The bioavailability of soluble oxalates in stir-fried silver beet leaves

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Abstract

Green leafy vegetables, such as spinach and silver beet, contain high to moderate levels of oxalate, which can be a cause of health concerns due to the formation of painful kidney stones and decreased bioavailability of minerals. In this study, 14 participants ingested 115 g of stir-fried silver beet leaves with or without standard yoghurt and low fat yoghurt, respectively. Stir-fried silver beet leaves contained 209.1 ± 0.1 mg of total oxalates/meal fresh weight (FW) and 109.2 ± 0.1 mg of soluble oxalates/meal FW. The proportion of soluble oxalates changed from 52 to 29% when standard yoghurt was added and to 30% after addition of low fat yoghurt. The absorption of oxalates was determined by measuring the output of soluble oxalate in the urine over a 6-hour period after ingestion of the meals. The mean absorption of oxalate from stir-fried silver beet leaves was determined to be 2.41%, which reduced to 1.10% when consumed with standard yoghurt. Addition of low fat yoghurt to the test meals decreased the absorption of oxalates even more, to 0.89%. This study confirms that the addition of calcium from yoghurt to stir-fried silver beet leaves decreases the bioavailability of soluble oxalates by converting most of the soluble oxalate to insoluble calcium oxalate.

Key words: Soluble oxalates, silver beet leaves, stir-frying, calcium, fat, pH, urine analysis.

Sammanfattning

Spenat och mangold är exempel på grönbladiga grönsaker som innehåller höga till måttliga halter av oxalater. Dessa kan orsaka hälsoproblem eftersom det kan ge upphov till bildning av smärtsamma njurstenar samt orsaka minskad biotillgänglighet av mineraler. I denna studie intog 14 deltagare 115 g wokad mangold som serverades antingen som den var eller tillsammans med standard yoghurt eller fettsnål yoghurt. Studien utfördes för att studera absorptionen av oxalater baserat på mängden oxalater funnet i urin 6 timmar efter intag av ovanstående tre dieter. Wokad mangold innehöll 209,1 ± 0,1 mg oxalater/diet och 109,2 ± 0.1 mg lösliga oxalater/diet. Andelen lösliga oxalater minskade från 52% till 29 respektive 30% efter tillsats av standard yoghurt och fettsnål yoghurt. Biotillgängligheten av oxalater påvisades genom mätning av antalet lösliga oxalater i urinen efter intag av respektive diet. Medelvärdet av de absorberade oxalaterna fastställdes till 2,41% vid intag av wokad mangold medan absorptionen minskade (1,10%) vid intag med standard yoghurt. Genom att tillsätta yoghurt med låg fetthalt minskade absorptionen ytterligare av oxalater (0,89%). Denna studie bekräftar att tillsats av kalcium från yoghurt till wokad mangold minskar biotillgängligheten av lösliga oxalater genom att omvandla de flesta av de lösliga oxalaterna till olösliga kalciumoxalater.

Nyckelord: Lösliga oxalater, mangold, wok, kalcium, fett, pH, urinanalys.

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1. Introduction

1.1 Background

Several green leafy vegetables are known to be good sources of nutrients even though some also contain moderate to high amounts of oxalate, causing some health concerns. Oxalates can give rise to painful kidney stones and decrease the bioavailability of minerals in the plants. Silver beet (*Beta vulgaris* var. *cicla*) and spinach (*Spinacia oleracia*) are examples of green leafy vegetables that contain high levels of oxalates (Zarembski and Hodgkinson, 1962; Savage *et al.,* 2004). The levels and bioavailability of oxalates have been well studied in many foods, although only limited studies have been undertaken on silver beet leaves.

Reduction of oxalates in the diet can be achieved by avoiding foods rich in oxalate, or by processing the foods in different ways to reduce the oxalate content. Soaking and boiling are efficient ways of reducing the level of soluble oxalate in a food if the cooking water is discarded (Savage *et al.,* 2000; Chai and Liebman, 2005a). Stir-frying is a common cooking method used mainly in Asia, and usually involves high temperatures and short processing times. However, there appears to be no recent studies on the effect of stir-frying on the oxalate content of plants. In this study, the effect of stir-fried silver beet leaves was investigated. Consumption of green leafy vegetables is widespread in Asia and, therefore, the effect of stir-frying silver beet leaves is investigated in this study. Furthermore, addition of calcium can make soluble oxalates unavailable for absorption by complex-binding (Hanson *et al.,* 1989). By consuming a food high in calcium together with foods containing oxalates, the insoluble calcium oxalate formed passes through the intestinal tract without absorption and thereby decreases the risk of kidney stone formation. Other studies have shown that fat and oils can bind oxalate and thereby make soluble oxalates unavailable for absorption (Liebman *et al.,* 1999). Recent studies have shown that the pH of the food affects the form of oxalate found in the food and this has an impact on whether it will react with free calcium in the food or not. Until recently, these two effects have been confused due to the addition of both calcium and fat in milk products to the food.

1.2 Aim

The aim of this study was to measure the bioavailability of oxalates from silver beet leaves after stir-frying. A comparison of the effects obtained when fat or calcium were added to the leaves

was conducted and, at the same time, the pH of the final test meals was monitored. A feeding experiment was designed to obtain useful information about how green leafy vegetables can be consumed without the risk of kidney stone formation and decreased bioavailability of minerals. The effect of consuming three test meals was determined by measuring the output of soluble oxalates in the urine as oxalates absorbed into the body are quickly excreted in the urine.

