, celery and beet leaves juice.

Eight randomised beetroot juice samples were sent to an external laboratory for Total coliforms test and the results are attached in **Appendix F**. A significant reduction to from > 1500 cfu/ml (Beetroot raw juice-A&B) to <1 cfu/ml (Beetroot pasteurised juice-C&D) in coliform counts was found suggesting that most of the coliforms were killed during the pasteurisation process. As coliform organisms can be easily killed by heat, these bacteria can also be used as an indicator of heat treatment failure as well as post heat treatment contamination (Chong, 2008). However pasteurisation does not kill all the bacteria and hence post storage conditions should be followed to ensure product safety (Morgan et al., 1996).

Pasteurisation at over 70 °C for 15 s should inactivate vegetative spoilage organisms such as yeasts, mould and *Lactobacillus fermentum* in a high acid juice. Fruit juices have a natural pH below 4.5 so are not high risk, but low acid juices such as pear juice, banana puree, and un-acidified beetroot juice (pH= 6.3) would be a higher risk. More severe conditions such as 87 °C for 15 s would be needed for inactivation of spoilage organisms in un-acidified juices (Wilbey, 2003a, 2003b).

Table 4.7 also shows microbial reduction in celery and beet leaves post pasteurisation. Raw celery juice had a comparatively lower total plate count than raw beetroot and beet leaves juice. This may be because celery grows above ground level making it less prone to contamination from microbes in the soil. The vegetables were washed thoroughly but not peeled before juicing to reduce nitrate loss. Beetroot had more soil attached to them than celery hence they would be more prone to higher microbial counts than celery. In general root crops are more susceptible to contamination than leaf crops such as lettuce. Some bacteria such as *E coli O 157:H7* survive longer in close proximity to the root area of certain plants, rhizosphere (Johannessen et al., 2005). The results shown here indicate that after pasteurisation, all the bacterial counts were within limits (10^5 cfu/ml of sample at 35°C) for beetroot, celery and beet leaves juice (New Zealand Food safety Authority, 1995).

The pH of the product significantly affects the lethality of heat treatment. Less heat is needed to inactivate microorganisms as the pH is reduced or increased from their optimum pH of growth which is generally pH 7.0 (Jay, 1996, Bari et al., 2005). The aim of the thermal processing is to prevent microbial and enzymatic activities in the final product. Food with a pH greater than pH 4.5 is considered to be a low acid food and microorganisms such as *Clostridium botulinum* can grow under these conditions. This organism requires heating to 121.11°C for the required length of time for destruction. If the pH of the product is lower than pH 4.5 then milder heat treatments (100°C or less) are effective (Bari et al., 2005).

4.3.3. Quantification of nitrates and nitrites and evaluation of quality parameters of vegetable juices

The results obtained from quantification on HPLC for nitrates (mg/L) and nitrite (mg/L), the quality readings derived for pH, titratable acidity and total soluble solids (°Brix) are presented in **Table 4.8**.

Table 4.8: Nitrates (mg/L), Nitrite (mg/L), pH, titratable acidity and °Brix results of beetroot, beet leaves and celery juice from the pilot plant trial. Data are means±SE (n=15) for nitrates and nitrites and data are means±SE (n=3) for pH, titratable acidity and °Brix.

JUICE	NITRATES (mg/L)	NITRITES (mg/L)	pH	TITRATABLE ACIDITY (g citric/100ml)	°BRIX
Beetroot lot 1	1417 ± 1	96 ± 1	6.34 ±0.1	0.20 ± 0.01	11.2 ± 0.1
Beetroot lot 2	1266 ± 1	59 ± 1	6.3 ± 0.1	0.20± 0.02	11.1 ± 0.1
Beetroot lot 3	1294 ± 1	47 ± 1	6.32± 0.1	0.20± 0.01	11.00 ± 0.1
Celery	1765 ± 2	20 ± 1	6.33 ±0.1	0.20± 0.01	3.5 ± 0.1
Beet leaves	953 ± 1	57 ± 1	6.27 ±0.1	0.20 ± 0.03	4.3± 0.1

It was observed that there were no significant differences between the pH ($P > 0.05$) and acidity ($P > 0.05$) values between beetroot, beet leaves and celery juice. Total soluble solids for celery and beet leaves were lower than beetroot. Reams (2005) explain the °Brix of 6.00 is considered average °Brix for celery and a °Brix of 10-11 is considered

acceptable for beetroot. Nitrate and nitrite concentrations in beetroots and celery purchased from growers were significantly lower than the nitrate and nitrite concentrations observed from the vegetables purchased from supermarket.

Since the pH of the juice was close to neutral and no preservatives were added, it was essential to acidify the juice with an acidity regulator to prevent microbial growth over time. Blending of different juice with acidification and flavour balance are explained in Chapter 5. All juices were stored frozen at -20±1°C until further blending.

CHAPTER 5

JUICE BLENDING & CONSUMER SENSORY EVALUATION

Chapter 5 describes the blending of different juice formulations (beetroot, beet leaves, celery), produced as described in Chapter 4, with single strength crisp apple juice and other flavours. Selected blends were tested by consumer sensory evaluation for overall product liking, acidity, sweetness and overall flavour.

5.1 Factors to be considered before blending juices

Unless a single varietal juice is required, blending of different juices can be carried out to off-set the high cost of some juices (Bates et al., 2001), to balance out undesirable flavours (for e.g. strong earthy flavour of beetroot juice), to correct low soluble solids level (°Brix), to give a desirable colour stability or to adjust the juice's °Brix/Acid ratio (Ashurst, 1999). Blending also helps in adjusting compositional imbalances in the juice from a single harvest or cultivar which can influence the quality of the juice (Ashurst, 1999).

One of the primary considerations in choosing individual components and preparing juice blends is the °Brix/Acid ratio (Harrill, 1998). Depending on the juice involved in the blending process, this ratio determines the sugar and acid balance and influences the perception of sweetness and sourness in the juice (Ashurst, 1999; Shachman, 2005). For example, a 10 % solution of sucrose is moderately sweet. An addition of 1 % citric acid for a °Brix/Acid ratio of 10, produces an intensely sour sensation (Harrill, 1998). The solution requires a few percent more sugar before the sensation of sweetness is dominant (Ashurst, 1999). Hence the final juice blends were made to a similar °Brix/Acid ratio of vegetable/fruit juices in the market.

The °Brix/Acid ratio of vegetable and fruit juices from the supermarket tested at Massey University is listed in **Table 5.1**.

Table 5.1: °Brix/Acid ratios of some commercial vegetable and fruit juices

VEGETABLE/ FRUIT JUICE	°BRIX/ACID RATIO
V8 vegetable juice (Campbell's Food Service Ltd)	10 ± 0.1
BEET IT (James White Drinks Ltd)	10.5 ± 0.7
Keri Pineapple juice (Coca-Cola Ltd)	10.5 ± 0.1
Keri Tomato juice (Coca-Cola Ltd)	8 ± 0.5
James White Carrot juice (James White Drinks Ltd)	8.5 ± 0.5
Fresh Up Crisp Apple juice (Frucor Beverages Ltd)	10 ± 0.1

As shown in **Table 5.1**, the average °Brix/Acid ratio of vegetable and fruit juices is 9 ± 1. A secondary consideration is the pH of the juice or beverage. To discourage microbial spoilage and hence achieve a longer shelf life, the pH of the juice should be less than 4.5 (Koutsoumanis et al., 2006).

The spores of *Clostridium botulinum* are prevalent in most soils and contaminate many types of vegetables (Bates et al., 2001). To grow, the spores require a pH above 4.5 and anaerobic condition; they produce toxins which potentially could be fatal if consumed (Koutsoumanis et al., 2006). Washing vegetables prior to juicing will minimize the number of spores in the juice and reducing the pH by adding an acidic juice like lemon or lime juice or organic acids (citric, malic and tartaric acids) are recommended (Bates et al., 2001; Mander & Liu, 2010; Raju & Bawa, 2006). Citric acid is found in many citrus fruits such as orange, lemon, lime and grapefruit. These juices are reported to blend well with flavour systems and bring out natural flavours (Bigelis & Tsai, 1995; Mander & Liu, 2010). Lemon juice with its naturally occurring citric acid is not only used as a general acidulant but also flavour enhancer, pH regulator and preservative (Bigelis & Tsai, 1995).

Pasteurisation along with the added acid can assist with the preservation of juices by killing most of the spoilage and pathogenic microorganisms (Duan, 2012). Some pathogenic bacteria, including *Escherichia coli, Listeria monocytogenes*, and

Salmonella species are resistant to acid and low pH (Duan, 2012). Outbreaks of these pathogens have occurred in acid foods that were not thermally processed with pH values below 4.5, such as apple cider and orange juice (Bates et al., 2001).

Vegetable juices like beetroot, beet leaves and celery juices which were used in this study were pasteurised prior to blending, then acidified with heat treated lemon juice in clean aseptic conditions to avoid microbial contamination. Good Manufacturing Practices (GMPs) were followed during the blending process of all the vegetable juices in the laboratory.

5.2 Vegetable juice formulation development

The ingredients used for blending were pasteurised beetroot, beet leaves and celery juices (**Section 4.3**), lemon juice concentrate (45 °Brix) and clarified apple juice (Fresh Up) plus natural flavours such as apple, lemon, orange (**Section 3.2**). Apple, lemon and orange flavours were added because similar combinations were used for the commercial high nitrate products on the market and also to help mask the earthy flavour of the beetroot.

A mixture design from MINITAB 16 was used to determine different blending combinations with the various ingredients (**Appendix G**). The mixture design used dependent variables of nitrate content (mg/L) and % soluble solids (°Brix). Based on the mixture-method design, varying proportions of beetroot, beet leaves, celery and apple juice were substituted within a formulation. Fresh Up crisp apple juice was used for blending because it provided a mild sweet taste contributing the appropriate soluble solids (10-11°Brix) and contained negligible concentrations of nitrates and nitrites. All blends were produced in duplicate.

5.2.1 Vegetable juice blend and acidulant

Eight formulations (300 mls each) with a nitrate content of greater than or equal to 1.5 g/L and a soluble solids content of 10 to 11 °Brix (**Table 5.2**) were formulated for the first taste trial. Previous studies have shown that a nitrate content of greater than 1 g/L have been associated with sports related benefits (**Table 2.7**) (Bailey et al., 2009; Kenjale et al., 2011; Lansley et al., 2011; Vanhatalo et al., 2011). Since nitrate converts

to nitrite over time, a concentration of at least 1.5 g/L nitrate was targeted for in the formulation to ensure the concentration of nitrates required were retained in the juice over the entire shelf life period.

Table 5.2 shows the 16 formulations (150 ml each) combining vegetable juices such as beetroot, beet leaves and celery juices with apple juice to achieve a nitrate concentration of greater than 1.5 g/L and 10-11 °Brix. **Table 5.2** also shows the measured pH and °Brix values and nitrate concentration for the 16 formulation blends.

Table 5.2: Formulation blends calculated to achieve nitrate concentration ≥1.5g/L and 10-11°Brix. Value tests (pH, nitrate content and °Brix).

	PERCENTAGE (% weight/ volume of juice)						
FORMULATIONS	**BEETROOT JUICE (11.2 ° BRIX)**	**BEET LEAVES JUICE (4.5 °BRIX)**	**CELERY JUICE (3.2°BRIX)**	**APPLE JUICE (10.5°BRIX)**	**LEMON JUICE CONCENTRATE (45 ° BRIX)**	**TOTAL %**	**pH A**
1	85.3	4	0	9.88	0.82	100	
2	74.4	0	14.88	9.9	0.82	100	
3	59.5	0	29.75	9.93	0.82	100	
4	64.4	4.0	20.8	9.98	0.82	100	
5	61.1	26.2	10.9	0.99	0.82	100	
6	77.4	6.88	0	14.9	0.82	100	
7	64.5	19.8	0	14.9	0.82	100	
8	71.4	12.9	0	14.9	0.82	100	
9	85.35	4	0	9.9	0.74	100	
10	74.4	0	14.86	10	0.74	100	
11	59.6	0	29.8	9.86	0.74	100	
12	64.5	4	20.8	9.96	0.74	100	
13	61.1	26.2	10.96	1	0.74	100	
14	77.4	7	0	14.86	0.74	100	
15	64.5	19.85	0	14.9	0.74	100	
16	71.5	12.9	0	14.86	0.74	100	

In total 16 formulation blends were presented for informal tasting to two juice tasting experts. The samples were presented in random order for tasting and participants were asked to rate the blends for overall product liking on a hedonic scale of 1-9.

Table 5.3: Overall product liking scores for vegetable juice formulations

FORMULATION #	TASTE EXPERT 1	TASTE EXPERT 2	AVERAGE PRODUCT LIKING SCORES (Scale 1-9)
1	6	8	7
2	3	3	3
3	4	4	4
4	3	3	3
5	2	2	2
6	4	6	5
7	2	2	2
8	3	3	3
9	6	8	7
10	2	4	3
11	2	2	2
12	4	4	2
13	5	3	4
14	5	5	5
15	3	3	3
16	4	4	4

Scale of 1-9 (1= Dislike extremely, 2=Dislike very much, 3= Dislike moderately, 4= Dislike slightly, 5= Neither like or dislike, 6= Like slightly, 7=Like moderately, 8=Like very much, 9=Like extremely)

Samples were presented in a random order. As shown in **Table 5.3,** the blends from formulations 1 and 9 were preferred over the other samples. Formulations 1 was a blend of 85.3 % beetroot juice, 4 % beet leaves juice, 9.88 % apple juice and 0.82 % lemon juice concentrate and formulation 9 was a blend of 85.35 % beetroot juice, 4 % beet leaves juice, 9.9 % apple juice and 0.74 % lemon juice concentrate. Formulations 6

(blend of 77.4 % beetroot juice, 6.88 % beet leaves juice, 14.9 % apple juice and 0.82 % lemon juice concentrate) and 14 (blend of 77.4 % beetroot juice, 7 % beet leaves juice, 14.86 % apple juice and 0.74 % lemon juice concentrate) were neither liked nor disliked. As the percentage of beet leaves juice was increased in the formulation, the liking of the formulation decreased, likely due to an increase in bitterness from the beet leaves. In the past, the juice from beet leaves has been described as pungent, grassy, chlorophyll like odours leaving a bitter after taste by trained panellists (Bianchi et al., 2010). The bitter taste of the juice containing 4 % beet leaves juice in the formulation appeared to be masked by the higher proportion of other juices and the lemon juice concentrate.

All the formulations containing celery juice had a strong celery flavour and scored below 5, and were disliked by the panellists. It was, therefore, decided to reject all the formulation blends containing celery juice as an ingredient. Celery has been described as slightly sweet and has been associated with bitter aromatics (Bianchi et al., 2010). The compounds 3-isobutylidene-3a, 4-dihydrophthalide; 3-isovalidene-3a,4-dihydrophthalide; cis-3-hexen-1-yl pyruvate and diacetyl in celery have been associated with strong celery flavour and aroma in combination with other juice blends (Gold & Wilson, 1963).

Based on the scores in **Table 5.3**, formulation 1 with a lemon juice concentrate (0.82 % weight per volume of juice) was selected and formulation 9 was rejected for further development. Formulation 1 also had a slightly lower pH.

Comments from the tasters suggested that further re-formulation was required in order to increase the acidity of the product with more lemon juice concentrate to ensure the pH was below 4.5 and also to incorporate additional flavours to improve the overall flavour of the juice.

5.2.2 Further development of recipe

Table 5.4 shows eight formulations formulated with two varying concentrations of lemon juice concentrate (1.00 % and 1.5 %, weight per volume of juice) with a combination of different added flavours: orange, lemon and apple (**Table 3.3**). All formulations were made to 100 % (ml ingredients/100 ml) with a nitrate content of at

least 1.5 g/L nitrate and soluble solids content 10-11°Brix. Tastings were conducted again by taste experts to who rated the juice blends on a 9 point hedonic. The ratings of individual formulations are shown in **Table 5.5**. It was observed that all the formulations containing the lemon flavour were disliked compared to the formulations with the apple and orange flavours. Formulations containing the natural lemon flavour were perceived to have a synthetic artificial taste which was not found to be acceptable. A combination of the lemon and orange flavour in formulations 4 and 8 were also disliked having a score of below 5.

Formulation blends 1 and 6 were liked slightly compared to the other formulations (**Table 5.5**). The amount of lemon juice concentrate in formulation 5 was too high, as it was perceived as too acidic, the formulation had a mean score of 5 which meant it was neither liked nor disliked by the taste experts. It was decided to reduce the amount of lemon concentrate from 1.5 % weight per volume of juice to 1.25 % weight per volume of juice. Formulations 1 and 5 had the same juice base except for the amount of lemon juice concentrate, which explains the difference between the overall liking scores. Formulations 2 and 6 were also the same juice base with different concentrations of lemon juice concentrate. The panellist's commented that, the blend from formulation 2 did not taste very acidic but had a strong orange flavour.

Hence it was decided to reduce the lemon juice concentrate in formulation 5 to 1.25 % weight per volume of juice. For formulation 2, it was decided to increase the lemon juice concentrate to 1.25 % weight per volume of juice and decrease the orange flavour to 0.03 %. Final formulations for informal consumer tasting evaluation are presented in **Table 5.6**.

Table 5.4: Formulation blending development II from MINITAB based on nitrate ≥1.5g/L and 10-11°Br
(n=3) *

FORMULATIONS	BEETROOT JUICE (11.2 ° BRIX) %w/w	BEET LEAVES JUICE (4.5 ° BRIX) %w/w	APPLE JUICE (10.5 °BRIX) %w/w	APPLE FLAVOUR %w/w	LEMON FLAVOUR %w/w	ORANGE FLAVOUR %w/w	LEMON JUICE CONCENTRATE (45 ° BRIX) %w/w
1	84.8	4	10	0.2	0	0	1
2	84.95	4	10	0	0	0.05	1
3	84.93	4	10	0	0.07	0	1
4	84.92	4	10	0	0.04	0.04	1
5	84.3	4	10	0.2	0	0	1.5
6	84.42	4	10	0	0	0.05	1.5
7	84.42	4	10	0	0.08	0	1.5
8	84.4	4	10	0	0.06	0.04	1.5

Table 5.5: Overall product liking scores for batch II vegetable juice formulations.

