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Oxalate content of green juices

and strategies for reduction of soluble oxalate content

A thesis

submitted in partial fulfilment

Of the requirements for the Degree of

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Leo Vanhanen

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Abstract of a thesis submitted in partial fulfilment of the

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Abstract

Oxalate content of green juices

by

Leo Vanhanen

Green juices are an example of a modern food innovation that has developed partly by itself in response to general nutritional advice in the media imploring people to consume more fruits and vegetables. Unfortunately, the risks of developing acute oxalate nephropathy is high following the consumption of some green juices and fruit juices.

This study investigates the occurrence and levels of oxalates in green juices made from common fruit and vegetables available in New Zealand. The influence of the type of juicer used to prepare the juices, and the use of treatments and/or additions to reduce the oxalate content of these mixed juices, was also investigated.

A Design of Experiments multi-factor approach was used to investigate the extraction of oxalates from a green juice. Fresh spinach samples were juiced in a high speed blender and the oxalate contents were extracted using seven levels of pH, eight levels temperature and six extraction times. A statistically significant quadratic model with an overall level of significance of $P < 0.0002$ was achieved by using 20 experimental runs performed over one day. The optimum conditions for extracting all the oxalate from the model green spinach juice was at pH 0.93, with a temperature of 65°C and at any time greater than 15 minutes. The least amount of oxalic acid was extracted at pH 4.59 with a temperature of 25°C.

To investigate the levels of oxalates found in homemade green juices, five green juice recipes containing between three and nine ingredients were made. Fresh spinach was the common component, and ranged from 20.1% to 37.9% w/w. The oxalate content ranged from 90.34 to

152 and 13.0 to 82.85 mg/100 g fresh weight (FW) for total and soluble oxalates, respectively. The percentage of soluble oxalate varied from 11.9 to 67.8%. The juices were made using a masticating juicer (MJ), which separates out a waste pulp fraction that is normally discarded. This fraction contained large amounts of oxalic acid, 134.6 to 348.09 mg/100 g FW in total. The waste pulp also had a higher concentration of minerals of all 11 minerals present, apart from Al, compared to the green juice. It is proposed there is an interaction between the oxalate, calcium and the pulp fraction of the green juice. A 200 g glass of green juice containing a mix of apple, celery and spinach, contained 165.7 mg of soluble oxalate; this is a considerable amount of soluble oxalate to ingest.

In the home, juicing commonly uses two basic types of juicer, a high speed blender (HSB) or a masticating juicer (MJ); the MJ separates out a pulp fraction while the HSB does not. High and low spinach containing juices were made using both juicers. The MJ had a concentrating effect during processing, resulting in 528.41 mg total oxalate/100 g FW compared to the HSB, at 369.47mg total oxalic acid/100 g FW. They both produced green juices that had similar ratios of soluble oxalate, for both the high and low spinach juices. A significant amount of the oxalate and minerals were separated out into the waste pulp fraction from the juice made using the MJ. The waste pulp contained 238.10 to 55.67 mg total oxalate/100 g FW and 61.7 to 74.7% of the calcium content of the original spinach leaves.

Two strategies to lower the soluble oxalate content of green juices were investigated; direct addtion of a food grade calcium salt, and soaking the raw materials used to make the fresh green juice. Four calcium salts tested. Calcium chloride was the most effective, reducing the soluble oxalate by 98.3% after the addtion of 500 mg/100 g to the fresh juice. Fresh spinach leaves were soaked in either tap water, a 1% w/v/ NaCl or a 1%w/v CaCl solution, prior to processing into a juice. The tap water and 1% w/v NaCl showed no loss of soluble oxalate but the 1% w/v CaCl treatment showed a 50% reduction in soluble oxalate and a 28.% increase in insoluble oxalate. This treatment had the added benefit of increasing the bioavailabilty of calium in the soaked spinach leaves. This study has shown that there are high levels of oxalate in green juices and, if consumed, this could pose a risk for diet induced nephropathy.

Keywords: Oxalic acid, soluble oxalic acid, oxalate, juice, green juice, masticating juicer, high speed blender, calcium chloride, soaking, kidney stone, calcium oxalate, green leafy vegetables, oxalate nephropathy

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I would finally like to devote this thesis to all of my family. Especially to my mother, my sister and my sister in-law, who are no longer with us. A special mention too for my Dad who turned 90 this year.

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Chapter 1
Introduction

1.1 Research background

Oxalic acid is an end-product of metabolism and has no known metabolic role. Once passed into the blood, it is filtered through the kidneys and excreted in the urine. The oxalic acid excreted by the kidney tubules can combine with the calcium excreted at the same time to form crystals and these, then, combine to form kidney stones. Calcium oxalate crystals are responsible for the formation of 70 - 80% of all kidney stones (Lewandowski & Rodgers, 2004). Kidney stone disease is an increasingly common disease in industrialised nations, affecting 15% of men and 6% of women (Bihl & Meyers, 2001). In the United States, the overall stone prevalence doubled between 1964-1972 (prevalence 2.6%) and 1988-1994 (prevalence 5.4%) (Romero *et al.*, 2010). Dietary and lifestyle factors are believed to be the cause (Neisius & Preminger, 2013), with people consuming more oxalate-containing foods, or foods being cooked in different ways that allow more oxalate to be retained in the food. It has been estimated that the financial burden from kidney stone disease in New Zealand was 18 million dollars in 2002 (Davidson *et al.*, 2009).

The development of kidney stones is a complex and multifaceted disease in which diet is one of the most important determinants (Lewandowski & Rodgers, 2004). As approximately 75% of all kidney stones are composed of calcium oxalate (Chai & Liebman, 2005), the oxalate content of foods and their metabolism in the body are important risk factors. Oxalates excreted by the kidney come from only two sources: 1) from ascorbate, glyoxylate and glycine metabolism in the liver (Hodgkinson, 1977); or 2) from oxalate-containing foods. The consumption of high oxalate-containing foods can increase the excretion of oxalate in urine, leading to an increased risk of kidney stone formation (Massey, 2003; Siener & Hesse, 2002; Holmes & Assimos, 2004). The risk of consuming foods high in soluble oxalates has been well documented (Noonan & Savage, 1999; Massey, 2003) but many studies measuring the oxalate content of foods have not always recognized that two forms of oxalate exist (soluble and insoluble) and the relative amounts of each are governed by their calcium contents and the pH of the foods (Holmes *et al.*, 2001; Holmes & Kennedy, 2000).

The oxalic acid content of commonly-eaten green vegetables and fruit, in a 'typical' Western diet has been reasonably well documented (Morrison & Savage, 2003), but there are many foods that have not been fully investigated. Food security for a growing population and a lack of diversification in crops are recent issues that have been highlighted that could make the problem worse (Kahane *et al.*, 2013). This study suggests that agro-biodiversity as a way to increase food security in the future as it will positively influence the nutritional status and, thus, increase human productivity.

Throughout human history it has been estimated that between 40-100,000 species of plants have been used by humans to provide food, fibres, and other uses. Around 7,000 cultivated species are used today, but only 30 crop species are used extensively and they form the basis of today's global agriculture (GFAR, 1999).

Many research programmes have been initiated globally to investigate modern cultivation techniques and the use of other ancient and rediscovered crops. This has not only been an attempt to improve food security, but also to enable the production and introduction of 'new crops'. These new crops may offer nutritional benefits that have not been appreciated so far (Hoeschle-Zeledon *et al.*, 2009; Jaenicke, 2013). In addition to the nutritionally beneficial aspects of these new crops, it would be remiss not to consider the potential anti-nutritive factors they may contain. Many of the new crops being introduced into Western diets are green leafy vegetables, while, in some cases, stems and roots are also eaten.

The Asian Vegetable Research and Development Center (AVRDC-The World Vegetable Center; Chavasit, 2012) focuses on the research and development of vegetables to alleviate poverty and improve nutritional status of the population in developing countries. At a recent symposium, co-organised by AVRDC (Holmer *et al.*, 2013), local and indigenous plants were promoted as a strategy to improve nutrition security (Chavasit, 2012). More specifically, Ebert (2012) promoted high value speciality vegetables, such as sprouts, microgreens and flowers, as they are widely eaten in certain parts of the world. These present an opportunity to diversify and enrich diets for both home and restaurant use.

The Vegetarian Resource Group (Wasserman, 2013) has regularly polled North Americans about their vegetable eating habits. Until recently, they have limited the poll questions, asking the basics, such as, "Do you never eat meat, fish, seafood or poultry?" Since 1994, this type of polling has resulted in very similar results each year, reporting that approximately 4% never

ate meat, fish, seafood or poultry. Recently, they have added extra questions, such as "would you eat one or more vegetarian meals per week?" Not including vegetarians and vegans in this response, 43% of respondents said "yes." This has been interpreted as a clear signal that vegetable-based meals are growing in importance in Western culture.

The objective of increasing fruit and vegetable consumption in the Western world has resulted in many innovative ideas. Recent innovations or trends in vegetable and fruit consumption have focused around delivery mechanisms to allow the freshest and most nutritiously possible products available using minimal processing. Examples include, modified atmospheric packing (MAP) of salad greens, irradiated tomatoes, apple chips and juicing vegetables and fruits.

Some of these innovations have been proposed since the 1930's. Norman W. Walker has been attributed with being the person to first propose vegetable juicing and to be the inventor of the first vegetable and fruit juicer (Walker, 1936). However, very little research has been carried out to measure the biochemical, nutritional and anti-nutritional aspects of this technique. More recently, juices are being promoted as not just for quenching the thirst and for refreshment, but also as a source of beneficial nutrients and vitamins (Sáenz, 2001; Singh, 2013).

Green juices are an example of a juice being able to deliver nutrients and vitamins as well as being a refreshing drink, but they can also, potentially, have anti-nutritional properties. Kang *et al.* (2004) fed 20 smokers 240 mL of green juice per day for eight weeks and suggested that the results supported the hypothesis that a green vegetable drink exerted a cancer protective effect, as their experiments measured a decrease in oxidative DNA damage. Using the same technique and assay researchers demonstrated that a reduction in oxidative DNA damage was due to the extra daily consumption of 480 mL of grape juice in 67 healthy volunteers over eight weeks (Park *et al.*, 2003).

The risk of acute oxalate nephropathy is high with the consumption of green juices and some fruit juices. Several cases have been reported in the literature. Syed (2105) reported values for iced tea (*Camellia sinensis*), taro (*Colocasia esculenta*) leaf consumption (Omura *et al.*, 2014), star fruit juice (Carambola, *Averrhoa carambola)* (Neto, 2014), bilimbi juice (tree sorrel, *Averrhoa bilimbi*) (Nair *et al.*, 2014) and green juices (Lien, 2013; Getting *et al.*, 2013).

This thesis will investigate the occurrence and levels of oxalates in green juices made from common fruit and vegetables available in New Zealand. The influence of the type of juicer used to prepare the juices, potential recipes and the use of treatment and/or additions to reduce the oxalate content of these mixed juices, will also be investigated.

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Chapter 2

Literature review

2.1 Oxalic acid in plants

Oxalic acid is an end product of metabolism and it accumulates in plant cells either as the free acid or as a salt of sodium or potassium. It can also be found as an insoluble salt, commonly calcium oxalate in the tissues of the plant cells. Oxalate is present in all plants at low levels the actual amounts depend on plant taxa- type-variety and location in the plant, ie: leaves compared to roots. McNair (1932) records it as present in up to 215 different plant families. There are several food-based plant family's that contain high amounts of oxalic acid, Amaranthus (Hoover & Karunairatnam, 1945), Colocasia (Adeniyi *et al.*, 2009) Oxalis (Liu *et al.*, 2009) Rheum (Nuss & Loewus, 1977), Portullaca (Palaniswamy *et al.* 2004), , Tetragonia (Ahmed & Johnson, 2000) and Atriplex (Al-Wahsh *et al.*, 2012).

The variability of oxalic content within species can be demonstrated by reported values of oxalic acid in spinach ranging from 217.9 to 490.5 mg/100 g fresh weight (Ruan *et al.*, 2013) compared to other reports of between 1634 to 2285 mg/100 g fresh weight (Siener *et al.*, 2006). There are also differences in seasonal cultivars. Watanabe *et al.* (1994) reports greater amounts of oxalic acid in a summer cultivar (Magic) compared to an autumn cultivar (Lead), 749 mg/100 g and 560 mg/100 g fresh weight, respectively.

The distribution of oxalic acid within plants is also not evenly distributed. Generally, it is highest in the leaves, then seeds and lowest in the stems and roots (Osweiler *et al.*, 1985; Libert & Franceschi, 1987).

The biosynthesis of oxalic acid is well known and researched. Glyoxylate, glycolate and ascorbic acid are the precursors for oxalic acid production on most plants (Hodgkinson, 1977).

Some of the proposed functions of oxalate in plants have been calcium regulation in plants. Studies have shown a relationship between the concentration of calcium in the nutrient medium and the number and size of calcium oxalate crystals in plants. (Zindler-Frank *et al.*, 2001; Volk *et al.*, 2002; Mazen *et al.*, 2003).

Kostman & Franceschi (2000) studied the growth of raphide crystals in idioblasts in *Pistia stratiotes* and showed that the idioblast in the leaf tissue acted as a high-capacity calcium sink. The growth of the oxalate crystals in these tissues are regulated and a bi-directional process.

Another proposed role of oxalate in plants is a defence mechanism from herbivores. Calcium oxalate crystals have five basic morphologies in plants, prisms, styloids, raphides, druses and crystal sand. The raphide shape crystals can cause irritation and pain through penetration of skin and by ingestion. (Sakai *et al.*, 1972; Bradbury & Nixon, 1998; Finley, 1999; Saltz & Ward, 2000). Besides the physical irritant of the oxalate crystals, there is the inherent toxicity to the animal following the consumption of oxalic acid, which interferes with mineral absorption and contributes to renal disorders. Deaths of large numbers of sheep and cattle have been reported after the consumption of high oxalate containing plants (Halogeton) (James & Butcher, 1972; James & Cronin, 1974). Similarly, food consumed by humans with high levels of oxalic acid have caused similar pathologic conditions relating to renal function. (Holmes *et al.*, 2001; Massey, 2003; Siener *et al.*, 2003).

Some plants also use oxalic acid as a mechanism to protect themselves from excessive absorption of heavy metals, such as Cu or Zn from the soil (Hall, 2002). Plants use organic acids such as citrate, malate and oxalate, as a metal detoxifying mechanism, by form metal crystals (Zheng *et al.*, 1998). There are two basic mechanisms, exclusion or internal mechanisms. The exclusion mechanism is where the plant releases oxalate into the surrounding soil via the roots and the oxalate binds any heavy metals in the soil making them insoluble and therefore unavailable for the plant. The internal mechanism is where the metal-oxalate complex within the plant cell is sequestered into specialised cells, enabling other normal functions to be carried out (Ma *et al.*, 1998).

Other less well research proposed functions of oxalic acid in plants are tissue support and rigidity, pH regulation, osmo-regulation and ion balance in the plant cell (Franceschi & Horner, 1980, Franceschi & Nakata, 2005).

Oxalic acid can be found in small amounts in just about every plant species, however there is large variation in the distribution between species and where in the oxalic acid is distributed within the plant itself.

The importance to human health and nutrition is significant, since plant material can make up a significant part of the diet. Levels of oxalates in plants have been reported to range from 121 to 1679 mg 100g fresh weight, for red beetroot and purslane leaf respectively (Noonan & Savage, 1999). On a dry weight basis herbs and spices have high amounts of oxalates, Ghosh Das & Savage (2013) measured a range of Indian-origin spices, reporting a range of 194 to 4014 mg/100 dry weight for nutmeg and green cardamom respectively.

Morrison & Savage (2003) classified foods into 3 categories based on their oxalate to calcium content ratio. Foods with a ratio greater than 2, a ratio approximately 1 and a ratio less than 1. With the consumption of plants with a ratio higher than 2, posing the most risk of pathological renal related disease. The presentation of dietary risk, as a ratio of oxalate to calcium has not been widely adopted, but is important as the soluble oxalic acid in a plant will readily combine with available calcium, to form an insoluble complex. Plants with high available Ca will generally have a much lower oxalate to calcium ration, this highlights a significant synergistic effect and the importance of levels of calcium in plants and subsequent foods.

2.2 A high oxalate containing diet

As an end-product of metabolism, oxalic acid has no energy value for human metabolism, is not an essential nutrient and therefore must be quickly excreted in the urine. An intake of large amounts of soluble oxalate can increase the risk of kidney stone development in susceptible people because of the increased concentration of oxalate in the urine. Oxalic acid excreted by the kidney tubule can combine with calcium excreted at the same time. These calcium oxalate crystals can then combine to form kidney stones. Calcium oxalate crystals are responsible for 70 - 80% of all kidney stones (Lewandowski & Rodgers, 2004).

Kidney stone disease is an increasing disease of affluent civilizations, affecting 15% of men and 6% of women (Bihl & Meyers, 2001). In the United States overall stone prevalence has doubled since 1964-1972 (prevalence 2.6%) to 1988-1994 (prevalence 5.4%) (Romero *et al.*, 2010). Dietary and lifestyle factors are believed to be the cause (Neisius & Preminger, 2013), with people consuming more oxalate containing foods, or foods being cooked in different ways that allow more oxalate to be retained, being suggested as the primary factors. In 2002, it was estimated that the financial burden of kidney stone disease in New Zealand was $18 million dollars (Davidson *et al.*, 2009). Development of kidney stones is a complex

multifaceted disease in which diet is one of the most important determinants of urine chemistry (Lewandowski & Rodgers, 2004). As approximately 75% of all kidney stones are composed of calcium oxalate (Chai & Liebman, 2005a) so the oxalate content of foods and their metabolism in the body are important risk factors. Oxalates excreted by the kidney comes from only two sources; metabolism in the liver from endogenous substrates or oxalate containing foods. The consumption of high oxalate containing foods can increase the excretion of oxalate in urine, leading to an increased risk of kidney stone formation (Massey, 2003). The risk of consuming foods high in soluble oxalates has been well documented (Noonan & Savage, 1999; Massey, 2003).

2.3 Green leafy vegetables in your diet

The oxalic content of commonly eaten green vegetables and fruit, in a "normal" Western diet has been reasonably well documented (Hesse & Siener, 1997; Holmes & Kennedy, 2000; Morrison & Savage, 2003) but there are many foods that have not been fully investigated. Food security for a growing population and lack of diversification of crops are recent issues that have been highlighted as issues that can make the problem worse (Kahane *et al.*, 2013). Kahane *et al.* (2013) suggests agro-biodiversity as way to increase food security in the future. Additionally, a more diverse diet, will also positively influence the nutrition status and thus increase human productivity.

Throughout human history between 40-100,000 species of plants have been estimated to have been used by humans to provide food, fibres and other uses. Around 7,000 cultivated species are used today, with approximately, only 30 crop species used intensively and form the basis of today's global agriculture (GFAR, 1999).

Globally, many research programmes have been initiated to investigate modern cultivation and use of these ancient and rediscovered crops. Not only for the objectives of food security, but also for the production of "new crops", that may offer nutritional benefits from their consumption, that historically, they may not have been aware of (Hoeschle-Zeledon *et al.*, 2009; Jaenicke, 2013).

In addition to the nutritionally beneficially aspects of "new crops", it would be remise to not consider potential anti-nutritive factors in these new crops. Many of the "new crops" and crops not often used in Western diets are green leafy vegetables. In some cases stems and

roots are often eaten as well. In addition, some of the more unusual fruits can potentially be a surprise source of anti-nutritive factors.

The Asian Vegetable Research and Development Center (AVRDC-The World Vegetable Center; Chavasit, 2012) focuses on the research and development of vegetables to alleviate poverty and improve nutrition in developing countries. At a recent symposium co-organised by AVRDC (Holmer *et al.*, 2012), the promotion of local/indigenous plants is promoted as a strategy to improve nutrition security (Chavasit, 2012). More specifically Ebert (2012) promotes high value speciality vegetables, such as sprouts, microgreens and flowers.

The Vegetarian Resource Group (Wasserman, 2013) have regularly polled North Americans about their vegetable eating habits. Until recently they have had limited the poll questions asking the basics, such as "do you never eat meat, fish, seafood or poultry". Since 1994 this type of polling has resulted in very similar results each year reporting that approximately 4% never ate meat, fish, seafood or poultry. Recently they have added extra questions, such as "would you eat one or more vegetation meals per week". Not including vegetarians and vegans in this response, 43% of respondents said "yes". This has been interpreted as clear signal that vegetable-based meals are growing in importance in Western culture.

The logistics of increasing fruit and vegetable consumption in the Western world, has resulted in many innovate ideas to increase the shelf life of fresh produce using for example, modified atmosphere packaging (MAP) of salad greens. These are now found in all supermarkets in all developed countries. Recent innovation or trends in vegetable and fruit consumption have mainly focused around delivery mechanisms to allow the freshest possible product available with minimal processing. The other main area of innovation is rethinking how people consume vegetables and fruits, perhaps in a less traditional manner. For example, green juices, apple chips and irradiated tomatoes. These innovations may require the processing of the food(s) to a lesser or greater extent.

2.3.1 Green juicing

The invention of the first juicer or food mixer has been attributed to Rufus M. Eastman who patented a "mixer for cream, eggs and liquors", in 1885 (Eastman, 1885). It was not until 1924, when Poplawski (1924) patented a "beverage mixer", which clearly has similarities to the modern day macerating juicer commonly used (Figure 2.1).

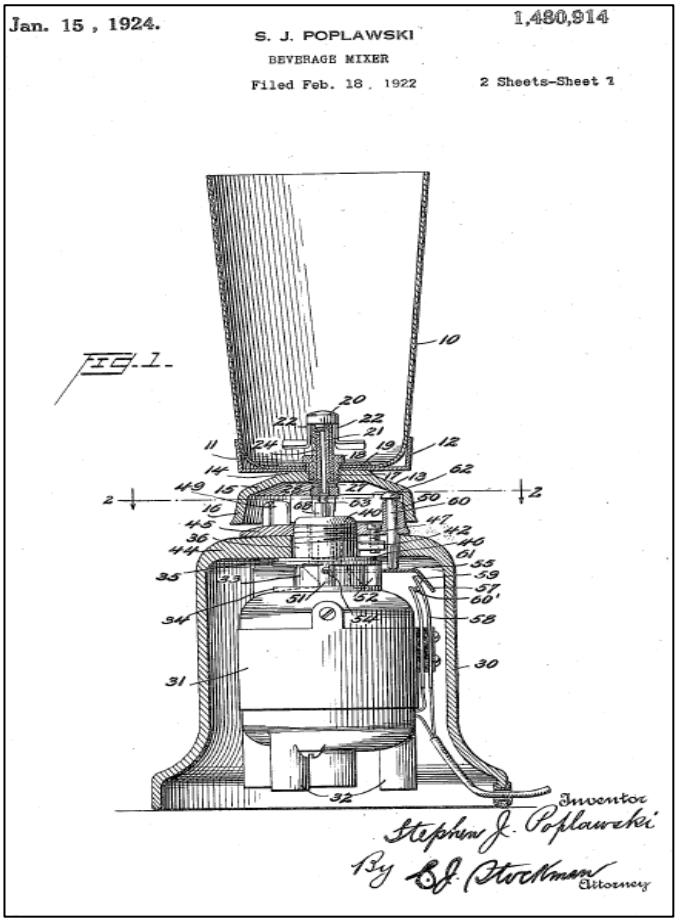

Figure 2.1 Schematic of beverage mixer patented in 1924. (Poplawski, 1924).

Juicing has recently become a popular health trend. The term “juicing” refers to a period of three to ten days when a person’s diet consists mainly of fruit and vegetable juices. It is widely promoted as providing health benefits, such as encouraging weight loss and flushing toxins from the body, even though there is no strong scientific evidence to support these claims. It is frequently claimed that during the juicing process the nutrients from fruit and vegetables will be exposed and, therefore, be absorbed more easily by the digestive system. Nutritionists and dietitians universally encourage increased consumption of fruit and vegetables in the diet, but no scientific evidence is available to suggest that juicing has any extra advantages.

The juicing of a mixture of green leafy vegetables along with several fruits is believed to be the basis of a healthy diet as these fruits and vegetables contain a wide range of essential amino acids, organic acids, vitamins and minerals (Hu, 2003). It is important to note that the vegetables and the juices are not heated or cooked during juicing and this is seen as an effective way to preserve the positive nutrients in the juices (Sussman, 2013) but it does not allow for the possibility of reducing potentially toxic anti-nutrients, such as oxalates.

Juicing or "juice cleansing" was first claimed to meet the body's requirements for enzymes for optimal health by Norman W. Walker in 1936 (Walker, 1936). Norman W. Walker was the first person to introduce the concept of juicing fruits and vegetables and to have "invented" carrot juice in 1936. Walker has written nine books on the subject spanning the years 1936 to 1981. These books extoll the virtues of consuming fruit and vegetable juices for optimum health and longevity (Walker, 1936, 1940, 1949, 1961, 1972, 1974, 1977, 1979, 1981). It is understood that he consumed various juices over his lifetime and this may well have maintained a healthy lifestyle. He is said to have died at 119 years of age. In 1936 Walker invented the first Norwalk juicer (Norwalk, Inc., Bentonville, AR). This juicer has a 2-stage operation. First the fruit or vegetables are macerated, then the juice portion is separated by placing the pulp in a muslin bag, which is then pressed by a small hydraulic press. The same original design of juicer is still available. During the1920 and 30's Max Gerson a German doctor proposed a cancer therapy based on a juice diet and various dietary supplements to treat patients for cancer (Ward, 1988). His choice of juicer was the Norwalk 280. The Gerson Institute (Convoy Court, San Diego, CA, USA) currently recommends the Norwalk juicer as a juicer for their therapy treatments but the Institute now also endorses other types of macerating juicers. The main part of the Gerson therapy includes a strict diet. The Gerson diet advocates 13 glasses of fresh fruit and vegetable juices per day, including green leaf juices. This is in addition to prescribed supplements and a whole grain vegetarian diet. The potential negative effects of consuming large amounts of vegetable juices are rarely discussed and warrants investigation into the potential oxalate intake.