Overall, this study will lead to increased understanding of the effect of stir-frying on oxalate content in stir-fried silver beet leaves and also on the effect of the addition of milk on the bioavailability of oxalic acid.

2. Literature review

2.1 Oxalic acid

Oxalic acid is a decarboxylic acid (Libert, 1987) that occurs either as a free acid, a water-soluble salt formed with K^+ and Na^+, or as a water-insoluble salt of Ca^{2+}, Fe^{2+} and Mg^2 (Noonan and Savage, 2000). Insoluble oxalates are predominately found as calcium oxalate (CaOx) and are formed through the binding of calcium to oxalate. Mineral binding to oxalate is pH dependent and binding prevents the minerals from being available for absorption. The free oxalate ion ($C_2O_4^{2-}$) is available to bind to calcium preferentially while the binding capacity is reduced if the semi-dehydro-oxalic acid ($HC_2O_4^-$) or oxalic acid ($H_2C_2O_4$) species are present. The effect of the pH on the relative abundance of each formed oxalate was investigated by Simpson *et al.,* (2009).

Oxalic acid is absorbed from the diet (exogenously) but can also be biosynthesised (endogenously) through oxidation of glyoxylate by glycolic acid oxidase, or through the cleavage of ascorbic acid (Wagner and Loewus, 1973). The catalysis of glyoxylate can be inhibited by the oxalate end product (Richardson and Tolbert, 1960). The combination of dietary oxalate and its formation from glyoxylate and ascorbic acid contributes to the output of urinary oxalate (Holmes *et al.,* 2001).

2.2 Oxalates in silver beet and other green leafy vegetables

Silver beet (*Beta vulgaris* var. *cicla*) belongs to the Chenopodiaceae family; it contains high levels of oxalate that varies among species. The oxalate content may also depend on the growing conditions, type of soil, season and time of harvesting (Hodgkinson, 1977). Fresh vegetables generally have lower levels of oxalates than dried ones (Aletor and Adegon, 1994) and younger leaves contain significantly lower levels of oxalates than mature leaves (Simpson *et al*., 2009). Regrowth tissue contains higher levels of soluble oxalates than mature leaves, ranging from 58% of total oxalate for the mature leaves and up to 89% for regrowth tissue (Simpson *et al.,* 2009). Savage *et al.,* (2004) determined that raw silver beet leaves contained 792.7 ± 22.9 mg oxalate/100 g wet matter (WM) and 350.0 ± 24.1 mg of soluble oxalate/100 g WM. Siener *et al.,* (2006) determined the oxalate contents in raw silver beet leaves to be 874 mg/100 g total oxalate (FW) and 327 mg/100 g soluble oxalate (FW).

Moreover, the distribution of oxalic acid is uneven within the plants and several studies show that the leaves generally contain higher levels of oxalate than petioles and roots (Santamaria *et al*., 1999; Savage *et al*., 2000). Harvesting of silver beet (also known as Swiss chard) occurs primarily in autumn in New Zealand and is, like spinach, generally served boiled. Oxalate levels in spinach have been well studied and, therefore, in contrast to previous studies, silver beet leaves were chosen to clarify whether stir-frying affects the oxalate content and whether an additional calcium and fat source affects the bioavailability of oxalates.

2.3 Occurrence of oxalates in food

Silver beet, spinach, rhubarb, nuts, multi-grain flours, chocolate, black tea and parsley contain high levels of total and soluble oxalates (Zarembski and Hodgkinson, 1962; Fasett, 1973; Brinkely *et al.,* 1981; Hönow and Hesse, 2002; Chai and Liebman, 2005b; Siener *et al.,* 2006). Due to the high levels of soluble oxalates these foods should be taken into consideration for avoiding kidney stone formation. In a study published by Zarembski and Hodgkinson (1962), oxalic acid contents in various English foods were calculated to range from 70 to 150 mg/day. Archer *et al.* reported, in 1957, that daily oxalate intake ranged from 1190 to 1370 mg/day. The difference in ranges of oxalate content is probably due to a diversity of preparation methods and differences in analytical methods. The oxalate levels found in vegetable foods are unlikely to cause oxalate poisoning during normal consumption of food as a lethal intake of oxalate is thought to be 2-30 g oxalic acid (Libert and Franceschi, 1987). However, poisoning has occurred when rhubarb leaves, which contain very high amounts of oxalate and other toxins, have been consumed (Hodgkinson, 1977).

2.4 Oxalate content in food – effect of processing

There are several ways of reducing the amounts and bioavailability of oxalic acid in food. Soaking, steaming and boiling are effective ways of decreasing oxalate content due to leaching of the biologically significant soluble oxalates into the cooking water. Most reductions of oxalate content occur when cooking water is discarded (Savage *et al.,* 2000; Chai and Liebman, 2005a). However, the content of oxalate increases during baking due to loss of moisture from the food (Albihn and Savage, 2000; Chai and Liebman, 2005a).