FORMULATION #	TASTE EXPERT 1	TASTE EXPERT 2	AVERAGE PRODUCT LIKING SCORES (Scale 1-9)
1	6	6	6
2	5	5	5
3	4	2	3
4	4	4	4
5	5	5	5
6	6	6	6
7	2	2	2
8	4	2	3

Scale of 1-9 (1= Dislike extremely, 2=Dislike very much, 3= Dislike moderately, 4= Dislike slightly, 5= Neither like or dislike, 6= Like slightly, 7=Like moderately, 8=Like very much, 9=Like extremely)

Table 5.6: Final formulations for consumer sensory evaluation

INGREDIENTS	APPLE FLAVOUR LOW ACID %w/w	ORANGE FLAVOUR LOW ACID %w/w	APPLE FLAVOUR HIGH ACID %w/w	ORANGE FLAVOUR HIGH ACID %w/w
BEETROOT JUICE	84.8	84.72	84.55	84.45
BEET LEAVES JUICE	4	4	4	4
FRESH UP CRISP APPLE JUICE	10	10	10	10
LEMON JUICE CONCENTRATE	1	1.25	1.25	1.5
APPLE FLAVOUR	0.2	0	0.2	0
ORANGE FLAVOUR	0	0.03	0	0.05
TOTAL (%)	100	100	100	100

The blends made from each of the formulations were analysed for microbial counts before being presented for sensory evaluation to ensure the blends were under the acceptable limits for total plate counts. **Table 5.7** summarises the quality parameters (pH, acidity titratable acidity, total soluble solids), nitrates and nitrites concentration and microbial counts of the four formulations (**Table 5.6**).

Table 5.7: Summary of quality parameters, nitrates (mg/L) and nitrites (mg/L) and microbial counts (TPCs and Y &M) of final formulation before sensory evaluation. Values are Mean ± SE (n=3)

Formulations	**pH**	**Titratable acidity g/100 ml (as citric acid)**	**Total soluble solids (°Brix)**	**Nitrates (mg/L)**	**Nitrites (mg/L)**	**TPCs (cfu/ml)**	**Y & M (cfu/ml)**
Apple Flavour low acid	3.9 ±0.1	0.5±0.1	10.8±0.1	1556 ± 6	91± 1	<50	<10
Orange Flavour low acid	3.8 ±0.1	0.5±0.1	10.7±0.1	1572± 5	89± 1	<50	<10
Apple Flavour high acid	3.65±0.1	0.51±0.1	10.9±0.3	1566± 5	92± 1	<50	<10
Orange Flavour high acid	3.5 ±0.1	0.52±0.1	10.8 ±0.1	1570 ± 2	90± 1	<50	<10

5.3 Consumer sensory evaluation of formulated nitrate rich juice beverages

The final four formulation blends presented in **Table 5.6** and a commercial product containing high concentrations of nitrates were evaluated with consumer sensory evaluation. The commercial product was BEET IT, which is a combination of 85 % beetroot juice and 15 % apple juice.

A randomised complete block design was used for the vegetable juices presented to consumers during the sensory evaluation to minimise the effects of uncontrollable sources of variation or error and to eliminate bias. The data obtained from sensory evaluation was to be

used to determine which juice samples were least and most preferred on a hedonic level. Nine point and seven point scales were used to evaluate the overall product liking and attributes such as flavour, acidity and sweetness liking, respectively.

The recommendation for the number of consumers required for consumer sensory evaluation of a product is minimum sixty (Lawless & Heymann, 1988). Seventy consumers (36 males and 34 females) participated in this study. Consumer tests typically involve 100 or more participants but as the participants recruited for this sensory evaluation were from the gym sector and Massey university they could as well be called in house non trained consumers (30-50 needed normally) (Watts et al., 1980).

5.4 Consumer sensory evaluation results

Five formulations namely *apple flavour low acid*, *orange flavour low acid*, *apple flavour high acid*, *orange flavour high acid* and *BEET IT* were assessed by consumers for different attributes including overall product liking, sweetness liking, acidity liking and flavour. The mean score results of attribute liking for the five formulation blends measured from the consumer sensory evaluation are represented in **Table 5.8**.

Table 5.8: Mean scores of 'liking' for five formulations used for consumer sensory evaluation. Values are mean scores ± SE (n=70)

SAMPLE	OVERALL PRODUCT LIKING	ACIDITY LIKING	SWEETNESS LIKING	FLAVOUR LIKING
Apple flavour low acid	5.9 ± 0.2^{a}	4.9 ± 0.1^{a}	4.8 ± 0.2^{a}	4.6 ± 0.2^{a}
Orange flavour low acid	7.0 ± 0.2^{b}	5.0 ± 0.1^{a}	4.3 ± 0.2^{b}	5.0 ± 0.2^{a}
Apple flavour high acid	5.7 ± 0.2^{a}	4.6 ± 0.2^{a}	4.6 ± 0.2^{a}	4.5 ± 0.2^{a}
Orange flavour high acid	6.1 ± 0.2^{c}	4.8 ± 0.2^{a}	5.0 ± 0.2^{a}	4.9 ± 0.2^{a}
BEET IT	5.0 ± 0.2^{d}	4.2 ± 0.2^{b}	4.3 ± 0.2^{b}	3.9 ± 0.2^{b}

Mean ±SE. Similar letters in each column represent that there is no significant difference (P > 0.05) between the mean scores of the formulations.

It was observed that the *orange flavour low acid* formulation was the most liked over-all, having a mean overall product liking score of 7.0 ± 0.2 whereas the commercial product, BEET IT had the lowest score of 5.0 ± 0.2 and was the least liked formulation presented to the consumers. There was no significant difference in the mean scores for overall product liking between the *apple flavour low acid* and *apple flavour high acid* formulation (P > 0.05). A significant difference was observed between *apple flavour high acid* and *orange flavour high acid* (P <0.05), and between *apple flavour low acid* and *orange flavour high acid* formulation. However, based on the mean scores, it was observed that orange flavour was preferred over the apple flavour in both high and less acid formulation blends. A significant difference in the mean scores for overall product liking was also observed between *BEET IT* and *apple flavour low acid* (P <0.05), *orange flavour low acid* (P < 0.05) and *orange flavour high acid* (P < 0.05).

There was no significant difference between the mean scores for acidity for *apple flavour low* and *high acid* and *orange flavour low* and *high acid*. However, these formulation blends were significantly different from *BEET IT* in terms of acidity (P < 0.05) (**Appendix H**). A mean sweetness score for *orange flavour low acid* and *BEET IT* was 4.3±0.2. However these were the least liked for sweetness as compared to the apple flavour less and high acid and orange flavour high acid formulation blends. For overall flavour liking, no significant difference (P > 0.05) in the mean scores was observed for the *apple* and *orange low* and *high acid* formulations. It was, however, observed that the flavour liking for *BEET IT* was the least preferred (3.9 ± 0.2) and there was a significant difference between the flavour preference between *BEET IT* and both the *orange flavour low* and *high acid* formulations (P < 0.05). Consumers were also asked if they had consumed beetroot juice before. From the completed sensory forms, 95 % of the population had never tried beetroot juice before.

5.5 Statistical analysis of results

The Ryan-Joiner test for normality was used to determine the normality of the data collected from 70 consumers for each attribute tested (**Appendix I**).

Critical values for the Ryan-Joiner test of normality from the MINITAB reference manual (Ryan & Joiner, 1976) are tabulated in **Appendix J**. The summary of test statistic values (r)

from the Ryan Joiner normality plots which indicated how close the population represents a normal distribution is shown in **Table 5.9**. The formula for Ryan Joiner test in **Appendix J** gives 0.986 as the critical value that captures lower-tail area 0.10 under the r sampling distribution curve when n=70, and the underlying distribution is actually normal.

Table 5.9: Test statistic values of attributes for final formulations

	TEST STATISTIC VALUE (r)			
Formulations	**Overall Product Liking**	**Acidity Liking**	**Sweetness Liking**	**Flavour Liking**
Apple flavour low acid	0.990	0.996	0.988	0.984*
Orange flavour low acid	0.996	0.999	0.993	0.995
Apple flavour high acid	0.984*	0.995	0.985*	0.986
Orange flavour high acid	0.980*	0.989	0.985*	0.981*
Beet It	0.996	0.998	0.998	0.995

*less than critical value (0.986) calculated from **Appendix J**

The data is normally distributed for all samples except for the *orange flavour high acid* formulation (**Appendix I**). For overall product liking and sweetness liking, all formulations except *apple flavour high acid* and *orange flavour high acid* were normally distributed (**Table 5.9**). For acidity liking, the r values of all samples were greater than 0.986 which confirms that the data was normally distributed for all samples (**Appendix I**). All formulations except *apple flavour low acid* and *orange flavour high acid* were normally distributed. The P values derived from normality plots in **Appendix I** were higher than 0.05 for the 'not normally distributed data' for the above formulations and hence it is customary to state that the result was not statistically significant though some of the r values from **Table 5.9** were less than 0.986.

Analysis of Variance (ANOVA) of sensory data was conducted to determine if there were any significant differences in the results obtained for each product attribute such as overall product liking, sweetness, acidity and flavour liking. (More details in **Section 3.9.6**). The F value for samples with 4 Degree of Freedom (DF) in the numerator and 345 DF in the denominator at $P<0.05$ is 0.000 (**Table 5.10**).

Table 5.10: ANOVA results for overall product liking

Overall Product Liking	DF	Seq SS	Adj MS	F	TABULAR (P<0.05)
Sample	4	136.069	34.017	11.18	0.000
Error	345	1050.00	3.043		
Total	349	1186.069			

The calculated F value must exceed the Tabular F value in order to be considered significant at the 5 % level. Since the calculated F value of 11.18 exceeded the tabular F value of 0.000, it was concluded that there was a significant difference among the mean hedonic scores for overall product liking between samples.

ANOVA was also conducted for acidity, sweetness and overall flavour liking (**Table 5.11**). Since the calculated F values for acidity liking (F=3.45), sweetness liking (F=2.56) and flavour liking (4.68) exceeded the tabular F values of 0.009, 0.0038 and 0.001, respectively (**Table 5.11**), it was concluded that there was a significant difference (P<0.05) between *apple flavour high acid, orange flavour high acid, apple flavour low acid, orange flavour low acid* and *BEET IT* for acidity (F =0.009), sweetness (F= 0.0038) and flavour (F = 0.001) liking.

A Tukey Simultaneous test was performed on overall product liking, acidity liking, sweetness liking and flavour liking. Individual P values were determined from the Tukey test and tabulated in **Appendix H**.

Table 5.11: ANOVA results for acidity, sweetness and flavour liking

ACIDITY LIKING	**DF**	**SEQ SS**	**ADJ MS**	**F**	**TABULAR (P<0.05)**
Sample	4	26.429	6.607	3.45	0.009
Error	345	660.786	1.915		
Total	349	687.214			
SWEETNESS LIKING	**DF**	**SEQ SS**	**ADJ MS**	**F**	**TABULAR (P<0.05)**
Sample	4	22.383	5.596	2.56	0.038
Error	345	753.457	2.184		
Total	349	775.840			
FLAVOUR LIKING	**DF**	**SEQ SS**	**ADJ MS**	**F**	**TABULAR (P<0.05)**
Sample	4	47.811	11.953	4.68	0.001
Error	345	881.286	2.554		
Total	349	929.097			

Figure 5.1 shows the multivariate data for overall product liking consisting of different samples recorded for each consumer. Because it is hard to visualise multi-dimensional space, Principal components analysis is used to reduce dimensionality of multi-attributes to two or three dimensions (Kohler & Luniak, 2005). Biplots were first described thoroughly by Gabriel (1971) and are heavily used in the context of Principal component analysis as a useful tool for data inspection in statistical modelling. Biplots consists of lines and dots. Lines reflect the variables of the dataset whereas the dots are used to show the observations. From **Figure 5.1**, the observations of this dataset are consumers and the variables are the overall product liking for individual sample formulations. A score plot was derived from the composite scores computed for each consumer in the bi-plot.

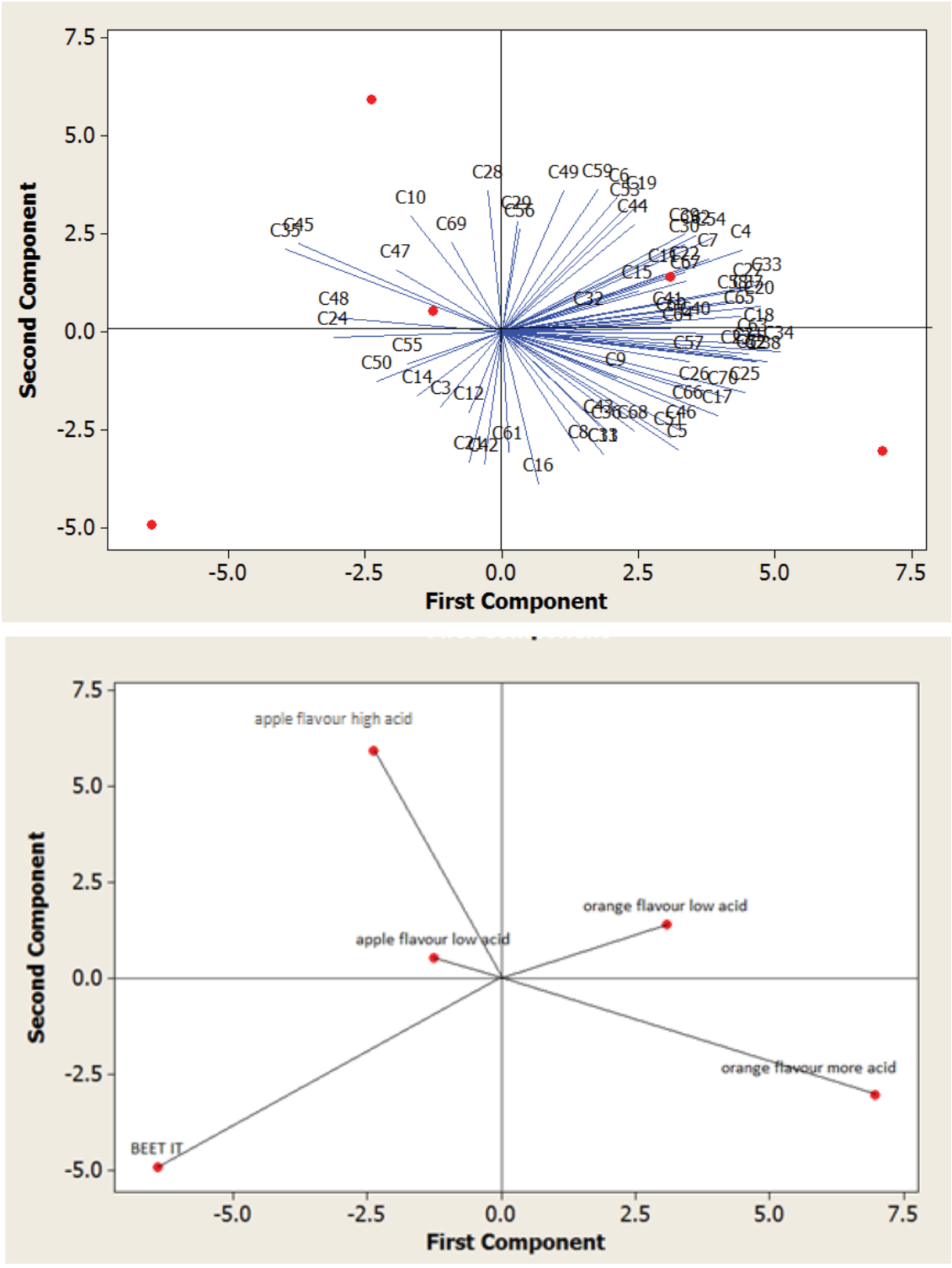

Figure 5.1: PCA bi-plot (top) and score-plot (bottom) for overall product liking for five sample formulations.

Inferring from **Figure 5.1**, the overall product liking for the *orange flavour high acid* blend had by far the highest variance among the variables in the bi plot and the overall product liking for *BEET IT* was the lowest. *BEET IT* fell in the negative scale of the first and second

component and hence it was the least preferred as compared to other formulations. The orange flavoured formulations fell on the positive scale of the first component and hence they were preferred than the apple flavoured formulations. The apple flavoured formulations fell on the positive scale of the second component hence they were preferred that BEET IT but not preferred as much as the orange flavoured formulations (positive scale, first component). The score plot in **Figure 5.1** shows a strong relationship between consumers and their preference for orange flavour low acid and a weak relationship between the consumers and their preference for *orange flavour high acid, apple flavour low acid* and *apple flavour high acid*.

The distance between two points approximates the Euclidean distance between two observations in the multivariate space. Observations that are far away from each other have a high Euclidean distance and vice versa (Kohler & Luniak, 2005). In the biplot, the highest Euclidean distance was observed between *orange flavour low acid* and *BEET IT, orange flavour high acid* and *BEET IT* and *apple flavour high acid* and *BEET IT. Apple flavour high acid* and *orange flavour high acid* were the other extremes. It was observed that a large cluster of the population pointed towards to the *orange flavour low acid* formulation (most preferred formulation). *BEET IT*, on the other hand, was the least preferred formulation. In general, orange flavour formulations were preferred to the apple flavour formulations.

Based on the statistical evidence, the *orange flavour low acid* formulation was finalised for further testing including a storage trial (Chapter 6) and consumer sensory triangle test (Chapter 7) against a placebo formulation (beverage with less nitrates).

5.6 Conclusion

Five formulation blends, *apple flavour high acid, orange flavour high acid, apple flavour low acid, orange flavour low acid* and *control-BEET IT* were finalised for consumer sensory evaluation. The formulations were quantified for nitrates (mg/L) and nitrites (mg/L), tested for quality parameters such as pH, titratable acidity and total soluble solids and also for bacterial and fungal growth. It was observed from the consumer sensory evaluation results that the *orange flavour low acid* blend had a significantly higher mean score for overall product liking compared to the other formulation blends. The *orange flavour low acid* formulation also had a high mean score for acidity and flavour liking. *BEET IT* had the lowest mean score for overall product liking, acidity and flavour liking. No significant differences in the mean scores were observed for the sweetness liking ($P > 0.05$) between

orange flavour low acid and *BEET IT*. Overall, orange flavoured formulations were preferred over the apple flavoured blends.