2.3.2 Positive effects of juicing

Some research has been carried out to try to quantity any positive effects of consuming green juices. Oliveira *et al.* (2013) fed rats green juice as a supplement over 15 days and concluded the green juice reduced weight gain, lipooxidatation and catalyse activity. Kang *et al.* (2004)

fed 20 smokers Angelica keiskei juice every day for 8 weeks. They showed a significant reduction on lymphocyte DNA damage and concluded that this supported their hypothesis that green juice exerts a cancer protective effect. Aiso *et al.* (2014) compared the blood parameters of 16 men split into two groups. With one group consuming a commercial apple juice while the other group consumed a freshly prepared komatsuna (*Braassica rapa* L. var. *perviridis*), commonly known as Japanese mustard spinach) green juice mixture. After 4 weeks of consuming 210 and 200 g of the komatsuna and commercial juices, twice daily, the komatsuna juice drinkers showed a slight but significant drop in blood levels of T-Cholesterol and LDL-Cholesterol, 220 to 211 and 143 to 134 mg/dL respectively. These three studies demonstrate the difficultly in researching this topic, as the variability in the composition of the juices in large, making it hard to come to any definite conclusions. In addition, the number of test subjects was very low. There is little scientific evidence at present that concludes the consumption of green juices will improve health outcomes for regular consumers.

2.3.3 Negative effects of juicing

Consuming green juices or juice type products in general, may not always have a potential positive health outcome. Getting *et al.* (2013) reported a case of acute renal failure in an adult male following six weeks consumption of a mixed fruit and vegetable juice diet. The mixed juice contained significant amounts of spinach and they estimate the patient consumed an average of 1260 mg oxalates/day (range 35 – 5000 mg/day). It is presumed total oxalates, as it is not stated by the authors.

There is one other case of acute oxalate nephropathy directly attributable to the consumption of green juices. Doctors from the Division of Nephrology, Nassau University Medical Center, NY, USA (Makkapati *et al.*, 2018) report on a 65-year-old woman with no history of kidney stones or use of nephrotoxic agents. She presented at the hospital with two weeks of refractory nausea, decreased appetite and weakness. She informed doctors she was on self-prescribed weight loss diet, 1 month prior that replaced all her normal meals for 10 days, with a diet of juices only. This included consumption of two cups of spinach per day. A kidney biopsy confirmed diagnosis of acute oxalate nephropathy. Makkapati *et al.* (2018) conclude that juicing can cause oxalate nephropathy even in a patient with normal kidney function in the presence of other risk factors.

In a letter to the New England Journal of Medicine, doctors, Syed, Mena-Gutierrez & Ghaffar (2015), informed readers that they treated a 56-year-old male who presented with oxalate nephropathy. He had consumed 16 x 8-oz glasses of iced black tea daily. They estimated that he had consumed 1500 mg oxalate per day, it was not clear for how long, however he was first presented in the Hospital with illness in May 2014, but also had medical records in February, 2014 suggesting no illness.

Star fruit (*Averrhoa carambola*) juice has often been identified as the source of acute oxalate nephropathy (Chen, 2001; Tsai *et al.*, 2005; Wu, 2007; Fang, 2008; Neto, 2014; Cader *et al.*, 2016). Chang *et al.* (2000) reported fatal outcomes for eight dialysis patients over a 10-year period, after eating star fruit. Levels of oxalic acid in Star fruit have been determined to be very high, O'Hare (1993) reports that two varieties of Star fruit, Arkin and Golden star to have levels of 1 and 5.8 mg oxalate/g respectively. Wilson *et al.* (1982) analysed 10 cultivars of Star fruit grown in Florida which ranged from 0.08 to 0.73 g oxalate/100 g. Huynh & Nguyen (2017) reports a total oxalate of 190.98 mg oxalate/100g FW in the fresh fruit of carambola, with the resultant juice having only 56.56 mg oxalate/ 100g FW. An important observation by these researchers is they report the soluble oxalate of carambola juice to be 83.24% of the total.

Nair *et al.* (2014) reported of two cases of acute oxalate nephropathy due to the consumption of bilimbi juice (*Averrhoa bilimbi*). In one case the patient had consumed approximately only 100 ml of a locally made juice. In the other case, the patient had consumed approximately 150 ml over two consecutive days before becoming unwell. Both patients had no history of oliguria and it was concluded the source of hyperoxaluria was the consumption of the juices.

Other beverages that have also been identified as responsible for hyperoxaluria are almond milk products. Doctors from the children's hospital of Pittsburgh, University of Pittsburgh Medical Center, PA, USA (Ellis & Lieb, 2015) reported that three children, aged three, 9 and 10 years of age, presented with symptoms of and were subsequently diagnosed with hyperoxaluria. The children had a diet high in almond based products, such as almond butter, yoghurt and milk. One child consumed up to 4 cups of almond milk daily. The plant-based milk substitutes were identified as the cause in their diet, as they had not eaten or consumed any other high oxalate containing foods and they had not consumed any vitamin C supplements.

2.3.4 Green juice high in oxalates

The consumption of any juice with high amounts of oxalate is has more inherit risk to health compared to consuming similar foods not juiced due to the mechanism of the absorption in the intestinal lumen (Lien, 2013). Soluble oxalate is transported from the lumen to the blood via a passive paracellular pathway (Figure 2.2). The rate is determined by oxalate and calcium concentration in the lumen and the water flux.

A green juice can potentially have a high oxalate content, high water content and low calcium concentration. These conditions could make the inflow of soluble oxalate into the blood above normal. Two factors influence the driving force of oxalate transport across the paracellular pathway, the concentration gradient of oxalate between the lumen and blood, and the water flux. If the water flux from the lumen to the blood is high more calcium oxalate complex can remain soluble and be absorbed into the blood via the paracellular pathway. If the calcium concentration in the lumen is low, any regulation of oxalate, by the precipitation of excess oxalate-Ca in the lumen, is negated, resulting in a higher concentration of oxalate in the lumen. The combination of these factors increases the oxalate and calcium oxalate in the blood.

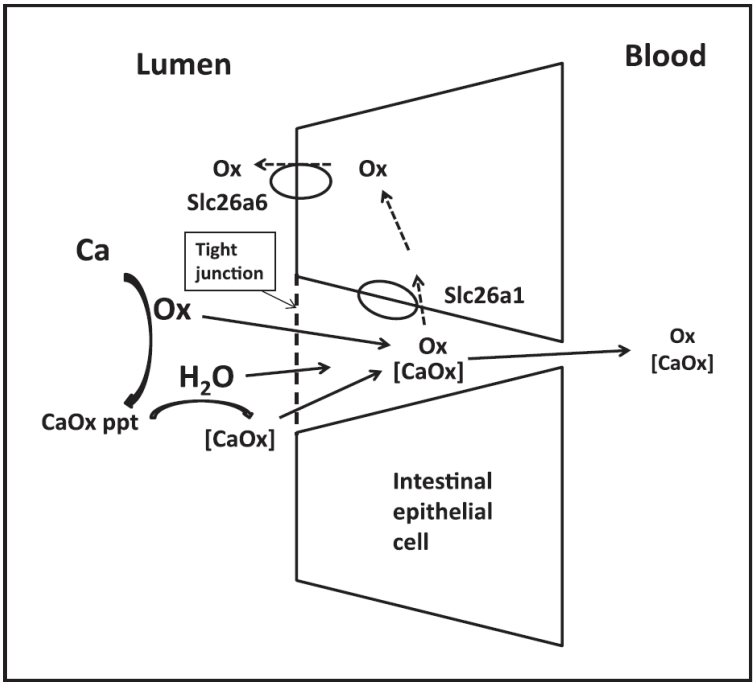

Figure 2.2 Intestinal oxalate absorption (adapted from Lien, 2013; Knauf *et al.,* 2011).

Overall, juicing, without considering whether high oxalate fruit and vegetables were included in the mix, is a risky undertaking, particularly if the juices are consumed over a sustained period of time and have high proportion of high oxalate containing foods in the mixture. Spinach (*Spinacia oleracea*) is a common constituent of juicing diets. It is well known to contain high levels of oxalate, with fresh or frozen spinach contents reported to range from 320-1260 mg/100 g wet matter (Brinkley *et al.*, 1981; Watanabe *et al.*, 1994; Noonan & Savage, 1999; Savage *et al.*, 2000; Brogren & Savage, 2003) and higher levels are being reported in fresh, summer-grown plants (Watanabe *et al.*, 1994).

2.3.5 Spinach as a green juice base

Spinach (*Spinacia oleracea*) is a common green leafy vegetable base used for both commercial and home production of green juices. It is usually very cheap in markets when in season and grows easily in a home garden. Spinach leaves are recognised to contain high amounts of vitamins, essential minerals and phytochemicals (i.e. carotenoids and flavonoids) (Liu *et al.*, 2015). It is thought to have been first grown for human use in Persia (modern Iran) and then was introduced by Arab traders into India and China. Earliest records (AD 647) of spinach can be found in Chinese herbals which revealed Nepal as the origin of the introduction of spinach into China. Its green leaves contain a wide range of important nutrients, such as protein, chlorophyll, beta-carotene, vitamins A, B1, B2, C, E and K,) and minerals (Ca, Fe, K, Mg, Na and S) (Alam, 2011; Zhang *et al.*, 2009). Small immature leaves can be eaten raw but larger mature leaves have a more bitter taste and these are more commonly cooked. The bitter taste in mature spinach leaves is presumed due to the presence of oxalates (Tsai *et al.*, 2005). Apart from the bitterness, astringency and scratching feeling of spinach, oxalates also generate anti-nutritive effects by inhibiting calcium (Ca), magnesium (Mg) and Iron (Fe 2+) absorption into the body. This condition may lead to an increase in mineral deficiency and health diseases, particularly kidney stones and renal dysfunction (Alam, 2011; Liu *et al.*, 2015).

Many studies have determined the oxalic content of spinach (Table 2.1). It can be seen from Table 2.1 that the amounts can range from 105.1 to 2677.0 mg/100 g fresh weight of spinach for spinach grown in a wide range of countries. An important distinction to make is whether total or soluble oxalate has been reported or not. In 1989 (Holloway *et al.*, 1989) first developed a method to determine total and soluble oxalates in taro leaves. The difference

between the soluble and total oxalate being, the total is extracted from the taro leaf using acid and the soluble using water. This technique was then adapted, improved on and applied to the determination of oxalic acid in spinach leaves by Savage *et al.* (2000), who reports the total, soluble and insoluble oxalic acid content of spinach leaves. The insoluble oxalate is the difference between the total and soluble.

The importance of quantifying soluble oxalic acid is this is the form that is readily absorbed in the blood stream (Stegelmeier *et al.*, 2013) when a plant is consumed, causing toxic effects, therefore the soluble ratio in a plant is important.

It can be seen in Table 2.1 that the ratio of soluble to total oxalate can vary, from 20 to 85%.

Table 2.1 List of studies that have determined oxalates in spinach (*Spinacia oleracea*).

No.	Location	Year	Oxalate Content (mg/100 g FW)		Reference
			Acid Extraction	Water Extraction	
1	Italy	1994	–	230.9 – 1010.8	Santamaria *et al.* (1999)
2	Italy	1995	–	105.1 – 699.9	Elia *et al.* (1998)
3	China	1997	752 - 764	–	Weaver *et al.* (1997)
4	Japan	1998	650.38 – 1111.85		Ombódi *et al.* (2000)
5	New Zealand	2000	1764.6	364.6	Savage *et al.* (2000)
6	Brazil	2001	559.0 – 674.0	–	Sotomayor *et al.* (2001)
7	China	2002	760.00 – 940.00	610.00 – 780.00	Zhang *et al.* (2005)
8	Japan	2003	–	614.90 – 1092.90	Kaminishi & Kita (2006)
9	Germany	2005	1634.00 – 2285.00	800.00 – 1257.00	Siener *et al.* (2006)
10	Italy	2005-2007	1462.30 – 1583.50	–	Merusi *et al.* (2010)
11	USA	2006	–	647.20 – 1286.90	Mou *et al.* (2008)
12	China	2006	620.00 – 940.00	490.00 – 760.00	Zhang *et al.* (2009)
13	India	2008	691.68	643.50	Savage & Mårtensson (2010)
14	Pakistan	2009	973.00 – 983.00	541.00 – 545.00	Akhtar *et al.* (2011)
15	New Zealand	2011	685.43	645.23	Ghosh Das & Savage (2013)
16	China	2012	217.90 – 490.50	–	Ruan *et al.* (2013)
17	China	2014	1968.00 – 2677.00	1667.00 – 2287.00	Liu *et al.* (2015)

2.4 Determination and optimisation of extraction of oxalic acid in green juice

2.4.1 Oxalic acid determination

It is important to have an accurate analytical method to measure the oxalate content of plant foods. The first measurement of oxalate content was performed using wet chemistry techniques (Zarembski & Hodgkinson, 1962; Hodgkinson, 1977) and permanganate titration (Franco & Krinitz, 1973). However, inaccuracy was the major issue in performing these methods, thus leading to other determination methods, namely gravimetric methods. These methods were first introduced in 1980's involving colorimetric or atomic absorption analysis, which unfortunately were time and labour intensive (Massey, 2007). Since then, many other studies have been carried out using alternative analysis methods, such as, enzymatic assays using oxalate oxidase (Kasidas & Rose, 1980; Chai & Liebman, 2005a); gas chromatography (Ohkawa, 1985); gas chromatography-mass spectrometry (GC/MS) (March *et al.*, 2003); HPLC (Holloway *et al.*, 1989; Savage *et al.*, 2000); HPLC-enzyme-reactor (Honow *et al.*, 1997; Honow & Hesse, 2002); ion chromatography (Ishii, 1991; Von Schnakenburg *et al.*, 1994); capillary electrophoresis (Trevaskis & Trenerry, 1996; Chai & Liebman, 2005b); bi-enzymatic optode (optical fiber chemical sensor) detection system (Sotomayor *et al.*, 2001); ion electrophoresis (Holmes & Kennedy, 2000); amperomeric biosensor (Sezgintürk & Dinçkaya, 2003; Pundir *et al.*, 2011) and an *in-vitro* digestive - HPLC assay (Savage & Mårtensson, 2010).

Shimada (2014) analysed a commercially available vegetable juice for oxalic acid using an ion chromatographic system and reports an oxalic acid concentration of 1.9 mg/100 ml. The composition or brand of the vegetable juice, or whether it could be classified as a green juice or not, was not reported on. There is also no distinction between the total and soluble oxalic acid content, so it is presumed to be total oxlate.

Siener *et al.* (2016) analysed 13 vegetable and 19 fruit juices, nectars and drinks, commercially available in Bonn, Germany, for levels of total and soluble oxalate, using a HPLC-enzyme reactor method. None of the vegetable juice, nectars or drinks tested, could be classified as a green juice. The closest could be sauerkraut juice (*Brassica oleracea*), however this is likely to be the juice exudate from the manufacture of sauerkraut, the fermented cabbage, popular in Germany and Eastern Europe.

Green juices have never been analysed for oxalic acid before, however spinach, a common vegetable has been and it is often used as a base for green juice juices. The studies on spinach summarised in Table 2.1 used a wide range of analytical techniques to measure oxalates: ion chromatography; permanganate titration, HPLC; bi-enzymatic optode detection; HPLC-enzyme-reactor; capillary electrophoresis; UV/VIS spectrophotometer and enzymatic assays.

According to Massey (2007), regardless of any other limiting factors, and advantages and disadvantages of each particular method, the determination of oxalate content has been recognized to be highly correlated with the extraction method used. Extraction with water releases most of the free oxalate ions of $C_2O_4^{2-}$ and extraction with acidic solutions releases the dicarboxylic oxalic acid of $H_2C_2O_4$, semi-dehydro-oxalate anions of $HC_2O_4^-$ and also free oxalate ions.

Regardless of the analytical techniques used to determine the oxalic acid content, the other important factor in the determination of oxalic acid, is the extraction conditions from the plant material prior to quantitative analysis. This determines whether the total or soluble oxalic acid are being measured (Holloway *et al.*, 1989). Table 2.2 summarises the extraction conditions used to determine oxalates in spinach. There is a large variation in extraction time, temperature and whether water or HCl is used, or both. To obtain full information about the total, soluble and insoluble oxalate content in a plant, the plant will need to be extracted with both acid and water to obtain the total and soluble oxalic acid contents (Holloway *et al.*, 1989; Savage *et al.*, 2000)

Previously reported, water extraction may extract about 59-87% of total oxalate content in spinach (Savage *et al.*, 2000; Jaworska, 2005) and almost all of the results from these listed studies are similar to this. However, two studies showed unexpected percentages of soluble oxalates, as low as 49% and higher than 94% of total oxalate content in spinach from the studies by Siener *et al.* (2006) and Ghosh Das & Savage (2013), respectively. This variation could be explained by variations in factors such as cultivation and harvesting conditions of spinach, the origin and species, location and climate condition, soil condition, irrigation process, and the maturity at harvesting time. Apart from these factors, extracting fresh spinach may also contribute to these varying results in terms of the fineness of grinding fresh spinach, the purity of the water, the concentration of acid solvent, the extraction procedures, the incubation temperature and time used to extract the food product. It is also important to point

out the type of extraction solvent, either a water or acid extraction solvent will extract all the soluble salts of oxalic acid, this includes Na and K. The more acidic extraction solvents will extract all the oxalate salts, including the less soluble, such as calcium oxalate and magnesium oxalate. (Simpson *et al.,* 2009). Therefore, it is important to investigate a set of extraction conditions that are suitably optimised for the food matrix involved.

Table 2.2 The extraction conditions used for the determination of oxalates in spinach (*Spinacia oleracea*).

No.	Location	Extraction conditions						Reference
		Solution	Concentration	pH	Volume (mL)	Temperature (°C)	Time (mins)	
1	Italy	$NaHCO_3$	1.7 mM	–	50	–	–	Elia *et al.* (1998)
2	Italy	$NaHCO_3$	1.7 mM	–	50	25	30	Santamaria *et al.* (1999)
3	Japan	HCl	0.05 N	1.30	–	–	–	Ombódi *et al.* (2000)
4	New Zealand	HCl	2 M	0.30	50	80	15	Savage *et al.* (2000)
5	Brazil	HCL	6 M	0.78	55	100	15	Sotomayor *et al.* (2001)
6	China	HCl	6 M	0.78	2	100	15	Zhang *et al.* (2005)
7	Japan	Deionized water	-	7.00	10X weight	RT[a]	5	Kaminishi & Kita (2006)
8	Germany	HCl/distilled water[b]	2 N	0.30	4	21	15	Siener *et al.* (2006)
9	USA	Deionized water	–	7.00	5	–	6	Mou *et al.* (2008)
10	China	HCl	6 M	0.78	2	100	15	Zhang *et al.* (2009)
11	Italy	HCl	2 N	0.30	4	21	15	Merusi *et al.* (2010)
12	New Zealand	HCl/distilled water [b]	2 M	0.30	50	80	15	Savage & Mårtensson (2010)
13	Pakistan	HCl/distilled water [b]	2 M	0.30	50	RT	20	Akhtar *et al.* (2011)
14	New Zealand	HCl/distilled water [b]	0.2 M	0.69	40	80	15	Ghosh Das & Savage (2013)
15	China	HCl/distilled water [b]	0.5 M	0.30	2.6	100	20	Ruan *et al.* (2013)
16	China	HCl	0.5 M	0.30	5	100	20	Liu *et al.* (2015)

2.4.2 Speciation of oxalates

The different forms of oxalates extracted from food samples are fundamentally related to the speciation or dissociation of oxalic acid at different pH values (Johansson & Savage, 2011; Simpson *et al.*, 2009). Acid and water extraction solvents release different fractions of the oxalates, which is due to the different pH value between acid and water solutions. pH affects the speciation or dissociation of oxalates in food samples (Hernández *et al.*, 2013; Simpson *et al.*, 2009). Figure 2.3 depicts the speciation of oxalates in different pH conditions resulting in different fractions of oxalates, which are semi-dehydro-oxalate anions of $H(C_2O_4)^-$ and free oxalate ions of $(C_2O_4)^{2-}$. The dissociation of oxalates occurs when pH shifts from acidic to neutral state by donating a single proton hydrogen ion (H+) of each dissociation step. The dissociation of oxalates is always in the equilibrium state (Figure 2.3). In a high acidic condition (pH < 1.5), oxalates present predominantly in their forms of dicarboxylic oxalic acid of $H_2C_2O_4$ and start to undergo speciation to form semi-dehydro-oxalate anions of $H(C_2O_4)^-$ by donating proton (H+). These oxalate fractions are further dissociated into free oxalate ions of $C_2O_4^{2-}$ in the pH range of 2.5 to 6.0. As the pH gets higher than 6.0, the dissociation of oxalates is completed and the free oxalate ions of $C_2O_4^{2-}$ are present as the predominant fraction of oxalates in food samples (Hernández *et al.*, 2013).

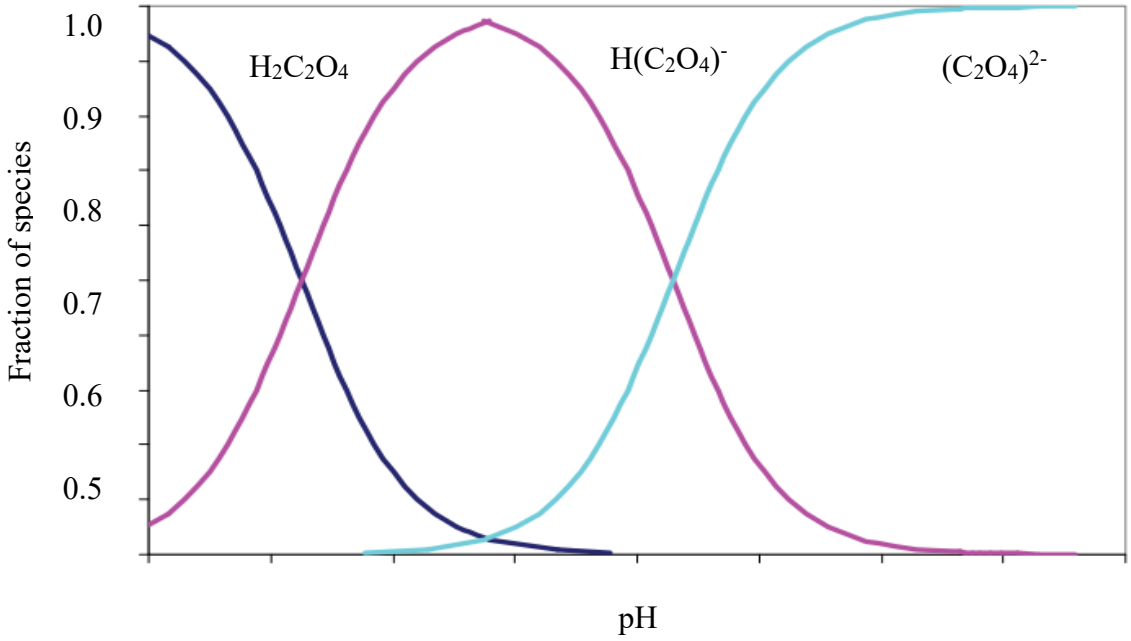

Figure 2.3 Speciation of oxalic acid with pH (Simpson *et al.*, 2009).

The fraction of free oxalate ions of $(C_2O_4)^{2-}$ is of concern because of their preferential binding capacity with calcium causing the formation of calcium oxalates and development of kidney

stones within the kidneys or urinary tract (Morrison & Savage, 2003; Simpson *et al.*, 2009). On the other hand, the semi-dehydro-oxalate anions of $HC_2O_4^-$ have less binding capacity to calcium, therefore the possibility of the formation of calcium oxalates after ingestion of food samples may be reduced compared with free oxalate ions (Simpson *et al.*, 2009). However, the available semi-dehydro-oxalate anions in food samples may still potentially be dissociated at the time they reach the intestine with increased pH of 8.0. As a consequence, this may enhance the free oxalate ions to bind to calcium and form calcium oxalates, thus increasing the risk of kidney stone formation in the human urinary tract (Morrison & Savage, 2003; Simpson *et al.*, 2009).

The free oxalate ions can also form a complex with other available cations in the food matrix, however their concentration and solubility constant (K_{sp}), determine whether they are likely to form a complex or not. Simpson *et al.* (2009) measured the concentration of Ca ions in silver beet leaves and compared to the other ions in the leaves, Mg, Fe, Mn, Zn and Cu, it was concluded because the concentration Ca was far greater than the other ions and the K_{sp} of calcium oxalate is the lowest (2.7×10^{-9}) the insoluble oxalate was most likely calcium oxalate.

2.5 Addition of Ca to bind oxalic acid

Most high oxalate-containing foods contain both soluble (bound to Na^+, K^+ and NH^{4+}) and insoluble (bound to Ca^{2+}, Mg^{2+} and Fe^{2+}) oxalates (Savage *et al.*, 2000). The soluble forms were available for absorption from the intestine. However, soluble oxalates can bind to Ca^{2+}, Mg^{2+} and Fe^{2+} ions to become insoluble salts during processing and cooking. There is some evidence to suggest that oxalates can become bound to fibre and if this fraction is removed during processing then the levels in the juice may be significantly reduced.

A strategy to decrease the absorption of soluble oxalate from food is to encourage it to form insoluble oxalates. Studies have found that soluble oxalate levels decreased when foods with high levels of calcium were consumed along with foods containing high levels of soluble oxalates. The addition of calcium from milk during the cooking process, or calcium salts during manufacturing, was also able to convert the soluble oxalates into insoluble oxalates (Savage *et al.*, 2003; Brogren & Savage 2003; Oscarsson & Savage, 2007; Savage *et al.*, 2009; Faudon & Savage 2014).

Faudon & Savage (2014) reduced the soluble oxalate by up to 60%, in a rhubarb mitsumame dessert, by the addition of either calcium carbonate or calcium chloride.