Oxalate bioavailability depends on pH, and the fat and minerals present in the food. High levels

of calcium, magnesium and iron decrease the bioavailability of oxalates due to the formation of insoluble oxalate salts (Hanson *et al.,* 1989). Soluble oxalates are biologically significant due to their ability to be absorbed, while insoluble oxalates are not absorbed by the intestine and are, therefore, not considered to be biologically significant (Albihn and Savage, 2000). Addition of foods high in calcium decreases the levels of soluble oxalates (Brogren and Savage, 2003). Recently, it was found that silver beet leaves cooked in tap water contained 1783 ± 187 mg total oxalate/100 g DM and 342 ± 48 mg soluble oxalate/100 g DM, while silver beet leaves cooked in standard milk contained 1912 ± 36 mg total oxalate/100 g DM and 102 ± 13 mg soluble oxalate /100 g DM (Simpson *et al.,* 2009).

Liebman *et al.,* (1999) performed a study which showed that the presence of fat lowers the absorption of oxalate. Mårtensson and Savage (2008) went on to show that soluble oxalates are beneficially reduced in taro when consumed with additional calcium and plant oil. In contrast, Bailly *et al.,* (2000) suggested that the amount of soluble oxalates that may be absorbed could be increased when fat is consumed with soluble oxalates and this could lead to an increase in the formation of kidney stones. Bailly *et al*., (2000) suggested that fatty acids could bind to calcium in the gastrointestinal tract to form insoluble soaps that are excreted in the faeces. This would mean that less calcium would be available to bind to soluble oxalates to convert them to insoluble calcium oxalates.

2.5 Absorption, bioavailability and effects in humans

Oxalate absorption is not yet fully understood; however, it is believed that the main absorption takes place in the proximal small intestine (Prenen *et al.,* 1984). The urinary oxalate peak is generally found 1-6 hours after consumption of an oxalate containing food, indicating that the upper part of the intestine acts as the major absorption site (Barilla *et al.,* 1978; Brinkley *et al.,* 1981). Previous studies have shown that the upper intestine absorbed more oxalate than the lower intestine (Hanes *et al.,* 1999). However, the stomach has also been suggested to be significant for oxalate absorption (Chen *et al.,* 2003), although it is commonly assumed that the main absorption still takes place in the small intestine (Barilla *et al.,* 1978; Holmes *et al*, 1995; Hanes *et al.,* 1999). Gastrointestinal oxalate absorption has been reported to range from 5 to 15%, depending on the co-ingestion of divalent minerals and fibre (Holmes *et al.,* 1995), while a more recent study suggested that the absorption ranges from 2.2 to 18.5% (Unruh *et al.,* 2003).

Several factors influence oxalate absorption such as bioavailability of oxalate (Holmes *et al.,* 1995), the amount of oxalate present and microbial oxalate degradation (Allison *et al.,* 1986). *Oxalobacter formigenes* is a gut bacterium that can metabolise oxalates in the small intestine and is thought to have an impact on the total oxalate absorption. Absence of *O. formigenes* is believed to be related to increased absorption of dietary oxalates (Allison *et al.,* 1986). Insoluble oxalates are not degraded by bacteria but are excreted in faeces due to their low ability to be absorbed.

Addition of calcium decreases the urinary oxalate excretion and significantly increases calcium levels in the urine (Barilla *et al.,* 1978). A decrease in dietary calcium at a constant oxalate level will cause less calcium oxalate to be formed due to the reduced calcium content in the food eaten, which therefore results in increased levels of soluble oxalate and enhanced intestinal absorption of oxalates (Marshall *et al.,* 1972). Moreover, high oxalate intake and low calcium intake increases the urinary oxalates, thought to be associated with kidney stone formation (Massey *et al.,* 1993).

It should be remembered that the acidic conditions in the stomach may have a considerable effect on what form of oxalate will predominate and this will affect the binding of oxalates with the free calcium in the stomach (Simpson *et al.,* 2009). The free oxalate ion ($C_2O_4^{2-}$) is available to bind with calcium preferentially and is present in the highest proportions at high pH. The binding capacity is reduced at a low pH as most of the oxalate species are present as the semi-dehydro-oxalic acid ($HC_2O_4^-$) or oxalic acid ($H_2C_2O_4$) (Simpson, *et al.,*2009).

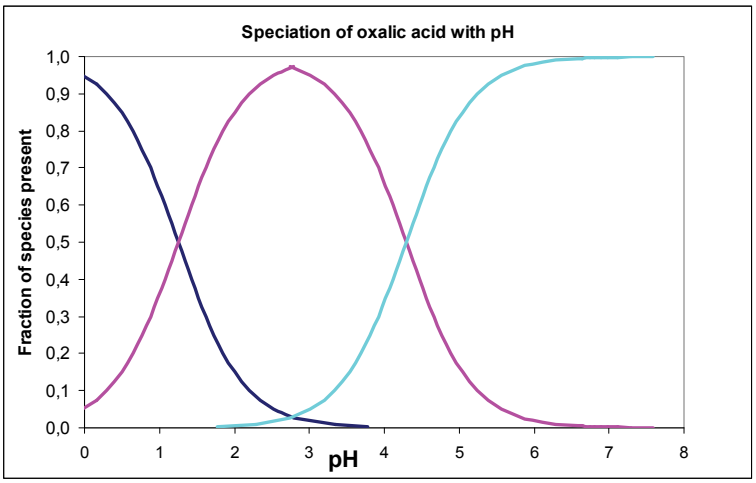

Figure 1. The pH speciation diagram for the three fractions of oxalate species present; free oxalate ion ($C_2O_4^{2-}$), semi-dehydro-oxalic acid ($HC_2O_4^-$) and oxalic acid ($H_2C_2O_4$). (Modified after Simpson *et al.,* 2009).