CHAPTER 6
SHELF LIFE TRIAL

6.1 Introduction

An investigation was undertaken of the shelf life stability of the high nitrate beetroot based beverage, formulated in Chapter 5. The beverage was stored in conditions approximating that of the retail environment for beverages, over an eight week period. Key factors studied during this eight week storage trial were microbial growth, chemical changes and loss of nutrients, colour change (browning) and development of off flavours (Sewald & DeVries, 2011).

The two main methods are used in the shelf life testing, the direct and indirect methods.

Direct method: This method is commonly used for testing of stored products under preselected conditions for a period of time longer than the expected shelf life and checking the product at regular intervals to see when it begins to spoil (Sewald & DeVries, 2011).

Indirect method: This approach uses the accelerated storage and /or predictive microbiological modelling to determine shelf life (Sewald & DeVries, 2011).

A shortened direct method was used to monitor changes during storage in the beetroot based beverages. As detailed in **Section 3.10.1**, the *orange flavour low acid* (finalised blend from consumer sensory testing) was divided into two equal parts and stored at 4±1°C and 20±1°C under light and dark conditions each for eight weeks). The *orange flavour low acid* juice beverage was analysed for microbiological growth, physical appearance, chemical and biochemical changes, nitrate and nitrite concentration and differences in the sensory profile. Juice beverages were stored in 200 ml clear glass bottles and stored in an open cardboard tray box under light storage and in an enclosed cardboard box covered with an A4 size black paper under dark storage.

The result of the beetroot juice beverage's shelf life quality investigation is divided into the following sections:

- Standard juice beverage properties: juice beverage pH, titratable acidity, total soluble solids

- Microbiological growth
- Nitrate and nitrite content
- Sensory changes: off-flavours, colour and appearance

6.2 Standard juice beverage properties during storage trial

6.2.1 Juice beverage pH

A change in the pH of the beetroot juice beverage can indicate possible microbiological activity (Tamme et al., 2009). The pH of the beetroot juice beverage was measured at 20±1°C and the results for juice beverage shelf life trial treatments are presented in **Figure 6.1**.

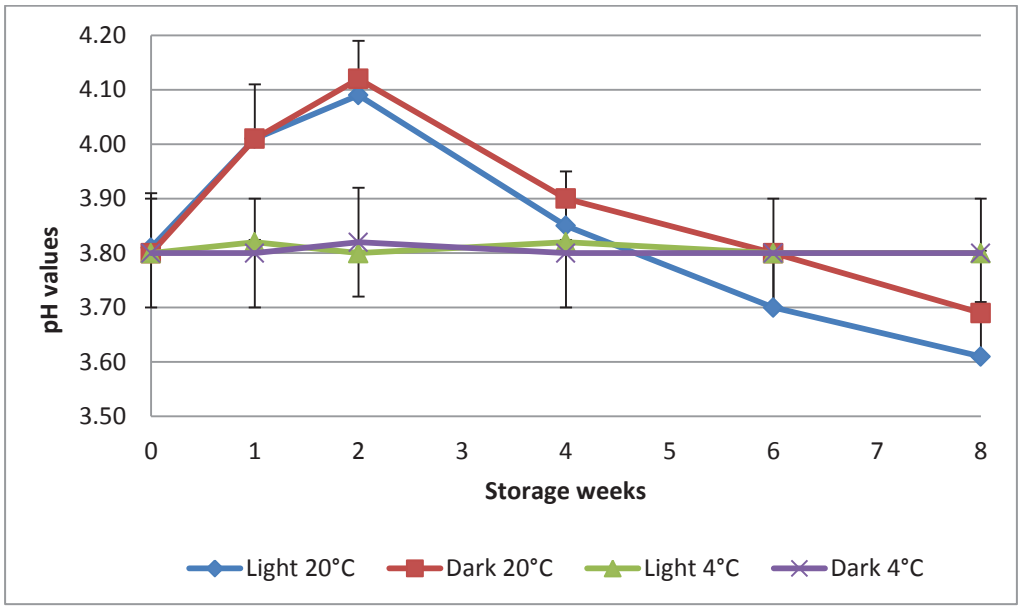

Figure 6.1: pH values of beetroot juice beverage stored at 4±1°C and 20±1°C under light and dark conditions over an eight week storage period. Data are presented as mean± SE (n=4)

There was a significant difference ($P < 0.05$) between the mean pH of the juice beverages stored at 20±1°C and at 4±1°C. There was no change in the pH of the juice beverage samples stored at 4±1°C under light (mean pH= 3.81 ± 0.01) and dark (mean pH= 3.8 ± 0.01) conditions throughout the eight week storage period. The pH of the juice beverage stored at 20 ± 1°C increased gradually from 3.8 ± 0.01 from week zero to about 4.10 ± 0.02 at week two and then declined to 3.6 ± 0.01 and 3.7 ± 0.01 under light and dark conditions, respectively. Wisal et al. (2013) explains the decrease in pH in strawberry juice in presence

of preservatives may be due to conversion of pectin found in juice beverage into pectinic acid, which increases acidity and therefore decreases the pH of the juice beverage.

6.2.2 Juice beverage titratable acidity

The acidity of a food is dependent on the amount of acid (titratable acidity), the pH and the type of acid present (Friedrich, 2001). The pH of a juice beverage is an indication of the concentration of free H_3O+ dissociated from the acids in the juice beverage, whereas the titratable acidity is the total acid content of a juice beverage, determined by titration of all the acid in the juice with a standard-base usually NaOH (Friedrich, 2001). A number of organic acids are present in plant-based juice beverages, results in this research are presented in terms of citric acid.

The titratable acidity results of the shelf life trial are found in **Figure 6.2**. The titratable acidity of the juice beverage stored at 4±1°C remained constant (P value= 1) over the eight week storage trial under light [mean titratable acidity = 0.5 ± 0.01 g/100 ml (as citric acid)] and dark [mean titratable acidity = 0.5 ± 0.01 g/100 ml (as citric acid)] conditions. The titratable acidity of juice beverage stored at 20 ± 1°C also did not change significantly over the eight weeks, and on average was 0.5 ± 0.01, for both light and dark storage. These minor changes in acidity shown in **Figure 6.2** correlate with the decreasing pH observed after two weeks of storage (**Figure 6.1**). No significant difference ($P > 0.05$) was observed between juice beverage samples stored under the light or dark conditions at both 4±1°C and 20±1°C.

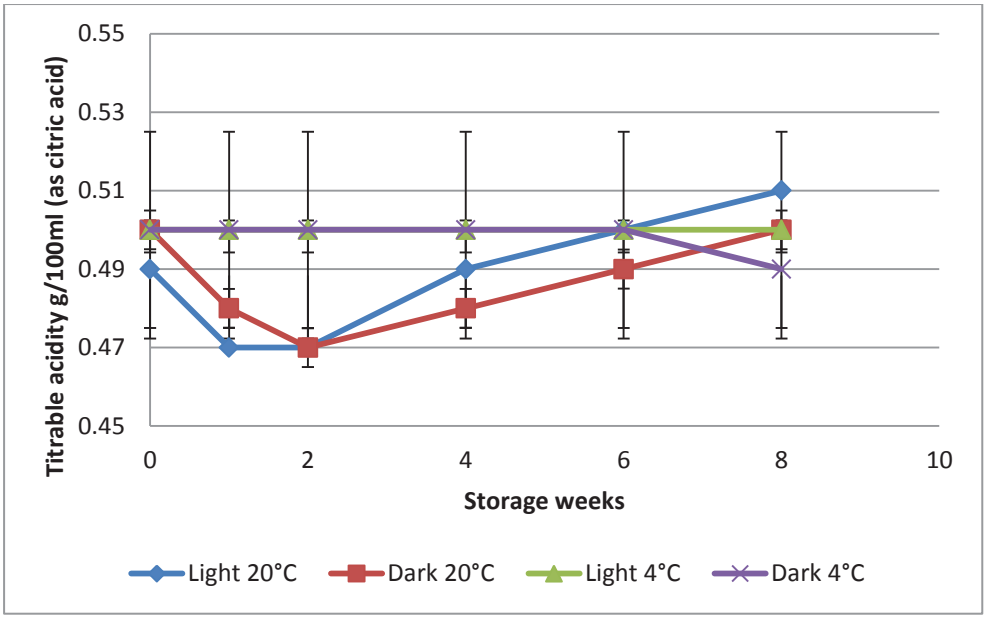

Figure 6.2: Titratable acidity g/100 ml (as citric acid) of juice beverage at 4±1°C and 20±1°C under light and dark conditions over an eight week storage period. Data are presented as mean± SE (n=4)

The juice beverage stored at 20±1°C in the light showed a decrease and then an increase in the acidity after two weeks of storage.

Safdar et al. (2010) observed gradual increase in acidity during storage of tomato concentrate at 25°C, 6°C and -10°C. Safdar et al. (2010) claims the increase in titratable acidity in tomato paste stored at 25°C may be due to the oxidation of alcohol and aldehyde during processing and is influenced by storage temperature, higher the temperature greater the increase in acidity (Gould, 1992). Hussain et al. (2008) explains the rise in acidity may be due to the increase in the concentration of weakly ionised acid and their salts during storage. Ayub & Khan (2001) are in agreement with results found in storage of the beetroot juice beverage. Similarly, an increase in the acidity of pomegranate syrup, stored under light conditions with clear packaging material was observed at room temperature over a four month storage period. This increase might be due to the acidic compounds formed by degradation or oxidation of reducing sugars at high temperature, breakdown of pectin substances and high temperature (Hussain et al., 2008).

6.2.3 Juice beverage soluble solids content (°Brix)

A hand held digital refractometer calibrated with a sugar concentration (°Brix) scale was used to measure the soluble solids content of the juice beverage blends stored at 4±1°C and 20 ±1°C.

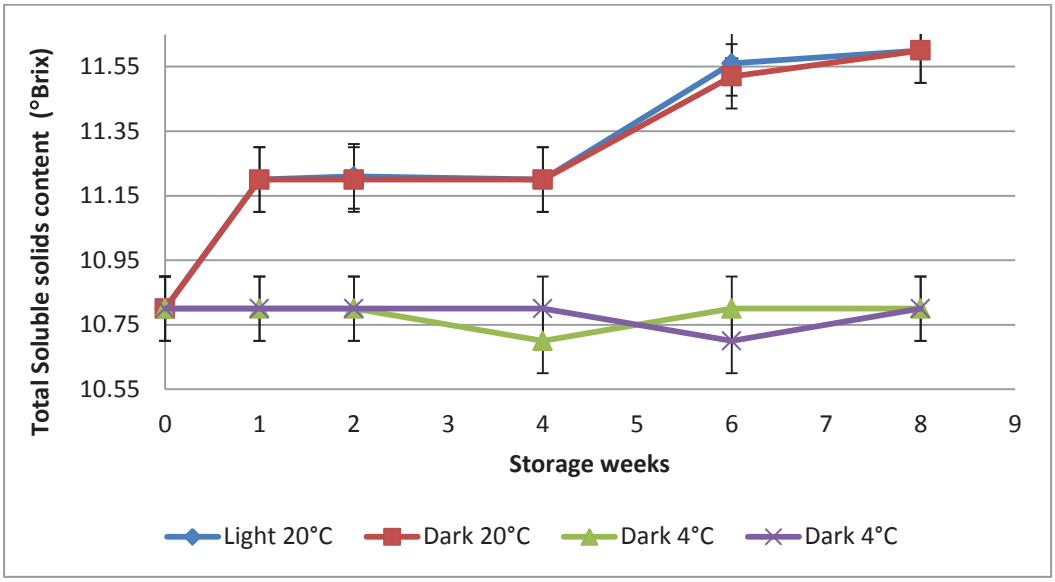

Figure 6.3: Soluble solids content (°Brix) of juice beverage at 4±1°C and 20±1°C under light and dark conditions over an eight week storage period. Data are presented as mean ± SE (n=4)

The refractive index is the ratio of the speed of light in a vacuum to its speed in a substance and is used as a measure of concentration of solutes in solution (Varnam & Sutherland, 1994). The °Brix has been correlated to the percentage (w/w) of sucrose in solution (Varnam & Sutherland, 1994).

The mean soluble solids of the juice stored at 20±1°C was greater than the mean soluble solids for the juice stored at 4±1°C but the difference was not significant ($P > 0.05$, **Figure 6.3**). This observation was not expected. It was observed that the soluble solids of the juice beverage stored at 20±1°C increased from 10.8 ± 0.0001 °Brix (week zero) to 11.6 ± 0.0001 °Brix (week eight) under light and dark conditions, however, the difference was not significant ($P > 0.05$). No significant difference ($P > 0.05$) was observed at 4±1°C throughout the eight week storage period in juice beverages stored under light (mean °Brix= 10.8 ± 0.0002) and dark (mean °Brix= 10.8 ± 0.0001) conditions. Karim (1996) also reported increase in reducing sugars during canning and storage at room temperature. Ali (1965)

reported that the increase in reducing sugars in canned orange juice during storage at room temperature could be due to the conversion of non-reducing sugar to the reducing sugars.

6.3 Microbiological growth in juice beverage during the storage trial

Microbiological testing was carried out primarily to ensure the juice beverage from shelf life trial was safe for consumption.

Table 6.1 shows the results obtained for the growth of aerobic plate counts (APCs) and yeasts and moulds of beetroot juice beverage stored for eight weeks at 4±1°C and 20±1°C under light and dark conditions. For weeks 4 and 6, samples were not tested for microbial counts as there were not enough samples to extend for the full eight weeks. Overall, it was observed that the microbial counts of the beetroot juice beverages stored at 20±1°C were higher than the juice beverages stored at 4 ±1°C. The pasteurisation conditions (90 ± 1 °C for 15 s) and storage at 4±1°C were effective at reducing and inhibiting the microbiological growth of APCs to within acceptable limits during the eight week shelf life trial. The microbial counts observed for the juice beverage samples stored at 20±1°C were also within acceptable limits (total plate count =10^5 cfu/ml), approved by the New Zealand Food Safety Authority until week eight when the counts increased to 6 x 10^5 cfu/ml for light and 5 x 10^5 cfu/ml for dark conditions (New Zealand Food Safety Authority, 1995).

Table 6.1: Microbiological test results for beetroot juice beverages stored at 20±1°C and 4±1°C under light and dark conditions.

STORAGE TIME (WEEKS)	BEVERAGE STORAGE TEMPERATURE (±1°C)	LIGHT OR DARK CONDITION	AEROBIC PLATE COUNTS (cfu/ml)	YEAST AND MOULD (cfu/ml)
0	20	Light	50	<10
1	20	Light	75	<10
2	20	Light	150	<10
3	20	Light	285	25
5	20	Light	450	40
7	20	Light	3×10^4	53
8	20	Light	6×10^5	78
0	20	Dark	42	<10
1	20	Dark	64	<10
2	20	Dark	135	<10
3	20	Dark	270	<10
5	20	Dark	485	31
7	20	Dark	4×10^4	46
8	20	Dark	5×10^5	69
0	4	Light	25	<10
1	4	Light	55	<10
2	4	Light	50	10
3	4	Light	59	<10
5	4	Light	72	<10
7	4	Light	80	<10
8	4	Light	85	<10
0	4	Dark	20	<10
1	4	Dark	50	<10
2	4	Dark	50	<10
3	4	Dark	62	<10
5	4	Dark	70	<10
7	4	Dark	79	10
8	4	Dark	84	10

There was a negative relationship between the pH and microbial counts of the beetroot juice beverage stored at 20 ±1°C. As the microbial counts increased, the pH of the beetroot juice

samples stored at 20±1°C decreased. The decrease in the pH at 20±1°C was caused by the microorganisms' vital activity products, as indicated by the increase of microorganism multiplicity. A decrease in pH is assumed to correlate with the nitrogen consumption of the studied microorganism's strains (Akin et al., 2008). The nitrogen concentration does not influence the pH itself, but during fermentation the consumption of nitrogen yeasts produces H+ ions (Castrillo et al., 1995).

6.4 Quantification of nitrates (mg/L) and nitrites (mg/L) during the eight week storage trial

Figures 6.4 and **6.5** show the nitrate (mg/L) and nitrite (mg/L) concentration results obtained for the beetroot juice beverage stored at 4±1°C and 20±1°C under light and dark treatments over an eight week storage period. It was observed that the nitrate concentration increased significantly at 4±1°C from 1586±16 mg/L to 1895±22 mg/L under light storage and from 1584±9 mg/L to 2901±15 under dark storage conditions, respectively (**Figure 6.4**). The sudden increase of nitrate content from 1584±9 mg/L to 2901±15 under dark conditions at 4 ±1°C was unusual and it was not clear why this occurred. The nitrite concentrations increased slightly but not significantly after week 1 from 90±1 mg/L to 100±3 mg/L on week 2 and remained stable up to week 8 at 101±2 mg/L at 4±1°C in light storage (**Figure 6.5**). A slight increase in the nitrite content was observed at week 8 under dark storage at 4±1°C from 101±0 mg/L (week 0) to 108±4 mg/L (week 8). However, there was no significant difference in the nitrate and nitrite concentrations during storage in light and dark conditions at 4±1°C ($P>0.05$).