When taro leaves are baked with milk, compared to raw and baking without milk, up to a 73% reduction in soluble oxalate was reported by Oscarsson & Savage (2007). They concluded the calcium in the milk binds to the soluble oxalate to form insoluble calcium oxalate salt. The addition or presence of calcium in a recipe or mixture used for the preparation of food, clearly reduces the soluble oxalate content.

2.6 Soaking to remove oxalates

Fruit and vegetable pre-treatments are used either soon after harvesting or prior to further processing, to clean or pre-prepare the food prior to the next stage of processing, i.e. rinsing with chlorine to sterilise fresh greens (Lee *et al.*, 2004) before packaging. The type of pre-treatments depends on the type of processing, but can be classified broadly into as: blanching, osmotic pre-treatments, brining and soaking/rinsing.

2.6.1 Blanching

Blanching is a process by which heat is applied using water or steam, to sterilise and inactivate heat labile enzymes, this helps retains colour and turgidity of a vegetable (Lee, 1958). Mosha (1995) blanched collard greens for up to 10 minutes in 98ºC water and observed a significant reduction (32.7%) in total oxalic acid, but overall commented the oxalic acid reduction was not significant in most of the treatments.

Abiodun (2014) pre-treated yams by blanching/soaking diced yam tubers in 60ºC water for 10 minutes then soaked for 12 hours at an unspecified temperature, prior to drying and grinding into a flour. The total oxalate content of the yam flour was reduced by 96% and suggests the blanching step may have ruptured cells to allow leakage of soluble oxalate.

Shimada (2014) showed that there was a 70% reduction in oxalic acid when 1 cm^2 pieces or whole spinach was soaked for 60 minutes at 80ºC. Shimada also observed there was a time-dependency in reduction of oxalic acid from spinach, with the rate increasing, to a maximum rate of 71.7% at 80 minutes and 77.5% at 60 minutes, for the whole and 1cm^2 spinach respectively, soaking in 80ºC water. After 20 hours the reduction rate was observed to be 0.3% and 2.0 % for the whole and 1cm^2 spinach respectively.

Zhou & Zhou (2014) pre-treated balsm pears in 100ºC for 3 minutes and got an average reduction in total/soluble oxalate of 39.76%. They also used a salt water (no concentration given) pre-treatment, placing the balsam pear in salt water for 3 minutes and got an average oxalate reduction of 37.41%.

Blanching has also been reported to increase the calcium content of the food being blanched , due the blanching water being high in calcium, this would only occur if hard water was used for blanching. (Jones & Etchells, 1944).

Bengtsson (1969) compared the mineral and oxalate content of blanched and unblanched spinach from a factory production line. The temperature of blanching was not given. A 60.9% loss of soluble oxalate is reported and notes that the water was very hard (360 ppm $CaCO_3$). Expanding on this Bengtsson (1969) compared blanching in tap water, distilled water and softened water. Although omitting to determine the oxalate content of the raw spinach, it was reported that a there was a decrease in soluble oxalic acid and corresponding increase in insoluble oxalic acid, which attributed to the oxalic acid binding to the calcium.

It should be noted that heating/blanching vegetables is in effect cooking the vegetable.

2.6.2 Osmotic pre-treatments

Brining

Brining is a specific type of osmotic pre-treatment that refers to the treatment of vegetables with high concentrations of a salt (NaCl) solution to preserve or to enable the selection of salt tolerant microbes naturally present, that go on to ferment the vegetable. Salt concentrations can range from 0.5% w/w for sauerkraut making to 15% w/w for preservation as in pickling (Jones & Etchells, 1944). Changes in oxalate levels of green vegetable due to brining have not been reported, however Wadamori *et al.* (2014), did observe a 22.86% reduction in soluble oxalates, in a traditional South Korean kimchi fermentation using silver beet and a brining pre-treatment of 10% NaCl. The reduction in oxalate was attributed to the oxalotrophic activity of the fermentation bacteria involved.

Soaking / rinsing

There have been no studies reporting the effect of soaking or rinsing with any type of aqueous solution that is not blanching, on the oxalate content of green vegetables. Ordinary soaking has been reported to have an effect on other anti-nutritive compounds such as phytic acid, saponins and trypsin in beans. Khokhar & Chauhan (1986) showed a 46 to 50% reduction on phytic acid for moth bean (*Vigna aconitifolia*) soaked in ordinary water for 12 hours at 37ºC. Saponins, and trypsin also showed reductions but to a lesser extent. Akande *et al.* (2015) reports a loss of ascorbic acid from lettuce of between 49.7 to 93.2%, using whole leaf, sliced, salted, unsalted, squeeze washed, 1 and 2 litre volumes and time as process variables. They also noted that soak time had a significant effect on the loss of ascorbic acid, the higher the soak time the higher the loss of ascorbic acid.

The effect of rinsing and /or soaking with plain cold water has not been well researched as a method of removing oxalates from foods.

2.7 Summary

Green juices are a popular, innovative way of increasing green vegetable intakes, the product that can easily be made at home or bought online, delivered fresh or from café style retail outlets.

There is little to no scientific data on the compositional analysis of green juices and similarly, researching the interaction of green juice consumption and medical outcomes.

One of the reasons why compositional data is hard to quote, is green juice is often a "homemade" product with inherit large variation in recipe components and is subject to seasonal availability of the fruits and vegetables used.

There are documented case studies of acute nephropathy due directly to the consumption of green juices and other fruit and vegetable type juices too.

With increased awareness of the benefits of extra fruits and vegetables in the diet, it is important to understand all the implications of this and with a concentrated product such a green juice.

Consumption of additional vegetables using green juices may have benefits to a person's health overall, but anti-nutritional factors need to be considered as well.

2.8 Research objectives

Experimental work was carried out with the following objectives:

1) To investigate the optimum extraction conditions, with respect to time, temperature and pH for the extraction of oxalic acid from a green juice, using spinach as an analogue.

2) To determine whether the composition of a green juice recipe has a relationship between recipe components and oxalic acid content of the final juice produced.

3) To investigate if using different types of juicers influence the outcome, with respect to oxalic acid content of the juice.

4) To identify and quantify potential strategies that could reduce the oxalic acid content in a green juice.

2.9 Research outline

This research was carried out in two major stages, firstly investigating the juices made and then secondly investigating strategies to reduce oxalic acid in juice making. This is best summarised in the diagram found in Appendix A. The numbers to the left-hand side of each main heading relate to the thesis chapter numbers.

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Chapter 3
General materials and methods

3.1 Sample extraction and preparation for HPLC analysis of oxalates

The extraction process of oxalates from samples of fresh green leafy vegetables or vegetable juices was performed according to the method published by Savage *et al.* (2000) with some modifications. Approximately 2 to 10 g of fresh sample was weighed into a 125 mL Erlenmeyer flask and either 40 mL of 0.2 M HCl (Aristar, BDH Chemicals, Ltd., Poole, Dorset, UK) or 40 mL of high purity water (HPW, GenPure Pro™ Thermo Scientific Barnstead International, Dubuque, Iowa, USA, 18.2 MΩ-cm) was added into the Erlenmeyer flask. Each flask was then incubated for 20 minutes, in a shaking water bath (Grant Instruments Ltd., Royston, UK) set at 80°C. The solutions were removed from the water bath and allowed to cool to 20°C and then quantitatively transferred to a 100 mL volumetric flask and made up to volume with the corresponding extraction solvent. Samples were then filtered through a 0.45 µm cellulose acetate syringe filter (dismic-25cs, Advantec, California, USA) into a 1 mL glass HPLC vial. Samples were analysed on the same day of extraction. Any repeat samples were re-extracted from their original sample.

3.2 HPLC analysis of oxalic acid

HPLC was carried out using a 300 x 7.8 mm Rezex ROA ion exclusion organic acid column (Phenomenex, Torrance, CA, USA) attached to a Cation-H guard column (Bio-Rad, Richmond, CA, USA) analytical column, held at 25ºC with a Shimadzu CTO-10A column oven (Shimadzu, Kyoto, Japan).

Analysis was performed by injecting 20 µL of sample or standard on to the column using 25 mM sulphuric acid (HPLC grade Baker Chemicals, Phillipsburg NJ, USA) as the mobile phase, pumped isocratically at 0.6 ml/min, with peaks detected at 210 nm. The HPLC equipment consisted of a Shimadzu LC-10AD pump and SPD-10Avp UV-Vis detector (Shimadzu, Kyoto, Japan) and a Waters 717plus Autosampler (Waters, Milford MA, USA).

Data acquisition and processing was performed using PeakSimple Chromatography Data System (model 203) and PeakSimple software version 4.54 (SRI Instruments, Torrance CA,

USA). The oxalic acid peak was identified by comparison of the retention time to a standard solution and a visualized spike from a standard solution with known oxalic acid concentration.

3.3 Standard calibration and recovery

Two standard curves of oxalic acid (99.99% oxalic acid, Sigma-Aldrich Co., St. Louis, USA) were constructed and used for quantification of oxalic acid in samples. The following concentrations: 1.0, 2.0, 5.0, 10, 15.0 and 25.0 mg oxalic acid/100 mL were made up in the corresponding extraction solutions (0.2 M HCl or HPW). All blank and standard solutions were passed through a 0.45 µm cellulose acetate filter prior to analysis. The R^2 of the acid and HPW were 0.9995 and 0.9991 respectively.

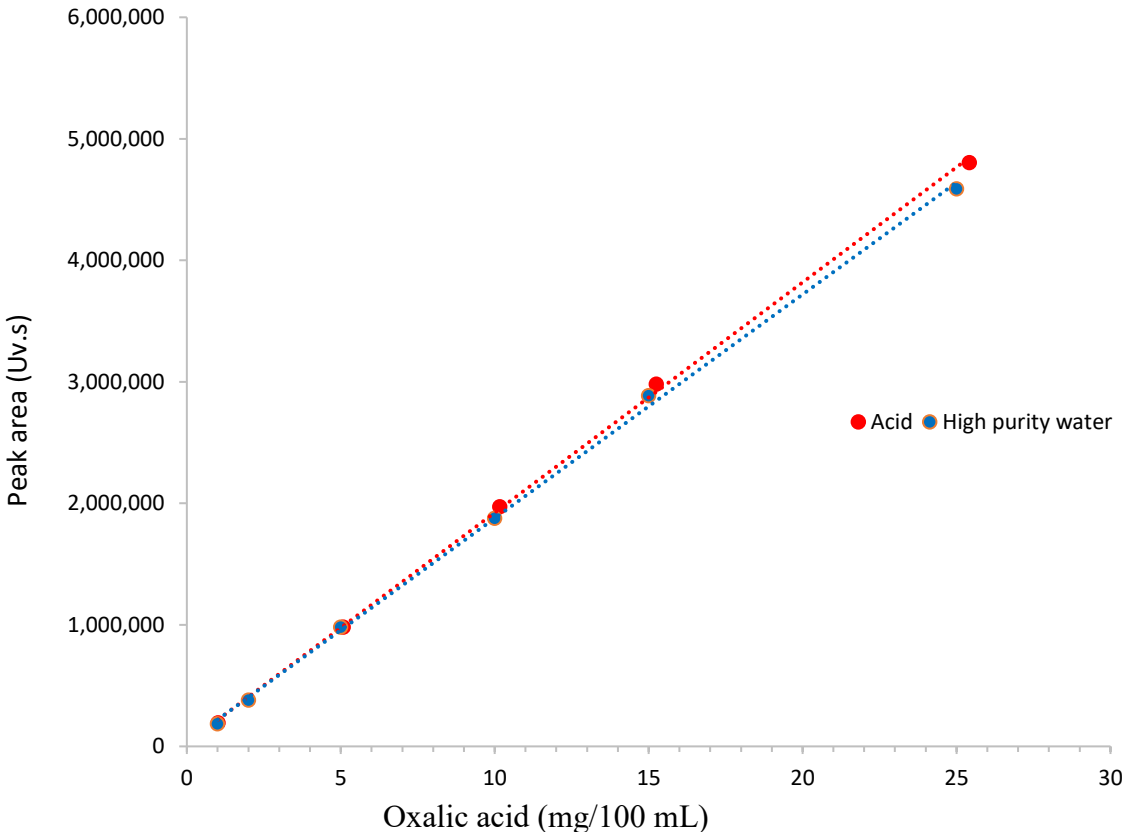

Figure 3.1 Oxalic acid standard curves for acid and high purity water extraction.

A recovery experiment was then performed, 1g fresh weight of spinach was then spiked with 10 mg of oxalic acid which was then extracted using the method outlined in 3.1, using both

acid and water extraction solvents. This was done in quadruplicate. The recovery for the acid extract was 99.6% ±0.13 and for the water extract 98.4 ± 0.15%.

Table 3.1 Recovery of oxalic acid added to fresh spinach.

Extraction method	Measured oxalic acid content (mg/g fresh weight*)		Recovery (%)
	1 g fresh weight spinach	1 g fresh weight spinach plus 10 mg added oxalic acid	
Water	6.85	16.17	98.48
	7.03	16.36	97.92
	6.55	15.88	98.56
	7.78	17.11	98.56
Mean			98.4 ±0.15#
Acid	10.44	20.62	99.49
	10.62	20.81	99.96
	8.91	19.09	99.33
	9.19	19.37	99.53
Mean			99.6 ±0.13#

* values rounded to 2 decimal places for table, 8 decimal places used in calculations.

standard error

3.4 Glassware cleaning protocol

All laboratory glassware used for the extraction and determination of oxalic acid, sections 3.1, 3.2 and 3.3 were cleaned thoroughly using the following protocol.

1. General rinse or scrub to remove debris using ordinary tap water
2. Soaked in 2% v/v Decon® 90 (Decon Laboratories Limited, East Sussex, UK) overnight (16 hours).
3. Rinsed with hot tap water
4. Soaked in 5% v/v/ HCl (Aristar, BDH Chemicals, Ltd., Poole, Dorset, UK) overnight (16 hours).
5. Rinsed with deionised (filter cartridge system) water
6. Dried in fan forced air oven set at 65ºC

3.5 Mineral determination

Samples were extracted by accurately weighing from 0.200 to 1.000 g into a 75 mL Teflon PFA® Kevlar-shielded digestion vessel (CEM Corporation, Matthews, NC, USA). To this 5 mL of acid mixture was added (4:1 nitric acid (BDH, 69%, Aristar®): hydrogen peroxide (BDH, 30%, AnalaR®)). The digestion vessels are then mechanically capped and the samples digested using a MARSXpress™ microwave digester (CEM Corporation, Matthews, NC, USA), which was programmed to ramp from ambient to 90ºC over 10 minutes then from 90ºC to 170ºC over 10 minutes and then held for 10 minutes at 170ºC. Once cooled, 10 mL of high purity water was added to make a final volume of 15 mL.

Mineral analysis was carried out on a Varian Axial 720 Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES, Varian, Palo Alto, CA, USA) with SP3 auto-sampler. Minerals were identified and quantitated using an ICP multi-element standard solution (CertiPUR, Merck, KGaA, Darmstadt, Germany) containing 23 elements or a single element standard, as required. Data and standard curves were processed using ICP-Expert™ II (Varian, Palo Alto, CA, USA). A water test standard and the ICP multi-element standard

solution containing 0.50 μg/g of each element were run in triplicate with each batch to determine the standard error. The overall standard error was ±0.01343 mg/kg. The limit of quantitation (LOQ) was performed using 10 times the standard deviation of the blank (5% nitric acid), for each mineral. Individual mineral LOQ values ranged from 0.12 to 12.24 μg/L, with a mean of 1.81 μg/L.

3.6 Dry matter

The dry matter (DM) of samples was determined using a general gravimetric method as described by Ruiz (2001). Ten gram of sample was accurately weighed on a 3-place balance (PB303, Mettler Toledo, Schwerzenbach, Switzerland) then placed in a Watvic forced air fan oven (Watson Victor Ltd., Wellington, NZ) set at 105°C for 16 hours. Subsequently the samples were placed in a desiccator, until they reached the same temperature as the balance, then weighed.

3.7 Proximate analysis.

Acid detergent fibre (ADF) was determined using AOAC method 973.18 (AOAC, 1990) and neutral detergent fibre (NDF) were determined gravimetrically using the method described by Van Soest *et al.* (1991). Total lipid content was determined gravimetrically, using an automated soxhlet extraction unit (Buchi E-816HE, Switzerland) using petroleum ether as the solvent (AR, Labserv Pronalys, Thermo Fischer Scientific Inc., Auckland, New Zealand). The nitrogen contents were determined using the Dumas method (Sweeny, 1989) using a rapid Variomax CN analyser (Elementar, Langenselbold, Germany) and multiplied by 6.25 to determine the protein content according to AOAC method 920.177 (AOAC, 2002). Total carbohydrate was determined using Anthrone reagent (Acros organics, Thermo Fischer Scientific Inc., Auckland, New Zealand), and extracted, and analysed using methods described by Jermyn (1956), Graham (1963) and Pollock & Jones (1979). All measurements were carried out in triplicate.

3.8 pH and titratable acidity

The pH of samples was determined using a SevenEasy™ pH meter (Mettler Toledo, Schwerzenbach, Switzerland) and a Ag/AgCl sealed reference Eutech pH probe (part number ECF7252101B, Thermo Fischer Scientific Inc., Auckland, New Zealand). Standardisation of the pH probe was carried out using pH 4.00 and 7.00 ±0.02 at 25ºC, buffer solutions, (Labserv Pronalys, Thermo Fischer Scientific Inc., Auckland, New Zealand).

Titratable acidity was determined using a 670 Metrohm titroprocessor using 0.1M NaOH (Labserv Pronalys, Volusol, Thermo Fischer Scientific Inc., Auckland, New Zealand, ±0.0005N) as the titrant and an Ecotrode gel pH electrode (part number 6.0221.100, Metrohm, Switzerland).

3.9 Statistical analysis

All calculations and raw data manipulation was performed using Excel 2016 (Microsoft Inc., Redmond, WA, USA). Statistical analyses were performed using Minitab 17.2.1 (Minitab Inc., State College, PA, USA) and graphs were drawn using Sigmaplot 13 (Systat software, Inc. GmbH, Erkrath, Germany). Unless otherwise indicated all analyses were carried out in triplicate and the results are presented as mean, with the standard error of the mean.

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Chapter 4

Optimisation of extraction of oxalates from green juice

4.1 Introduction

The Design of Experiment (DOE) method is a general and flexible way to provide a comprehensive understanding about the development of processes and/or systems. DOE also serves as a useful assessment of input factors and their interactions, necessary for the determination of the outputs (Goos & Jones, 2011; Khan, 2013). According to Khan (2013), the use of DOE may vary in its type and number of runs depending on the objective(s) of the experiment. Types such as, cover screening DOE, characterization DOE, response surface designs and Taguchi designs. Screening DOE reduces a complex and large number of factors to focus on the few most significant and relevant factors; characterization DOE assesses the main factors and their interactions to calculate a predicted equation; response surface designs construct the curvature as a design's output and Taguchi designs focus is to reduce the variability and sensitivity caused by errors within the experimental data (Khan, 2013).

The major benefit of conducting an experiment based on DOE is that the optimization can be applied to determine the number of experimental runs necessary to perform a statistically sound experiment, while considering several factors (parameters) applied in the experiment (Khan, 2013).

Using the DOE technique, an experiment was designed to optimise the extraction of oxalates and to identify the significant factors.

Three extraction factors for oxalic acid from green juices, were considered; pH, time and temperature. The minimum number of experimental runs needed to be carried out to give an accurate prediction of optimum extraction conditions was determined using Design Expert DX9 Software (Stat-Ease Inc., Minneapolis, MN, USA).

4.2 Materials and methods

4.2.1. Sample collection and preparation

Fresh spinach samples were purchased from a local supermarket (New World, Lincoln, Christchurch, NZ) and were not washed prior to homogenization. The stems of the fresh spinach were removed about 10 mm from their root tips and the remaining edible portion of the fresh spinach samples (stems and leaves) were chopped into approximately 10 mm lengths. The chopped spinach samples were then transferred into a food extractor container, up to the maximum line (NutriBullet, Briscoes Homeware, NZ). Each sample was then homogenized for 10 seconds, then shaken within the food extractor, then homogenized a further 10 seconds to produce a homogenous juice. This uniform homogenate was used as a green juice analogue to determine the optimum extraction conditions.

4.2.2. Preparation of extraction solutions

A series of HCl extraction solutions were prepared in the pH range of 1 to 7 (concentration range of 1×10^{-1} to 1×10^{-7} M). The first HCl solution of 1×10^{-1} M or pH 1, was prepared by diluting 8.73 mL of 36.46% HCl 1 N (Sigma Aldrich, St. Louis, MO, USA) with high purity water (HPW) in a 1000 mL volumetric flask. The other HCl solutions were prepared through a serial dilution process from the first 1×10^{-1} M or pH 1 solution into 1000 mL volumetric flasks with high purity water. The actual pH values of the extraction solutions were measured using a pH meter (SevenEasy, Mettler Toledo, Schwerzenbach, Switzerland), equipped with a Eutech pH probe (part number: ECFC-7252101B, Thermo Fischer Scientific, Auckland, NZ), which was calibrated with pH buffers 4.0 and 7.00 ± 0.02 (Pronalys, Thermo Fischer Scientific, Auckland, NZ). The measurements were conducted in triplicate and the recorded values were used for designing the experimental runs using the Design Expert DX9 Software (Stat-Ease, Inc., Minneapolis, USA).

4.2.3. DOE experimental design, method, data analysis, and verification

A 3 X factorial experiment was designed using seven levels of pH, 8 x levels of temperature and six points of time. The seven levels of pH were chosen, by using a step increase of a factor of 10, from an initial concentration of 1×10^{-1}, relating to the pH range from pH 1 to 7. The actual measured pH values are tabulated in Table 4.1. The 8 x levels of temperature used for extraction were chosen by taking 10ºC increments from 25ºC to a maximum of 95ºC, which was deemed close enough to boiling an extracted sample. Non-trial samples of spinach

juice were measured at 25ºC and 95ºC extraction temperatures. It was determined that it took approximately five minutes for the sample to reach the start extraction temperature. Therefore, five minutes was added to each extraction time to account for this. Previous researchers have used times ranging from six minutes (Mou *et al.*, 2008) to 300 minutes (Weaver *et al.*, 1997; Sotomayor *et al.*, 2001) for oxalic acid extractions. More often the extraction times used were 15 minutes (Savage *et al.*, 2000; Zhang *et al.*, 2005; Siener *et al.*, 2006; Merusi *et al.*, 2010) or 20 minutes (Akhtar *et al.*, 2011; Ruan *et al.*, 2013; Liu *et al.*, 2015). Therefore, a minimum time of 15 minutes was chosen, with 15-minute steps until 60 minutes, to a maximum extraction time of 120 minutes (Table 4.1).

Table 4.1 Extraction parameters: levels of pH, temperature and time.

No.	Parameter	Levels	Total levels
1.	pH (actual)	0.93, 1.91, 2.96, 4.04, 4.98, 5.62, 5.81	7
2.	Temperature (ºC)	25, 35, 45, 55, 65, 75, 85, 95	8
3.	Time (minutes)	15, 30, 45, 60, 90, 120	6

For a complete 3 X factorial experiment, at multiple levels, this would require 336 individual experimental runs, with no replication of points. This was recognised as excessive to achieve in one day. Conducting all the experimental runs in one day was considered to be statistically sound, as this would minimize any variation that might occur if the experiment was carried out over several days. Therefore, using these factors and levels (Table 4.1) as parameters, a DOE experimental design was created using Design Expert software, based on a DOE method using response surface methodology (RSM) for optimal (custom) design for the three discrete numeric factors. The design was divided into design modelling verification and design response analysis. Design modelling verification was conducted by inserting the three experimental parameters: pH of the extraction solutions, temperature and extraction time for the extraction process (Table 4.1) and it was then evaluated based on a quadratic model design. This experimental model was optimally designed for a one-day experiment period without blocking and was constructed with five estimated lack of fit points, 10 model points and five replicates. Total experimental runs were determined to be 20, to give a statistically valid experiment using the DOE experimental model. (Table 4.2). The DOE experimental model was analysed by modelling simulation in Design Expert, for its VIF (Variance Inflation Factor) and Ri2 (correlation coefficient of determination) values. According to Allen (2010), VIF is the statistical measure for the evaluation in determining the possibility of experimental parameter(s) reliably fitting the model based on the experiments objective(s). The VIF value

provides a clarification for the power of the model to determine any statistical differences. The Ri2 value describes the levels of correlation of the extraction parameters and their interactions, to determine the power level of the experimental model design. The ideal values for VIF and Ri2 values are 1.0 and 0.0, respectively.

The response analysis of the experimental design (oxalate concentration) was performed after all experimental runs had been completely carried out.

The first statistical analysis in the DOE method was an ANOVA using a quadratic model design to determine the significance of the model and the significant factors (parameters) of the model. The determination process provided the calculated values of R^2, adjusted R^2 and predicted R^2 of the experiments model design. From these three values, a mathematical equation was generated from the numerical optimization process. This optimization process was used to plot the graph of the relationships of the three extraction parameters and the extracted oxalate content from the fresh spinach homogenate. The mathematical equation was also expected to be useful to calculate the oxalate content that could be extracted from different combinations of extraction process in addition to the 20 key experimental runs.

4.2.4. Sample extraction

The extraction process for oxalates from fresh spinach juices was performed according to the method outlined in Chapter 3, section 3.1 with minor modifications. Two g of fresh spinach juice was accurately weighed into an Erlenmeyer flask and 40 mL of the appropriate extraction solution (Table 4.2) was added into the Erlenmeyer flask. The accuracy levels of the pH of extraction solutions, extraction temperature and extraction time were ± 0.02, ± 0.2°C and ± 0.05 seconds, respectively. After each extraction, the flask was allowed to cool in cold running water (11.5°C) for five minutes. Each sample solution was then quantitatively transferred into a 100 mL volumetric flask and made up-to volume with the corresponding extraction solution. Each sample solution was held at room temperature for 15 minutes with periodical shaking every five minutes prior to the next step.