Stomach pH ranges from 1.5-2.0 while pH in the intestine has a mean value of 8.1 (Savage and Mårtensson, 2010). Re-formation of insoluble oxalate may take place when solubilised oxalate passes from the acidic stomach to the alkaline intestine. The high pH might lead to decreased absorption of oxalate due to the re-formation of insoluble calcium oxalate crystals. When solubilised oxalates pass through the acidic gastric tract to the alkaline intestine, some re-form insoluble oxalates and so will not be absorbed. The binding capacity increases at pH above 4.0 and slows as the pH rises (Siener *et al.,* 2001).

Humans with low calcium intakes, or who are lactose intolerant, should avoid food containing high levels of soluble oxalates as they will significantly reduce the bioavailability of calcium in the food eaten (Savage *et al.,* 2000). In order to avoid mineral deficiency silver beet and spinach are not considered good food sources for these people.

Another health concern is kidney stone formation. This is known relate to a high intake of soluble oxalates where the soluble oxalates can bind the urinary calcium and form crystals of calcium oxalate in the body. The risk for kidney stone formation is higher when urine is supersaturated with calcium oxalate (Holmes *et al.,* 2001), and increased excretion of urinary oxalates are believed to have a greater impact on kidney stone formation than the equivalent calcium changes (Robertson and Hughes, 1993).

The risk factors for kidney stone formation were evaluated and summarised in a review by Lewandowski and Rodgers (2004), where the main factors were low urine volume, high oxalate concentration and presence of inhibitors such as citrate, magnesium and uric acid.

2.6 Urine oxalate extraction

Excretion of oxalate in urine varies between individuals with a daily range of 15 to 50 mg/day (Hodgkinson, 1977). Previously, 40 to 50% of urine oxalates were considered to originate from non-enzymatic breakdown of ascorbic acid and dietary oxalate was thought to contribute only 10-20%. Later studies have shown that 24-53% of urine oxalates (daily intake from 10 to 250 mg) are derived from intestinal absorption from the diet (Holmes *et al,* 2001) and therefore restriction of dietary oxalate should be taken into consideration. Small amounts of insoluble calcium oxalate (2.2%) were absorbed in rats and the same absorption rate might occur in humans (Hanes *et al.,* 1999). Women excrete less oxalate than men which seems to be due to women's higher urinary citrate and lower urinary calcium content (Parks and Coe, 1986).

3. Materials and Methods

3.1 Sampling the silver beet

Fully grown silver beet plants (Beta vulgaris var. cicla) were harvested from Lincoln Horticulture Research Area, Christchurch NZ (43°38'S, 172°27'E) and cooked on the 22nd and 25th of May, 2009. Whole bunches of silver beet plants (total height between 0.2 – 0.3 m) were cut with a knife just above soil level and were placed upright in plastic buckets filled with tap water to maintain freshness. Harvesting took place in the morning and sample preparation took place in the afternoon. Young and mature silver beet leaves (5 kg) were detached, torn into pieces (2 cm^2) and put in stainless steel buckets filled with tap water at12°C. The leaves were mixed by hand to obtain a representative sample.

3.2 Cooking procedure

The silver beet leaves (460 g) were randomly collected and soaked in 2740 mL fresh tap water for 30 minutes for each of the three test meals. After soaking, the silver beet leaves were boiled for 2 minutes in a 16 L pot under constant stirring after the water reached 100°C. The cooking element was then turned off and the silver beet leaves were steamed for 1 minute with the pot lid on. Prior to stir-frying the leaves were drained and subdivided into serving sizes (115 g per serving). The remaining leaves were weighed for the calculation of water loss and freeze-dried for further analysis.

Single servings of silver beet leaves (115 g) were then stir-fried for 2 minutes in a electric wok (model: EW 30 Breville Health Smart Wok, Australia) at 200°C together with 28 mL of canola oil (25.8 g) (Pam's Salad and Cooking Oil, Pam's Products Ltd., Mt. Roskill, Auckland, NZ) under constant stirring. The cooked silver beet leaves were left in the wok for 1 minute with the lid closed before being transferred to 1.2 L plastic containers. A timer and temperature probe were used to monitor time and control the cooking temperature. In one of the test meals, 12 mL of soya sauce (14.5 g) (Lee Kum Kee, Hong Kong, Food Co, Ltd., China) was added to the leaves during stir-frying to enhance the flavour. The other two test meals were cooked the same as above, except that no soy sauce was added and, instead, yoghurt was added at serving. The stir-fried silver beet leaves were then randomly distributed to each of the three test meals, weighed, and frozen at -20°C immediately after preparation.

3.3 Yoghurt making procedure

Both the standard yoghurt (EasiYo´s Real Base & Culture, Natural, unsweetened, 3.6 % fat; EasiYo Products Ltd., Albany, North Shore, Auckland, NZ) and low fat yoghurt (EasiYo´s Real Base & Culture, Low Fat Greek, unsweetened, 1.3 % fat; EasYio Products Ltd., Albany, North Shore, Auckland, NZ) were prepared the day before serving. The yoghurt was prepared exactly to the manufacturer's instructions. Briefly, the yoghurt culture (140 g) was dissolved in tap water and made up to a final volume of 1 L. The plastic container with the well shaken yoghurt mix was transferred to a yoghurt maker and boiling water was added to the marked level in the outer container. The yoghurt maker was kept at room temperature until the yoghurt had set. The fresh yoghurt was then kept at 4°C for a minimum of 2 hours before serving.