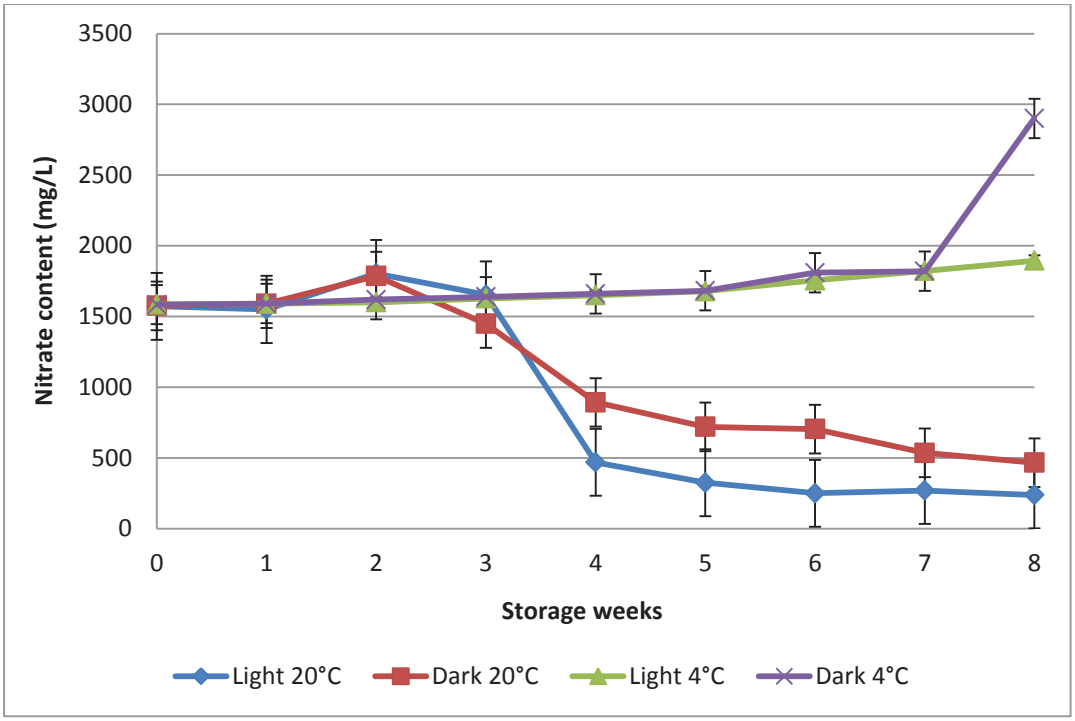

Figure 6.4: Nitrate content (mg/L) of juice beverage at 4±1°C and 20±1°C under light and dark conditions over an eight week storage period. Data are presented as mean ± SE (n=6)

Significant decreases in nitrate content were observed for juice beverage samples stored at 20±1°C from 1572±31 mg/L (week zero) to 238±25 mg/L (week 8) under light storage and from 1575±30 mg/L (week zero) to 466±12 mg/L (week 8) under dark storage conditions ($P < 0.05$) (**Figure 6.4**). The nitrate content at 20±1°C at week 4 in beetroot juice beverages stored under light (469±12 mg/L) was significantly less than in the sample stored in the dark at the same time (893±13 mg/L) ($P < 0.05$). Nitrate content decreased significantly ($P<0.05$) after week 4 from 469±12 mg/L to 238±25 mg/L at week 8 under light storage and from 893±13 mg/L (week 4) to 466±12 mg/L (week 8) under dark storage conditions.

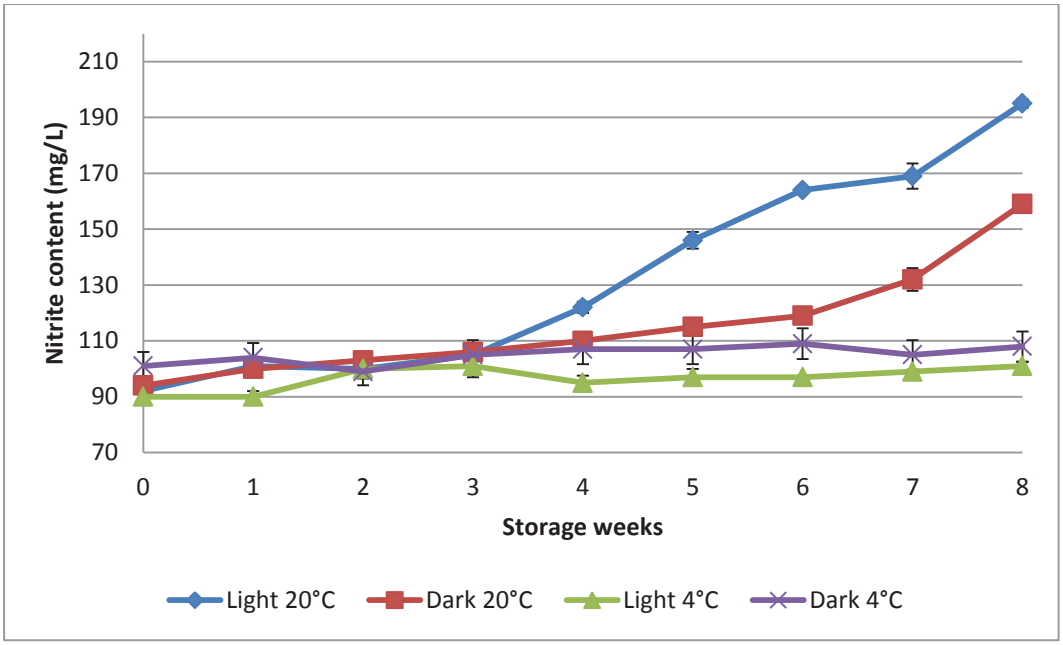

Figure 6.5: Nitrite content (mg/L) of juice beverage at 4±1°C and 20±1°C under light and dark conditions over an eight week storage period. Data are presented as mean ± SE (n=6)

Correspondingly nitrite concentration increased significantly after four weeks of storage at 20±1°C from 122±2 mg/L (week 4) to 195±2 mg/L (week 8) under light and from 110±0 mg/L (week 4) to 159±0 mg/L (week 8) under dark storage conditions. Similar correlations between decreasing nitrate and increasing nitrite content were found in vegetable juices after storage at room temperature (Chung et al., 2004; Nabrzyski & Gajewska, 1994) as explained by microbiological reduction of nitrite from the nitrate ion. The nitrite concentration and the total viable microbial counts increased during storage at 20±1°C, it may be concluded that microbial activity is likely to be the main factor in the nitrate-reduction process.

Burstrom (1943) showed that nitrate reduction was directly linked to a photochemical reaction which occurs in the presence of light, and nitrate reductase was shown to be dependent on and was most active under light conditions (Lorenz, 1978).

Phillips (1968) also showed the initial nitrate concentration present in vegetable juice beverage was significantly reduced to nitrite (reduced to approximately 64% of its initial level) under storage at 20±1°C. Nabrzyski & Gajewska (1994) reported the decrease of nitrate from 261 to 46 mg/kg with consequent increase of nitrite from 0.14 to 83 mg/kg for juice beverages prepared from carrot and stored at >20±1°C temperatures during 30 days of storage. Chung et al. (2004) also showed a decrease in nitrate concentrations and dramatic

increase in nitrite concentration at 22±1°C. Refrigerated storage (5±1°C) did not lead to significant changes in the concentrations of nitrate and nitrite levels in juice (Chung et al., 2004).

6.5 Changes in the colour and odour of the juice

Lower pH values over time at 20±1°C and high microbial counts unfavourably affected the stability of juice beverage colour as well as flavour (Walkowiak-Tomczak & Zielinska, 2002). Juice beverages stored at 20±1°C were observed visually to undergo a colour change from the characteristic red-violet colour of beetroot to red-brown from week four (**Figure 6.6**). No colour changes were observed for beetroot juice beverage samples stored at 4±1°C under both light and dark conditions.

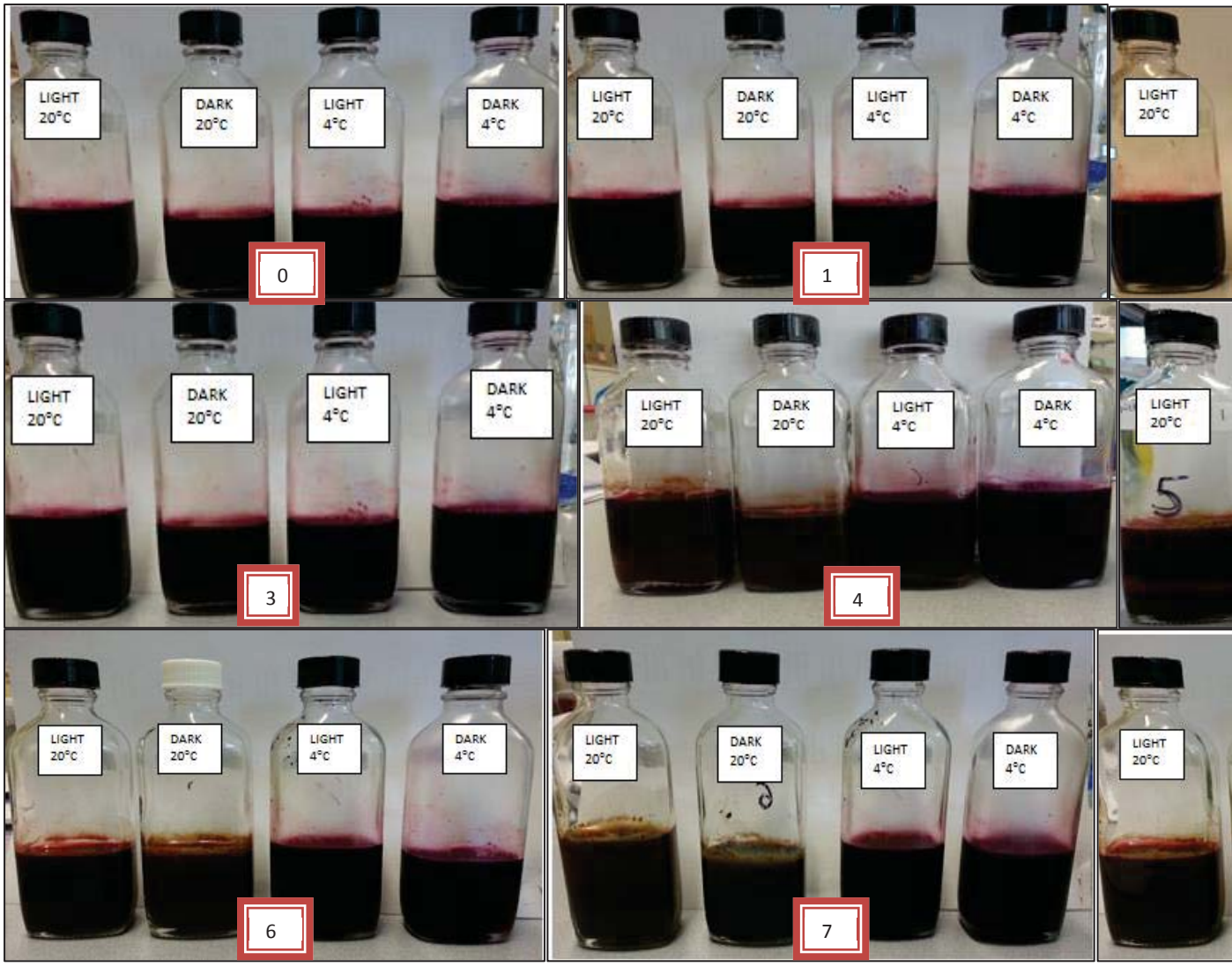

Figure 6.6: Photos of beetroot juice beverages stored at 4±1°C and 20±1°C under light and dark conditions o

Floating residues were observed at the top surface of the beetroot juice beverage from the week four onwards when stored at 20±1°C under both light and dark storage conditions (**Figure 6.6**). No residues were observed for any of the juice beverage samples stored at 4±1°C over the eight week storage period under both light and dark conditions. Similar observations were made by Walkowiak-Tomczak & Zielinska (2002), who concluded the colour change in beet juice beverage at 25-30 °C was due to denitrification by *Paracoccus denitrificans* in their juices. Since the nitrate concentrations were reduced at 20±1°C under light and dark storage conditions, it is highly possible that the denitrification could have been caused due to the presence of *Paracoccus denitrificans.* Denitrification at 25-30°C results in a lower ratio of red pigments to brown yellow pigment content (betalain pigment) found in beet juice beverage and therefore a reddish brown colour was observed in the juice beverage samples stored at 20±1°C.

The odour of the juice beverage stored at 4±1°C could be described as fresh, fruity and earthy over the eight week storage period. The juice samples stored at 4±1°C showed no signs of visual colour (**Figure 6.6**), flavour or odour change throughout the eight week storage period. The juice beverage samples stored at 20±1°C for weeks seven and eight under the light or dark conditions were not tasted as the microbial counts indicated microbial growth over the acceptable limits. Walkowiak-Tomczak & Zielinska (2002) found the natural juice beverage odour was also observed to change from red beet to caramel or worty (cereal) (Langstaff & Lewis, 1993) in juice stored at 25-30°C. The odour of the juice beverage stored at 20±1°C between week 4 and week 6 could be described as grassy, malty and burnt (caramelised) whereas those stored at 7 and 8 could be described as stale, oxidised and musty. The desirability of odour of the juice beverage stored at 20±1°C would be scored lower than that of colour due to the unattractive, stale odour in the red beet juice.

Figure 6.7 summarises the changes to pH, titratable acidity levels, total soluble solids, microbial counts (total plate counts and yeasts and moulds) and nitrate and nitrite content of *orange flavour low acid* formulation during a simulated retail storage over eight weeks at 4±1ºC and 20±1ºC under light and dark conditions.

	20±1°C		4±1°C	
	LIGHT	DARK	LIGHT	DARK
Week eight photos				
Microbiological (cfu/ml)	↑	↑	No Δ	No Δ
Juice pH	↗↘	↗↘	No Δ	No Δ
Titratable acidity g/100 ml (as citric acid)	↘↗	↘↗	No Δ	→↘
Soluble solids (°Brix)	↑	↑	No Δ	No Δ
Nitrates (mg/L)	↓	↓	↑	↑
Nitrites (mg/L)	↑	↑	↔	↔
Colour	Δ	Δ	No Δ	No Δ
Flavour change	↓	↓	No Δ	No Δ

Figure 6.7: Shelf life trial summary of trends

Key: Data are presented as means± SE

↑	Increasing trend over time	↗↘	Initial increase then decrease over time
↓	Decreasing trend over time	↘↗	Initial decrease then increase over time
No Δ	No change over time	↔	Variable data over time but not significant and no increasing or decreasing trend
Δ	Change over time		

6.6 Conclusion

Based on these results, it can be concluded that the juice beverage has to be stored at 4±1°C or less to prevent chemical changes, nitrate loses and unacceptable microbial increases within the juice beverage. From this storage trial, the beetroot juice beverage can be safely consumed after eight week storage if stored at 4±1°C. Over this period, there were less changes in the physical and microbiological parameters of the beetroot juice beverage.

CHAPTER 7
PLACEBO DRINK DEVELOPMENT

Chapter 7 describes the development of a placebo drink, low in nitrate content, suitable to match the current high nitrate formulation developed in **Chapter 5**. A sensory evaluation test to determine if consumers could detect a difference between the placebo and non-placebo formulations is also reported on.

In an effort to disguise 'nitrate' in placebo drinks, other studies have employed various methods, such as the development of nitrate depleted beetroot juice (Lansley et al., 2010), substituting blackcurrant cordial instead of beetroot juice (Bailey et al., 2009; Cermak et al., 2012; Vanhatalo et al., 2010) and using sodium chloride solution instead of sodium nitrate (Larsen et al., 2011, Lundberg et al., 2007). The placebo drink is then validated by assessing the subjective effects reported by the participants (Finnigan et al., 1995), comparing the placebo drink with nitrate rich juice beverage (Fillmore et al., 1998) or simply asking the participants if they thought they had received a standard drink or a placebo (Hammersley et al., 1998). These checks often reveal that the formulation was successful and a credible placebo drink was provided. For this research project it was decided to conduct a consumer triangle sensory evaluation test to determine whether consumers could detect a difference between a placebo juice drink containing less nitrates and the beetroot juice beverage developed in **Section 5.2**.

The terminology used in this chapter is the "standard beverage" refers to the formulation of beetroot juice beverage formulation with high nitrate content (more than 1.5 g/L), called *orange flavour low acid* in Chapter 5. The placebo drink was formulated to contain less than 300 mg nitrate/L but to taste the same as the "standard beverage".

7.1 Development of placebo

In subsequent research following this project, it is anticipated the standard beverage will be tested with athletes to determine if the increased nitrate content can improve

performance. Therefore a placebo drink was required to be developed in order to provide a control beverage which could be given to the athletes.

Placebo versions of some manufactured foods can be easy to make, for instance not fortifying otherwise fortified foods, however, making placebos from whole foods is much more difficult. Nitrates can be removed from water using nanofiltration (NF) and reverse osmosis (RO) (Mahvi et al., 2011). It was decided to use a lower percentage of nitrate rich beetroot juice in combination with water in the placebo drink formulation. The flavour and colour was boosted with beetroot juice concentrate which contained a low concentration of nitrate. The placebo drink was produced to match the standard beverage quality parameters in **Table 5.7**.

Table 7.1 shows the two placebo formulations that were produced for an informal triangle test trial with the taste experts. The suppliers for the ingredients used in the development of placebo formulations are listed in **Table 3.3**.

Table 7.1: Placebo formulations with their respective nitrate content (mg/L), total soluble solids (°Brix) and titratable acidity g/100 ml (as citric acid) results. Values are mean± SD (n=3)*

INGREDIENTS	PLACEBO FORMULATION 1 (%)	PLACEBO FORMULATION 2 (%)	STANDARD BEVERAGE (%)
WATER	38.97	35.77	0
APPLE JUICE (10.5 °Brix)	31	29	10
BEETROOT JUICE (11.2 °Brix)	25	30	84.72
BEET LEAVES JUICE (4.5 °Brix)	0	0	4
WHITE SUGAR	3.45	3	0
LEMON JUICE CONCENTRATE (45 °Brix)	1.2	1.2	1.25
BEETROOT JUICE CONCENTRATE (69°Brix)	0.35	1	0
ORANGE FLAVOUR	0.03	0.03	0.03
TOTAL	100	100	0
NITRATE CONTENT (mg/L)	159 ± 3.5*	181± 4	1572 ± 5
TOTAL SOLUBLE SOLIDS (°BRIX)	10.5 ± 0.1	10.8 ± 0.1	10.7 ± 0.1
TITRATABLE ACIDITY g/100 ml (as citric acid)	0.5 ± 0.05	0.5 ± 0.05	0.5 ± 0.05

The placebo formulation contained significantly less nitrate than the standard beverage (**Table 7.1**). The nitrate concentration in the placebo formulations was on average 9.2 times lower than the standard beverage. No significant differences were observed in the total soluble solids ($P > 0.05$) and titratable acidity ($P > 0.05$) between the placebo formulations and the standard beverage. Beetroot juice concentrate provided the beetroot colour in the placebo drinks as it is used as a natural source of colouring agent in applications such as candies and ice cream (Manoharan et al., 2012). The colour of the placebo drink was visually matched to the standard beverage as colour (appearance) is the first attribute that is perceived by panellists/consumers before consuming the beverage (Vazquez et al., 2013). Beetroot juice and the beetroot juice concentrate contributed to the earthiness flavour from the beetroot. The earthiness from beetroot was partially masked by orange flavour in both placebo and standard beverages.