4.2.5. Oxalate analysis

The extracted samples were analysed using the standard method for oxalic acid with no alterations, as described in Chapter 3, the experimental response.

Table 4.2 DOE experimental model for extraction of oxalates from spinach juice.

Run	Buffer HCl solution (pH)	Solvent concentration (M)	Incubation temperature (°C)	Extraction time (minutes)
1	4.04	10^{-4}	25	60
2	4.04	10^{-4}	65	120
3	2.96	10^{-3}	95	15
4	4.04	10^{-4}	65	120
5	5.81	10^{-7}	45	15
6	4.04	10^{-4}	65	45
7	5.81	10^{-7}	55	90
8	5.81	10^{-7}	95	15
9	4.04	10^{-4}	25	60
10	2.96	10^{-3}	35	120
11	5.81	10^{-7}	25	120
12	5.81	10^{-7}	95	90
13	4.04	10^{-4}	65	45
14	0.93	10^{-1}	65	60
15	0.93	10^{-1}	25	120
16	0.93	10^{-1}	65	60
17	4.04	10^{-4}	65	45
18	0.93	10^{-1}	95	120
19	0.93	10^{-1}	25	15
20	1.91	10^{-2}	95	60

4.3 Results

4.3.1. pH measurement of extraction solutions

The measured pH of the extraction solutions ranged from 0.93 to 5.81 (Table 4.3). The actual and calculated pH values were different due to the slightly acidic pH of the high purity water used (pH 5.90) in this experiment.

Table 4.3 pH measurement of HCL extraction solutions.

Extraction solution	Solution concentration (M)	Theoretical pH	Measured pH
HCl - 1	10^{-1}	1	0.93 ± 0.36
HCl - 2	10^{-2}	2	1.91 ± 0.17
HCl - 3	10^{-3}	3	2.96 ± 0.11
HCl - 4	10^{-4}	4	4.04 ± 0.14
HCl - 5	10^{-5}	5	4.98 ± 0.12
HCl - 6	10^{-6}	6	5.62 ± 0.06
HCl - 7	10^{-7}	7	5.81 ± 0.06
HPW water	n/a	n/a	5.90 ± 0.10
0.2 M HCl	2×10^{-1}	0.69	0.85 ± 0.39

4.3.2. DOE statistical modelling

From the 20 experimental runs performed, the quadratic model of the three extraction parameters gave VIF and Ri2 values ranging from 1.07 to 1.24 and 0.069 to 0.191, respectively (Table 4.4).

Table 4.4 Calculated VIF and Ri^2 values for a 3 x factor, 20 run experiment.

Parameter 1	Parameter 2	VIF	Ri^2
pH	-	1.07	0.0689
Temperature	-	1.08	0.0707
Time	-	1.16	0.1403
pH	Temperature	1.11	0.0977
pH	Time	1.12	0.1107
Temperature	Time	1.15	0.1281
pH	pH	1.12	0.1057
Temperature	Temperature	1.14	0.1201
Time	Time	1.24	0.1909

4.3.3. Response measurement

The total oxalate content in the fresh spinach homogenate for all extraction conditions ranged from 424.95 to 856.57 mg/100 g FW (Table 4.5).

Table 4.5 Response values (oxalic acid concentration of the spinach juice).

Run	Buffer solution (pH)	Solvent concentration (M)	Extraction temperature (°C)	Extraction time (minutes)	Oxalic acid (mg/100 g FW)
1	4.04	10^{-4}	25	60	479.34
2	4.04	10^{-4}	65	120	604.34
3	2.96	10^{-3}	95	15	652.13
4	4.04	10^{-4}	65	120	592.74
5	5.81	10^{-7}	45	15	561.98
6	4.04	10^{-4}	65	45	606.31
7	5.81	10^{-7}	55	90	635.26
8	5.81	10^{-7}	95	15	663.97
9	4.04	10^{-4}	25	60	424.95
10	2.96	10^{-3}	35	120	427.01
11	5.81	10^{-7}	25	120	465.72
12	5.81	10^{-7}	95	90	659.17
13	4.04	10^{-4}	65	45	620.07
14	0.93	10^{-1}	65	60	824.96
15	0.93	10^{-1}	25	120	785.60
16	0.93	10^{-1}	65	60	801.58
17	4.04	10^{-4}	65	45	610.85
18	0.93	10^{-1}	95	120	856.57
19	0.93	10^{-1}	25	15	775.44
20	1.91	10^{-2}	95	60	684.48

Using the data from Table 4.5 a quadratic model was constructed (Equation 1).

$$y = 22.16\ A2 - 0.04\ B2 + 7.74\times10\text{-}6\ C2 + 0.39\ AB - 0.03\ AC + 9.14\times10\text{-}6\ BC - 213.18\ A + 4.43\ B - 0.69\ C + 863.10 \quad \text{Equation 1}$$

Where:

y = oxalic acid (mg/100 g FW)
A = extraction solution pH (pH)
B = extraction temperature (°C)
C = extraction time (minutes)

The accuracy and significance of the overall quadratic model and its factors can be seen in Table 4.6.

Table 4.6 Level of significance of quadratic model design.

Source	P value	Significance
Quadratic model	< 0.0002	***
pH	< 0.0001	***
Temperature	0.0010	**
Time	0.6822	NS
pH × Temperature	0.1150	NS
pH × Time	0.8703	NS
Temperature × Time	0.4049	NS
pH^2	0.0003	***
$Temperature^2$	0.0911	NS
$Time^2$	0.9360	NS

NS = not significant. * P<0.05, ** P<0.01, *** P<0.001

The DOE program can graphically express the quadratic model in a two-dimensional space. The pH vs extraction temperature is shown in Figure 4.1, at 20 minutes extraction time, with contours depicting the response value (oxalic acid). If desired a similar plot can be obtained for every combination of factors.

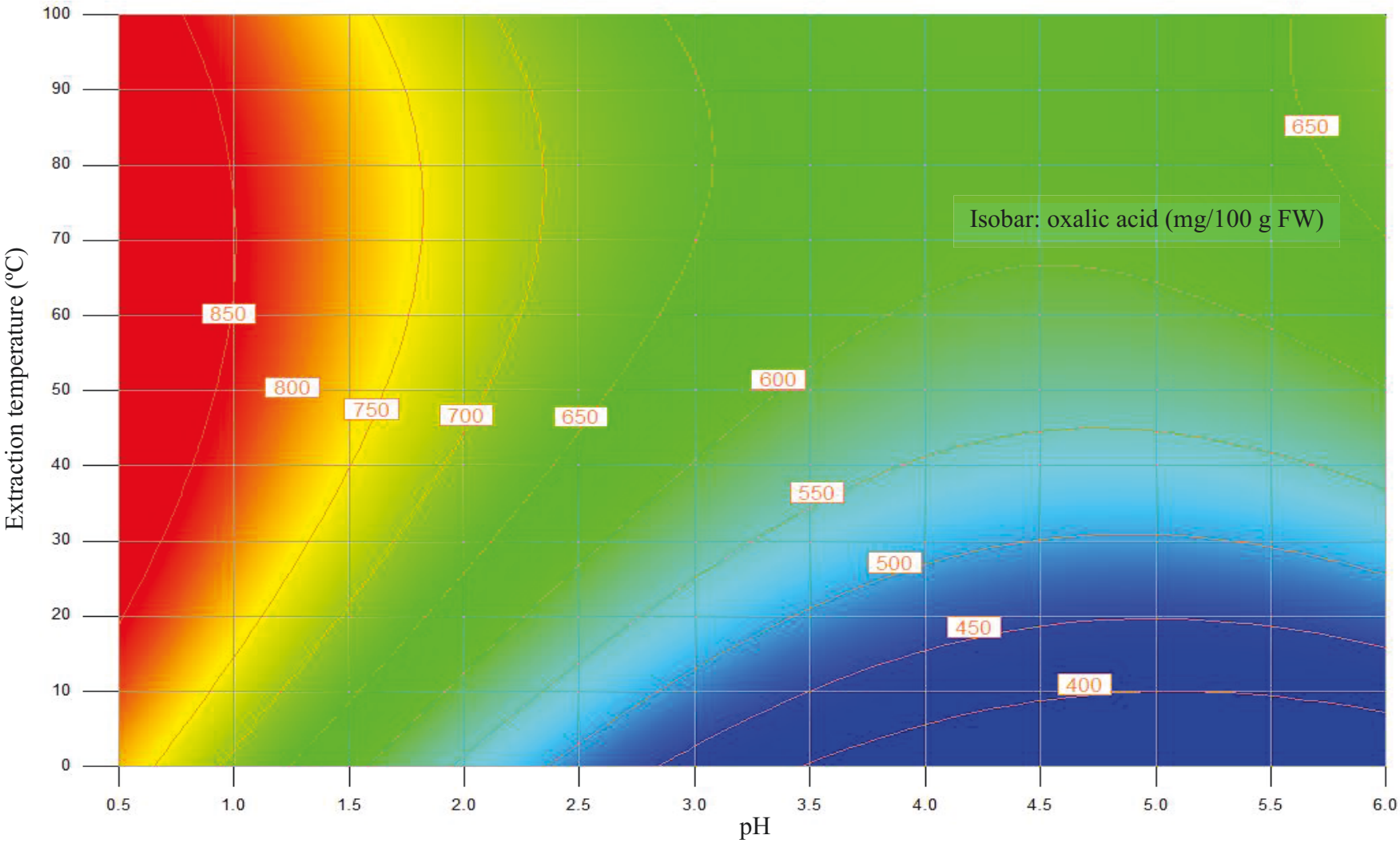

Figure 4.1 Two dimensional (pH vs extraction temperature) contour plot of the response variable (oxalic acid), from the spinach homogenate extracted for 20 minutes.

4.4 Discussion

4.4.1. pH of extraction solutions

The actual and calculated pH values were different due to the slightly acidic pH of the high purity water used (pH 5.90) in this experiment. Theoretically, the pH of high purity water should be pH 7.0 because all the dissolved ions have been removed, resulting in no ionic interaction between the ions to yield neither acidic nor alkaline conditions. However, high purity water quickly reacts with CO_2 in the air and any dissolved CO_2 will shift the pH value lower. It has been calculated at an air CO_2 concentration of 350 ppm, the pH of a pure water solution would be pH 5.68 at 20ºC (Byck, 1938). The high purity water used in this experiment was pH 5.90 ± 0.10 with a reported CO_2 concentration of the atmosphere in New Zealand of 398.43 ppm (NIWA, 2015). All the extraction and buffer solutions prepared for this experiment used standard laboratory glassware that had been carefully cleaned using the protocols outlined in Chapter 3, section 3.4.

4.4.2. DOE modelling

The main reasoning for using a DOE method in this experiment was to optimize the experimental factors and determine a statistical model using 20 experimental runs, compared to a complete factorial combination of 336 runs. The VIF values of all parameters and their interactions in this quadratic model design were close to 1.0 (Table 4.4). This indicates that the quadratic model design was powerful and fit for determining any significant differences once the model response values (oxalic acid concentration) were determined from the experimental runs. On the other hand, the Ri2 value is expected to be as close to 0.0 which means the experimental parameters were less correlated (significantly different) between each other and the model design was well-fitted or powerful. The Ri2 values in this experiment indicated that the levels of correlation of the extraction parameters were considerably low, thus resulting in a powerful and fit quadratic model design with only slightly correlated parameters.

4.4.3. Response values

Spinach grown in New Zealand contains higher amounts of oxalates compared with plants cultivated in other countries in Asia, Europe and USA (Table 2.1). Previous reports have shown that the amount of oxalic acid from acid and water extractions ranges from 364.6 and 1764.7 mg/100 g FW (Savage *et al.,* 2000). In this experiment, the total oxalate content in the

fresh spinach homogenate from all extraction conditions ranged from 424.95 to 856.57 mg/100 g FW (Table 4.5) and these are comparable to previously reported values (Savage *et al.,* 2000).

Statistical analysis of the quadratic model generated from the response values (Table 4.6) showed that the level of significance of the quadratic model design was 99.98% and the significant parameters in this experiment were pH and temperature. Based on these results the R^2 was calculated to be 0.926, indicating that this quadratic model design was well-fitted for this experiment and all the results obtained were shown to be in a normal distribution pattern. However, the difference between the adjusted R^2 (0.859) and the predicted R^2 (0.558) of about 0.3 was noted to be slightly higher than the recommended difference of 0.2 or less, for this type of experiment (Anderson & Whitcomb, 2016).

Using the general mathematical equation as shown in Equation 1 the extracted oxalate content (response value) from any combination of pH, temperature and time in extraction process can be estimated with a high degree of confidence. In a practical sense this information could be used estimate the oxalate levels in a food prepared using spinach or a spinach homogenate and helps to determine if more or less heat is required, or whether pH may influence the recipe.

4.4.4. Effect of extraction time

Extraction time was the parameter with the least significant effects (Table 4.6). With time, pH x time, temperature x time and time^2 all having $P > 0.05$ on the extracted oxalate contents from fresh spinach homogenate in this experiment (Table 4.6). This was evidenced from the results of Run 8 – Run 12 and Run 15 – Run 19 (Table 4.5). Within the same extraction conditions of pH 5.81 and temperature 95°C, the oxalate content from Run 8 with 15 minutes extraction (663.97 mg/100 g FW) was not significantly different from Run 12 with a 90 minute extraction (659.17 mg/100 g FW). In accordance with these two runs, the extracted oxalate content from Run 15 with a 120 minute extraction (785.60 mg/100 g FW) was similar to Run 19 with a 15 minute extraction (775.44 mg/100 g FW). Both extraction runs were performed with an extraction solution of pH 0.93 at 25°C. In regarding to this condition, the two dimensional plot of the extracted oxalate contents from fresh spinach homogenate from any extraction times illustrated the similar pattern, as shown in Figure 4.1. Therefore, it was determined to be plotted at the 20 minute extraction time, which was the same as the extraction time used by Juajun *et al.* (2012) for a range of Thai vegetables.

Figure 4.1 illustrates the areas with different oxalate contents extracted from fresh spinach homogenate, in which the highest oxalate content is indicated with the reddish-coloured area and the lowest oxalate content is in the bluish-coloured area. From this curve, it can be seen that to extract the highest oxalate content from fresh spinach homogenate, the extraction process should be carried out at pH 0.93 and at a temperature of approximately 65°C. On the other hand, the lowest amount of extracted oxalate content from fresh spinach homogenate was obtained at a pH of about 4.59 and lower temperature of approximately 25°C. Based on this curve, an adjustment for the combination of pH and temperature may be necessary, in comparison to the standard method used by Savage *et al.* (2000), for the oxalate extraction process from fresh spinach homogenate, in order to adequately release the highest amount of oxalate from fresh spinach homogenates.

4.4.5. Effects of pH and temperature

The extraction time did not significantly affect the extracted oxalate content from fresh spinach homogenate ($P > 0.05$), however, pH ($P < 0.001$) and temperature ($P < 0.01$) were determined as the significant parameters in the extraction process (Table 4.6). The extraction with HCl solutions at pH 0.93 (0.1 M), pH 0.69 (0.2 M) and pH 0.09 (2 M) resulted in extracted oxalate contents of 856.57, 886.59 and 1764.7 mg/100 g FW, respectively. It was shown that decreasing the pH of the extraction solution significantly increased the extracted oxalate content. Even though pH may be the statistically more significant parameter compared to temperature, in this extraction process, the noticeable differences in response values due to changes to the temperature of the extraction process should not be overlooked.

In this experiment, the much lower temperature applied (25°C) compared to the extraction temperature of 80°C used by Savage *et al.* (2000), is assumed to be the reason for the incomplete extraction process. This means that there might be some portion of oxalate in fresh spinach homogenate, still bound to the food matrix or that exists as complexes with other compounds within the spinach matrix. It has been reported earlier that a temperature higher than 60°C is the lowest temperature to initiate the breakdown and disintegration of cell structures in food, thus allowing the release of nutrients from the food matrix (Uusiku *et al.,* 2010). Therefore, the extraction temperature of 25°C in this experiment was thought to be insufficient to exert enough of a synergetic effect with the low pH value in the oxalate extraction process to result in complete oxalate extraction.

Previous research by Nguyen & Savage (2013) has suggested that oxalates in vegetables or fruits may bind to the food matrix of fibre complexes (fibre-mineral-oxalate complexes), and exposure to high acidic (pH < 2) and temperature (50-90°C) may enhance the breakdown of molecular bonds of these fibre-complex molecules.

The influence of the food matrix of spinach with respect to the bioavailability of other compounds has also been demonstrated by Castenmiller *et al.* (1989). They showed the bioavailability of β-carotene was changed dependant on whether the spinach was whole, liquefied or minced. The liquefied spinach, when feed to 72 men and women over three weeks, significantly increased the blood serum concentrations total β-carotene. Aside from this, the buffering capacity of fresh spinach homogenate might have influenced the oxalate extraction process. A more concentrated buffer solution increases the buffering capacity of foods and leads to greater retention of any chemical compounds in the food matrix (Averill & Eldredge, 2012). The greater retention of oxalate in the matrix of fresh spinach homogenate resulted in lower extracted oxalate content, which was 424.95 mg/100 g FW at pH 4.04.

4.5 Conclusions

The outcome of this experiment provides important information on the bioavailability of oxalate from spinach in the small intestine. The extraction process performed in this experiment could represent the digestion process in the human body, particularly in the stomach, after the ingestion of spinach. HCl is the sole acidic fluid secreted to maintain the pH of the stomach, in the range of 1.0 to 2.0 (Scanlon & Sanders, 2014). Once ingested and transported into the stomach, spinach undergoes enzymatic and chemical digestion and the low pH condition (pH 1.0-2.0) of the stomach will enhance oxalate extraction from the spinach to its optimum. This experiment has shown that a lower pH enhances the oxalate extraction process and results in the highest extracted oxalate content to be at pH 0.93. Chemical digestion of spinach in the stomach may provide for greater bioavailability of oxalates to the human body, particularly in the form of free oxalate ions of $(C_2O_4)^{2-}$, as the pH of the fresh spinach homogenate ranged from 6.4 to 6.5 (data not shown). Based on Figure 4.1, at pH > 6.0, oxalates are predominantly present in their free-ion forms, which are recognized to possess high binding capacity with the available calcium in the body, but could also bind to any other available cations, dependant on their concentration and K_{sp} relative to calcium. The binding of free oxalate ions to calcium could occur once the oxalates are absorbed in the intestine (pH 8.0) as the body tries to get rid of the excess oxalates via the

kidneys. The greater bioavailability of oxalate in the human body, the higher the potential risk of having health issues like renal dysfunction and mineral deficiencies.

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Chapter 5

Differences between green juice recipes

5.1 Introduction

Fruit and vegetable juices are becoming popular as an easy way to consume a range of fruits and vegetables. Green, yellow or orange juices are widely promoted as healthy drinks because they contain a wide range of essential amino acids, vitamins and minerals from different combinations of green leafy vegetables, together with many kinds of fruits (Hu, 2003). Fruit and vegetable juices are often promoted as an excellent way to flush the kidneys and, even, as a way to lose weight, although there is no strong scientific data to support these reports. There are very few scientific papers to support any of these claims. Nainwal *et al.* (2011) studied rats feed fruit and the juice from *Lagenaria siceraria* and the results showed no lowering in the serum chemistry of the rats, although they did lose weight. Shils & Hermann (1982) reviewed several diet-based cancer treatment programmes, including the Gerson Therapy, which has 4 x 8 oz green juices per day as part of the diet. They discuss two potential causes of harm to patients; first, being nutritional depletion or toxicity due to the dietary programme; and, secondly, by the patients' failure to adopt more conventional treatments, such as surgery. Juice processing normally uses fresh raw fruits and vegetables in order to preserve the health benefits of the extracted nutrients. The removal of the fibre fraction and processing the extracted juices without heating to preserve the 'goodness' of the juice is an important part of the process. The fibre fraction, which is commonly discarded, contains bound oxalates together with high levels of many other minerals that are an important part of human diets (Vanhanen & Savage, 2015). However, this processing cannot prevent or reduce potentially toxic anti-nutrients, such as oxalates, in the juices (Savage & Klunklin, 2018).

Homemade and commercial juices can be prepared using a wide range of fruits and vegetables, and the proportions and ingredients used are not often recorded on the labels of commercially-prepared juices. The pH of the juice mixes covers a wide range so the juices often do not store very well. It is of considerable concern that 'green mixes' often contain large amounts of 'cheap' vegetables, particularly spinach (*Spinacia oleracea*) and silver beet (chard or Swiss chard, *Beta vulgaris* subsp. *vulgaris*, Cicla), which can contain large amounts of oxalate (Noonan & Savage, 1999; Nguyen & Savage, 2013; Savage & Klunklin, 2018). The majority of fruits and vegetables in a Western diet, which Nguyen & Savage (2013) have studied, contained low to moderate concentrations of oxalate. However, the soluble oxalate

concentrations of green juices made from rhubarb nectar and beetroot juices have been reported to be very high, with values of 197.1 mg/100 mL and 54.3–65.2 mg/100 mL, respectively. (Siener *et al.*, 2016). The study by Siener *et al.* (2016) showed that the consumption of two cups, or 500 mL, a day of certain vegetable juices can increase the daily oxalate intake, and this could cause acute oxalosis if consumed on a regular basis.

Oxalates occur in two forms in plants; soluble oxalates in plant foods are found bound to Na^{+}, K^{+} and NH^{4+}, and insoluble oxalates are commonly bound to Ca^{2+}, Mg^{2+} and Fe^{2+} (Savage *et al.*, 2000). Soluble oxalates from foods that remain unbound are readily absorbed from the gastrointestinal tract but, as oxalates cannot be utilised in the body, they are quickly excreted by the kidneys. When oxalates are being excreted they can combine with calcium that is being excreted at the same time. This leads to the formation of kidney stones. Approximately 63% of all kidney stones comprise calcium oxalate (Kittanamongkolchai *et al.*, 2018). The oxalate content of foods, which depend on plant families and plant organ systems, plays an important role in kidney stone formation in the human body (Siener *et al.*, 2016; Savage & Klunklin, 2018). Kittanamongkolchai *et al.* (2018) studied the prevalence of kidney stones from 1984 to 2012 among more than 10,000 residents of Minnesota, USA. Their research found kidney stones have been on an upward trend, especially in young women aged 18 to 39, with an increase from 62 to 252 cases (per 100,000 person-years) from 1984 to 2012. After the age of 25, kidney stones became more common among men. There are only two reliably documented reports of acute oxalate nephropathy due to the consumption of green juices. In 2013, Getting *et al.* (2013) reported that an 81-year-old man presented with a sudden decrease in renal function that had occurred after juicing all his meals, most of which were juices made from high-oxalate vegetables; his rationale for this being to lose weight. In the second case of acute oxalate nephropathy, a 65-year-old woman had replaced all her normal diet with a diet of juices only, for 10 days, again, in an attempt to lose weight. Part of her diet included the consumption of two cups per day of juiced spinach. She presented at the Nassau University Medical Center (NY, USA) with nausea, decreased appetite and weakness. Subsequently, she was confirmed to have a diagnosis of acute oxalate nephropathy (Makkapati *et al.*, 2018).

An example of the potential problems that can occur from excessive consumption of juices containing high levels of soluble oxalate are from the studies of star fruit juice (Tse *et al.*, 2003). Star fruit, carambola (*Averrhoa carambola*), is a tasty fruit that contains high levels of soluble oxalates, 301.1 mg/100 g fresh weight, (Nguyen & Savage, 2013). In a period between July 2001 and May 2013 eight out of 20 patients died in two Hong Kong hospitals

following regular consumption of star fruit juices. The patients were brought into hospital with a range of acute kidney and kidney stone problems. Indian gooseberry (*Phyllanthus emblica*) has also been promoted as a new nutritious juice. This is unwise, as the fruits have been shown to contain 1167.2 mg soluble oxalate/100 g fresh weight, 99% of which is soluble: this is very unique (Vanhanen *et al.*, 2011). So far, there have been no reports of problems from the regular consumption of this tasty, but rather, acid fruit or juice made from the fruit.

While several vegetable juices are known to contain substantial amounts of soluble oxalate and are therefore more likely to be absorbed in the gastro intestinal tract, e.g., spinach, celery and parsley ranging from 78 to 364.09 mg/100 g FW (Vanhanen & Savage, 2015), comprehensive and reliable data on the oxalate content of formulations of green juices are lacking. Recently, total, soluble and insoluble oxalates of spinach leaves were reported to be in the range of: 329.6–1764.7, 266.5–736.6 and 40.2–1400.1 mg/100 g fresh weight, respectively (Noonan & Savage, 1999; Savage *et al.*, 2000). Vanhanen & Savage (2015) noted that typical green juices may contain 40% spinach leaves and so the juice will contain 364.2 mg soluble oxalate/100 g of juice in a standard 200 mL glass. There are reports of people consuming six glasses/day, which could result in the consumption of 4.4 g of soluble oxalate/day. There are also informal reports of people commonly consuming these drinks for several days or, even, weeks. This would result in a very significant intake of soluble oxalates.

The risk undertaken by consuming moderate to large amounts of soluble oxalates (Vanhanen & Savage, 2015) from spinach leaves in vegetable juices has driven studies to compare widely promoted recipes to evaluate the soluble oxalate contents. The absorption of soluble oxalate from foods can be reduced when foods contain high levels of calcium as these modify soluble oxalate to form insoluble oxalates (Bong *et al.*, 2017). Additional factors, including calcium and other minerals, may influence urinary oxalate excretion. Therefore, the aim of this study was to determine the oxalate concentration and mineral compositions of five different green juices produced using spinach leaves as a significant constituent of the fruit and vegetable mix.