3.4 Proximate analysis

Dry matter of all samples was determined in triplicate according to the AOAC method 925.10 (AOAC, 2002); drying the processed silver beet leaves to constant weight in an oven (105°C for 16 hours). Each sample was then ground to a fine powder in a coffee mill (Sunbeam, model: EM0400, China). The fat from each sample was extracted using a Tecator Soxtec HT6 fat extractor with petroleum ether as the solvent; defatted samples were required for the oxalate determination. Fat, ash, protein, acid detergent fibre (ADF) and neutral detergent fibre (NDF) were determined in triplicate on the freeze-dried meal samples. All results were calculated on a dry matter basis since the freeze-dried samples were not totally dry. Determination of pH was carried out in triplicate on the freshly thawed test meals using a Metler Toledo S20 (GmbH, Schwerzenback, Switzerland) pH meter with a Eutech 9406 probe. See Appendix 1a for the raw data of all samples and Appendix 1b for the pH values.

3.5 Extraction of total and soluble oxalates

Total and soluble oxalate contents were determined in quadruplicate by homogenizing 0.2 g (DW) of finely ground silver beet leaves with 40 mL of 0.2M HCl (total oxalates) or with 40 mL of Nanopure water (soluble oxalates). The extraction was performed in a shaking water bath (80°C) for 15 minutes. The samples were then allowed to cool before being quantitatively transferred to a volumetric flask and the volume made up to 100 mL with Nanopure water. The extract was then centrifuged at 3500 rpm for 15 minutes and the supernatant filtered through a cellulose nitrate filter into 2 mL HPLC vials for further analysis using HPLC chromatography.

3.6 HPLC analysis

Oxalate extracts were analyzed using a Phenomenex Rezex 300 x 7.8 mm ion exchange column (Phenomenex, USA). The HPLC comprised a tertiary pump (Spectra-Physics, SP 8800, CA, USA) and an UV/VIS detector (Spectra-Physics, SP, 8450, CA, USA) set at 210 nm. Separation was carried out using a degassed aqueous solution of 25 mM sulphuric acid as the mobile phase.

Samples (20 µl) were injected onto the column and eluted at a flow rate of 0.6 mL/min. The oxalate peaks were identified by comparison with the retention times of an oxalate standard (see below). Elution of the oxalic peak occurred after approximately 8.9 minutes. Data capture and peak manipulation were performed using Peak Simple S/W version 3.78 (SRI, Inc. USA).

3.7 Standard calibration

The oxalate concentration was calculated using a standard calibration curve prepared from 99.99 % oxalic acid (Sigma-Aldrich, USA) dissolved in 0.2M HCl or Nanopure water then filtered through a cellulose nitrate filter into HPLC vials. Oxalic acid concentrations of 0.4, 1.0, 2.4, 5.0, 10.0 and 20.0 mg/100 mL were used for the standard curve. The insoluble oxalates were calculated from the difference between the total oxalate content and then soluble oxalate content, according to Holloway *et al.,* (1989). The results from the chemical analysis are presented as mg/100 g DW.

3.8 Bioavailability assay

Fourteen volunteers (seven females and seven males) aged from 21-32 were recruited, from students at Lincoln University, Canterbury, NZ, to participate in the study. All participants were asked to maintain their normal diets throughout the experimental period, but to avoid foods and drinks known to contain high levels of oxalate and calcium. Each volunteer was also instructed not to have breakfast on each data-collection day. A half a cup of water every hour was recommended during the test day to ensure adequate urine production. See Appendix 2 for further dietary restrictions.

Each participant consumed the following meals, in a random order, on three different occasions: stir-fried silver beet leaves with soy sauce, standard yoghurt meal and low fat yoghurt meal (Table 1). A tray with 115 g of processed silver beet leaves was served as breakfast (9 am) after overnight fasting on each occasion. The silver beet test meals were defrosted and heated to 80°C

using a stainless electric fry pan (Sunbeam, China) on each collection day before ingestion. Daily 6 hour urines were collected in individual plastic containers on each collection day, starting exactly from the time when test meal was consumed. A urinary control sample was also collected during the test period. This was used as the reference blank for each person.

Table 1. Composition of the three test meals used.

Test meal	Content
Control	No silver beet leaves eaten
Stir-fried silver beet leaves	115 g of silver beet leaves
Stir-fried silver beet leaves and standard yoghurt meal	115 g of silver beet leaves served with 115 g standard yoghurt
Stir-fried silver beet leaves and low fat yoghurt meal	115 g of silver beet leaves served with 115 g low fat yoghurt

3.9 Urine analysis

Urine weight was recorded and 10 mL of 35.4% HCl (BDH, UK) was added immediately after collection to prevent the conversion of ascorbic acid to oxalate and to inhibit microbial growth. Sub-samples (100 mL) were taken from the collected urine, in duplicate, and kept at 4ºC until total urinary oxalate concentrations were determined at the Canterbury Health Laboratories (Christchurch Hospital, Riccarton Avenue, Christchurch, NZ). The enzymatic method used was based on oxidation of oxalates by oxalate oxidase and the measurement of the hydrogen peroxide produced by the catalytic activity of oxalates, see Appendix 3. The oxalate concentration was presented as μmol/L.