Placebo formulation 2 had a more earthy beetroot flavour from the beetroot juice and beetroot juice concentrate compared to placebo formulation 1. It was decided to test the placebo formulation 2 against the standard beverage using a consumer triangle sensory evaluation discrimination test with 25 panellists.

7.2 Triangle sensory consumer test

Two beverages (standard versus placebo) were evaluated to determine if consumers could detect a difference using a triangle test. The main purpose of conducting this discrimination test was to determine if the panellists could identify the placebo drink with low nitrate from the high nitrate rich beverage. Twenty-five consumers were asked if they could detect a difference.

The sensory test measures if any differences detected are truly significant by analysing the sensory data for statistical significance. After statistical analysis, a meaningful interpretation from the results of the sensory data can be successfully made (Meilgaard et al., 1999). A panellist's preference, acceptance or degree of difference after initial selection of the odd sample was not asked because the selection of the odd sample could bias the reply to any additional questions. A comment section asking why the choice was made was included for the panellists' remarks.

7.2.1 Consumer triangle test results

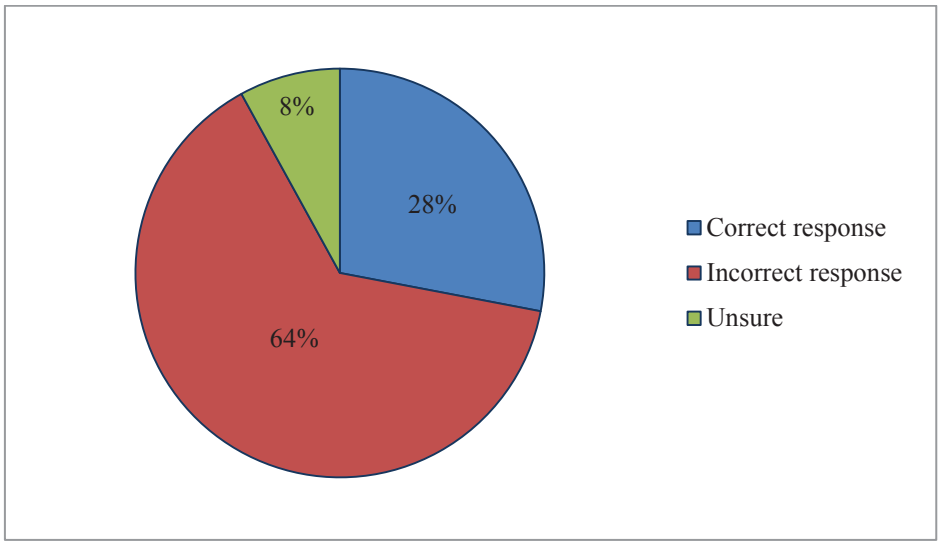

Figure 7.1: Pie chart illustrating the results from triangle consumer sensory test

Out of 25 consumers tested with the placebo drink and standard beverage using a triangle sensory test, seven people could taste a difference between the placebo and standard beverage (correct responses), 16 people could not differentiate between the two beverages and two people were not sure about the difference.

7.2.1.1 Analysis of sensory results

The minimum number of correct responses needed to conclude that a perception difference exists based on a triangle test has been summarised by Meilgaard et al. (1999) as shown in **Appendix K.** If the number of correct responses was greater than or equal to the number given in **Appendix K** (corresponding to the number of assessors and the α-risk level chosen for the test), it could be concluded that a perceptible difference existed between the samples (Meilgaard et al., 1999).

Seven panellists correctly identified the odd sample which is sufficient according to Meilgaard et al. (1999) to conclude that the two samples are not perceptibly different (with 95% confidence).

Comments of the panellists who could differentiate between placebo and standard beverages were analysed. Most of the comments stated ‘it was hard to tell, just guessed,

sweeter, preference, minor difference, cannot tell' which indicates that possibly the correct responses could be a 'correct by chance'.

7.3 Conclusion

The placebo formulation developed in this study, contained 181± 3.6 mg nitrate/L placebo juice, which was nine times less than the nitrate concentration observed in the standard beverage. No significant difference was observed in the titratable acidity and total soluble solids between the placebo and standard beverage ($P > 0.05$). Approximately 64 % of the population could not tell the difference between the placebo drink and standard beverage while, 28 % of the population could identify a difference and 8% of the population were unsure after the triangle sensory test.

CHAPTER 8
OVERALL DISCUSSION

8.1 Overall Discussion

Over recent years, health and environmental authorities have focussed on methods to reduce the concentration of nitrate in drinking water (Morgan et al., 2000). However, recent research suggests that dietary nitrate aids in the physical performance of athletes (Bailey et al., 2009; Vanhatalo et al., 2010). The main objective of this project was to produce a nitrate rich beverage which not only tasted good and provided with high concentrations of nitrates but was also safe to consume. In the future, the nitrate rich beverage described here will be tested in subsequent research at Massey on athletes or sports related consumers with the placebo beverage. Most of the high nitrate products currently on the market are a blend of beetroot and apple juices. There has been little published on studies involving the sensory evaluation of the high nitrate beverages, placebo development, biochemical properties and microbiological evaluation of high nitrate beverages and changes during storage trials.

This study demonstrated that the formulations that are acceptable to New Zealand consumers can be developed with naturally occurring nitrate (>1500 mg/L). The samples produced were palatable and the results from sensory analysis indicated that consumers preferred the low acid orange flavoured beetroot juice beverage over the commercial product, *BEET IT* after sensory evaluation. The *orange flavour low acid* beverage was formulated with beetroot juice, beet leaves juice, apple juice, lemon juice concentrate and natural orange flavour.

The placebo drink was formulated using beetroot juice, apple juice, beetroot juice concentrate and natural orange flavour. From the triangle sensory evaluation conducted at Massey University, the majority of consumers could not differentiate between the high nitrate beverage and the placebo drink. The following sections discuss the findings from each of the analysis methods utilised in the study with specific regard to the available literature.

8.2 Pilot scale production of juice

Low juice yields were obtained as all the vegetables were juiced using a laboratory scale juicer. Due to the volumes required and the resources available, a pilot scale production was not available for this project. The use of different equipment essential for cleaning, size reduction, juice extraction, mixing and pasteurisation may increase the yield and improve the efficiency of the process.

In order to produce eight litres of beetroot juice with the resources available, it took three days to process the required 30kg of beetroot. Beet leaf juice (2.5 L) was extracted on the fourth day along with celery juice. There were several constraints in the process resulting in delays that may have introduced sources of potential contamination, affected the nitrate and nitrite content and reduced the quality of the final juice. The constraints included the slow rate at which the beetroot could be juiced with hourly halts. This was a very slow process which means juice made at the beginning of the day was sitting around waiting to be pasteurised as this was carried out in a batch process. Juice was pasteurised on the same day as they were extracted to reduce possible microbial contamination followed by aseptic bottling.

The physical structure of beetroot required it to be hand cut into small pieces (4 cm × 1 cm) so as not to damage the food processor, this was followed by chopping into finer pieces (2 mm × 2 mm) in a food processor, leading to significant time required. These steps were essential to allow easy feeding into the extractor and to achieve reasonable juice yields from the extractor. The biggest time and process constraint was the capacity of the laboratory juicer. The juicer mesh also had to be cleaned every 10-15 mins as it would get clogged with beetroot fibre particulates creating high pressures on the juicer. The juicer also needed a 20 min rest cycle after every 3 hrs to prevent it from overheating. The juicer used, had several limitations including low juice yields and delays, potentially compromising microbial and compositional quality of the final juice. Nevertheless, the juice produced for the shelf life trial met the microbiological safety requirements, contained high nitrate concentrations and was palatable.

Commercially, the juice can be obtained by grinding the beetroots and pressing in a hydraulic press to achieve satisfactory yields (Nelson & Tressler, 1980). Beetroots were

also ground in a hammer mill and pressed in a Chisholm Ryder continuous press to macerate the beetroots to release the juice from nearly all the cells (Nelson & Tressler, 1980). A larger press that could extract the juice in one or two pressings is recommended. The extracted juice should be blended and pasteurised immediately after extraction in order to avoid excessive enzyme activity. Larger processes could also result in a continuous batch process thereby minimising the contamination factors. For this project, blending of juice was carried out after the pasteurisation process in a hygienic environment due to the laboratory experimental plan. However, in future, juice will be extracted and ingredients will be added together based on the formulation and the final juice will be pasteurised in a continuous batch process to 90±1°C for 15 s followed by aseptic bottling.

The beetroot juice was filtered through a 150 μm mesh to minimise the fouling in the pasteuriser. The filtering removed some of the total soluble solids and dietary fibre not already discarded as pomace which still included a mixture of whole and ruptured plant cells. The resultant juice (beetroot, beet leaves) was a homogeneous cloudy juice rather than a juice with noticeable particulates like freshly squeezed orange juice. Cloud stability is a key visual characteristic and quality attribute of cloudy juices that influence consumer acceptance (Sila et al., 2009). The cloud particles influence the mouth-feel, flavour and colour. To be a stable suspension, a juice cloud must have the appropriate specific gravity, particle size and charge (Sila et al., 2009).

8.3 Sensory evaluation of juices

Five formulations (*apple flavour low acid, orange flavour low acid, apple flavour high acid, orange flavour high acid* and commercial product *BEET IT*) were presented to the consumers in a randomised order, to be ranked on a sensory scale for attributes such as overall product liking, flavour liking, acidity liking and sweetness liking. Sensory evaluation results revealed useful information for the overall product liking attributes for the five beverages. The *orange flavour low acid* beverage formulation was the most liked whereas *BEET IT* was the least liked out of the five formulations presented to consumers. Although the mean score for sweetness liking of *BEET IT* was similar to the *orange flavour low acid* beverage formulation, *BEET IT* was still the least preferred formulation for sweetness liking attributes. *BEET IT* was also ranked lowest for acidity

and flavour liking attribute as compared to the formulations produced in the laboratory and a statistical difference was observed between their mean scores ($P < 0.05$). This is extremely encouraging as it demonstrates that the novel formulations produced were liked and preferred over the current product present on the market.

ANOVA and Tukeys test were also performed to determine the P values and to explain if a significant difference was observed between formulations for different attributes. It was observed that, in general, the orange flavoured beverages were liked over the apple flavoured beverages. *Orange flavour low acid* was the most preferred formulation with a mean score of 7 on a 9 point liking scale.

Comments from the panellists indicated that the beetroot juice formulations (*orange flavour low acid, apple flavour low acid, orange flavour high acid and apple flavour high acid*) had a "bright reddish purple beetroot colour", "tasted more appetising", "had a good flavour combination" and "seemed organic" whereas for *BEET IT*, it was "sweeter", "looked dull" and "not very appetising" (brownish red colour) and "tasted more synthetic". The brown colour of *BEET IT* could be due betalain instability, including peroxidase, polyphenol oxidase, β glucosidase and betalain oxidase, which may account for betalain degradation and colour losses (Tiwari et al., 2013).

Based on the consumer sensory evaluation results and comments, *orange flavour low acid* formulation was finalised for future comparisons with a placebo drink and evaluated for storage stability.

8.4 Juice in retail environment

The stability of the beetroot juice beverage was evaluated over eight weeks for the *orange flavour low acid* formulation stored at 4±1ºC and 20±1ºC under light and dark conditions.

8.4.1 Co-relation between pH, nitrate and nitrite concentrations (mg/L) and microbial counts at 4±1ºC and 20±1ºC

The pH, titratable acidity and total soluble solids of the juice stored at 4±1°C remained constant throughout the eight week storage; though a slight decrease in acidity was observed at week eight under dark storage. The decrease in acidity at 4±1°C was

however not significant (P = 0.90). Tamme et al. (2010) reported that no considerable change in the pH was observed in carrot, cabbage and beetroot juice when stored at 4ºC for three weeks.

It was observed that the nitrates (mg/L) increased over time by 16 % under light storage and 45% under dark storage at 4±1°C. No significant change was observed in the nitrite concentration (mg/L) under light (P=0.73) and dark (P=0.89) storage at 4±1°C. Similar trends were observed by Tamme et al. (2009), where the nitrates increased by 10 to 18% for canned vegetable based infant food under light storage conditions at 4 to 6 °C over a period of eight weeks. No significant changes in the nitrate and nitrite concentrations in homogenised leafy vegetables have been reported by Chung et al. (2004) at 4±1ºC. According to Ezeagu (1996) and Phillips (1968), the increase in nitrate content was reported to have been caused by the differences between species-specific nitrate-reducing activities and by the influence of levels of bacterial counts. Also, under refrigerated storage of vegetable juice, nitrite accumulation tended to be inhibited (Ezeagu, 1996). The microbiological counts in this study were under the recommended acceptable limits under both light and dark treatments at 4±1°C storage. Hence the vegetable juice beverage can be stored in a clear bottle under refrigerated storage conditions. No visual changes were observed in the colour or taste of the juice beverages stored at 4±1°C over the eight week storage period.

However, at 20±1°C, changes were observed in the pH, titratable acidity, total soluble solids, nitrate and nitrite content (mg/L) and microbial counts. A positive correlation was observed between pH and acidity. It was observed that the pH increased initially and decreased over time. Similar trends were observed by Tamme et al. (2010) in raddish juice where the pH decreased from an initial 6.4 to 4.2 at 20-22 ºC. The decrease in pH is caused by microbial activity, as indicated by the increase of microorganism multiplicity (Tamme et al., 2010). Akin et al. (2008) reported a decrease in pH was assumed to correlate with the nitrogen consumption of the studied microorganism strains. Castrillo et al. (1995) suggested that the nitrogen concentration did not influence the pH itself, but during fermentation, the consumption of nitrogen by yeasts produces H^+ ions. The acidity decreased initially and then increased over the

eight week storage period. However, on a statistical level, the changes in pH and acidity were not significant ($P > 0.05$).

Significant microbial reduction of nitrate leads to the accumulation of nitrite concentrations due to nitrate reductase activity caused by increasing microbes at ambient storage (Ezeagu, 1996). The juices were pasteurised to inhibit any enzyme activity therefore it is unlikely this enzyme activity occurred.

Cases of poor storage or beyond normal 'use by' dates or at increasing ambient temperatures (≥20 ºC) could probably result in bacterial growth, which could have contributed to the increasing accumulation of high nitrite concentration (Bosch et al., 1995; Ezeagu, 1996; Petersen & Stoltze, 1999). If a food contains high concentration of nitrate, it is a potential risk if the conditions during storage or processing are conducive to conversion to nitrite (European Food Safety Authority, 2008; Hill, 1996). Since both nitrite concentration and total viable counts increased during storage at 20-20 ºC, Tamme et al. (2010) concluded that microbial activity was the main factor in the nitrate-reduction process. This suggests that for consumers that nitrate rich juice purchased from the market must be stored immediately in a refrigerator (≤5 ºC) and consumed before its targeted best by date. Storage at appropriate refrigerated temperature would prevent bacterial nitrite formation and thus improve safety during its consumption.

8.5 Placebo formulation and consumer triangle sensory test

The placebo formulation containing less nitrate content was produced to match the *orange flavour low acid* formulation containing high nitrate content. The placebo formulation containing a combination of water, juices (beetroot, apple), concentrates (lemon juice, beetroot juice) and orange flavour was finalised for the triangle consumer sensory evaluation against the *orange flavour low acid* and tested on 25 consumer panellists. The placebo formulation was produced to match the specifications of total soluble solids and titratable acidity of the *orange flavour low acid* to result in similar taste, flavour and mouth feel between two products. The nitrate content of the placebo beverage was quantified as 181±3.6 mg/L and was about 9 times lower than the *orange flavour low acid* formulation. The beetroot juice concentrate was added to the placebo formulation for colour and flavour.

Based on the consumer sensory triangle test result, 64 % of the panellists could not differentiate between the placebo and *orange flavour low acid* beverage and 8 % were not entirely sure. Hence, at 95 % confidence interval, the placebo beverage could be substituted in place of *orange flavour low acid* beverage for exercise performance on athletes.

8.6 Consumption of nitrate rich beverage and safety

Nitrate content in vegetables exert a natural ergogenic or performance-enhancing effect (Cermak et al., 2012; Lansley et al., 2011). Sodium nitrate and nitrite have antimicrobial properties and are used for preservation in lunch meats, sausage and bacon (Food Standards Australia New Zealand, 2010). The dietary nitrate found naturally in beetroots does not play the same role as the artificially made chemical sodium nitrates and nitrites used for preservation of processed meats. This has caused confusion in the past because concerns over being cancer linked to artificial nitrates (Hill, 1994; Moller, 1995) used to preserve meat, resulted in World Health guidelines in the 1950's to be issued about all types of nitrates, despite there being no evidence that consumption of natural dietary nitrates has the same potential impact (European Food Safety Authority, 2008). The evidence amongst populations that consumed high concentration of natural dietary nitrates indicated that there was lower incidence of cancer (Addiscott, 2005; Shuval & Gruener, 1972). In 2008, European Food Standards Agency issued a scientific opinion on the matter that corrected this misunderstanding (European Food Safety Authority, 2008; McKnight et al., 1999). Ward et al. (2005) explained the intake of dietary nitrate was less likely to increase nitrosation due to presence of nitrosation inhibitors in vegetables. Hence the efficacy of certain vitamins as nitrosation inhibitors in vegetables provides a plausible explanation of epidemiologic findings that have shown a protective effect of fruit and vegetable consumption against various malignancies (Bartsch et al., 1988; Block et al., 1992).

8.7 Nitrate rich products in the market besides juice

Other products such as nitrate powders, bars and gels have also been formulated for endurance athletes for consumption aimed for exercise performance. GoPlus® nitrate gel was the first nitrate gel to improve energy efficiency during exercise. It contains

swiss chard as the main ingredient combined with maltodextrin, rhubarb juice, sweeteners, preservatives and gelling agents (Else, 2013). GoPlus® contains 250 mg nitrate per gel (Else, 2013) and is easy to digest. ZipVit® sport nitrate performance gel is another gel available in the market which is formulated to maintain health and significantly improve sport recovery and performance. The recommended intake for ZipVit® is two gels per day in three days before the event and one gel two hrs before the event (Audane, 2014). The cost of ZipVit® is USD $3.65 per 60ml gel tube whereas the cost of GoPlus® is USD $3.2 per 60ml gel tube (Audane, 2014).