5.2 Materials and Methods

5.2.1 Green juice preparation

Fresh vegetables were bought the local supermarket (Lincoln, New Zealand). Soil, dead leaves and stalks ends were removed using a stainless-steel knife. Each green juice type was prepared upon a w/w basis for each ingredient (Table 5.1) and then processed using a masticating juicer (Oscar 9000, Dongah Industrial Co., Ltd, Gyeongsangnam Do, South Korea). The juicer produced two fractions, a clear juice fraction and a pulpy fibre fraction, which was normally discarded. Both fractions were subsequently either analysed immediately or stored at -20ºC until analysis.

Table 5.1 Green juice ingredient profile (% w/w).

	GJ1*	GJ2	GJ3	GJ4	GJ5
Apples	-	34.6	42.8	-	33.6
Blueberry	-	-	-	27.8	-
Celery	14.9	-	26.7	-	7.5
Cucumber	14.9	28.8	-	-	-
Ginger	0.9	-	-	-	-
Green capsicum	9.0	-	-	-	-
Kale	7.5	-	-	-	13.9
Lemon	14.0	8.5	-	-	4.5
Pak choy	18.4	-	-	-	-
Parsley	0.4	2.8	-	-	2.6
Pear	-	-	-	41.0	-
Red capsicum	-	4.8	-	-	-
Spinach	20.1	20.6	30.5	31.2	37.9

GJ = green juice

5.2.2 Proximate analysis, pH and titratable acidity

Ten grams of fresh juice and fibre fractions of each of the recipes were, accurately weighed into 100 mL beakers and their pH and titratable acidity were determined in triplicate using the method described in Chapter 3, section 3.8.

Similarly, proximate analysis was carried out using the methods described in Chapter 3, section 3.7.

5.2.3 Dry matter determination

The dry matter (DM) content of each sample of fresh juice and fibre fraction was determined using the method detailed in Chapter 3, section 3.6, with no variation.

5.2.4 Oxalate extraction and analysis

Sample extraction for total and soluble oxalic acid determination of the green juices and fibre fractions were extracted and analysed in triplicate using the HPLC method described in Chapter 3, sections 3.1, 3.2, 3.3 and 3.4.

5.2.5 Mineral analysis

Mineral analysis was performed on the fresh juice and fibre samples using the method described in Chapter 3, section 3.5.

5.2.6 Statistical analysis

All statistical analysis were undertaken using the software programs and methods outlined in Chapter 3, section 3.9, with no variation.

5.3 Results

5.3.1 Fraction yield

Since each recipe was only made once, then its fractions analysed in triplicate there are no variation statistics available on the yield of the juices (Table 5.2). Juice production ranged from 71.7 to 84.3% yield in a fresh weight basis. Waste was low ranging from 2.1 to 5.5%.

Table 5.2 Green juice production yield (% w/w).

Yield (%)	GJ1	GJ2	GJ3	GJ	GJ5
Juice	73.2	80.3	80.8	84.3	74.3
Pulp	26.2	14.2	15.6	12.9	22.9
Waste	2.1	5.5	3.6	2.8	2.8

GJ = green juice recipe

5.3.2 pH, titratable acidity and proximate analysis

The pH of each juice differed at most by 0.77pH units (Table 5.3). The titratable acidity was expressed as expressed milli-equivalents of acids, as there was a mixture of vegetables. It ranged from 2.66 to 7.28 mEq. acid /100g FW of juice.

Table 5.3 Green juice pH and titratable acidity for five different green juice recipes

	pH	mEq. acid / 100g FW
GJ1	4.35 ± 0.01	7.28 ± 0.14
GJ2	4.18 ± 0.02	7.07 ± 0.04
GJ3	4.95 ± 0.01	2.66 ± 0.01
GJ4	4.26 ± 0.02	3.41 ± 0.04
GJ5	4.54 ± 0.01	6.18 ± 0.12

GJ = green juice recipe

The dry matter of the different juices varied from 5.32 to 10.32% DM and for the pulp, 15.89 to 28.56 %DM (Table 5.4).

Table 5.4 Dry matter of juice and pulp for five different green juice recipes

Dry matter (%w/w)		
	Juice	Pulp
GJ1	5.32 ± 0.04	15.89 ± 0.15
GJ2	7.14 ± 0.09	19.83 ± 0.63
GJ3	7.94 ± 0.03	18.61 ± 0.73
GJ4	10.32 ± 0.58	28.56 ± 0.68
GJ5	8.41 ± 0.05	21.55 ± 0.21

GJ = green juice recipe

No statistical tests of mean value correlations or significance can be made between the recipes, as they are not true replicates of each other and there is a high variability among the types and amounts of vegetables. However, in the green juices made, the pulp removes significant amounts of fibre, protein fat and carbohydrates (Table 5.5). GJ3 has the lowest fibre content, 0.92 and 1.44, ADF and NDF, respectively. GJ1 has twice as much total protein compared to the other recipes.

The total, soluble and insoluble content of the juices and waste pulp can be seen reported in Table 5.6. GJ3 (82.85 mg/100 g FW) had more than six times the amount of soluble oxalic acid compared the lowest juice, GJ1 (13.07 mg/100 g FW). The absolute amounts do not necessarily reflect the true impact of the amount of soluble oxalic present.

Table 5.5 Proximate analysis of juice and pulp from five different green juice recipes (g/100g dry matter).

	ADF		NDF		Total protein		Total Fat		Total Carbohydrates	
	juice	pulp	juice	pulp	juice	pulp	juice	pulp	juice	pulp
GJ1	3.29	26.45	4.06	29.06	4.39	2.86	1.71	1.18	44.3	44.4
GJ2	2.32	26.47	2.81	32.11	1.84	2.28	0.67	3.18	45.1	45.9
GJ3	0.92	26.51	1.44	34.36	2.02	2.54	0.51	1.85	42.6	44.6
GJ4	4.92	34.76	8.27	45.60	1.29	1.95	0.48	1.80	43.8	47.4
GJ5	2.02	22.37	2.89	29.27	2.86	2.70	2.00	1.82	44.4	44.6

GJ = green juice recipe

Table 5.6 presents the results as ratio of the total oxalate present. The soluble oxalate content of the five different green juices ranged from 11.9 to 67.8%. This shows GJ2, 3 and 4 all have high ratios of soluble oxalate, 52.7, 67.8 and 63.1%, respectively. The fibre fraction contained appreciable amounts of soluble and insoluble oxalate.

Twelve different minerals were detected in all the juices and waste pulp produced (Table 5.7). No minerals were not-reported even though there were present only at trace levels or at levels close to the limit of quantitation (LOQ, 0.12-12.4 μg/L). Copper, at 0.5 mg/L, in the juice, GJ3, had the lowest level of the minerals, at 40 to 4000 times more than the LOQ. For all recipes of green juice there was a large amount of calcium in the waste pulp, ranging from 65 to 90% of the total on a fresh weight basis.

5.3.3 Oxalic acid

Table 5.6 Oxalate content (mg/100g fresh weight) of juice and pulp fractions of green juices.

	Juice			Pulp		
	Total	Soluble	Insoluble	Total	Soluble	Insoluble
GJ1	109.24 ± 12.11	13.07 ± 0.54 (11.9)	96.17	135.05 ± 11.56	29.21 ±10.07 (21.6)	105.84
GJ2	95.74 ± 7.82	50.45 ± 0.12 (52.7)	45.29	134.60 ± 19.14	126.25 ± 24.16 (93.8)	8.35
GJ3	122.14 ± 2.53	82.85 ± 7.87 (67.8)	39.29	291.47 ± 21.48	123.65 ± 9.72 (42.4)	167.82
GJ4	90.34 ± 5.58	56.95 ± 2.76 (63.1)	33.39	236.12 ± 17.43	114.98 ± 25.53 (48.7)	121.14
GJ5	152.00 ± 1.56	42.74 ± 0.24 (28.1)	109.26	348.09 ± 90.05	86.99 ± 16.74 (24.9)	261.10

GJ = green juice recipe

Values in brackets are percent of total oxalate

Table 5.7 Mineral content of five different green juices and waste pulp.

Mineral	GJ1		GJ2		GJ3		GJ4		GJ5	
(mg/L)	Juice	Pulp	Juice	Pulp	Juice	Pulp	Juice	Pulp	Juice	Pulp
Al	10.3 (73)	3.7 (27)	10.1 (43)	13.5 (57)	12.1 (57)	9.2 (43)	15.14 (51)*	14.4 (49)	14.8 (51)	14.0 (49)
B	0.7 (8)	7.7 (92)	1.1 (14)	7.2 (86)	0.63 (7)	8.5 (93)	1.49 (16)	7.7 (84)	1.5 (11)	12.1 (89)
Ca	983.3 (35)	1,865.4 (65)	164.8 (10)	1,420.9 (90)	266.4 (18)	1,182.6 (82)	170.8 (14)	1,026.3 (86)	651.2 (16)	3,539.3 (84)
Cu	0.7 (43)	0.9 (57)	0.4 (33)	0.9 (67)	0.5 (26)	1.5 (74)	1.03 (24)	3.2 (76)	0.6 (36)	1.1 (64)
Fe	11.6 (51)	11.0 (49)	7.8 (31)	17.5 (69)	9.5 (34)	18.3 (66)	11.07 (30)	25.9 (70)	13.8 (41)	19.5 (59)
K	2,824.8 (42)	3,913.3 (58)	2,164.5 (36)	3,876.5 (64)	2,828.2 (37)	4,915.1 (63)	2,299.4 (33)	4,770.6 (67)	3,050.9 (40)	4,649.8 (60)
Mg	237.5 (44)	302.8 (56)	183.1 (28)	475.5 (72)	201.9 (31)	446.3 (69)	190.9 (30)	450.8 (70)	286.9 (38)	460.9 (62)
Mn	2.0 (35)	3.7 (65)	1.0 (21)	3.9 (79)	1.4 (23)	4.8 (77)	1.8 (23)	6.1 (77)	1.1 (18)	5.0 (82)
Na	119.4 (39)	183.9 (61)	46.0 (23)	157.5 (77)	82.4 (32)	177.1 (68)	33.5 (32)	72.2 (68)	115.7 (30)	263.9 (70)
P	411.7 (37)	687.5 (63)	234.9 (30)	547.6 (70)	301.8 (28)	788.0 (72)	253.3 (21)	930.9 (79)	364.9 (30)	863.3 (70)
S	375.7 (37)	647.8 (63)	117.6 (26)	338.4 (74)	166.4 (27)	460.4 (73)	129.0 (20)	529.3 (80)	300.1 (25)	890.3 (75)
Zn	2.4 (38)	3.8 (62)	1.8 (27)	4.8 (73)	2.4 (32)	5.0 (68)	2.3 (26)	6.7 (74)	2.7 (31)	6.1 (69)
Mean Ratio	40	60	27	73	29	71	27	73	31	69

Number in brackets (%), percent ratio in fraction.
GJ = green juice recipe

5.4 Discussion

The obvious differences between pH/TA, dry matter and proximate analysis parameters for the green juices were not unexpected (Table 5.3, Table 5.4, Table 5.5), as the recipes varied from three components (GJ3 and GJ4) to nine components (GJ1). There has been very little or no research undertaken on the composition of green juices. Choi *et al.* (2014) juiced 12 different fruits and vegetables using six different juicers, and found their juice yields were very variable across all fruits and vegetables (12.4 to 76.8%), but were in not as good as the results reported in this experiment that were between 73.2 and 84.3% juice yields (Table 5.2). Hostetler *et al.* (2012) made vegetable juice, using parsley and celery. The parsley juice had a yield of 71.9% and the celery juice 79.1%; these results were more consistent with the results we obtained.

Other parameters that have been reported when making different vegetable juices mainly relate to antioxidant activity or enzyme activities (Hostetler *et al.*, 2012; Choi *et al.*, 2014; Kim *et al.*, 2015; Park & Kim, 2016; Kim *et al.*, 2017; Park *et al.*, 2018).

There are no reports of the oxalate content of freshly made green juice recipes. There are reports on freshly made kiwifruit juice, 21.2, 6.9 and 14.3 mg/100 g FW for total, soluble and insoluble oxalates (Nguyen & Savage, 2013). Siener *et al.* (2015), determined the total and soluble oxalate of 32 different commercially available fruit and vegetable juices from local establishments in Bonn, Germany. None of samples could be classified as green juices, although vegetable juices and nectars were analysed but with unknown compositions. The vegetable nectars were reported to contain 198.1 and 197.14 mg/100 mL, total and soluble oxalate for a rhubarb nectar (60% juice) and a celeriac nectar (50%), 1.11 mg/100 mL soluble oxalate only. The vegetable juices were all 100% juice and ranged from 70.1 to 3.64 mg/100 mL total oxalate for beetroot and multi-vegetable juice, respectively. Bong & Savage (2018) made a green juice, with a predominance (49 %w/w) of a poplar South East Asian fruit, bitter gourd (*Momordica charantia*). The fruit itself is moderately high in oxalate (83.32 to 102.06 mg total oxalate/100 g FW) and when made into a juice, produced a juice with a low to moderate content of oxalate (27.11 mg total oxalate /100 g FW). The soluble oxalate ratio was high for all of the three types of juice made (79.7% to 92.4%).

GJ3 had the highest soluble oxalic acid level (82.85 mg/100 g FW). This recipe had 30.5% spinach and only two other components, apple (42.8%) and celery (26.7%), both low oxalic containing foods. GJ4 and GJ5 had 31.2 and 37.9% spinach, so a similar level would be expected, if not more, of soluble oxalate. However, the concentration was lower in both cases, at 56.95 and 42.74 mg/100 g FW, respectively. This aberration can be partly answered if the ratio of soluble oxalic was used instead. GJ3 and GJ4 have similar ratios of soluble oxalic acid of 67.8 and 63.1%, respectively (Table 5.7) and GJ5 had only 28%. This indicated that there were interactions between the oxalate and the various other components of the juices, other than the juice fraction being separated from the pulp, and with the pulp fraction discarded.

The soluble oxalic acid concentration across the five juices ranged from 13.07 to 82.85 mg/100g FW. If a single 200 g glass of green juice was consumed, then between 26.14 to 165.70 mg of soluble oxalate per serving could be consumed. There have been no definite RDIs for oxalate consumption published. The American Dietetic Association (Marcason, 2006) gives a general guideline to reduce the risk of urolithiasis, as <40 to 50 mg of oxalate per day. The normal daily consumption of oxalates is highly variable. This was shown by Taylor & Curhan (2007) who extracted dietary oxalate intake and nephrolithiasis risk data from three major cohort studies in the United States, the Nurses´ Health Study I and II, and Health Professionals Follow-up Study. The 10^{th} to 90^{th} percentile range for daily oxalate intakes for men, older and younger women, ranged from 106 to 342 mg, 86 to 291 mg and 81 to 293 mg, for both stone and non-stone formers. From this study, they concluded there were only modest associations between dietary oxalate intake and kidney stone formation. Other research has also confirmed the wide range of daily dietary consumption of oxalate, Holmes & Kennedy, (2000) measured the daily intake of oxalate for five individuals to be 44 to 352 mg/day. As mentioned in all the preceding reports, the dietary intake of oxalate alone, does not solely affect the risk of urolithiasis, other factors in the diet, such as protein, calcium, sodium and other ions, vitamin supplements and calorie intake, have major effects too. This point was also made by Norton (2018) in a recent general review of oxalate consumption. Another point made by Norton (2018) is that urolithiasis is not the only toxic effect on the human body that can occur. There is also the possibility of oxalate accumulation in non-renal tissues, such as thyroid and breast. There are also functional body issues, such as, inflammation, neurotoxicity, digestive health, and connective tissue instability to be considered. Therefore, one 200 g serve of green juice could give close to the maximum daily

population intake of oxalate, observed to be from a normal diet and this could, potentially, contribute to a range of negative health outcomes.

The fresh weight yields from the production of the green juices are different for each fraction and for each green juice (Table 5.2). If this is taken into account and if it is assumed the insoluble oxalate content of each different green juice and in each fraction Table 5.6 is calcium oxalate. Using the total calcium ion concentration measured in each green juice and fraction, the available un-bound calcium, in the green juice and pulp can be estimated (Table 5.8).

Table 5.8 Available calcium in homemade green juices (%).

	Juice	Pulp
GJ1	69.4	82.2
GJ2	14.0	98.2
GJ3	53.8	55.6
GJ4	38.8	63.0
GJ5	47.5	76.9

GJ = green juice recipe

This clearly shows there is less un-bound calcium in the juices compared to the waste pulp.

It was important to note that the waste pulp fraction had considerable amounts of total oxalic acid in it, between 134.6 and 348.09 mg/100 g FW (Table 5.6). Since this is a waste stream, this is a positive outcome. One pulp sample, GJ2, had a considerably higher ratio of soluble oxalic acid, at 93.8%. The only ingredient component that was not in any of the other recipes was red capsicum. Capsicum is not known to have high oxalic acid levels, so perhaps there was another synergistic affect occurring.

One of the interactions that was likely to occur was the formation of calcium oxalate, due to the mixing of components of the different recipes, some high in calcium and some low.

The waste pulp across all five green juice recipes contained 11 minerals (apart from Al) at a higher concentration compared to the juice (Table 5.8). This was also reflected in the absolute

amounts. If the mg amounts of calcium in the juice and pulp were calculated (data not shown) across all five recipes, the ratio of calcium in the pulp fraction ranged from 40.9% to 62.7% on a fresh weight basis.

It has been well documented that there was an interaction between fibre consumed in the diet and mineral bioavailability/absorption. Although increased fibre in diets is recommend for improved general health, fibre has been shown to alter the mineral balance in the body. (Harland, 1989). Southgate (1987) reports a drop in the potassium, calcium, magnesium and phosphorous absorbed, for young and elderly men, and women, after a dietary modification from a low to a high fibre diet. The exception in this study was for young women. The proportion of calcium absorbed for this group, increased from 0.180 to 0.248, after increasing the dietary fibre intake from 6.2 to 29.9 g per day.

The interactions between fibre, oxalate and the mineral balance were studied by Kelsay & Prather (1983), who demonstrated a lower mineral balance with diets high in fibre. They also showed significantly lower mineral balances in diets with high fibre and spinach in the diet, and go on to suggest there is a combined effect from oxalic acid and fibre in the diet that requires further investigation.

These are all diet related interactions; however, the interaction between fibre, oxalic acid and minerals in the plant have not been well researched. Kamchan *et al.* (2004) determined the calcium bioavailability, dietary fibre and oxalate contents for 14 vegetables, two legumes and two seeds. They showed the five foods with the highest oxalate content also had the lowest calcium bioavailability, with dietary fibre having a strong negative correlation as well. No interactions between fibre, oxalate and calcium or any other minerals were observed.

This confirmed the observations made in our experiments about the high amounts of minerals in the pulp were due not only to the high fibre content but also the high oxalic acid content of the fibre.

5.5 Conclusions

Five different green juice recipes with a spinach base, had between 135.05 and 348.09 mg/100g FW total oxalic acid in the pulp fraction, which was normally discarded. The soluble oxalic acid in the juices ranged from 13.07 to 82.85 mg/100g FW.

The high fibre content of the discarded pulp fraction had high concentrations of all minerals apart from Al, while from 40.9 to 62.7% of the calcium on a fresh weight basis was lost to the pulp.

There was no clear trend or pattern to the division or loss of oxalate into the pulp. The total oxalate concentration in the pulp had a large range, between 135.05 and 348.09 mg/100g FW, and the ratio that was soluble in each different recipe also varied widely, between 21.06 to 93.8%.

While the assumption that the recipe containing the largest amount of spinach would contribute to largest amount of oxalate to the juices, this was not clearly reflected in the results. In addition, there appeared to be an interaction between the soluble oxalate, minerals and the pulp.

Further research needs to be undertaken to isolate, identify and understand the interactions between vegetable fibres, mineral and soluble oxalic acid content.

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Chapter 6
Comparison of oxalate contents from two green juices prepared using a masticating juicer and a high speed blender

6.1 Introduction

Home juicing using readily available juicing machines is now common. It is promoted not only as a mechanism to quench thirst and provide refreshment, but is also purported to be a source of extra nutrients, vitamins and minerals (Roberts, 2015; Knight *et al.*, 2017).

Vegetable juicers can be classified into two basic types, based on whether they retain or remove the fibrous portion from the juice. A high speed blender (HSB), does not remove any components of the juice, whereas a masticating juicer (MJ), separates out a fibrous portion (pulp) of the juice, which is then normally disposed of and the retentate is the juice that is consumed (Calbom, 2013).

Very little research has been carried out to compare the differences between the types of juicing method and the resultant nutritional qualities of the juices. Choi *et al.* (2014) compared two types of blender with four types of low and high speed juicers. In this experiment, yield, enzyme activity, antioxidant and anti-inflammatory activities and polyphenol content were measured for each juicing method. Unfortunately, only fruits and not green vegetables were used in this study.

Kim *et al*. (2015), compared a tomato juice made using two different types of juicers, a low-speed masticating (LSM) and a high speed centrifugal (HSC) juicer. Again, this research only used fruit juice, but Kim *et al.* (2015) did conclude there was an advantage to using the LSM compared to the HSC method. Juices prepared using the LSM scored better for sensory parameters, and had higher amounts of antioxidant and phytochemical measures, overall the juices were more homogenous. This clearly demonstrates there are measurable differences in the quality of juices made from different types of juicers.

Green juices prepared using commonly available green vegetables can contain high levels of soluble oxalates, which will vary with the type and proportion of vegetables used and whether

the pulp fraction is retained during processing. If spinach is included in the mix, then the juice may contain significant quantities of oxalates. Total oxalate levels in spinach have been reported to range from 217.90 to 2,285.00 mg/100 g FW (Table 2.1).

It is well documented that oxalate can affect the balance of calcium in a diet. The use of spinach to evaluate the bioavailability of calcium has been carried out many times with varying results. By replacing spinach with kale in a diet, Fincke & Garrsion (1938) report a negative calcium balance for two women over two three-day periods. However, there are also reports that show no alteration in the Ca balance. Johnson *et al*. (1952) added spinach to a basal diet of six women over eight weeks and noted no significant differences in their Ca balances.

A more recent study by Heaney *et al*. (1988) determined the Ca absorbability in a diet high in spinach compared to a milk-based diet and found the absorption of Ca from spinach was 10 times lower compared to the milk diet. They suggest that the explanation for this is that the Ca becomes complexed with the oxalate found in spinach and they estimate the availability of Ca in the spinach-based diet to be about 5.1%.

In this study the oxalate composition of green juices prepared using a high speed blender and a masticating juicer were investigated. Two separate green juice recipes were prepared using two different methods of making a green juice, either using HSB or MJ.

The mineral profile of the juices was determined and a comparison of the two different methods of making the juices on the mineral levels was made, taking particular notice of the overall calcium contents of the juices.

6.2 Materials and methods

6.2.1 Source of materials and preparation

A vegetable juice recipe consisting of spinach, apple, celery, cucumber, green pepper, red capsicum, lemon and parsley was juiced using a HSB and MJ juicer (Table 6.1). This mixture of vegetables is typical of recipes published in the popular press (Roberts, 2015; Knight *et al.*, 2017).

All vegetables and fruits were purchased fresh from the New World Supermarket, Lincoln, Canterbury, New Zealand. Soil, dead leaves and excess stems were removed using a stainless-

steel knife and the remaining edible portions were chopped, weighed and processed using a masticating juicer (Oscar 9000, Dongah Industrial Co., Ltd, Gyeongsangnam Do, South Korea) or a high speed blender (Vitamix 5200, Vita-Mix Corp. Cleveland, OH, USA). Two juice mixes were prepared, one containing low levels of spinach leaves while the other contained high levels of spinach leaves. Each mix contained other commonly used ingredients. Each juice type was prepared in triplicate following the weight schedule shown in Table 6.1.

Table 6.1 Composition of the two juicing mixes.

Ingredients	Low spinach mix (g)	High spinach mix (g)
Spinach	300	600
Apple	300	225
Celery	225	168.75
Cucumber	225	168.75
Green pepper	150	112.5
Red capsicum	150	112.5
Lemon	120	90
Parsley	30	22.5

6.2.2 Dry matter

The dry matter (DM) content of each sample was determined using the method detailed in Chapter 3, section 3.6.

6.2.3 Determination and extraction of total and soluble oxalic acid

The extraction and measurement by HPLC of total and soluble oxalates was performed using the method detailed in Chapter 3, sections 3.1, 3.2, 3.3 and 3.4. Each juice sample was measured in triplicate.

The insoluble oxalate content of each sample was calculated by the difference between the total and the soluble oxalate contents (Holloway *et al.*, 1989). The oxalate data was presented as mg/100 g fresh weight (FW) as this was how these products are commonly consumed.

6.2.4 Mineral profile

The pulp and juice samples were analysed for minerals using the method described in Chapter 3, section 3.5, with no variation.

6.2.5 Statistical analysis

All statistical analysis were undertaken using the software programs and methods outlined in Chapter 3, section 3.9, with no variation.

6.3 Results

Three replicate recipes for each juice type were prepared (Table 6.1). The total weights of the ingredients for the low and high spinach juices were 1502.9 ± 2.4 and 1502.5 ± 0.9 g, respectively.

The mean recovery of juice using the high speed blender was 98.0 ± 0.1%, as a small amount of material could not be recovered from the cutting blades and from the inside of the juicer jar (Table 6.2). The mean recovery of juice using the masticating juicer was 75.7 ± 0.2%. This represented the material remaining in the juicer mechanism and the intentional removal of the pulp fraction by the juicer. This also resulted in the mean dry matter of the juice fraction yielded by the masticating juicer to be significantly lower (7.12%, $P<0.001$) compared to the juice produced by the high speed blender (8.31%).

Table 6.2 Mean recovery of juice and pulp from the high speed blender and the masticating juicer.