3.10 Bioavailability calculation

The oxalate bioavailability was determined using each participant's urinary oxalate output after ingestion of the test meals subtracted from each participant's reference blank, expressed as a percentage of total oxalates ingested. Urinary output of oxalates that were below zero after subtraction of the reference values were made up to zero to compensate for individual variations. Mean values for each test meals were determined after correction.

3.11 Mineral analysis

Mineral content was analysed, in triplicate, in the three test meals and the two yoghurt cultures using a Varian Axial ICP-OES with a SP3 autosampler. All equipment was soaked in 10% HCl for 48 hours and left to dry before the procedure begun. The Teflon microwave digestion vessels used were soaked in Decon solution and then rinsed with acid, to eliminate contamination.

The test meals and yoghurt mixes (0.5 g DM) were accurately weighed and placed into 100 mL Teflon digestion tubes. Two mL of hydrogen peroxide (30%) and 5 mL of nitric acid (35%) was added to all samples and then left to digest overnight. Three standard solutions were prepared and run at the same time, see Table 2. The ICP Multi-element standard solution (STD) contained 23 elements (1.000 mg/L) and was diluted to 20 ppm with 1 M nitric acid.

A recovery study was done using a spike of 20 mg/kg of the standard mineral mixture and ranged from 92.2 - 123.4%. The spikes were extracted and analysed using the same method as for the samples.

Table 2. Volumes of reagents added for wet digestion of food samples for the analysis of minerals

	Test meal/ yoghurt (g) (DM)	**HNO_3 (mL)**	**H_2O_2 (mL)**	**Multi-element STD (mL)**	**Water (mL)**
Silver beet/yoghurt	0.5	5	2	--	--
Silver beet/yoghurt + Spike	0.5	5	2	1	--
Acid blank	--	5	2	--	--
Water blank	--	5	2	--	1
Standard blank	--	5	2	1	--

The samples were digested in a microwave oven (Milestone Ethos Sel, Sorisole, Italy) at 800 W for 15 minutes; see Table 3 for the digestion procedure. After digestion, the samples were allowed to cool then quantitatively transferred to a 25 mL volumetric flask and made up with DDI water (18.2 megaohms). A Cetac 5000 UT Ultrasonic Nebulizer was attached to the autosampler for increased sensitivity of the mineral analysis.

Table 3. Microwave digestion operating conditions

Step	Temperature ºC		Run time (min)	
	Start	Finish	Start	Finish
1	Ambient		0	10
2			10	15
3	200	Ambient	15	25

3.12 Calculation of oxalate speciation

The concentration and the K_{sp} values were calculated for Ca^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+} and Mn^{2+} to determine the cation likely to be bound to the insoluble oxalates found in the test meals (Lide, 2007-2008; Simpson *et al.,* 2009). Six selected cations found in the greatest levels in the test meals are presented in mg/100 g DM and in mg/100 g FW, respectively (Appendix 5).

3.13 Statistical analysis

All calculations were performed using Microsoft Excel and the results are presented as mean values ± standard error. Statistical analysis of the output of urinary oxalate from the different test meals was performed using Minitab version 15.1 (Coventry, U.K.) using a two-way analysis of variance.

4. Results

4.1 Chemical composition

The chemical compositions of the three test meals were determined on the freeze dried whole meals. The results were then calculated after allowing for any residual dry matter in the freeze-dried samples and expressed on a wet matter basis as mean values ± SE, Table 4.

Table 4. Chemical composition of the three test meals (g/100g FW)

Test meal	DM	Ash	Fat	Protein	Acid detergent fibre	Neutral detergent fibre
Stir-fried silver beet leaves	21.0 ± 0.9	2.0 ± 0.01	9.6 ± 0.20	4.2 ± 0.01	1.1 ± 0.01	5.7 ± 0.01
Stir-fried silver beet leaves and standard yoghurt meal	16.2 ± 0.4	0.9 ± 0.01	6.0 ± 0.01	4.2 ± 0.01	0.6 ±0.01	4.1 ± 0.01
Stir-fried silver beet leaves and low fat yoghurt meal	16.8 ± 0.4	1.1 ± 0.01	5.0 ± 0.01	4.9 ± 0.01	0.4 ± 0.01	4.2 ± 0.01

4.2 Oxalate content and its bioavailability in the test meals

Mean values of oxalates were calculated in quadruplicate for the three test meals and can be seen in Table 5. Total, soluble and insoluble oxalates in stir-fried silver beet leaves were found in lower levels than in previous data reported for boiled silver beet leaves (Simpson *et al.,* 2009).

The proportion of soluble oxalates decreased in the test meals when yoghurt was added. The stir-fried silver beet leaves contained higher levels of soluble oxalate (109.2 ± 0.1 mg/meal FW) than

the two yoghurt meals (60.6 ± 0.1 mg/meal FW and 62.9 ± 0.1 mg/meal FW). The proportions of soluble oxalates for the standard and the low fat yoghurt meal were similar, while the stir-fried silver beet leaves test meal contained higher proportions of soluble oxalates.