Pure clean® beet juice powder recommends addition of two scoops of beetroot powder equivalent to the nitrate content in about 300-500ml beetroot juice (1-1.2 g nitrate per L) to water or juices about two-three hrs prior to exercise for maximal exercise benefit (Overholt, 2013). Pure clean® beet juice powder is sold in 15 or 40 count single serve packs for USD $1.40 each pack or in 40 and 80 beet bulk powder for USD $46.75 and USD $85.50, respectively (Overholt, 2013).

BEET IT has also developed beetroot and oat sports bar with natural nitrate beetroot concentrate (James White Drinks, 2014). Each BEET IT bar contains natural dietary nitrate content of 0.4 g and is similar to the nitrate content in the BEET IT sports shot. The BEET IT bar contains 50% oat content and is an excellent source of slow energy release. The cost of BEET IT sports bar is USD $3.30 per 60 g bar (James White Drinks, 2014). **Figure 8.1** shows the packaging of the high nitrate beetroot products mentioned.

Figure 8.1: Nitrate rich products available in the market for exercise performance. Top left: ZipVit® gel, Top right: Pure clean beet juice powder, Bottom left: GoPlus® nitrate gel and bottom right: BEET IT sports bar (Audane, 2014; Else, 2013; James white drinks, 2014; Overholt, 2013).

8.8 Cost of producing the final product formulation

Indicative ingredient costs of the *orange flavour low acid* formulation have been calculated per 100ml. **Table 8.1** gives a breakdown of cost based on the *orange flavour low acid* formulation.

Table 8.1: Cost of ingredients for *orange flavour low acid* formulation

INGREDIENTS	%	COST PER 100ML (NZD $)
Beetroot juice	84.8	0.69
Beet leaves juice	4	0.00
Apple juice	10	0.13
Lemon juice concentrate	1.25	0.20
Orange flavour	0.03	0.12
TOTAL	100	$1.14/100 ml
		$11.40/ 1000 ml

Beet leaves had no cost since the leaves provided by Freshmax Ltd were discarded as waste. Only beetroots were charged when they were purchased from Freshmax Ltd. The cost of packaging and manufacturing costs (labour, bottles, overheads etc.) were not included in the final cost. The current cost of *BEET IT* as sold in the supermarket (Huckleberry Farms, New Zealand) was NZD $13.80 /1000ml, purchased in January,2014.

CHAPTER 9
CONCLUSIONS AND RECOMMENDATIONS

9.1 Conclusions

The results from this research indicate that a consumer acceptable safe vegetable juice beverage with a stable nitrate content is achievable with refrigerated (4±1°C) shelf life of two months under light storage. The following conclusions can be made from this research:

- The vegetable juice beverage required heat treatment at 90±1°C for 15 s.

- Acidification required 1.25 g/L of lemon juice concentrate (@ 45°Brix) to reduce the pH to below 4.5.

- The vegetable juice beverage stored for eight weeks under normal light conditions maintained microbiological safety levels below the minimum standard for ready to eat food products.

- The pH, titratable acidity g/100 ml (as citric acid), total soluble solids (°Brix) did not change during storage at 4±1°C under light and dark storage. No changes in colour (visual) and taste were observed for juice beverages stored at 4±1°C under light and dark storage.

- The *orange flavour low acid* consisted of a blend of beetroot juice, beet leaves juice, apple juice, lemon juice concentrate and orange flavour. The *orange flavour low acid* was the most preferred formulation whereas the commercial product *BEET IT* was the least preferred. The *orange flavour low acid* formulation also had high mean scores for acidity, sweetness and flavour liking.

- Production of a vegetable juice beverage with a stable nitrate content of ≥1000 mg/L is achievable. At these levels, beetroot beverages have been reported to deliver improved exercise related performances. The nitrate content observed at week eight under light storage (1895 ± 22 mg nitrate/L) was comparatively less than that observed under dark storage (2901 ± 16 mg/L). Hence a light protective packaging would be recommended. No significant increase was observed in the nitrite content under light and dark conditions.

- A placebo formulation can be developed to match the flavour and colour of *orange flavour low acid*. The placebo formulation contained nine times less nitrate than the *orange flavour low acid* formulation. From the consumer sensory triangle test, it was observed that only 28 % of the population could differentiate between the placebo and *orange flavour low acid* formulations which concludes that the placebo beverage containing less nitrates could be used in place of the high nitrate beverage for placebo controlled future sports studies to study the benefits of nitrate in exercise performance. The nitrate rich juice beverage and the placebo beverage with less nitrates will be tested on athletes or consumers on two different days, hence it is highly unlikely that the consumers could detect a noticeable difference.

9.2 Recommendations

Process optimisation and development work is recommended to make the beetroot juice production commercially viable. This research was carried out using traditional processing technologies. This can be used as the baseline to develop processing conditions that will optimise the nutritional components in the high nitrate juice. Factory trials should be carried out on 100 L juice runs to determine if the scaling up affects the texture, colour and flavour of the final product. The pilot-scale trials may also suggests any modifications and improvements needed for further research work. Since all the ingredients were added post pasteurisation, it would be essential to conduct a trial by blending all the ingredients before pasteurisation to result into an industrially made commercial product.

This research shows it is possible to produce a beneficial sports juice using basic processing and storage methods, whilst still retaining a usable concentration of nitrates, one of the most labile water soluble compounds. Further research into retention of high level of biologically accessible antioxidants in beetroot juice such as ferulic acid and betanins as well as other nutritional health promoting compounds such as potassium, magnesium, folic acid, iron, zinc, calcium, phosphorus, sodium, niacin, biotin, B6 and soluble fibre would be recommended. Existence of these naturally occurring components in beetroot juice would further assist in promotion of the juice beverage.

Additional tests to determine ascorbic acid levels, dietary fibre and pectin levels would be recommended. The influence of chlorophyll and carotenoid levels on the colour changes in juice could be determined.

Advanced consumer sensory testing on the juice should be conducted in Massey University using a sensory trained panel team as well as athletes targeted to use this product in the future. This would be very helpful to aid in the success of this product as it would help gauge how well the juice may be received by the target market. Focus group discussions could also provide with ideas for different flavour combinations with beetroot juice or changes to any of the concepts and could help narrow down on options for commercial production.

Further, nitrate rich powder could be developed from the nitrate rich juice beverage with the aid of spray drying process and quantification through HPLC could be conducted to check if the nitrate concentrations are sustained after the processing. This would help extend the shelf life on the final product.

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APPENDICES

Appendix A: Specification sheets of ingredients

1. **Product Name:** Lemon Juice Concentrate 400 GPL – Clear

   Company Name: Directus

Section One: Ingredients and Nutritional Information

Determinations	Minimum	Maximum
Brix (refractometer at 20 °C)	41	46
Acidity % (w/w as anhydrous citric acid)	32	33
Acidity GPL (w/v)	396	404
Brix (corrected)	47	52
Ratio (Brix correted/Acidity %)	1.430	1.630
Density (g/cc)	1.215	1.243
Viscosity (cps - Brookfield Spindle#4 12rpm)	-	3000
Pulp (% v/v at 8°Brix refractometer)	-	2
Pulp on 60 Mesh screen (g/24 fl oz in reconstituted)	-	3
Pulp on 20 Mesh screen (g/24 fl oz in reconstituted)	-	0
Defects (USDA/AMS score in recontituted)	18	20
Color	Light Yellow	
Oil Content (g/kg)	-	0.05
pH (in reconstituted juice)	2.25	2.45
Sodium Content (mg/kg in reconstituted juice)	-	30
Limonin (ppm at 4.5 % acidity)	-	20
Aminic Nitrogen (mg/100g in concentrated)	120	-
Ascorbic acid (mg/100g in concentrated)	220	-
Citric acid / Isocitric acid	170	220
Cloud (at 7 days)	Stable	
Stability % (at 1.6 °Brix refract)	55	-
Imhoff test (ml at 0.32 °Brix refract)	-	5
Jelly test	Negative	
Sodium benzoate (mg/Kg)	None	
Sulphur dioxide (mg/Kg)	None	
Total plate count (cfu/ml of concentrated)	-	100
Yeast and mold (cfu/ml of conccentrated)	-	50
Heat resintant mold spores (cfu/100ml at 8°Bx)	-	5
Howard Mold Count %(in reconstituted juice)	-	<10

Packaging:

250 kg drums

Aseptic bag in drum

Nutritional Information:

Available on request

Allergen Information:

Available on request

HACCP certification:

HACCP certified and available on request.

2. Product Name: Beetroot juice Powder

SENSIENT

Sensient Technologies New Zealand
5 Doraval Place Mt Wellington
P O Box 22-451 Otahuhu
Auckland New Zealand
Tel:+649 270 8510 Fax:+649 270 8520

Product Specification

Product Code	DR3262
Product Name	DR3262 BEETROOT JUICE POWDER WATER SOLUBLE
Description:	Beetroot Juice from pressing of Beta vulgaris, concentrated and spray-dried by maintaining the typical vegetable-like taste and flavour.
CAS. No:	7659-95-2
Manufacturer:	Sensient Food Colors- Germany.
Allergens:	No Allergens.
GMO Status:	No Genetically modified ingredients /additives.

Quality Parameters

Appearance:	Bluish red powder.
Ingredients:	Beetroot Concentrate, Maltodextrin, Citric Acid E-330.
Taste & Odour:	Typical of vegetable-like.
Halal:	Compliant.
Kosher:	Certified.
Application:	We recommend the flavour and dosages be determined by the customer in their product using their process conditions.

Physical

Colour Intensity:	E 1%:530 = 4.6 - 5.1 in water at pH5.
Others:	Water (Karl Fischer): Max.5 %
pH:	4 - 5 in 10% aqueous solution

Chemical

Nitrate	Max. 0.6%
Total acid (calc. As citric acid):	Approx. 2%
Arsenic:	Max 3 ppm
Lead:	Max 2 ppm

Others:	Mercury: Max. 1 ppm
Others:	Cadmium: Max 1ppm
Others:	Aromatic and Halogenated Carbohydrates: Not detectable
Others:	Pesticides: The product complies with the German and European guidelines for residues of pesticides.
Others:	Mycotoxins: Not detectable
Others:	The product complies with the FAO/WHO purity requirements for food additives.

Microbiological

Standard Plate Count:	<1000/g
Yeast and Moulds:	<100/g
Enterobacteriaceae:	<1/g
Salmonella:	Negative/25g

Storage and Packaging

Shelf life & Storage:	12 months. Store cool, dry,protected from light and tightly closed.
Packaging:	Aluminium bags, 10 kg net, paper bags 25kgs.
Dangerous Goods Class:	Non Hazardous.

3. Product Name: Red beet juice concentrate

DISCOVER AND DELIVER

COLOUR SPECIFICATION

Product Name	:	**Red Beet Juice Concentrate**
Sample Code	:	**615**
Sales Code	:	
Product Description	:	**Red to dark purple liquid**

Application Information

Typical Usage Rate	:	Application	Dose	Unit
		Candies	1 – 3	Kg : 1000 Kg
		Ice Cream	1 – 3	Kg : 1000 Kg
		Test Medium (water, demineralized)	1	Kg : 1000 Kg
Direction for Use	:	Depends on application		

Regulatory Information

Regulatory Status	:	Colouring Foodstuff (Beetroot Juice Concentrate)			
Ingredient List	:	Beetroot Juice Concentrate, Citric Acid (E330)			
		Suitable	**Certified**	**Not Suitable**	**Information available on request**
Kosher	:	☐	☐	☐	☒
Halal	:	☐	☐	☐	☒
GMO Status*	:	Non-GM sourced materials.			
Allergen Status**	:	None			
Country of Origin	:	Germany			

* In accordance with Australian and New Zealand Food Standards Code Standard 1.5.2 – Food produced using Gene Technology
**According to table to clause 4 of the Food Standards Australia New Zealand Standard 1.2.3. All due care given to reduce any cross contamination at the point of manufacture but this is not a 100% guarantee of allergen absence as per the manufacturers full allergen and cross contamination policy.

Physical / Chemical Data

		Target	Min	Max	Unit	
Brix	:	69.000	66.000	72.000	°Bx	Refractometric (20°C)
pH	:	4.500	4.000	5.000		Potentiometric (20°C)

Microbiological Data

		Max	Unit
Aerobic, mesophilic total viable count	:	<1000	cfu/g
Coliforms and E.coli	:	Neg	/g
Yeasts	:	<100	cfu/g
Moulds	:	<100	cfu/g

Shelf life and Storage

Storage and Shelf Life	:	Refrigerated (+5° to +8°C) in unopened containers	:	270	Days
		Frozen (-18°C)		730	Days
Hazardous Goods	:	Non-Hazardous			

4. **Product Name: Orange Flavour N264-DG**

Product Specification

Product Code	961827
Product Name	ORANGE FLAVOUR N264 - DG
Country of Origin:	New Zealand manufacture with local and imported ingredients.
Allergens:	No Allergens.
GMO Status:	No Genetically modified ingredients /additives.
Usage:	Start at 0.03 % and adjust as required.

Quality Parameters

Appearance:	Clear straw colour liquid.
Taste & Odour:	Typical of orange. Free from off or objectionable flavours.
Regulatory:	Synthetic. Product complies with Australia/New Zealand Food Standards.BATF Reg.# 5305.
Halal:	Not Compliant.
Kosher:	Compliant.
Ingredients:	Flavouring.

Physical

Refractive Index:	1.379 - 1.382 at 20ºC.
Specific Gravity:	0.80 - 0.82 at 20ºC.
Flash Point:	13ºC (Closed Cup).

Storage and Packaging

Shelflife & Storage:	12 months from the date of manufacture when stored at 20ºC in full closed containers out of sunlight.
Packaging:	HDPE non-returnable 2,5,15,25 L containers DG approved
Labelling:	Product is labelled with Code, Name, Batch Number, Best Before Date and quantity.
Lot Number:	Sequential lot number for tracebility, and date code

	(batch/yr.day.mo)
Availability:	5 litres minimum ex stock Auckland. Larger quantities may have a lead time of 8-10 weeks, please check.
Dangerous Goods Class:	Hazardous Class 3.

5. Product Name: Lemon Flavour N326-DG

Product Specification

Product Code	962007
Product Name	LEMON FLAVOUR N326 -DG
Country of Origin:	New Zealand manufacture with local and imported ingredients.
Allergens:	No Allergens. Ref. FSANZ Standard 1.2.3.
GMO Status:	Free from Genetically modified ingredients and components.
Usage:	Start at 0.10 % and adjust as required.

Quality Parameters

Taste & Odour:	Typical of lemon. Free from off or objectionable flavours.
Appearance:	Clear colourless liquid.
Regulatory:	Natural. Product complies with Australia/New Zealand Food Standards.
Halal:	Not Compliant.
Kosher:	Compliant.
Ingredient Declaration:	Flavouring.

Physical

Refractive Index:	1.369 ± 0.002 at 20ºC.
Specific Gravity:	0.915 ± 0.015 at 20ºC.
Flash Point:	18ºC (Closed Cup).

Storage and Packaging

Shelf life & Storage:	12 months from the date of manufacture when stored unopened in original container under airtight ambient conditions away from direct sunlight.
Packaging:	HDPE non-returnable 2,5,15,25 L containers DG approved
Labelling:	Product is labelled with Code, Name, Batch Number, Best Before Date and Quantity.

Lot Number:	Sequential batch number for traceability, and date code. (batch/yr day mo).
Dangerous Goods Class:	Hazardous Class 3.

6. Apple Flavour NAT 407540-DG

Product Specification

Product Code	965217
Product Name	APPLE FLAV NAT 407540 -DG
GMO Status:	No Genetically modified ingredients /additives.
Allergens:	No Allergens. Ref. FSANZ Standard 1.2.3.
Usage:	Start at 0.10 % and adjust as required.
Country of Origin:	U.S.A.

Quality Parameters

Appearance:	Clear colourless liquid.
Taste & Odour:	Typical of apple. Free from off or objectionable flavours.
Ingredients:	Flavouring.
Halal:	Not Compliant.
Kosher:	Certified.
Regulatory:	Natural. Product complies with Australia/New Zealand Food Standards. Legal in Japan.

Physical

Refractive Index:	1.349 ± 0.01 at 20ºC.
Specific Gravity:	1.009 ± 0.02 at 20ºC.
Flash Point:	60ºC (Closed Cup).

Storage and Packaging

Shelf life & Storage:	12 months from the date of manufacture when stored unopened in original container under airtight refrigerated conditions away from direct sunlight.
Packaging:	HDPE non-returnable 2,5,15,25 L containers DG approved
Labelling:	Product is labelled with Code, Name, Batch Number,and quantity.

Lot Number:	Begin with letter B which designates manufacturing location, and are followed by a six (6) number that is assigned sequentially.
Dangerous Goods Class:	Hazardous Class 3.

Appendix B: Questionnaires for two sensory trials

Sensory Evaluation Questionnaire

Please tick the right box:

Q1. What is your gender?

Female ☐

Male ☐

Q2. What age are you?

18 –25 ☐

26 – 35 ☐

36 – 45 ☐

46 – 55 ☐

56 – 65 ☐

65 + ☐

Q3. Have you tried beetroot juice before?

Yes ☐

No ☐

If Yes, how many times do you drink beetroot juice and what brand

_____

_____

We would like to ask your opinion about different flavoured beetroot juice by answering few questions. Please take 20 s gap between samples and rinse your mouth with water.

Sample 820

In front of you is a coded **sample 820** of sports beverage. Please answer the following questions.

Q1. Please tell us your overall impression of the beverage after having looked at, smelled and tasted the product. Please circle *one* response only.

Dislike extremely	Dislike very much	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**

Q2. What do you think of the acidity of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Q3. What do you think of the sweetness of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Q4. What do you think of the flavour of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Any additional comments

Sample 945

In front of you is a coded **sample 945** of sports beverage. Please answer the following questions.

Q1. Please tell us your overall impression of the beverage after having looked at, smelled and tasted the product. Please circle *one* response only.