Type	Spinach mix*	Juice yield (g)	Recovery (%)	Dry matter (%)	Discarded pulp (g)
High speed blender	Low	1448.98 ± 2.98	96.6 ± 0.2	8.62 ± 0.14	#
	High	1493.87 ± 2.41	99.4 ± 0.1	7.99 ± 0.23	#
Masticating juicer	Low	1146.18 ± 10.75	76.4 ± 0.1	6.88 ± 0.12	250.73 ± 2.10
	High	1124.95 ± 4.15	74.9 ± 0.3	7.36 ± 0.36	302.31 ± 13.14

* Amount of spinach in green juice recipe: low = 300 g and high = 600 g fresh weight

No pulp separated.

Overall, the juice prepared using the MJ contained more total and soluble oxalates (mg/100 g FW, $P < 0.001$) compared to the juice prepared using the HSB (Table 6.3). There was a small, but significant, difference between the insoluble oxalate contents of the juices prepared by the different juicers. The juice produced by the MJ contained significantly ($P < 0.05$) more insoluble oxalates than the juice produced by the HSB.

The juice prepared using the high level of spinach contained significantly more ($P < 0.001$) total and soluble oxalates (mg/100 g FW) when compared to the juice prepared using the low spinach recipe. The insoluble oxalate contents of the juices prepared from both recipes were very similar. There was a positive interaction effect for both total ($P < 0.001$) and soluble ($P < 0.01$) oxalates prepared from both high and low spinach content mixes. The interaction effect showed that the MJ was more efficient at extracting total and soluble oxalates into the juice for both recipe types.

Comparison of the amounts of total, soluble and insoluble oxalates between the high and low spinach recipe juices showed that, within the limits of experimental error, the high spinach mix contained approximately double the amount of total and soluble oxalates when compared to the low spinach mix. Overall, the main effect was that the pulp content remaining in the high speed blended juice effectively lowered the overall oxalate contents per 100 g of juice.

The discarded pulp from the MJ contained significant amounts of oxalates (Table 6.4). A total oxalate content of 238.10 and 555.67 mg/100 g FW, respectively, was found for the low and high amounts of spinach. The high spinach containing recipe was approximately twice that of the low spinach recipe, as would be expected.

The fraction of soluble oxalate differed between the low and high spinach recipes, at 36.2 and 62.5%, respectively. This was not an expected result, as it would be reasonable to presume that the fraction of soluble oxalates would remain unchanged even though the amount of spinach added to the mix had doubled.

Table 6.3 Mean oxalate content of the green juice prepared using two different types of juicers.

Type	Spinach level*	Oxalate (mg/100 g FW) Total	Soluble	Insoluble
High speed blender	Low	171.21 ± 8.35[a]	77.69 ± 5.26[a] (45.4)[#]	93.51 ± 4.96[a] (54.6)
	High	369.47 ± 11.49[b]	275.26 ± 16.13[b] (74.5)	94.21 ± 23.93[a] (25.5)
Masticating juicer	Low	209.60 ± 24.27[a]	97.73 ± 2.61[a] (46.6)	109.87 ± 24.42[a,b] (52.4)
	High	528.41 ± 17.03[c]	364.09 ± 22.44[c] (66.5)	164.32 ± 21.40[b] (31.1)
Source of variation	df			
Level high/low	1	***	***	NS
Juicer type	1	***	***	*
Level x type	1	***	**	NS

* Amount of spinach in green juice recipe: low = 300 g and high = 600 g fresh weight

[#] Values in brackets are % of total oxalate content

Means in the same column not sharing the same letter differ significantly using

Fisher's LSD ($\alpha = 0.05$).

Significance: NS = not significant; *P < 0.05; **P < 0.01; ***P < 0.001.

Table 6.4 Oxalate content of the discarded pulp from the masticating juicer (mg/100 g FW).

Level of spinach*	Oxalate content of discarded pulp (mg/100 g FW)	
Low	Total	238.10 ± 15.66[a]
High	"	555.67 ± 25.92[b]
Low	Soluble	86.10 ± 5.65[a] (36.2)
High	"	347.39 ± 11.3[b] (62.5)
Low	Insoluble	152.01 ± 13.34[x] (63.8)
High	"	208.28 ± 22.08[y] (37.5)

* Amount of spinach in green juice recipe: low = 300 g and high = 600 g fresh weight.
Mean values with superscripts [a] and [b] and, [x] and [y] are significantly different by $P < 0.001$ and $P < 0.05$, respectively.
Values in brackets are % of total oxalate content.

Fifteen minerals where detected in all the juices made (Table 6.5). Three were detected at very low levels, below 0.1 mg/100 FW, Cd, Cr and Ni, and these were not tabulated.

The most abundant mineral was K with the highest concentration across all treatments. It had values ranging from 279.99 to 359.36 mg/100 FW. The concentration between the low and high spinach recipes changed very little, 302.6 compared to 340.55 mg/100 FW.

The ratio of Ca was 38.3 and 25.3% on a dry weight basis for the low and high spinach juices, respectively (Figure 6.1). The majority of the Ca was in the discarded pulp fraction of the MJ juice, at 61.7% and 74.7%, for the low and high spinach juices, respectively.

Table 6.5 Mineral profile of juices made with high and low amounts of spinach, using either a high speed blender (HSB) or masticating juicer (MJ) (mg/100 g fresh weight ± SE)*.

Spinach amount	Fraction	Al	B	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
Low (300g)	HSB juice	0.55 ±0.17	0.57 ±0.03	124.49 ±9.55	0.08 ± 0.01	1.32 ±0.20	302.60 ±26.91	32.93 ±1.49	0.33 ±0.01	6.02 ±0.17	42.24 ±2.12	35.79 ±2.14	0.34 ±0.02
	MJ Juice	1.18 ±0.15	0.09 ±0.01	31.12 ±5.62	0.04 ±0.01	1.48 ±0.23	280.86 ±3.38	24.09 ±1.05	0.15 ±0.01	3.78 ±0.13	28.15 ±0.96	21.16 ±2.70	0.18 ±0.01
	MJ Pulp	1.80 ±0.07	0.19 ±0.02	57.09 ±6.25	0.05 ±0.01	2.46 ±0.05	279.99 ±9.19	26.98 ±1.67	0.20 ±0.02	5.88 ±1.99	32.06 ±1.32	27.44 ±1.77	0.22 ±0.02
High (600g)	HSB Juice	3.22 ±1.11	0.64 ±0.03	163.60 ±22.3	0.12 ±0.01	4.74 ±0.85	340.55 ±43.68	79.08 ±5.52	0.54 ±0.11	14.90 ±3.92	52.74 ±1.02	38.61 ±5.77	0.58 ±0.10
	MJ Juice	4.96 ±0.69	0.12 ±0.01	25.19 ±0.60	0.05 ±0.01	5.58 ±0.94	359.36 ±2.28	55.10 ±3.67	0.27 ±0.02	12.63 ±1.70	27.99 ±0.45	18.33 ±0.19	0.29 ±0.01
	MJ Pulp	4.40 ±0.49	0.19 ±0.01	35.69 ±2.76	0.06 ±0.01	5.53 ±0.64	317.72 ±0.99	46.12 ±1.80	0.25 ±0.02	22.89 ±0.59	34.62 ±0.54	19.71 ±0.42	0.22 ±0.01

*Minerals below 0.1mg/100g FW were detected but are not reported; Cd; Cr; Ni.

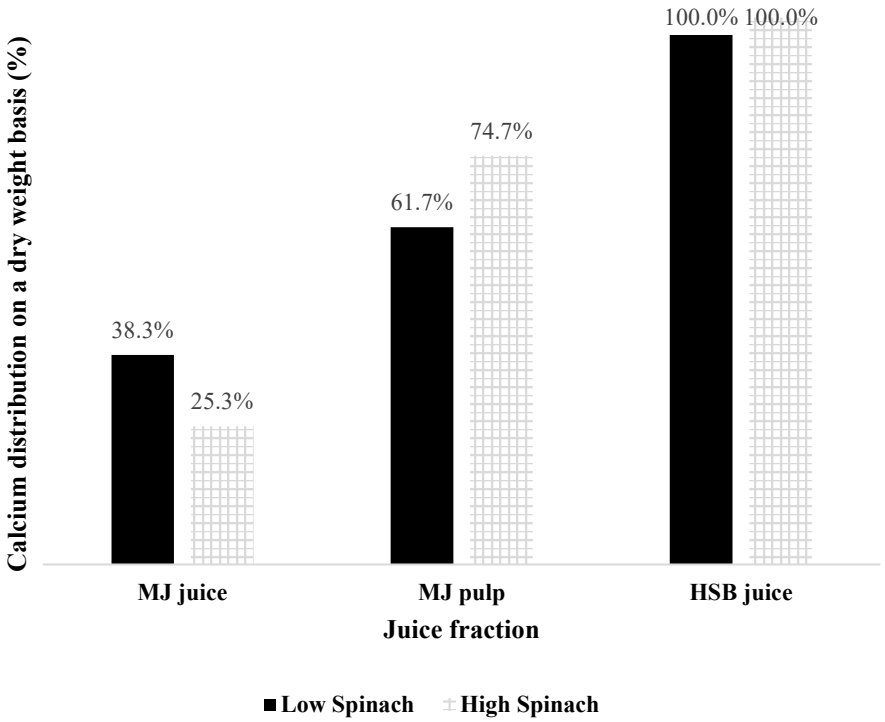

Figure 6.1 Distribution of calcium in juice fractions made by a high speed blender (HSB) and masticating juicer (MJ).

6.4 Discussion

The amounts of juice produced from the mean 1502.7 g of chopped green vegetables processed through each of the juicers were significantly different ($P < 0.01$). The high speed juicer yielded a mean of 1471.43 g of juice while the masticating juicer produced 1135.6 g. The most interesting effect observed in this experiment was that the masticating juicer removed a mean 367.4 g of pulp from both the high and low-level spinach mixes. The soluble oxalate content of the juice prepared using low levels of spinach inclusion were very similar when the values for the two juicers were compared. However, the juice prepared from the high-level spinach mix and processed by the masticating juicer contained significantly more ($P < 0.001$) soluble oxalates than the juice prepared using the high speed juicer. It should be noted that some of the other vegetables commonly used to make the juicing mixture may also contain very small amounts of oxalates. These individual values were not measured in this experiment because of their insignificant contribution to the total levels in the final juices.

The consumption of 200 grams of either green juice prepared using the HSB or MJ would result in the consumption of 155 or 728 mg of soluble oxalate, respectively. This, in itself, is not a significant intake of soluble oxalate from one glass of green juice; however, there are many recommendations that three or four and, even, six glasses of green juice should be consumed each day over a period of three to ten days (Gerson Institute, San Diego, CA, USA). Taylor & Curhan (2007) using a food frequency questionnaire study involving n=45,985 men, n=92,872 older women and n=101,824 younger women, documented mean daily intakes of oxalates to be 214 mg, 185 and 183 mg for the men, older and younger women, respectively. No distinction between total, soluble or insoluble oxalates was made in this study. They do report that spinach either cooked or raw made up between 40.4 and 44.2% of the diet contributing to the oxalate intake, among the test groups. Other researchers have estimated the daily intake of oxalates to be between 44 and 351 mg/day (Holmes & Kennedy, 2000) and the oxalate intake for vegetarians has between estimated to range from 80 to 2000 mg/day (Ogawa *et al.*, 2000).

Ca was the second most abundant mineral in the HSB and MJ green juice (Table 6.5). This is of significance due to the ability of the available Ca to bind to the soluble oxalate in the juice.

The HSB green juice retained all of the available Ca, and was available to bind to the soluble oxalate present in the juice (Figure 6.1); however, between 61.7 to 74.7% of the total Ca is discarded in the fibre fraction when the MJ green juice is made.

It was not surprising, then, that the consumption of similar green juices for up to six weeks can lead to significant damage to the kidneys as the body would attempt to excrete such large amounts of toxin. Though the efficiency of absorption in the body is low from dietary sources, there are a few well reported cases of oxalate nephropathy attributed to green juice consumption.

People who have other risk factors associated with the development of kidney disease, should be aware of the potential pathogenesis of oxalate nephropathy due to the consumption of moderate to large amounts of green juices on a regular basis.

6.5 Conclusions

Overall, this experiment has shown that green juices made using either HSB or MJ methods can contain significant amounts of soluble oxalates and the amounts found in particular mixes are directly related to the amounts of spinach used in the original mix. Removing the pulp from the juice using the MJ did not decrease the soluble oxalate levels but did decrease the amount of Ca in the green juice produced.

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Chapter 7

Reduction of oxalic acid in green juices by the addition of calcium

7.1 Introduction

Calcium salts are widely used in the food industry as acidity regulators, firming agents and stabilisers in processed foods (Wang, 2012; Msagati, 2013; Saltmarsh, 2013). Four of the most commonly used calcium salts in the food industry are calcium carbonate, calcium citrate, calcium sulphate and calcium chloride. Table 7.1 summarises their function and the typical products they can be used in.

Table 7.1 Calcium salts used in food processing (Saltmarsh, 2013).

Calcium salts	E number	Functional classes	Typical products
Calcium Carbonate	170	Acidity regulator, anticaking agent, colour, firming agent, flour treatment agent and stabilizer	Dairy based, frozen seafood, pastas, processed meats, wines
Calcium Chloride	509	Firming agent, stabilizer and thickener	Dairy based, processed and precooked fruits and vegetables
Calcium Citrate	333	Acidity regulator and source of calcium ion	Supplements and processed vegetables
Calcium Sulphate	516	Acidity regulator, sequestrant, firming agent, flour treatment agent and stabilizer	Dried pastas and noodles, frozen vegetables, dairy based

It is well documented that when calcium salts are added to a food high in oxalates it will bind to the soluble oxalate and form insoluble calcium oxalate (Kelsay & Prather, 1983; Weaver *et al.*, 1987; Charrier *et al.*, 2002; Brogren & Savage, 2003; Dahlgren & Savage, 2007). The insoluble oxalate will then precipitate, in whatever medium it is present in.

Faudon & Savage (2014) added increments of calcium chloride during the manufacture of a mitsumame-type dessert and showed that the soluble oxalate content of the ingredients could be efficiently converted into insoluble oxalates. In their study the authors concluded that the addition of calcium salts to foods was an efficient way to reduce the potential absorption of soluble oxalates from processed foods. The possibility of using other commonly-used calcium

salts during processing and manufacturing of food and green juices has not previously been investigated.

In a bioavailability study performed by Brogren & Savage (2003), they demonstrated that the addition of a calcium containing food (sour cream, milk) into a test meal, provided enough additional calcium to reduce the bioavailability of oxalate in the diet. The meal with the greatest amount of available calcium (spinach with sour cream and milk; 500 mg of Ca) significantly lowered the mean bioavailability of the oxalate in the cooked spinach from 1.9% to 0.7%, while other high calcium containing foods added to the cooked spinach also showed significantly lowered bioavailability of oxalate. Both these studies demonstrated it was possible to manipulate the bioavailability of soluble oxalate in a food by either adding calcium salts or high calcium containing foods to a test meal.

The objective in this experiment was to investigate whether similar methods could be used to reduce the soluble oxalate content of green spinach juice and evaluate the most effective calcium salt to add to juices to achieve an efficient reduction of soluble oxalate in the processed juice.

7.2 Materials and methods

7.2.1 Source of materials and preparation

Fresh spinach (*Spinacia oleracea* L.) was purchased from a local supermarket in Lincoln, Canterbury, New Zealand. Soil, dead leaves and stalk ends were removed using a stainless-steel knife and the remaining portions were chopped into 10 mm pieces and processed using a blender (NutriBullet, NBR-1207M, 600 Watts, Homeland Housewares LLC, Los Angeles, CA, USA) for 1.5 minutes at room temperature. The four calcium salts used in the experiment are shown in Table 7.2.

Table 7.2 Calcium additives.

Calcium additive	Manufacturer	Cost (NZ$/kg)	Calcium (%)
Calcium carbonate	Omya Australia Pty., Ltd. Sydney, Australia	5.10	33.71 ± 9.69
Calcium chloride dehydrate	Kirsch Pharma GmbH., Salzgitter, Germany	35.0	29.34 ± 2.46
Calcium citrate tetrahydrate	Jost Chemical Co. St Louis, MO, USA	45.0	24.71 ± 2.04
Calcium sulphate dehydrate	Kirsch Pharma GmbH., Salzgitter, Germany	28.0	25.60 ± 3.17

Three replicate 100 g samples of homogenised spinach were weighed into 250 mL conical flasks. A calcium salt was added to each of the triplicate set of flasks at the following rates 0, 50, 100, 200, 300, 400 and 500 mg/100 g of homogenised spinach, together with 100 mL of high purity water (Barnstead International, Dubuque, Iowa, USA, 18.0 MΩ·cm). Each conical flask was then placed in a shaking water bath at 25°C for 30 minutes. Four different trials were carried out using the same added amounts of calcium chloride, calcium sulphate, calcium citrate and calcium carbonate added to the homogenised spinach.

7.2.2 Test green juice

A realistic green juice was made to test the effectiveness of the best performing calcium additive. The green juice had a spinach base and seven other components commonly used to make a green juice that were sourced fresh from the local supermarket (Table 7.3).

Table 7.3 Composition of test green juice

Raw material	Weight (g)	%
Spinach	600	40.0
Apple	225	15.0
Celery	168.25	11.2
Cucumber	168.25	11.2
Green pepper	112.5	7.5
Red capsicum	112.5	7.5
Lemon	90	6.0
Parsley	22.5	1.5
Total	1488	100.0

A single batch of juice was made using a masticating juicer (Oscar 9000, Dongah Industrial Co., Ltd, Gyeongsangnam Do, South Korea) that separated the juice and pulp fractions, before the addition of the calcium salts and analysis. Additions of calcium chloride to the test green juice were made by weighing 100 g of juice into a conical flask in triplicate. To each replicate the required amount of calcium chloride was added, 0, 100, 200, 300, 400 and 500 mg/100 g, and the contents were then shaken vigorously for 1 minute. There was no incubation period and it was carried out at room temperature (25°C), no additional water was added.

7.2.3 Determination of total and soluble oxalic acid

Triplicate 10 g samples of the homogenised spinach, with and without the range of calcium additives, and the juice and pulp, made with and without a calcium additive, were all extracted using the methods in Chapter 3, sections 3.1, 3.2, 3.3 and 3.4, to measure the total and soluble oxalic acid content of the samples.

7.2.4 pH and titratable acidity

The pH and titratable acidity were determined in all samples using the same technique, as described in Chapter 3, section 3.8. The titratable acidity was expressed as equivalents of acid, as the ratios of the organic acids in the green juice mixture were not determined.

7.2.5 Calcium determination

All samples were analysed for calcium using the method described in Chapter 3, section 3.5, with only the calcium being reported in this Chapter.

7.2.6 Statistical analysis

All statistical analysis were undertaken using the software programs and methods outlined in Chapter 3, section 3.9, with no variation.

7.3 Results

For each calcium addition trial, a fresh batch of spinach was bought from the local supermarket. The total oxalate of the spinach used for this study ranged from 973.36 to 1089.37 mg/100 g FW with a mean of 1024.43 mg/100 g FW. The soluble oxalate ranged from 585.58 to 729.87 mg/100 g FW with a mean of 642.95 mg/100 g FW and insoluble oxalate ranged from 270.67 to 503.79 mg/100 g FW with a mean of 382.23 mg/100 g FW (Table 7.4). The spinach used for this study contained, on average, 62.93% soluble oxalate and 37.14% insoluble oxalate.

Table 7.5 shows the percent reduction in the soluble oxalate content of the homogenised spinach juice for each incremental addition of calcium carbonate, calcium chloride, calcium citrate and calcium sulphate. A graphical representation of this data can be seen in Table 7.1. The calcium chloride addition was most effective in reducing the soluble oxalate content. Table 7.5also shows the pH of the initial homogenised samples (mean 6.27 ± 0.01) and the values following each incremental addition of the individual calcium salts.

Table 7.6 shows the results of the test juice with incremental additions of calcium chloride. The soluble oxalate was reduced to 97.7% of the original amount, after the maximum addition of 500 mg of calcium chloride (Table 7.2). The pH and titratable acidity remained the same at all levels of addition of calcium chloride. Table 7.6 reports the total measured Ca in each green juice. It should be noted that calcium chloride contains 29.33% calcium (Table 7.2). Even after taking this into account, there was still a discrepancy of between 6.5% and 14.9% less Ca, when the actual measured amounts of calcium addition were compared to the amount added by calculation (Table 7.6).

Table 7.4 Oxalic acid content of homogenised spinach juice treated with calcium carbonate, calcium chloride, calcium citrate and calcium sulphate.

Calcium treatment (mg)	Calcium carbonate			Calcium chloride			Calcium citrate			Calcium sulphate		
	Oxalic acid (mg/100 g FW)											
	Total	Soluble	Insoluble	Total	Soluble	Insoluble	Total	Soluble	Insoluble	Total	Soluble	Insoluble
0	973.36 ± 9.76	592.62 ± 4.98	383.73 ± 14.35	1000.54 ± 8.23	729.87 ± 15.90	270.67 ± 23.87	1089.37 ± 11.92	585.58 ± 1.24	503.79 ± 1.24	1034.46± 21.11	663.74 ± 15.36	370.72 ± 36.46
50	953.93 ± 17.97	534.31 ± 18.84	419.62 ± 49.32	921.46 ± 73.41	496.16 ± 14.65	425.31 ± 66.96	1019.59 ± 5.83	464.02 ± 2.59	552.99 ± 6.11	1071.55 ± 34.52	494.14 ± 3.35	577.40 ± 64.94
100	955.84 ± 5.03	456.20 ± 3.26	499.64 ± 5.71	992.94 ± 31.82	319.96 ± 14.97	672.99 ± 23.34	988.04 ± 8.51	346.41 ± 3.81	641.63 ± 4.86	1063.68 ± 1.78	367.33 ± 2.23	696.35 ± 23.65
200	894.84 ± 37.41	383.98 ± 4.04	510.86 ± 39.73	1053.81 ± 42.48	94.29 ± 5.21	959.52 ± 40.42	1030.43 ± 10.11	159.80 ± 26.81	870.63 ± 35.49	1080.40 ± 2.17	117.05 ± 4.15	963.35 ± 3.74
300	897.33 ± 48.62	337.14 ± 13.06	560.19 ± 56.89	1065.16 ± 14.90	31.27 ± 1.00	1033.89 ± 14.36	1034.95 ± 15.62	120.14 ± 7.67	914.81 ± 12.30	1045.55 ± 25.75	53.98 ± 2.25	991.58 ± 2.25
400	1054.23 ± 1.63	312.52 ± 2.17	741.72 ± 2.30	995..85 ± 5.81	18.31 ± 0.79	977.54 ± 6.16	1033.29 ± 7.59	78.85 ± 2.46	954.44 ± 9.88	1097.05 ± 59.35	36.86 ± 2.26	1060.19 ± 38.72
500	1049.54 ± 5.45	300.42 ± 1.81	749.12 ± 5.53	1001.66 ± 6.66	12.81 ± 0.73	988.85 ± 7.08	1026.52 ± 7.71	61.25 ± 4.99	965.27 ± 4.72	1118.04 ± 36.25	31.30 ± 0.73	1086.74 ± 0.73

Table 7.5 Percentage remaining of soluble oxalate content ± SE of homogenised spinach juice following individual additions of calcium carbonate, calcium chloride, calcium citrate and calcium sulphate and pH ± SE.

	Remaining soluble oxalate (%)							
Calcium salt addition (mg/100 g spinach homogenate)	Calcium carbonate	pH	Calcium chloride	pH	Calcium citrate	pH	Calcium sulphate	pH
0	100	6.27 ± 0.01	100	6.27 ± 0.01	100	6.27 ± 0.01	100	6.27 ± 0.01
50	90.16 ± 3.18	7.28 ± 0.01	67.98 ± 1.16	6.13 ± 0.01	79.24 ± 0.44	6.30 ± 0.01	74.45 ± 0.50	6.20 ± 0.01
100	76.98 ± 0.55	7.74 ± 0.01	43.84 ± 1.18	6.09 ± 0.01	59.16 ± 0.65	6.38 ± 0.01	55.34 ± 0.34	6.20 ± 0.01
200	64.79 ± 0.68	8.03 ± 0.01	12.92 ± 0.41	5.92 ± 0.01	27.29 ± 4.58	6.49 ± 0.01	17.64 ± 0.62	6.20 ± 0.01
300	56.89 ± 2.20	8.13 ± 0.01	4.28 ± 0.08	5.77 ± 0.01	20.52 ± 1.31	6.52 ± 0.01	8.13 ± 0.34	6.16 ± 0.01
400	52.73 ± 0.37	8.16 ± 0.01	2.51 ± 0.06	5.68 ± 0.01	13.47 ± 0.42	6.49 ± 0.01	5.55 ± 0.34	6.10 ± 0.01
500	50.69 ± 0.31	8.26 ± 0.01	1.75 ± 0.06	5.62 ± 0.01	10.46 ± 0.85	6.44 ± 0.01	4.72 ± 0.11	6.06 ± 0.01

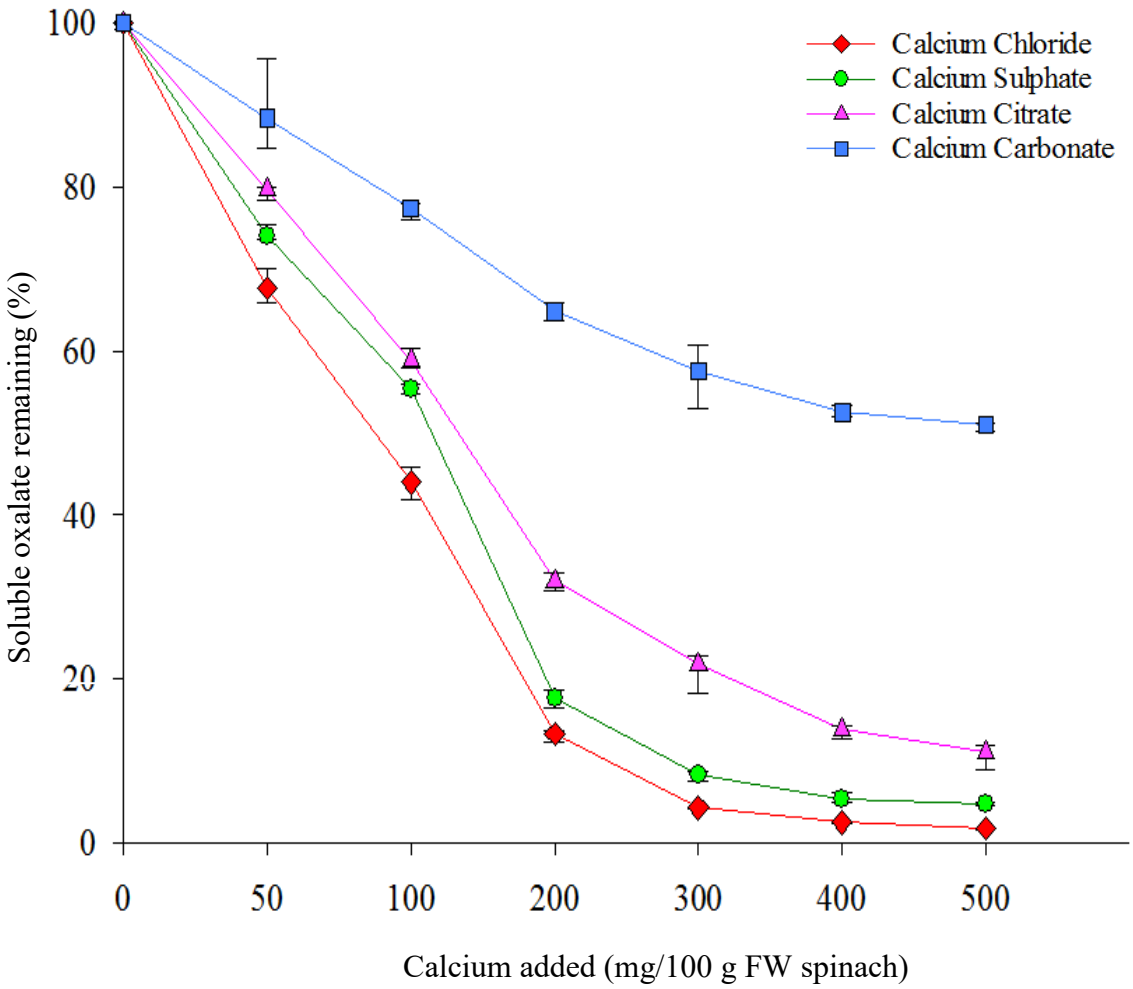

Figure 7.1 Effect of adding four different calcium ions on the percentage of soluble oxalate remaining in 100 g of homogenised spinach juice (error bars indicate ± SE).