Table 5. Total, soluble and insoluble oxalates in the three test meals (mg/meal FW ± SE)

Test meal	Total oxalate	Soluble oxalate Oxalate ion $C_2O_4^{2-}$	Insoluble oxalate Semi-dihydro-oxalic acid $HC_2O_4^-$	Ratio of soluble: insoluble oxalate
Stir-fried silver beet meal	209.7 ± 0.1	109.2 ± 0.1	100.5 ± 0.1	52:48
Stir-fried silver beet leaves and standard yoghurt meal	209.1 ± 0.2	60.6 ± 0.1	148.5 ± 0.1	29:71
Stir-fried silver beet leaves and low fat yoghurt meal	209.7 ± 0.2	62.9 ± 0.1	146.8 ± 0.2	30:70

4.3 Urine analysis

In order to compare the intake, excretion and bioavailability of oxalate in the urine, the intake was calculated as mg oxalate per meal. The mean urinary oxalate output in 6 hours can be seen in Table 6, where ingestion of the low fat yoghurt meal caused the higher excretion, followed by the stir-fried silver beet leaves then the standard yoghurt meal.

According to the urinary analysis performed at Canterbury Health Laboratories, each individual reference blank value ranged from 2.8-18.9 mg/6 hours. The mean urinary output of oxalate after 6 hours was determined to be 10.89 ± 0.96 mg/6 h in the stir-fried silver beet leaves, 6.86 ± 0.77 mg/6 hours in the standard yoghurt meal and 8.22 ± 0.93 mg/6 hours in the low fat yoghurt meal (Appendix 4). The mean urinary oxalate increase was calculated by subtracting each participant's individual value from each individual's reference blank. Some of the values corrected for the reference blank were negative after subtraction and, therefore, these values were taken as zero.

Table 6. Intake, excretion and bioavailability of oxalate in stir-fried silver beet leaves with additions of high and low fat yoghurt

Test meal	Mean oxalate intake (mg/meal) ± SE	Mean urinary oxalate increase above the reference values (mg/6h) ± SE	Oxalate bioavailability (% of intake)
Stir-fried silver beet leaves	209.7 ± 0.1	5.05 ± 0.89	2.41
Stir-fried silver beet leaves and standard yoghurt	209.1 ± 0.2	2.29 ± 0.66	1.10
Stir-fried silver beet leaves and low fat yoghurt	209.7 ± 0.2	1.87 ± 1.35	0.89
Analysis of variance	NS	***	***

Significance: *** P<0.001, ** p< 0.01, * P<0.05, NS = non significant

The mean bioavailability of total oxalates was 2.41% in the stir-fried silver beet leaves, which was not significantly different from either the standard yoghurt or the low fat yoghurt meals. The mean bioavailability was reduced to 1.10% after ingestion of the standard yoghurt meal and to 0.89% after consumption of the low fat yoghurt meal. None of the results were significantly different from each other. However, the addition of low fat yoghurt was only marginally effective in lowering soluble oxalates.

4.4 Mineral analysis

The cations analysed were selected after considering the likelihood of their binding to oxalate based on the solubility product constant (K_{sp}) and concentration of minerals present (Appendix 5). The K_{sp} values can be used to determine the conversion of soluble oxalates to insoluble oxalates by addition of metal ions and show the equilibrium existing between the ions in the saturated aqueous solution and the solid ionic solution. K_{sp} values used were based on calculated values for oxalate salts and metal ions in silver beet leaves (Lide, 2007-2008; Simpson *et al.,* 2009) and were obtained for Ca^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+} and Mn^{2+}. According to the low K_{sp}

value and the high concentration of calcium in food, it is most likely that calcium would bind to the oxalate ion and the insoluble oxalates were therefore assumed to be made up of calcium oxalate (CaOx). The other five cations analysed were not considered to be relevant due to high K_{sp} values or due to the low levels present in the test meals (see Table 7).

The mineral analyses on the six selected cations are presented as mg/100 g FW± SE. The highest levels of calcium were found in the low fat yoghurt meal (75.9 ± 0.1 mg/100 g FW) followed by the standard yoghurt meal (64.6 ± 0.01 mg/100 g FW) then the stir-fried silver beet leaves meal (42.8 ± 0.01 mg/100 g FW). The stir-fried silver beet meal contained 14.46 g soy sauce, which contributed 1.8 mg calcium (see Appendix 6). Overall, the mineral composition was similar for the different test meals. See Appendix 7 for results presented on a dry matter basis.

Table 7. Selected cations (mg/100 g FW ± SE) in the three test meals

Sample/mineral	Ca	Mg	Fe	Mn	Zn	Cu
Stir-fried silver beet leaves	42.8 ± 0.01	26.8 ± 0.01	1.2 ± 0.01	1.1 ± 0.01	0.2 ± 0.01	0.2 ± 0.01
Stir-fried silver beet leaves and standard yoghurt meal	64.6 ± 0.01	48.7 ± 0.01	1.2 ± 0.01	1.1 ± 0.01	0.2 ± 0.01	0.2 ± 0.01
Stir-fried silver beet leaves and low fat yoghurt meal	75.9 ± 0.10	48.8 ± 0.01	1.2 ± 0.01	1.1 ± 0.01	0.2 ± 0.01	0.2 ± 0.01*

* = only two determinations

The calcium contents were also calculated based on the calcium contents (136 mg/100 g DM for standard yoghurt and 205 mg/100 g DM for low fat yoghurt) stated on the table of contents on the yoghurt packages. The values provided by the manufacturer and the measured calcium content in the yoghurt can be seen in Appendix 8.