Dislike extremely	Dislike very much	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**

Q2. What do you think of the acidity of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Q3. What do you think of the sweetness of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Q4. What do you think of the flavour of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Any additional comments

Sample 569

In front of you is a coded **sample 569** of sports beverage. Please answer the following questions.

Q1. Please tell us your overall impression of the beverage after having looked at, smelled and tasted the product. Please circle *one* response only.

Dislike extremely	Dislike very much	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**

Q2. What do you think of the acidity of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Q3. What do you think of the sweetness of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Q4. What do you think of the flavour of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Any additional comments

Sample 718

In front of you is a coded **sample 718** of sports beverage. Please answer the following questions.

Q1. Please tell us your overall impression of the beverage after having looked at, smelled and tasted the product. Please circle *one* response only.

Dislike extremely	Dislike very much	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**

Q2. What do you think of the acidity of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Q3. What do you think of the sweetness of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Q4. What do you think of the flavour of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Any additional comments

_____

_____

_____

Sample 433

In front of you is a coded **sample 433** of sports beverage. Please answer the following questions.

Q1. Please tell us your overall impression of the beverage after having looked at, smelled and tasted the product. Please circle *one* response only.

Dislike extremely	Dislike very much	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**

Q2. What do you think of the acidity of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Q3. What do you think of the sweetness of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely

1	2	3	4	5	6	7

Q4. What do you think of the flavour of the beverage? Please circle *one* response only.

Dislike extremely	Dislike Moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like extremely
1	**2**	**3**	**4**	**5**	**6**	**7**

Any additional comments

_____

_____

_____

Triangle consumer sensory questionnaire

Instructions: Taste samples from left to right. Two are identical; determine which the odd sample is. If no difference is apparent, you must state that the odd sample is not detectable.

Triangle Sensory Test on Beverages

Please take a drink of water before tasting beverage samples. Drink beverage samples from left to right, and please take a sip of water between samples. **Place an "√" under the beverage which is different than the others.**

569 **820** **685**

_____ _____

_____

Comments:

Appendix C: Forms used during sensory evaluation (participant consent form, participant information form and ethics committee approval form)

Participants Information form

INFORMATION SHEET

TITLE of Work: Development of vegetable juice for health and sports sector.

Researcher(s) Introduction

Researchers Name:	Tejal Kolte	Supervisors Name:	Dr John Grigor
Contact Details:	02102783276	Contact Details:	(09) 4140800

You are invited to take part in a *consumer sensory* to taste vegetable juice formulations targeted to improve performance in sports and exercise.

The types of activities that this work involves includes*: tasting of juice samples and answering a sensory questionnaire.* Your participation in this activity will take approximately 10-15 mins.

The type of food that you will be testing is: Vegetable juice (Juice pressed from beetroots, apples and natural flavouring).

The information collected in this study will be used to complete a thesis in partial fulfilment of the Master of Technology in Food Technology. No data linked to an individual's identity will be collected. In some circumstances the research may be published.

You should not participate in this trial if you have any doubts concerning the appropriateness of this food with respect to your religious, ethical or cultural beliefs or any other reason.

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- *decline to answer any particular question;*
- *ask any questions about the study at any time during participation;*
- *provide information on the understanding that your name will not be used unless you give permission to the researcher;*

If you have any questions about this work, please contact one of the people indicated above.

> *"This project has been evaluated by peer review and judged to be low risk. Consequently, it has not been reviewed by one of the University's Human Ethics Committees. The researcher(s) named above are responsible for the ethical conduct of this research.*
>
> *If you have any concerns about the conduct of this research that you wish with someone other than the researcher(s), please contact Professor John O'Neill, Director (Research Ethics),telephone 06 350 5249, email humanethics@massey.ac.nz".*

Participant Consent form

PARTICIPANT CONSENT FORM

- I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

- I have advised and discussed the Researcher of any potentially relevant cultural, religious or ethical beliefs that may prevent me from consuming the Foods under consideration.

- I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: ..... **Date:** .....

Full Name - printed .....

Ethics Committee Approval form

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA

17 October 2013

Tejal Kolte
Unit 4Q
15 Nelson Street
AUCKLAND 1010

Dear Tejal

Re: Development of an Acceptable, Stable and Safe Nitrate-Rich Vegetable Juice Beverage for the Sports Performance Market

Thank you for your Low Risk Notification which was received on 10 September 2013.

Your project has been recorded on the Low Risk Database which is reported in the Annual Report of the Massey University Human Ethics Committees.

The low risk notification for this project is valid for a maximum of three years.

Please notify me if situations subsequently occur which cause you to reconsider your initial ethical analysis that it is safe to proceed without approval by one of the University's Human Ethics Committees.

Please note that travel undertaken by students must be approved by the supervisor and the relevant Pro Vice-Chancellor and be in accordance with the Policy and Procedures for Course-Related Student Travel Overseas. In addition, the supervisor must advise the University's Insurance Officer.

A reminder to include the following statement on all public documents:

> *"This project has been evaluated by peer review and judged to be low risk. Consequently, it has not been reviewed by one of the University's Human Ethics Committees. The researcher(s) named above are responsible for the ethical conduct of this research.*
>
> *If you have any concerns about the conduct of this research that you wish to raise with someone other than the researcher(s), please contact Professor John O'Neill, Director (Research Ethics), telephone 06 350 5249, e-mail humanethics@massey.ac.nz".*

Please note that if a sponsoring organisation, funding authority or a journal in which you wish to publish requires evidence of committee approval (with an approval number), you will have to provide a full application to one of the University's Human Ethics Committees. You should also note that such an approval can only be provided prior to the commencement of the research.

Yours sincerely

J. O'Neill

John G O'Neill (Professor)
Chair, Human Ethics Chairs' Committee and
Director (Research Ethics)

cc
Assoc Prof Marie Wong
IFNHH
Albany

Dr Ajmol Ali
IFNHH
Albany

Prof Richard Archer, HoI
IFNHH
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Dr John Grigor
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Appendix D: Randomisation charts used for sensory trials

Sensory Evaluation randomisation charts (n=70)

Consumer	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
1	718	820	569	433	945
2	433	820	945	718	569
3	945	569	820	433	718
4	820	945	569	718	433
5	945	718	433	569	820
6	433	945	820	569	718
7	820	718	569	433	945
8	718	820	945	569	433
9	945	433	569	718	820
10	718	433	945	820	569
11	820	945	569	433	718
12	569	433	820	718	945
13	945	569	433	718	820
14	820	718	433	945	569
15	945	718	569	820	433
16	820	433	945	569	718
17	569	433	820	718	945
18	433	820	718	945	569
19	820	718	569	945	433
20	569	945	718	433	820
21	569	433	945	820	718
22	718	820	569	433	945
23	945	820	433	569	718
24	569	718	820	945	433

25	820	945	433	718	569
26	569	820	433	945	718
27	718	945	569	820	433
28	569	433	718	945	820
29	718	569	433	820	945
30	569	945	433	820	718
31	433	945	820	718	569
32	718	433	569	945	820
33	820	718	433	569	945
34	433	945	820	569	718
35	945	820	718	433	569
36	569	945	433	820	718
37	820	433	569	718	945
38	433	718	945	569	820
39	718	433	945	820	569
40	820	569	945	718	433
41	433	945	820	569	718
42	718	433	820	945	569
43	569	945	820	433	718
44	569	820	433	718	945
45	945	718	569	820	433
46	569	433	820	945	718
47	820	718	433	945	569
48	945	433	569	718	820
49	433	718	569	820	945
50	820	569	433	945	718
51	433	718	945	820	569

52	945	820	718	569	433
53	718	433	945	569	820
54	820	718	569	433	945
55	433	820	569	945	718
56	820	945	433	569	718
57	569	820	718	945	433
58	945	718	569	433	820
59	433	820	945	718	569
60	718	569	433	820	945
61	945	820	718	433	569
62	569	945	433	820	718
63	945	569	820	433	718
64	820	945	569	718	433
65	945	718	433	569	820
66	433	945	820	569	718
67	433	945	820	569	718
68	820	718	569	433	945
69	718	820	945	569	433
70	945	433	569	718	820

Triangle Test randomisation chart (n=25)

Placebo codes: 416, 685

Standard juice codes: 298, 978

Order number	Sample codes (Order of presentation)	Panellist numbers
1	416, 298, 685	1, 7, 13, 19, 25
2	416, 298, 978	2, 8, 14, 20
3	298, 416, 978	3, 9, 15, 21
4	298, 978, 416	4, 10, 16, 22
5	416, 685, 298	5, 11, 17, 23
6	298, 416, 685	6, 12, 18, 24

Appendix E:

P values to determine if there was a significant difference in the nitrate and nitrite concentration of beetroot juice at three different temperatures over a period of two weeks

P VALUES						
Days	Nitrate concentration			Nitrite concentration		
	-80°±1°C	-20°±1°C	4°±1°C	-80°±1°C	-20°±1°C	4°±1°C
1	0.95	0.96	0.96	1	0.84	0.89
2	0.58	0.61	0.18	0.68	0.68	0.73
5	0.66	0.51	0.02	0.63	0.63	0.44
7	0.08	0.07	0.02	0.45	0.53	0.33
14	0.001	0.0001	0.000000001	0.29	0.29	0.23

P values to determine if there was a significant difference in the pH, titratable acidity and °brix values of beetroot juice over a period of two weeks

	P VALUES		
Days	pH	Titratable acidity	°Brix
0	0.9	0.99	0.96
1	0.9	0.99	0.96
2	0.86	0.95	0.95
5	0.9	0.98	0.97
7	0.9	0.95	0.96
14	0.84	0.95	0.96

P values to determine if there was a significant difference in the nitrate and nitrite concentrations, pH, titratable acidity and °brix of beetroot, celery and beet leaves juice from the pilot plant trial. P values for beetroot are mean scores of lots 1, 2 and 3.

P VALUES					
JUICES	Nitrate concentration	Nitrite concentration	pH	Titratable acidity	°Brix
Beetroot and celery	0.8	0.8	0.78	0.8	0.59
Beetroot and beet leaves	0.83	0.6	0.75	0.79	0.63
Celery and beet leaves	0.87	0.19	0.25	0.94	0.78

Appendix F: Total Coliforms (cfu/ml) lab report from Assure Quality

AsureQuality Auckland
131 Boundary Road
Blockhouse Bay
PO Box 41
Auckland
New Zealand

Phone: +64 9 626 8000
Fax: +64 9 626 8282
email: vlabauckland@asurequality.com

17 Dec 2013

Massey University Albany - Food Division
Private Bag 102-904
North Shore Mail Centre
Auckland 0745

Submitted By	Massey University Albany - Food Division
Customer ID	Beetroot 4/12/13
Job Type	Routine
Sampled By	Tejal Kotle
Date/Time Submitted	04 Dec 2013 00:00
Date/Time Received	04 Dec 2013 14:55
Order No.	PN 132433

Attention: **Helen Matthews**

Final LABORATORY REPORT - Job Number 1770893

Comments
Temperature of samples on arrival in laboratory: Ambient.

Lab Ref	Sample Description	Dates	Test	Test Result
1770893-1	Beetroot juice A	Manufactured: 04 Dec 13 Best Before: 04 Dec 13 Sampled: 04 Dec 13 11:45	Coliforms cfu/g	>1500
1770893-2	Beetroot juice B	Manufactured: 04 Dec 13 Best Before: 04 Dec 13 Sampled: 04 Dec 13 11:45	Coliforms cfu/g	>1500
1770893-3	Beetroot juice C	Manufactured: 04 Dec 13 Best Before: 04 Dec 13 Sampled: 04 Dec 13 11:45	Coliforms cfu/g	<1
1770893-4	Beetroot juice D	Manufactured: 04 Dec 13 Best Before: 04 Dec 13 Sampled: 04 Dec 13 11:45	Coliforms cfu/g	<1

Method Reference Coliforms / APHA 8.73

Preetika Prasad
Scientific Analyst

Appendix G: Blending protocol using MINITAB

The nitrate and brix content of individual juices (beetroot, celery, apple, beet stalk) were put into MINITAB with the final nitrate content to be ≥1 g/L and °Brix content of ≥10°Brix. The nitrate (g/L) and °brix values of beetroot, celery and beet leaves juice from **Table 4.8** were entered in MINITAB to determine the likely percentages (in Table below). The nitrate content of apple juice was <0.1 g/L as determined on HPLC and the brix was around 10. MINITAB calculated all the possible percentages of juices in order to meet the requirement. Ratios of different juices were chosen based on the highest nitrate content and formulations were finalised based on sensory evaluation.

Percentage % (w/w)					
Beetroot juice (w/w)	Celery juice (w/w)	Apple juice (w/w)	Beet leaves juice (w/w)	Total nitrate content (g/L)	°Brix
75.188	11.125	12.563	1.125	1.188	10.973
72.375	12.250	13.125	2.250	1.183	10.764
70.000	10.000	15.000	5.000	1.138	10.705
72.688	13.625	12.563	1.125	1.201	10.740
78.000	10.000	12.000	0.000	1.194	11.182
71.188	13.625	12.563	2.625	1.196	10.619
75.000	10.000	15.000	0.000	1.155	11.110
70.000	13.000	12.000	5.000	1.192	10.498
73.688	11.125	14.063	1.125	1.169	10.937
71.188	12.625	12.563	3.625	1.187	10.631
71.188	11.125	14.063	3.625	1.160	10.734
70.000	15.000	15.000	0.000	1.180	10.645
73.000	10.000	12.000	5.000	1.177	10.777
72.688	11.125	12.563	3.625	1.180	10.770
70.000	15.000	12.000	3.000	1.209	10.474
73.000	15.000	12.000	0.000	1.219	10.717
71.188	13.625	14.063	1.125	1.181	10.704
68.000	12.000	15.000	5.000	1.100	10.712

Beetroot juice	Celery juice	Apple juice	Nitrate content	Brix
76.667	11.667	11.667	1.207	11.000
75.833	10.833	13.333	1.181	11.000
75.000	15.000	10.000	1.245	10.800
75.000	10.000	15.000	1.155	11.100
75.833	13.333	10.833	1.226	10.900
78.333	10.833	10.833	1.213	11.100

80.000	10.000	10.000	1.220	11.200

Beetroot juice	Beet stalk juice	Apple juice	Nitrate content	Brix
86.000	4.000	10.000	1.156	11.836
85.667	4.167	10.167	1.153	11.819
85.167	4.167	10.667	1.147	11.807
85.333	4.333	10.333	1.151	11.801
85.000	4.000	11.000	1.143	11.812
85.000	5.000	10.000	1.153	11.775
85.167	4.667	10.167	1.152	11.778

Beetroot juice	Celery juice	Apple juice	Beet stalk juice	Nitrate content	Brix
65.125	20.125	10.125	4.625	1.253	10.000
65.625	20.125	10.125	4.125	1.255	10.000
65.250	20.250	10.250	4.250	1.253	10.000
66.000	20.000	10.000	4.000	1.256	10.000
65.000	20.000	10.000	5.000	1.253	9.900
65.000	21.000	10.000	4.000	1.261	9.900
65.125	20.125	10.625	4.125	1.248	10.000
65.000	20.000	11.000	4.000	1.243	10.000
65.125	20.625	10.125	4.125	1.257	10.000

Beetroot juice	Celery juice	Apple juice	Beet stalk juice	Nitrate content	Brix
60.125	25.125	10.625	4.125	1.273	9.500
60.000	25.000	10.000	5.000	1.278	9.400
60.125	25.625	10.125	4.125	1.282	9.400
60.250	25.250	10.250	4.250	1.278	9.500
60.000	26.000	10.000	4.000	1.286	9.400
60.00	25.000	11.000	4.000	1.268	9.500
60.125	25.125	10.125	4.625	1.278	9.400
60.625	25.125	10.125	4.125	1.280	9.500
61.000	25.000	10.000	4.000	1.281	9.500

Beetroot juice	Celery juice	Apple juice	Nitrate content	Brix
63	25	12	1.269	9.8

59.75	28.75	11.5	1.294	9.45
63.25	26.25	10.5	1.295	9.71
60.75	28.75	10.5	1.307	9.5
65	25	10	1.295	9.8
60	30	10	1.320	9.4
62.25	26.95	11.5	1.282	9.7
61.50	27.50	11	1.295	9.6
58	30	12	1.294	9.3

Appendix H: P values derived from Tukey's Test

	P VALUES			
Pairwise comparison between samples	Overall Liking	Acidity Liking	Sweetness Liking	Flavour Liking
Apple flavour high acid & orange flavour high acid	0.0002*	0.4660	0.6084	0.4721
Apple flavour high acid &apple flavour low acid	0.9504	0.7021	0.8912	0.9978
Apple flavour high acid &BEET IT	0.1691	0.0460*	0.8678	0.1919
Apple flavour high acid & orange flavour low acid	0.5296	0.9132	0.3561	0.5751
Orange flavour high acid & apple flavour low acid	0.0037*	0.9962	0.9859	0.6775
Orange flavour high acid& BEET IT	0.0000*	0.0087*	0.1147	0.0013*
Orange flavour high acid& orange flavour low acid	0.0521	0.9325	0.9946	0.9999
Apple flavour low acid & BEET IT	0.0260*	0.0279*	0.3241	0.0939
Apple flavour low acid& orange flavour low acid	0.9235	0.9930	0.8912	0.7723
BEET IT & orange flavour lowacid	0.0015*	0.0497*	0.0406*	0.0024*

*Significant at α=0.05

All the values with an asterisk (*) indicate that there is significant difference between the samples.