Table 7.6 Test green juice, soluble oxalate, pH, titratable acidity and calcium content after the addition of calcium chloride.

Calcium chloride addition (mg)	Soluble oxalate (mg/100 ml)	Reduction (%)	pH	Titratable acidity (mEq./100 g FW)	Measured Ca (mg/100 ml)
0	32.21 ± 1.88	0	4.59 ± 0.11	4.52 ± 0.02	36.26 ± 2.74
100	21.02 ± 0.46	34.7 ± 1.4	4.84 ± 0.02	4.68 ± 0.04	57.08 ± 2.55
200	13.52 ± 0.25	58.0 ± 0.8	4.88 ± 0.01	4.68 ± 0.04	82.66 ± 2.50
300	8.27 ± 0.62	74.3 ± 1.9	4.73 ± 0.05	4.68 ± 0.03	114.07 ± 2.42
400	2.89 ± 1.26	91.7 ± 0.8	4.64 ± 0.04	4.66 ± 0.04	142.56 ± 3.97
500	0.74 ± 0.05	97.7 ± 0.2	4.56 ± 0.03	4.68 ± 0.04	171.96 ± 3.41

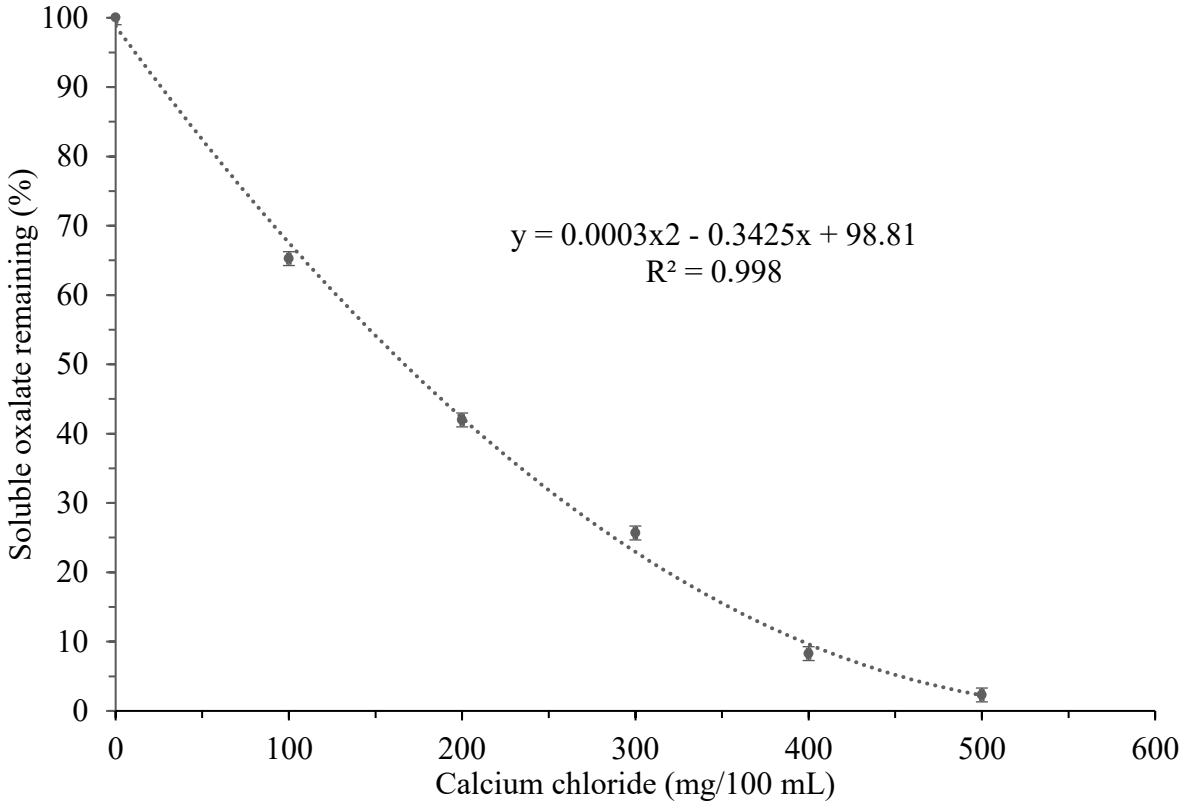

Figure 7.2 Effect of adding five different amounts of calcium chloride to a test green juice (error bars indicate ± SE).

7.4 Discussion

In this study, the homogenised spinach used contained a mean of 1024.43 mg/100 g FW total oxalate, 642.95 mg/100 g FW of soluble oxalate and 382.23 mg/100 g FW of insoluble oxalate; these are within the range of previous studies that have reported for the oxalate content in locally grown spinach (*Spinacia oleracea*). Brogren & Savage (2003) reported 957.7, 736.6 and 220.1 mg /100 g FW for total, soluble and insoluble oxalate respectively. Similarly, Savage *et al.* (2000) reported 329.6, 266.2 and 63.4 mg/100 g FW, for total, soluble and insoluble oxalate, respectively. These results also highlight that there is a wide range of reported values (320-1260 mg/100 g FW) for spinach (*Spinacia oleracea*, Noonan & Savage, 1999). Some of the major factors that can impact on the variation in the oxalate content will be the harvest age of the plant, growth conditions and climate (Noonan & Savage, 1999).

With each calcium increment the total oxalate content remained unchanged, ranging from 894.84 to 1118.04 mg/100 g FW across all treatments, with an overall mean of 941.66 ± 20.18 mg/100 g FW. The insoluble oxalate increased as increasing amounts of calcium were added to the juice. It was believed the additional calcium had bound with the soluble oxalates as the calcium oxalate had a very low solubility product contact (K_{sp}) of 2.7 x 10^{-9} (Simpson *et al.*, 2009). The effectiveness of the addition of small amounts of calcium ions into the homogenised spinach juice can be seen in Figure 7.1, which shows a reduction in soluble oxalate in all cases, with calcium chloride being the most effective.

In this experiment each increment of calcium salt was made on a fresh weight basis and this meant that there was a small difference in the amount of calcium actually added, as the calcium content of each salt varied from 24.7 to 33.7% FW of the additive (Table 7.2). Calcium chloride was the most effective additive to reduce the soluble oxalate content of the mixes as the addition of 500 mg to 100 g of spinach homogenate reduced the soluble oxalate content by 98.3%. In contrast, the addition of 500 mg of calcium carbonate only reduced the soluble oxalate content by 49.3%. There appeared to be no relationship between the calcium content of the four salts investigated and their potential to reduce the soluble oxalate content of the spinach mixture.

It was interesting to note that the pH change in the incremental mixtures was an indicator of the effectiveness of the reduction of soluble oxalate in each of the incremental mixes.

Addition of 500 mg calcium chloride to 100 g of the spinach homogenate gave a pH value of 5.62 ± 0.01, while the addition of 500 mg of calcium carbonate was the least effective at reducing the soluble oxalate content and also increased the mix pH to 8.26.

The speciation diagram published by Simpson *et al.* (2009) shows that, in an ideal pure water solution, pH influences the occurrence of the three possible forms of oxalate species. The pH of the original spinach homogenate was 6.7, which, in an ideal solution, would suggest that most of the oxalate would be $(C_2O_4)^{2-}$ and the addition of calcium carbonate would increase this further. The addition of calcium chloride changed the pH of the solution to 5.62, which would increase the proportion of $H(C_2O_4)^-$ so that, in theory, would bind less effectively to the added soluble calcium. In practice, calcium chloride was the most effective calcium salt to bind to the soluble oxalate. Additions of calcium citrate and calcium sulphate only made small changes to the pH values, but they still effectively reduced the soluble oxalate content in the spinach homogenate. These results strongly suggested that the homogenised mix of spinach was not responding in the same way as an ideal solution would behave in pure water. This suggests that a more complex interaction may be taking place between the added calcium, soluble oxalate and other constituents in the spinach leaf matrix, such as other minerals or complex carbohydrates, when the pH was altered.

This study confirmed that the addition of modest amounts of calcium chloride to a spinach homogenate was the most effective additive even though it was more expensive than the cheapest calcium additive, calcium carbonate, which was very ineffective at reducing the soluble oxalate content in the spinach mix. Calcium carbonate also has the unfortunate effect of making the juice more alkaline and this would have implications for the storage of the juice and could have an effect on the taste of the juice.

The test green juice followed a quadratic decrease in soluble oxalate ($R^2 = 0.998$, Figure 7.2), which was very similar to the spinach only juice. However, the overall effect of Ca additions to a realistic green juice was lower compared to the spinach by itself. At the 100 mg addition of calcium chloride to the green juice, there was 65.3% soluble oxalate remaining compared to 43.8% for the spinach juice alone. This is clearly due to the buffering and additional organic acids in a realistic green juice compared to plain spinach. This can be seen by the average pH of spinach being pH 6.27 compared to pH 4.59 for the test green juice (Table 7.5 and Table 7.6).

Overall, this study showed that a 50% reduction in the soluble oxalate content of a spinach homogenate could be achieved by the addition of 88 mg of calcium chloride to 100 g of spinach juice and, for the mixed green juice this would be 145 mg. This would have a significant effect on the soluble oxalate intake for a regular consumer of green juices containing spinach. In contrast, calcium sulphate, calcium chloride and calcium carbonate could achieve a 50% reduction in soluble oxalate content by the addition of 113, 125 and 500 mg/100 g FW, respectively.

This experiment showed that in a real-life scenario the addition of small amounts of calcium chloride would be an effective measure to reduce the soluble oxalate content of a green juice mixture containing spinach.

7.5 Conclusions

Spinach leaves and stems are often homogenised and then used as the base vegetable to make green juices. The green juices are not cooked or processed in a way that would remove or reduce the amount of soluble oxalate content. However, the addition of soluble calcium ions during juice processing at room temperature offered the opportunity to convert some of the soluble oxalates in the juices into insoluble oxalates that would, therefore, not be available for absorption in the digestive tract.

This study showed that the addition of four types of commonly used food grade calcium salts all reduced the soluble oxalate in the green juice. Calcium chloride was the most effective additive, with a 98.3% reduction in soluble oxalate after the addition of 500 mg/100 g juice. While calcium carbonate was the least effective additive, with a 49.3% reduction in soluble oxalate. Overall, the addition of even small amounts of soluble calcium would make the green juice considerably safer to consume by reducing the soluble oxalate content.

When testing the addition of calcium chloride to a realistic recipe of green juice, the reduction in soluble oxalate followed a polynomial reduction curve, reducing the soluble oxalate to 2.5% remaining after the addition of 500 mg of calcium chloride salt to 100 g of green juice. With no changes in pH or titratable acidity of the mixed juice little taste change would be expected.

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Chapter 8
Reduction of oxalic acid in green juices by soaking the raw materials

8.1 Introduction

Green leafy vegetables are a common source of oxalic acid in human diets. Dietary advice for people with kidney disease or susceptibility to kidney disease is to consume low oxalate containing foods. There are three approaches to the problem of reducing oxalate intakes: 1) identification and avoidance of vegetables that contain oxalate in their leaves; 2) selection and breeding of new cultivars with lower oxalate content; and 3) processing the cultivars currently in production to reduce the oxalate content in their leaves. Two forms of oxalate can be found in the leaves, soluble (bound to Na^+, K^+ and NH_4^+) and insoluble (bound to Ca^{2+}, Mg^{2+} and Fe^{2+}) (Savage *et al.,* 2000). Soluble oxalate is most likely to be leached from leaves but the location of oxalic acid in the leaf tissue will influence its ability to be leached or soaked out of the plant. Oxalic acid is synthesised in the leaves and is stored in specialised plant cells called crystal idioblasts (Franceschi & Horner, 1980; Franceschi & Nakata, 2005). Insoluble oxalate contained in the idioblast cells are unlikely to be leached from the leaf tissue, while soluble oxalate is much more likely to be leached from the leaf tissue if it is not bound to fibre in the leaf tissue.

Reduction of the soluble oxalate content of the raw materials used to manufacture green juices is even more important as many green juices are not often clearly labelled with the amounts of vegetables used, or the method used to process them. Pre-treatment by leaching soluble oxalate from the leaves or the conversion of soluble oxalate in the leaves to insoluble oxalate, are two possible approaches. These two processing methods, however, may affect other constituents of the leaves. Kelsay (1987) reviewed human clinical studies on the effects of oxalic acid, phytic acid and fibre on mineral bioavailability in the diet. They reviewed numerous studies on the effect of spinach in the diet and its influence on the calcium balance and whether it had a neutral or a negative effect when consumed regularly in the diet. They concluded that the consumption of oxalic acid may cause a reduction in mineral bioavailability. This is likely due to the interactions of soluble oxalic acid with cations in foods to form insoluble complexes. In contrast, Israr *et al.* (2013) undertook an experiment

with bran-type foods and demonstrated that minerals in wheat bran reduced the overall soluble oxalate content.

The unbound form of oxalic acid is highly soluble in water. Hussain *et al.* (2012) reported the solubility of oxalic acid at different water temperatures ranged from 30 g/L at 14°C to 75 g/L at 40°C. Therefore, in theory, oxalic acid can be easily leached or washed from a green leafy vegetable and can then be available to bind to any available cations to form insoluble complexes. Fruit and vegetable pre-treatments have been used, either soon after harvesting or prior to further processing, to clean or pre-prepare the food before the next stage of processing, i.e. rinsing with chlorine solutions to sterilise the fresh greens (Lee *et al.*, 2004) before packaging. The type of pre-treatment depends on the type of processing, but can be broadly classified as: blanching, osmotic pre-treatments, brining and soaking/rinsing.

Blanching is a process by which heat is applied, using water or steam, to sterilise and inactivate heat labile enzymes, and this helps to retain the colour and turgidity of a vegetable. Mosha *et al.* (1995) blanched collard greens for up to 10 minutes in 98°C water and observed a significant reduction (32.7%) in total oxalic acid in the leaves but, overall, Mosha *et al.* (1995) commented that oxalic acid reduction was not significant in most treatments.

Abiodun & Akinoso (2014) pre-treated yams by blanching/soaking diced yam tubers in 60°C water for 10 minutes then soaking them in tap water at room temperature for 12 hours prior to drying and grinding into a flour. The total oxalate content of the yam flour was reduced by 96% and Abiodun & Akinoso (2014) suggest that the blanching step may have ruptured cells to allow the leakage of soluble oxalate from the tubers.

Blanching has also been reported to increase the calcium content of the food being blanched, due the blanching water being high in calcium; this would only occur if hard water was used for blanching (Jones & Etchells, 1944). It should be noted that heating/blanching vegetables in any way is, in effect, a cooking process.

So far, no research has been carried out to measure whether osmotic pre-treatments of leafy foods would have any effect on the oxalate contents of the treated foods. Brining is an osmotic process using high concentrations of a salt (NaCl) solution to preserve or to pre-treat, enabling the natural selection of the correct microbes for fermentation. The salt concentration can range from 0.5% for sauerkraut making, to 15% for preservation (Jones & Etchells,

1944). Although changes in oxalate levels of green vegetables due to brining have not been reported, Wadamori *et al.* (2014) did observe a 22.86% reduction in soluble oxalates in a traditional South Korean kimchi fermentation using silver beet after a brining pre-treatment of 10% w/v NaCl. The reduction in oxalate in this case was attributed to the oxalotrophic activity of the kimchi fermentation bacteria involved.

Zhou & Zhou (2014) pre-treated balsam pears in 100°C tap water for three minutes and observed an average reduction of 39.76% soluble oxalate in the processed pears. They also used a salt water solution (no percentage given) pre-treatment, by placing the pears in salt water for three minutes and observed an average reduction of 37.41% in the soluble oxalate content.

Some experiments investigating the effect on oxalate due to soaking have used non-green leafy foods. Canadian pulses were soaked in distilled water for four hours at room temperature, and this showed losses of soluble oxalate for all 23 pulses trialled (Shi *et al.*, 2018). Kumoro *et al.* (2014) soaked taro corm chips in tap water and a sodium bicarbonate solution and observed a reduction in calcium oxalate of 6.63%. Soluble oxalic acid was not determined, and it seemed unusual they did not measure the total or soluble oxalic acid in their experiment. None of these soaking experiments were carried out using green leafy vegetables. One experiment was found in the literature using green leafy vegetables. Shimada (2014) soaked spinach leaves in tap water for 60 minutes at 80°C. Following this treatment, a 70% reduction in soluble oxalate was measured in the soaked spinach leaves. Shimada (2014) observed that there was a time dependency effect in the reduction of soluble oxalate from spinach due to soaking in the tap water as there were also differences due to the temperature of the soak water. The whole spinach leaves soaked at 15°C reached a peak reduction rate of 0.3% after 20 hours compared to a peak reduction rate of 71.7% reached after 80 minutes in 80°C in tap water. The researcher, in this case, may have misinterpreted the data, and the reduction rate, should actually mean a reduction in soluble oxalate.

Soaking in tap water at room temperature has been reported to leach out other anti-nutritive compounds from foods, such as phytic acid, saponins and trypsin inhibitors (Khokhar & Chauhan, 1986).

The risk posed by soluble oxalate in a diet has driven studies to identify methods to reduce the level of soluble oxalates during food processing. Savage *et al.* (2000) found that soaking and

cooking in water can reduce the soluble oxalate content of leafy foods as soluble oxalates easily leach into the water at room temperature.

Studies using the addition of calcium from milk (Brogren & Savage, 2003; Savage *et al.*, 2003; Oscarsson & Savage, 2007; Simpson *et al.*, 2009; Faudon & Savage, 2014) have also shown that significant reductions in soluble oxalates can be achieved in food mixtures. This has been attributed to the formation of a Ca-oxalate insoluble salt in the food mixture.

This experiment investigates the use of soaking spinach leaves, prior to any other processing steps, as a strategy to reduce the soluble oxalate content of the spinach leaves. The soaking mediums to be investigated will be tap water, a salt - sodium chloride - tap water solution and a calcium chloride - tap water solution, all at room temperature.

8.2 Methods

Fresh raw spinach leaves (*Spinacia oleracea* L.) were purchased from the New World Supermarket (Lincoln, Canterbury, NZ). Fresh spinach leave (150 g) were weighed in a plastic 2 L container. Into this container 1,500 mL of either tap water, food grade 1% w/v NaCl (Homebrand non-iodised table salt, Woolworths, Bella Vista, NSW, Australia), food grade 1% w/v CaCl (Kirsch Pharma GmbH., Salzgitter, Germany) or no liquid at all (raw), was added. The mixture was then left to soak for 16 hours at room temperature. The containers were then drained and the waste solution collected; then, the remaining spinach leaves shaken dry prior to being homogenised using a high speed blender (NutriBullet, NBR-1207M, Homeland Housewares LLC, Los Angeles, CA, USA).

8.2.1 pH and titratable acidity

Ten g of the freshly homogenised samples were accurately weighed into a 100 mL beaker, in triplicate. This sample was then analysed for pH and titratable acidity using the method described in Chapter 3, section 3.7. The endpoint for titration was pH 8.1 and titratable acidity was calculated and expressed in units of milliequivalents per 100 g fresh weight of sample.

8.2.2 Dry matter

The dry matter was determined on triplicate samples of the treated spinach leaves, using the method outlined in Chapter 3, section 3.5.

8.2.3 Oxalic acid

Ten grams of freshly homogenised sample from each treatment were extracted and analysed for soluble and total oxalic acid, in triplicate, using the methods outlined in Chapter 3, sections 3.1, 3.2, 3.3 and 3.4.

8.2.4 Mineral content

Approximately 10 g of a fresh leaf sample from each treatment was dried at 65°C in a fan forced air oven, then ground in a mortar and pestle before 1 g was accurately sampled for mineral analysis. The analysis was carried out using the method described in Chapter 3, section 3.5, with no variation. The results in this chapter are expressed as mg/g dry matter (DM).

8.2.5 Statistical analysis

All statistical analysis were undertaken using the software programs and methods outlined in Chapter 3, section 3.9, with no variation.

8.3 Results and Discussion

All treatments showed a significant reduction in dry matter content compared to the raw un-soaked leaves - 9.44% for raw compared to 6.53, 7.08 and 7.10% for the water, NaCl and CaCl treatments, respectively. Only the NaCl and CaCl treatments showed a statistically significant increase in pH; however, the real/actual numbers were fairly similar, with the largest difference between the raw and CaCl treatments being 0.16 pH units. The NaCl treatment did show a statistically different mEq acidity value compared to all other treatments, with a maximum value at 0.56 mEq. unit less than the raw (Table 8.1).

Table 8.1 Moisture content, pH and acidity of spinach leaves soaked in three different solutions.

Treatment	Dry matter (% ± SEM*)	pH (± SEM*)	Titratable acidity (mEq./100 g FW ± SEM*)
Raw	9.44 ± 0.09[a]	6.49 ± 0.021[a]	1.79 ± 0.10[a]
Water	6.53 ± 0.27[b]	6.61 ± 0.011[b]	1.54 ± 0.11[a]
NaCl 1% w/v	7.08 ± 0.07[c]	6.65 ± 0.017[c]	1.23 ± 0.05[b]
CaCl 1% w/v	7.10 ± 0.12[c]	6.56 ± 0.068[c]	1.52 ± 0.09[a]

* Means that do not share the same letter differ significantly, using Fisher's LSD, $p<0.05$.

Table 8.2 Oxalic acid content of soaking pre-treatments of spinach leaves.

	Oxalic acid (mg/100 g dry weight)				
Treatment	Total	Soluble		Insoluble	
Raw	11263.0 ± 262.0[a]	6823.1 ± 84.3[a]	(60.6)[#]	4439.9 ± 251.0[a]	(39.4)
Water	10418.2 ± 250.0[b]	7019.0 ± 8.2[a]	(67.4)	3399.2 ± 251.0[b]	(32.6)
NaCl 1% w/v	10469.2 ± 18.2[b]	7731.2 ± 38.1[b]	(73.8)	2738.0 ± 43.8[b]	(26.2)
CaCl 1% w/v	10379.5 ± 248.0[b]	3377.2 ± 198.0[c]	(32.5)	7002.3 ± 313.0[c]	(67.5)

* Means that do not share the same letter differ significantly, using Fisher's LSD, $p<0.05$.

[#] numbers in brackets are percentage of measured total oxalate.

There was no significant difference between the total oxalate levels in the treatments; however, all treatments were significantly lower than the raw un-soaked spinach leaves, at most by a measurement of 883.5 mg/100 g dry weight (Table 8.2).

The soluble contents of the raw, water and 1% NaCl treatments ranged from 60.6 to 73.8% of the total oxalate. Simpson *et al.*, (2009) cooked silver beet leaves in tap water and reported that 19.2% soluble oxalate remained in the leaves, which showed that heating improved the removal of soluble oxalic acid from the leaves. The 1% CaCl treatment contained significantly less soluble oxalic acid, having only 32.5% soluble oxalic acid, compared to cooking silver beet leaves; this is a very good result.

Table 8.3 compares the amount of Ca bound in the insoluble oxalate fraction of each treatment compared to the total calcium measured by ICP-OES for each treatment. The available calcium is defined as the proportion of total calcium not bound in the insoluble oxalate fraction. All treatments increased the available calcium, when compared to the

untreated raw spinach, from just over double for plain water, to three times for NaCl soaking and five times for CaCl soaking (Table 8.3). It is well documented that Ca will bind to soluble oxalic acid (Vityakon & Standal, 1989) and that this could lead to a reduction in the bioavailability of Ca in the diet. In an experiment adding milk products to spinach, Brogren & Savage (2003) reported that 76.7% of the Ca in spinach is unavailable, but did not analyse the Ca availability after adding the milk products, only the soluble oxalic acid bioavailability. When adding a Ca source to a recipe containing a high oxalate containing green leafy plant (purslane), Moreau & Savage (2009), observed a lowering in soluble oxalate and a reduction in Ca bioavailability. This indicates that it is important at what stage of processing Ca is added and how much is required, which is dependent on the amount of soluble oxalate present.

Table 8.3 Comparison of calcium availability for three different soaking treatments of spinach leaves (*Spinacia oleracea* L.).

Treatment	Insoluble oxalic acid (mg/g DW)	Bound calcium* (mg/g DW)	Total calcium[#] (mg/g DW)	Calcium availability (%)
Raw	44.39	13.90	15.91	12.6
Water	33.99	10.60	14.94	28.7
NaCl 1% w/v	27.38	8.57	14.78	42.0
CaCl 1% w/v	70.02	21.92	58.97	62.8

* Calcium – oxalate complex
[#] measured by ICP-OES

Table 8.4 Mineral profile of raw and soaked spinach leaves (mg/g DM ± SEM)*.

	Al	Ca	Fe	K	Mg	Na	P	S
Raw spinach	0.81 ± 0.02	15.91 ± 0.26	1.03 ± 0.04	156.91 ± 1.75	21.46 ± 0.95	1.43 ± 0.04	13.20 ± 0.19	11.23 ± 0.19
Soaking treatment								
Water	0.71 ± 0.04	14.94 ± 0.38	0.84 ± 0.04	54.42 ± 1.24	14.45 ± 0.48	1.04 ± 0.03	7.69 ± 0.21	7.21 ± 0.14
	(-11.8)[#]	(-6.1)	(-18.4)	(-65.3)	(-32.7)	(-26.9)	(-41.8)	(-35.8)
NaCl 1% w/v	0.65 ± 0.03	14.78 ± 0.26	0.86 ± 0.04	55.47 ± 0.70	15.65 ± 0.66	25.69 ± 0.24	9.06 ± 0.17	8.55 ± 0.28
	(-19.4)	(-7.1)	(-17.1)	(-64.7)	(-27.1)	(1701.1)	(-31.4)	(-23.9)
CaCl 1% w/v	0.71 ± 0.03	58.97 ± 1.59	0.82 ± 0.03	55.07 ± 1.05	15.33 ± 0.43	0.97 ± 0.03	8.63 ± 0.28	8.08 ± 0.20
	(-12.1)	(270.6)	(-10.9)	(-64.9)	(-28.5)	(-31.9)	(-34.7)	(-28.0)

* Below 1 mg/g DM data not reported
[#] Percentage (%) loss/gain in mineral content from raw, after soaking for 16 hours in the treatment solution.

In total, fourteen elements were detected in the raw and soaked spinach leaves using IPC-OES analysis. Eight elements are reported in Table 8.4, a further six elements (B, Cd, Cr, Cu, Mn and Zn) were detected by the instrument but, at levels below 1 mg/g DW, these later results were not included in further analysis. The mean loss of total minerals for the three soak solutions was 31.4, 27.0 and 29.6%, respectively, for water, 1% w/v NaCl and 1% w/v CaCl. As expected, soaking the fresh spinach leaves in 1% w/v CaCl increased the Ca content of the leaves by 270.6% and soaking in 1% w/v NaCl increased the Na levels in the leaves by 1701.1%.

All the soaking treatments removed minerals, ranging from a 6.1% loss of Ca following soaking in cold tap water, to a 65.3% loss of K from the same treatment. Just soaking in water reduced all minerals in the spinach leaves, ranging from a 11.8% loss of Al to a 65.3% loss of K. When a cation (Na^{+} or Ca^{+2}) was introduced into the soaking solution and, excluding the corresponding cation results, the loss of minerals due to soaking generally remained the same as the loses observed from soaking in tap water alone. For instance, the loss of K in the water, NaCl and CaCl treatments, was 65.3, 64.7 and 64.9%, respectively. The Al content of the leaves was affected by the addition of Na, giving a 19.4% loss compared to 11.8 and 12.1% for water and CaCl soaking, respectively. Less Fe was lost (10.9%) compared to the loss of 18.4 and 17.1% for water and CaCl soakings, respectively.

Losses of minerals due to processing have been fairly well documented. Watzke (1998) reviewed processing and bioavailability, in general, but did not make any comments about soaking, as it is often included or considered to be blanching process. There is very little research on the losses of minerals following cold water soaking. Kimura & Itokawa (1990) performed an experiment that typifies this, in that they determined nine different minerals across 14 different meals and observed a 60 to 70% reduction in all minerals compared to the raw or uncooked foods. Specifically, they carried out an experiment on raw and cooked (12 different ways) spinach and reported a reduction in Ca ranging from 24.0 to 74.8%, and from 25.0 to 71.9% for Na. The results from soaking raw spinach leaves are consistent with the literature, with respect to mineral losses. The 270.6% and 1701.1% increase in Ca and Na, respectively, just by soaking in a 1% w/v solution, could assist in the retention of these minerals even after further processing.

8.4 Conclusions

Soaking fresh spinach leaves at room temperature for 16 hours in water, 1% NaCl and 1% CaCl had little effect on the total oxalate content of the raw leaves. There was also little evidence for the loss of soluble oxalate in plain tap water or 1% NaCl solutions the leaves were soaked in. The most effective treatment for the reduction of soluble oxalate in the leaves occurred during soaking in the 1% CaCl treatment. A significant amount of soluble oxalate was converted into insoluble oxalate within the leaves (28.1% more). This confirmed that the 1% CaCl was absorbed into the leaf tissue. This treatment was a most effective way to reduce the soluble oxalate of spinach leaves prior to being cooked. This treatment might also prove to be an effective pre-treatment for green leafy vegetables prior to using the processed leaves to produce green juice mixtures.

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Chapter 9

General discussion and conclusions

9.1 General discussion

The aim of this thesis was to investigate and understand the occurrence of oxalic acid in green juices.

The original food innovation of "juicing" occurred in the 1940's and since then it has become increasingly mainstream, due to readily available and low cost of home juicing machines, this combined with a modern, popular press mantra to eat healthy and increase our vegetable / fruit intake, this has increased the use of juicing as a way to consume more fruits and vegetables.

Green juices are commonly promoted as a tonic with many health benefits that are generally unsubstantiated. Unconsidered is the potential negative effects of consuming green juices. This has recently been highlighted by reliably reported incidences of induced nephropathy following regular consumption of green juice (Getting *et al.,* 2013; Makkapati *et al.*, 2018).

Experiments surrounding the production of green juices were done and oxalic acid levels measured in the juices made. Two strategies to reduce oxalic acid in green juices were also investigated; by the addition of calcium ions and soaking the raw materials.

Objective 1: To investigate the optimum extraction conditions, with respect to time, temperature and pH for the extraction of oxalic acid from a green juice. Using spinach as an analogue.

There are always many ways to extract compounds from foods for further analysis, commonly they revolve around the theme of polarity of extraction solution, temperature and time of extraction. Commonly an experiment will vary one variable and monitor a response, for example time of extraction vs amount of oxalic acid extracted (the response). However, to address this objective a multi-variable statistical method called design of experiments (DOE) can be used. A three factorial experiment was performed using seven levels of pH, eight levels of temperature and six points of time were used. This allowed a balanced and valid experiment to be carried out using only 20 experimental runs were performed with statistically significant results a full factorial experiment would need 336 experimental runs to

give the same data. The resultant DOE quadratic of the extracted oxalic acid content using 20 targeted runs had a R^2 of 0.926. Therefore, from any combination of pH, temperature or time an extracted oxalic acid could be predicted with a very high degree of confidence. Overall low pH is more effective to extract all of the oxalates from spinach rather than high pH ($P<0.001$). Low temperature extractions do not fully extract the oxalates from spinach leaves compared to high temperature extraction ($P<0.01$). Extraction time did not have a significant response in the model, so is not as important when extracting oxalic acid from green leafy vegetables. The optimum conditions for extracting all the oxalate from the model green spinach juice was at pH 0.93, a temperature of 65ºC and at any time greater than 15 minutes. The least amount of oxalic acid was extracted at pH 4.59 and temperature of 25ºC.

Objective 2: To determine whether the composition of a green juice recipe has a relationship between recipe components and oxalic acid content of the final juice produced.

A homemade green juice by definition itself, is likely to have highly variable ingredients. An effort was made to create realistic and consumable green juices by basing them on popular media recipes (references not shown) and using a range of components. GJ1 had 9 different ingredients. Across all recipes the one common recipe component was the spinach. The spinach content of each green juice ranged from 20.1 to 37.9% w/w.

The juices made from the five different recipes had predictable differences in proximate, pH, titratable acidity and dry matters, due to their inherent ingredient differences, of 3 to 9 different fruits and vegetables. pH ranged from pH 4.18 to 4.54. The titratable acidity, from 2.66 to 7.28 mEq. Acid/100 g FW. The lowest titratable acidity juice, GJ3 had 42.8% apple, 26.7% celery and 30.5% spinach, all low acid fruit and vegetables, whereas GJ1, had the most ingredients (9), including 14% lemon, 9% green capsicum and 0.9% ginger, all high acid fruits and vegetables.

This is the first study to determine the oxalate content of homemade green juices of known composition. The oxalate content for all five recipes, ranged from 90.34 to 152 and 13.0 to 82.85 mg/100 g FW, for total and soluble oxalate. Of importance the ratio of soluble oxalate varied from 11.9% for GJ1 to 67.8% for GJ3.

In this study the green juices were made using a masticating juicer, which separates out a waste pulp fraction that is normally discarded. It is important to note that this fraction also contains large amounts of oxalic acid, 134.6 to 348.09 mg/100 g FW.

A mineral profile obtained for the juices and waste pulp for all recipes. For all of the five green juices the waste pulp had a higher concentration of minerals of all 11 minerals, apart from Al, compared to the green juice. The portion of Ca in the waste pulp ranged between 65 to 90%.

It was assumed the green juice recipe with the highest portion of spinach (GJ5) would also be the juice with largest amount of oxalate, but this was not the case.

The results of this experiment demonstrated there was an interaction between the oxalate, calcium and pulp fraction of the green juice. Interaction between minerals, oxalate and fiber are noted in research related to human diets (Southgate, 1987; Kelsay & Prather, 1983), however very little research has been done on these interaction with respect to the compositional make-up of a food.

A 200 g glass of GJ3, which contains, apple, celery and spinach, has 165.7 mg of soluble oxalate. Though there are no clear recommended dietary intake limits, this could be considered a considerable amount of soluble oxalate to consume and if multiple servings are consumed daily, and/or for an extended period of time, this would pose a risk of urolithiasis.

Objective 3: To investigate if using different types of juicers influence the outcome, with respect to oxalic acid content of the juice.

The two most common types of juicers, high speed blender (HSB) and masticating juicer (MJ) that are used to make green juices at home, operate quite differently yet they had variable outcomes with respect to the oxalic acid contents in the juices they made. Both types of juicers were used to make both a high and low spinach containing juice. They produced green juices that had similar ratios of soluble oxalate. The low spinach juice had 45.4 and 46.6% soluble oxalate for HSB and MJ, respectively. The high spinach juice had 74.5 and 66.5% soluble oxalate for the HSB and MJ, respectively. The MJ produced a juice with 528.41 mg total oxalic acid/100 g FW compared to the HSB juicer which produced a juice

which contained 369.47mg total oxalic acid/100 g FW. Clearly the MJ had a concentrating effect during processing. The MJ separates out a pulp, which is normally discarded, this fraction contained 238.10 to 55.67 mg total oxalate/100 g FW and 61.7 to 74.7% of the calcium content of the original leaves was recovered in the pulp.

These results indicate that an interaction occurred between the ratio of soluble oxalate and calcium content in the extraction process. It is also clear, that calcium and oxalate species reside within different parts of the plant cell and perhaps other interactions for instance, with the fibre fraction within the cell were also occurring. These interactions were out of the scope of this study but could warrant further investigation.

This study did establish that the consumption of 200 g FW of a green juice made by either a HSB or MJ would result in the consumption of 155 or 725 mg of soluble oxalate, respectively. This is a significant single consumption.

Objective 4: To identify and quantify potential strategies that could reduce or improve the oxalic acid content in a green juice.

The addition of four types of commonly used food grade calcium salts was the first method investigated to reduce oxalic content of green juices. Calcium carbonate, calcium chloride, calcium citrate and calcium sulphate were all tested against 100% spinach juice, then the most effective ion was tested again on a green juice made from eight vegetables, which spinach comprising 40% of the mix.

Calcium chloride was the most effective calcium salt as additions reduced the soluble oxalate by 98.3% after the addition of 500 mg/100 g juice. While the addition of 500 mg of calcium carbonate was the least effective additive, giving only a 49.3% reduction in soluble oxalate.

When testing the addition of calcium chloride to a realistic recipe of green juice, the reduction in soluble oxalate followed a polynomial reduction curve, reducing the soluble oxalate to 2.5% remaining after the addition of 500 mg of calcium chloride salt to 100 g of green juice. With no changes in pH or titratable acidity of the mixed juice little change of taste of the green juice would be expected.

Another technique investigated to reduce soluble oxalate in leaves, was to test soaking the raw material prior to juicing. Soaking is slightly different to blanching or pickling, no heat or high levels of salt are added. In this case a comparison was carried to investigate soaking overnight in plain water. 1% NaCl and 1% CaCl solutions at room temperature.

The plain water and 1% w/v NaCl solution showed no reduction of soluble oxalate. The leaves soaked in 1% w/v CaCl showed an increase in insoluble (28.1%) and a corresponding decrease in soluble oxalate content by converting more oxalate into its insoluble form this makes oxalate less bioavailable when eventually consumed either as a juice or any other type of food. This is a novel strategy that has not been proposed before. In addition to binding soluble oxalate early in the food production process, it has the added benefit of increasing the bioavailability of calcium, if excess to the amount of soluble oxalate present is added to the juice.

9.2 Conclusions

There is clear evidence of acute oxalate nephropathy directly attributable to the consumption of green juices. This study made and measured some typical green juices and confirmed the levels of total and soluble oxalate are high, but there are some differences depending on the type of juicing method used.

In this study it has been clearly demonstrated that simple strategies to decrease the risk of diet induced oxalate nephropathy due to the consumption of green juices can be achieved by adding calcium ions to the juice. This can be done directly by the addition a food grade powdered source of calcium ion. Calcium chloride dihydrate ($CaCl_2.2H_2O$) was found to be the most effective.

Another strategy that was effective was to soak the raw material, in this case leaves of spinach. Leaves soaked in an aqueous 1% w/v CaCl solution had half the amount of soluble oxalate, compared to the original raw leaves. The balance being converted to insoluble calcium oxalate within the leaves. The strategy of soaking the raw leaves with the addition of a calcium ion coverts a significant amount of the soluble oxalate to insoluble, as well as increasing the bioavailability of calcium, from 12.6 to 62.8 % for the raw and CaCl soaked leaves, respectively. The bioavailable calcium coming from the 1% w/v CaCl soaking solution.

9.3 Future research

Understanding the relationship and interactions of the conditions of oxalic extraction from a food are important, as this relates to the bioavailability of oxalates in the food system and the human digestion system. This could be different for different types of food groups, for example high starch containing vegetables (eg. taro) compared to berry fruits. Further studies to investigate a wide range different types of food groups should be conducted to gain a clear understanding of the occurrence of oxalate in common vegetables and its bioavailability when cooked and processed.

There is evidence to suggest the fibre content in food has an influence on the distribution of the oxalic content. Is this directly related to the fibre in food binding to key minerals, such as calcium or does the fibre bind directly to the oxalic acid? This warrants investigation and the implications on whether to supplement fibre in a healthy diet or not.

This study has highlighted the need to be vigilant and aware, when new and innovative ways of consuming existing, new and rediscovered foods that may have anti-nutritive factors in them. These could become a new health risk following increased consumption of a food when previously that particular food posed not risk. For example, Martínez-Hernández *et al.* (2013) vacuum boiled and sous vide broccoli and Shyamala *et al.* (2005) added leafy vegetable powder to heated oils. It would be useful to investigate potential future risks and to identify them.

There is a need to continue to monitor the uptake of new and re-discovered vegetables and fruits that could cause potential harm. For example, Ruan *et al.*, (2013) reported the total oxalate content of many foods commonly available in Southern China. Many of the foods they investigated had previously not been analysed. It is unfortunate that they did not analyse the soluble oxalate content as this is the most important fraction in foods.

Developing future strategies that can be easily adopted and understood by consumers or industry to reduce oxalate levels in food products that have a potential risk. This is an approach that has previously been ignored.

Another area of future research is the development of a low oxalate containing green leafy vegetable by selective breeding or gene manipulation. Morris *et al.* (2007) demonstrated

that a single gene in *Medicago trunatula* regulated the deposit of calcium oxalate in leaf tissue and this would significantly affect the calcium bioavailability. Approaching this differently, a similar gene manipulation could be used to produce a green leafy vegetable with changed ratios of soluble to total oxalic acid or an overall lower total oxalic acid amount. Traditional germplasm selection for lower oxalic acid levels has been investigated for spinach in the USA (Mou, 2008) by the USDA. Perhaps similar selection for other commonly used green leafy vegetable needs to be considered.

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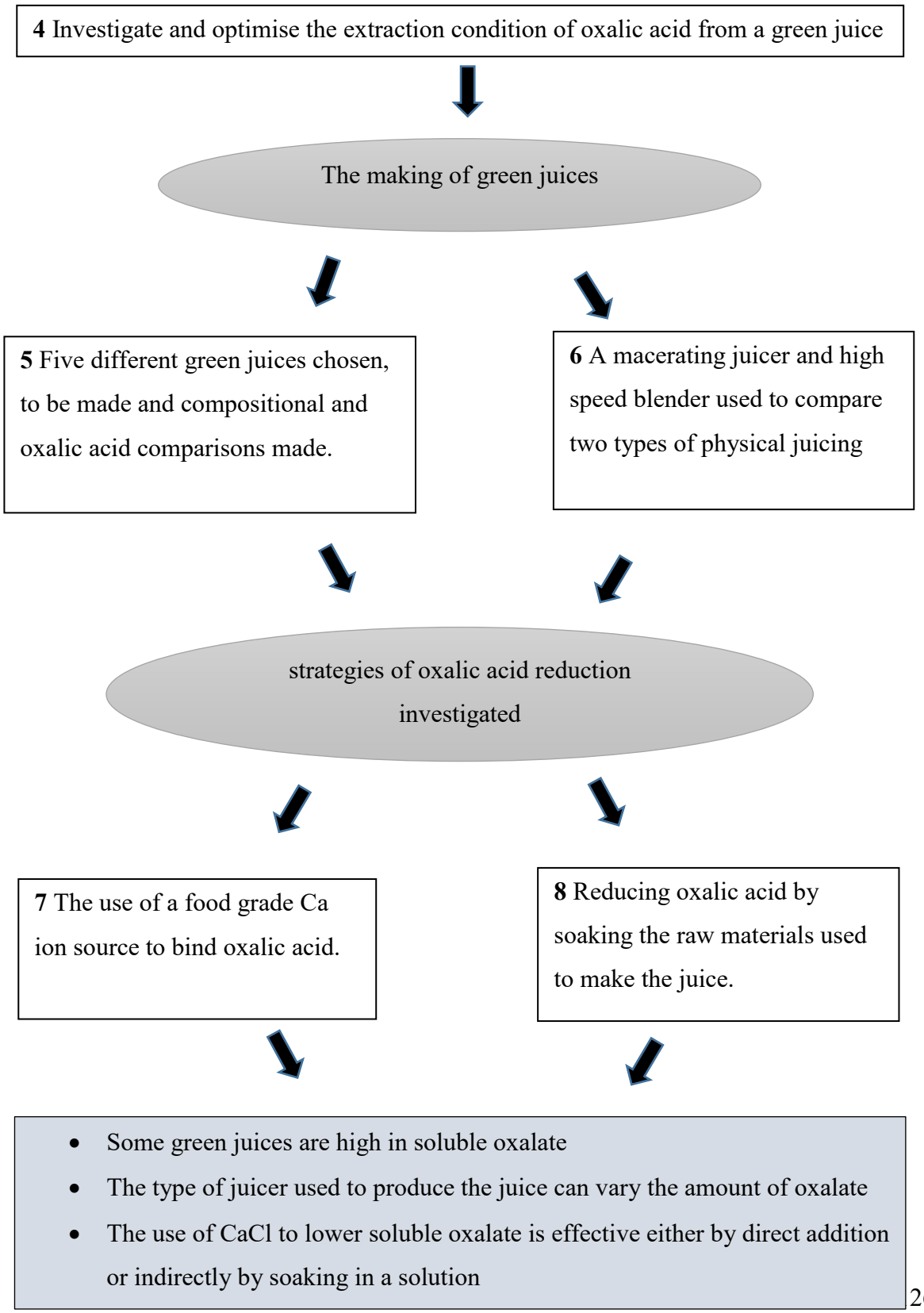
Appendix A

Research outline



Appendix B

Pictures of domestic juicers

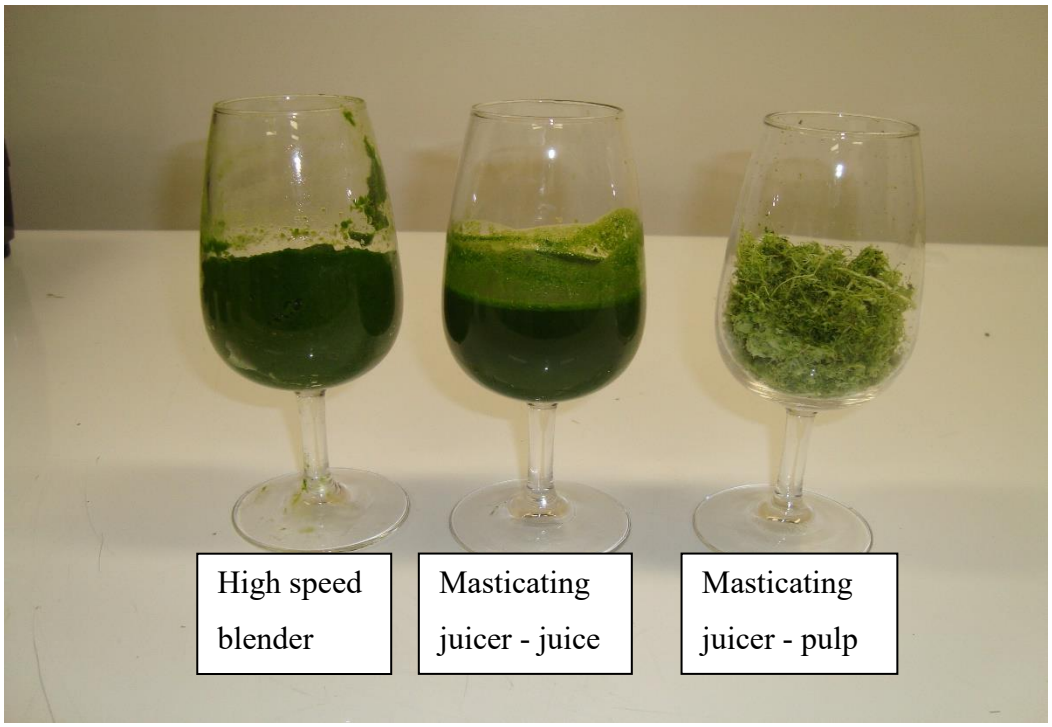
C.1 High speed blender

C.2 Masticating juicer

Appendix C

Pictures of green juice

D.1 Green juice made from a high speed blender and masticating juicer



Appendix D

List of publications from this thesis

D.1 Refereed Published Papers

Bong, W.-C., Vanhanen, L. P., & Savage, G. P. (2016). Addition of calcium compounds to reduce soluble oxalate in a high oxalate food system. *Food Chemistry, 221*, 54-57.

Kusuma, D. S., Vanhanen, L. P., & Savage, G. P. (2016). Evaluation of extraction parameters for total oxalate determination in spinach using Design of Experiment analysis. *Journal of Food Composition and Analysis, 51*, 9-14.

Savage, G. P., & Vanhanen, L. P. (2015). Calcium and oxalate contents of curly leaf (*Petroselinum crispum*) and flat leaf (*P. crispum* var. *neapolitanum*) parsley cultivars. *Food and Nutrition Sciences, 6* (16), 1565-1570.

Savage, G. P., & Vanhanen, L. P. (2015). Oxalate content of green juices produced by two different methods. *Proceedings of the Nutrition Society of Australia. Thirty ninth annual scientific meeting of the Nutrition Society of Australia in conjunction with The Nutrition Society of New Zealand, 39*, 133.

Vanhanen, L.P., & Savage, G.P. (2015). Comparison of oxalate contents and recovery from two green juices prepared using a masticating juicer or a high speed blender. *NFS Journal, 1*, 20-23.

D.2 Papers in progress -2019

Vanhanen, L.P., Klunklin, W., & Savage, G.P. (2019). Composition of five different green juice recipes produced using a masticating juicer. *Journal to be confirmed*.

D.3 Scientific Poster Presentations

Vanhanen, L. P., & Savage, G. P. (2016). Mineral content of green vegetable juice using two different juicers. *Annual conference of the Nutrition Society of New Zealand.* DoubleTree by Hilton, Christchurch, New Zealand, 8-9th December.

Vanhanen, L.P., & Savage, G.P. (2015). Oxalate content of green juices produced by two different methods. *Joint annual meeting of the Nutrition Society of New Zealand and the Nutrition Society of Australia.* Te Papa, Wellington, New Zealand, 1-4 December.

Vanhanen, L.P., & Savage, G.P. (2015). Drink your greens? *International Journal of Food Science & Technology, 50th Volume celebration.* Lincoln University, Lincoln, New Zealand, 17-19th February.