**Appendix I: Normality plots for five formulations (*apple flavour low acid, apple flavour high acid, oran*
***high acid* and *BEET IT*) for four attributes (overall product liking, acidity liking, sweetness liking and**

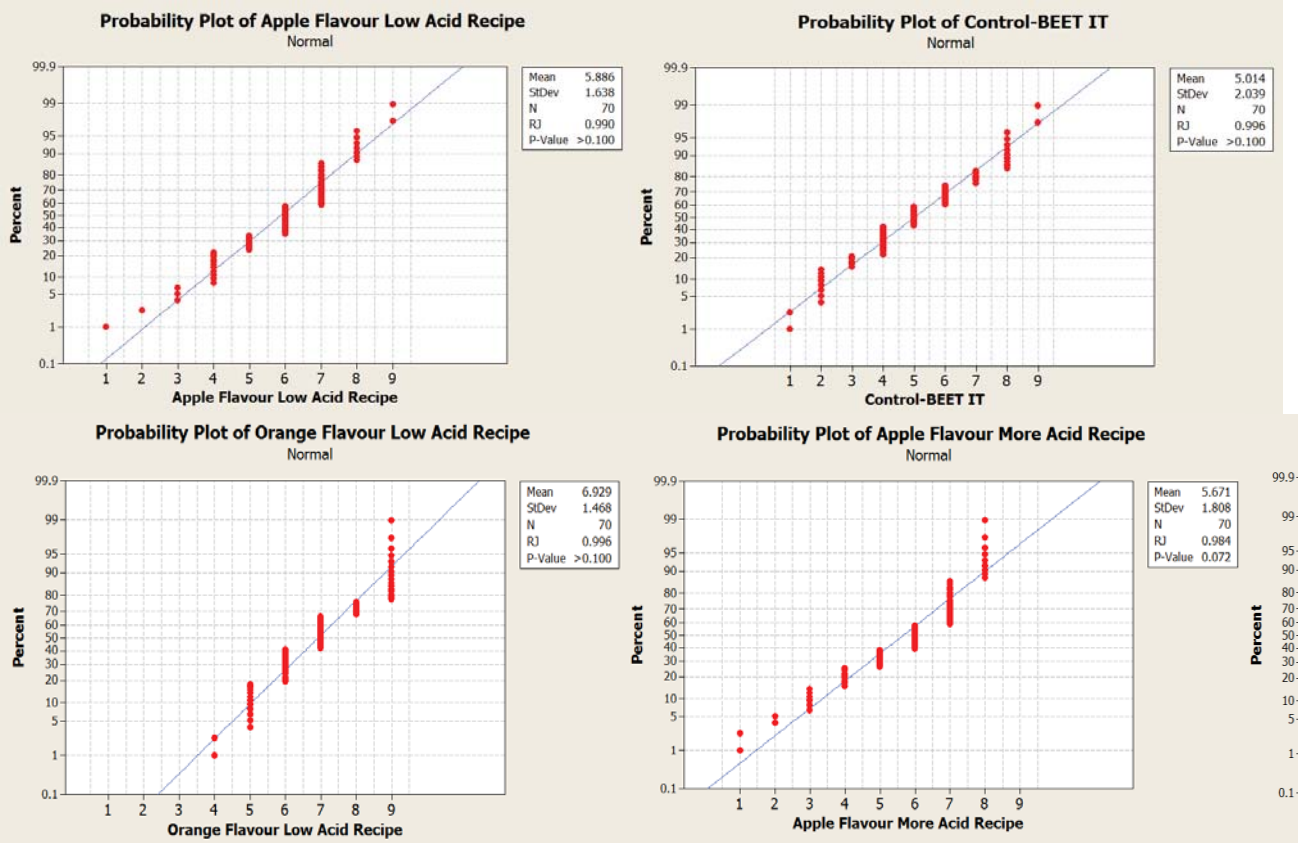

Figure 1: Normality distribution plots for overall product liking on five formulation blends (n=70)

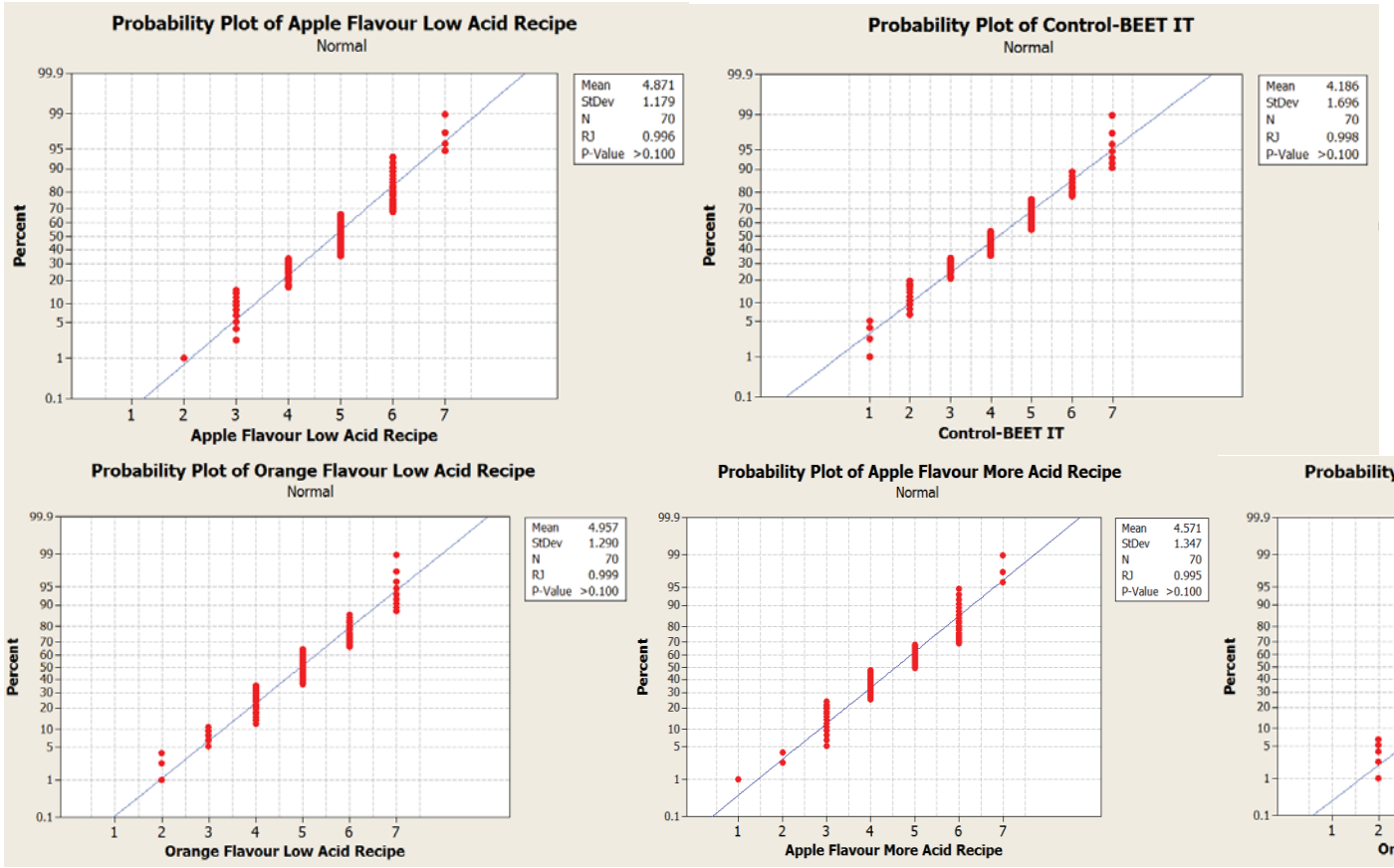

Figure 2: Normality distribution plots for acidity liking on five formulation blends (n=70)

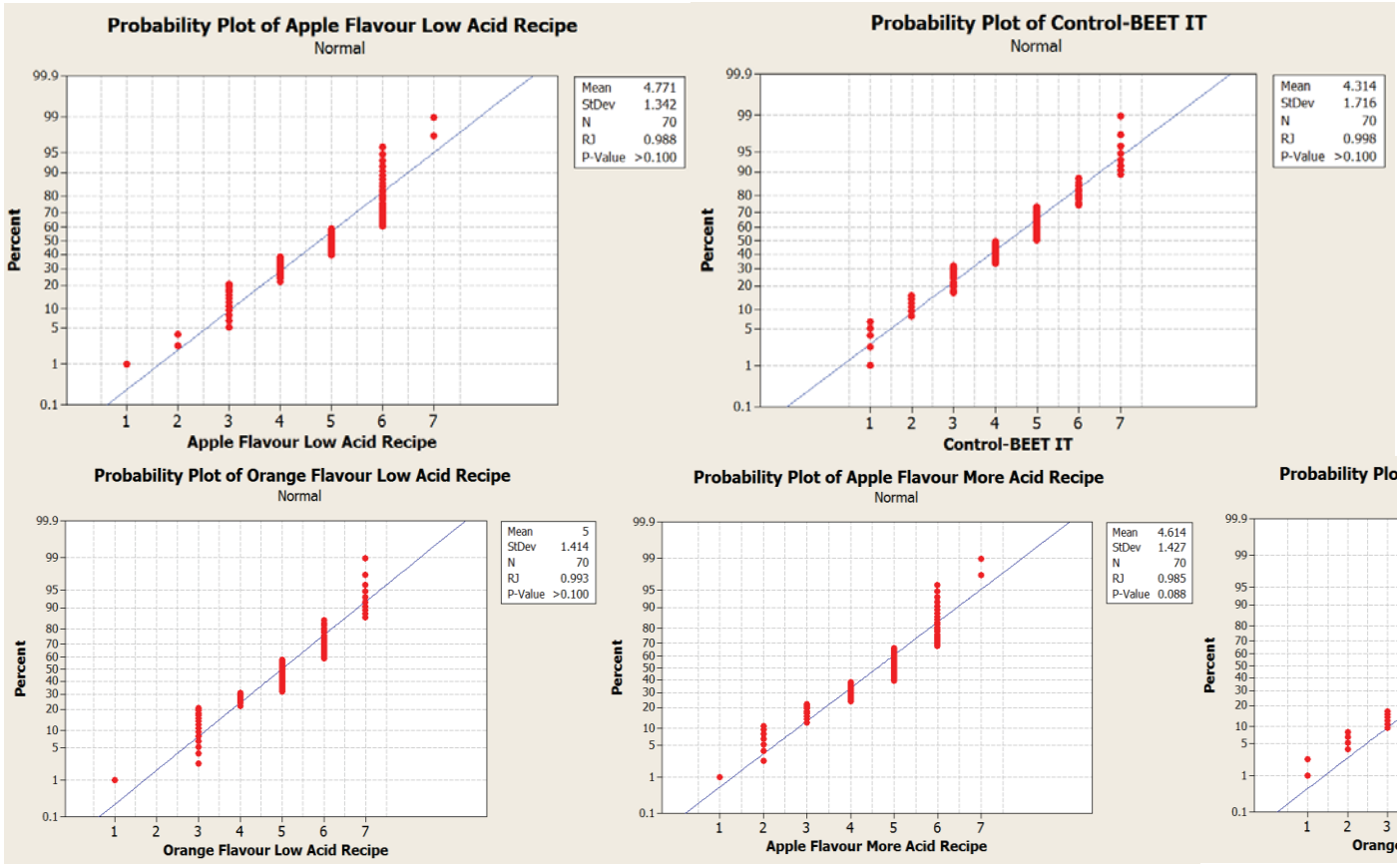

Figure 3: Normality distribution plots for sweetness liking on five formulation blends (n=70)

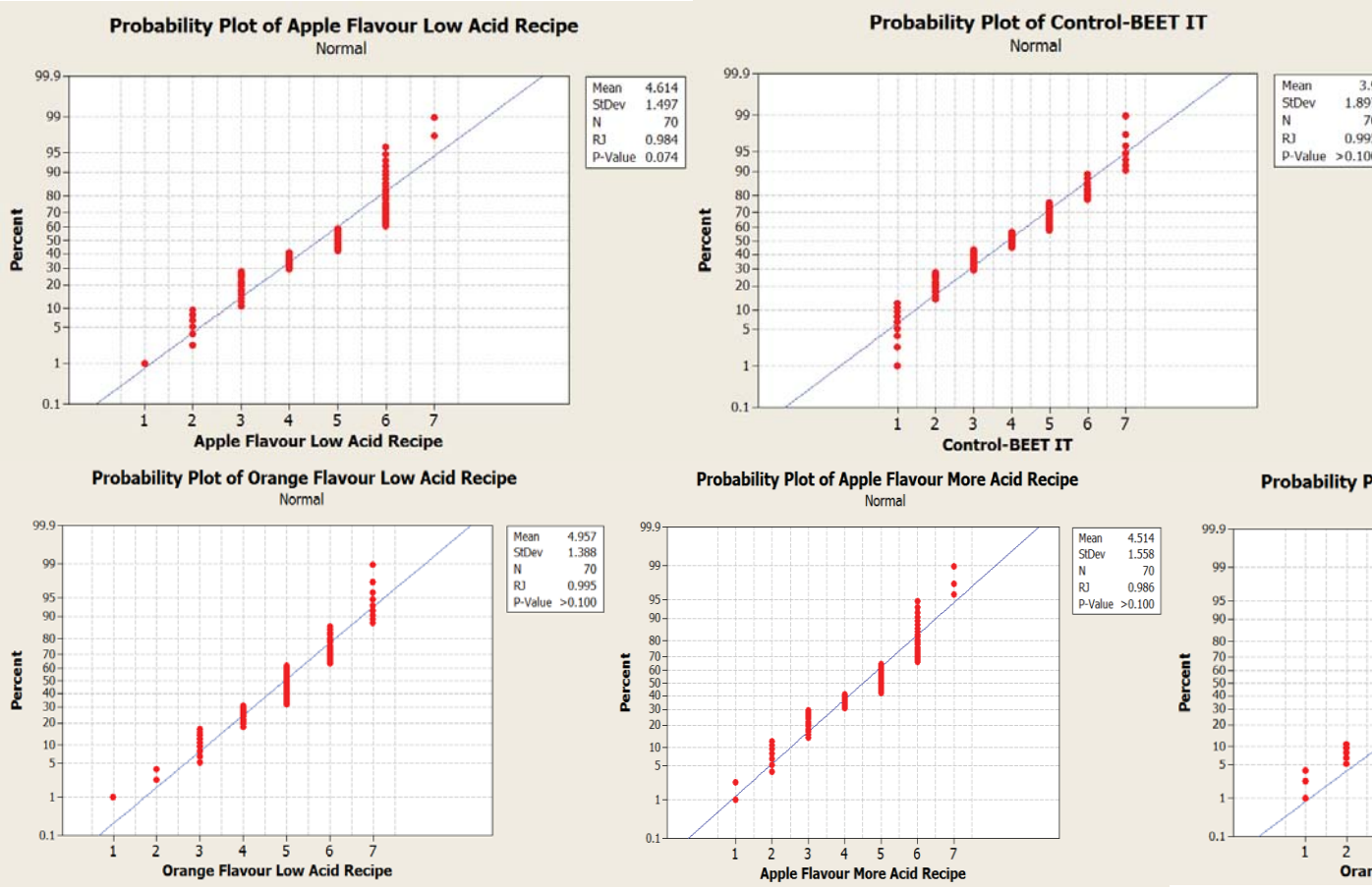

Figure 4: Normality distribution plots for flavour liking on five formulation blends (n=70)

Appendix J: Critical Values for Ryan-Joiner Test for Normality

		α		
		.10	.05	.01
n	5	.9033	.8804	.8320
	10	.9347	.9180	.8804
	15	.9506	.9383	.9110
	20	.9600	.9503	.9290
	25	.9662	.9582	.9408
	30	.9707	.9639	.9490
	40	.9767	.9715	.9597
	50	.9807	.9764	.9664
	60	.9835	.9799	.9710
	75	.9865	.9835	.9757

The critical values for significance level α=0.10,0.05 and 0.01 are approximated using the formula below (Ryan & Joiner, 1976):

$$CV(n) \approx 1.0071 - \frac{0.1371}{\sqrt{n}} - \frac{0.3682}{n} + \frac{0.7780}{n^2} \; [\alpha = 0.10]$$

$$CV(n) \approx 1.0063 - \frac{0.1288}{\sqrt{n}} - \frac{0.6118}{n} + \frac{1.3505}{n^2} \; [\alpha = 0.05]$$

$$CV(n) \approx 0.9963 - \frac{0.0211}{\sqrt{n}} - \frac{1.4106}{n} + \frac{3.1791}{n^2} \; [\alpha = 0.01]$$

Reference :http://www.statcato.org/doc/statcato-documentation.pdf

Appendix K: Minimum number of correct responses needed to conclude that a perceptible difference exists based on a triangle test (Meilgaard et al., 1999).

n	α					n	α				
	0,20	0,10	0,05	0,01	0,001		0,20	0,10	0,05	0,01	0,001
6	4	5	5	6	—	27	12	13	14	16	18
7	4	5	5	6	7	28	12	14	15	16	18
8	5	5	6	7	8	29	13	14	15	17	19
9	5	6	6	7	8	30	13	14	15	17	19
10	6	6	7	8	9						
						31	14	15	16	18	20
11	6	7	7	8	10	32	14	15	16	18	20
12	6	7	8	9	10	33	14	15	17	18	21
13	7	8	8	9	11	34	15	16	17	19	21
14	7	8	9	10	11	35	15	16	17	19	22
15	8	8	9	10	12						
						36	15	17	18	20	22
16	8	9	9	11	12	42	18	19	20	22	25
17	8	9	10	11	13	48	20	21	22	25	27
18	9	10	10	12	13	54	22	23	25	27	30
19	9	10	11	12	14	60	24	26	27	30	33
20	9	10	11	13	14	66	26	28	29	32	35
21	10	11	12	13	15	72	28	30	32	34	38
22	10	11	12	14	15	78	30	32	34	37	40
23	11	12	12	14	16	84	33	35	36	39	43
24	11	12	13	15	16	90	35	37	38	42	45
25	11	12	13	15	17	96	37	39	41	44	48
26	12	13	14	15	17	102	39	41	43	46	50

NOTE 1 Values in the table are exact because they are based on the binomial distribution. For values of n not in the table, compute approximate values for the missing entries based on the normal approximation to the binomial as follows. Minimum number of responses (x) = nearest whole number greater than

$$x = (n/3) + z\sqrt{2n/9}$$

where

z varies with the significance level as follows: 0,84 for $\alpha = 0{,}20$; 1,28 for $\alpha = 0{,}10$; 1,64 for $\alpha = 0{,}05$; 2,33 for $\alpha = 0{,}01$; 3,09 for $\alpha = 0{,}001$.

NOTE 2 Values of $n < 18$ are usually not recommended for a triangle test for a difference.

NOTE 3 Adapted from Reference [11].

In the row corresponding to n= 25 panellists and the column corresponding to α=0.05, 7 correct responses are sufficient to conclude that the two samples are not perceptibly different (less than correct responses=13;α=0.05 and n=25).