The calculated % of total calcium bound as insoluble oxalates ranged from 26-63%, if it is

assumed all insoluble oxalates were found as calcium oxalates (Table 8).

Table 8. Total calcium, bound calcium as calcium oxalate, unbound calcium and bound calcium as % of total calcium in the test meals (mg/meal FW)

Sample	Total calcium	Bound calcium (as calcium oxalate in the meal mg/meal)	Unbound calcium (mg/meal)	Bound calcium (% of total calcium)	Bound calcium (% of total calcium calculated from the pH speciation diagram
Stir-fried silver beet leaves	49.28 ± 0.13	31.45	17.85	63	72
Stir-fried silver beet leaves and standard yoghurt meal	148.48 ± 0.18	46.46	102.02	31	29
Stir-fried silver beet leaves and low fat yoghurt meal	174.81 ± 0.33	46.00	128.81	26	16

5. Discussion

5.1 Chemical composition

There was a higher fat content in the stir-fried silver beet meals than in the yoghurt meals. During stir-frying of the meals, 28 mL of fat (25.8 g) was added and was the source of the main fat content in the meals (3.6 and 1.3% fat were added to the standard and the low fat yoghurt meals, respectively). Addition of yoghurt diluted and changed the composition of nutrients in the yoghurt meals compared to the stir-fried silver beet meal. Yoghurt not only contained fat but it also contained protein and carbohydrates, which effectively reduced the fat content of the overall yoghurt containing meals.

5.2 Mineral analysis

Stir-fried silver beet leaves contained relatively high levels of calcium and magnesium; the levels of these two minerals increased following the addition of yoghurt. It should always be remembered that soluble oxalates reduce the amount of calcium which can be absorbed in the small intestine. This is important since calcium and magnesium are essential elements in the diet (Hodgkinson, 1977).

The overall calcium value was lower in the yoghurt test meals than in the stir-fried silver beet meals due to the dilution effect on the cooked silver beet leaves when yoghurt was added. Fat, protein and lactic acid, found in yoghurt, contributes to the dilution of calcium and oxalate contents.

The pH of the food and food mixes was a very important parameter. A low pH protonates the oxalate ion and, thereby, makes the oxalate unavailable for binding to divalent minerals such as calcium, iron, zinc and magnesium. The maximum bioavailability of the free oxalate ion ($C_2O_4^{2-}$) is found at a neutral pH (Simpson *et al.,* 2009). According to the low pH present in the standard yoghurt and low fat yoghurt meal (4.08 ± 0.02 respectively 4.25 ± 0.03) these test meals contained lower proportions of soluble oxalates than the stir-fried silver beet leaves test meal. This was due to the addition of yoghurt, which made the test meals more acidic and increased the proportions of the insoluble oxalic acid ($H_2C_2O_4$) and the semi-dehydro-oxalic acid ($HC_2O_4^-$). The proportions of soluble oxalates were only slightly higher in the standard yoghurt meal than in low fat yoghurt meal.

Calcium was assumed to be the only mineral constituent in the insoluble oxalates due to the high concentration present in the leaves and yoghurt, and the low K_{sp} value which meant that it will combine strongly with soluble oxalates in the food. In contrast, iron, zinc and magnesium were present in low concentrations in these foods and have low K_{sp} values so were unlikely to combine with soluble oxalates at these pH values.

The calculated % of total calcium bound, as calcium oxalate, ranged from 26 - 63% of the insoluble oxalate content in the test meals. This was lower than the value (76.7%) previously reported by Brogren and Savage (2003) for grilled spinach.

The fractions of oxalate species present might also change, due to pH changes, when the food goes into the acidic stomach and again when the food goes into the alkaline conditions found in the small intestine.

The expected and the measured fractions of oxalates are presented in Table 9. A pH speciation diagram for oxalic acid (Figure 1) was used to determine the fraction of oxalate species, depending on the pH of the three test meals (Simpson *et al.,* 2009). For further calculations, see Appendix 9. The low fat yoghurt meal had a lower pH and contained a higher level of calcium; therefore lower proportions of soluble oxalates were expected.

Table 9. Comparison of the fractions of calculated oxalates with the measured oxalates for the three test meals

		Fraction of oxalates calculated from the speciation diagram			Fraction of measured oxalates	
Test meal	**pH**	**Oxalic acid** $H_2C_2O_4$*	**Insoluble oxalates** **Semi-dihydro-oxalic acid** $HC_2O_4^-$	**Soluble oxalates** **Oxalate ion** C_2O4^{2-}	**Insoluble oxalates** **Semi-dihydro-oxalic acid** $HC_2O_4^-$	**Soluble oxalates** **Oxalate ion** C_2O4^{2-}
Stir-fried silver beet leaves	5.67 ± 0.01	-	0.04	0.96	0.48	0.52
Stir-fried silver beet leaves and standard yoghurt meal	4.08 ± 0.02	-	0.61	0.39	0.71	0.29
Stir-fried silver beet leaves and low fat yoghurt meal	4.25 ± 0.03	-	0.52	0.48	0.70	0.30

*The pH of the stir-fried diets where too low for this fraction to be present.

Bound calcium, as a percentage of total calcium, was also calculated based on the calculated fractions of oxalates from the pH speciation diagram from Figure 1. The percentage of total calcium bound as calcium oxalate calculated from the pH speciation diagram ranged from 16-72% and differs from the determined values (26-63%) shown in Table 8. In the case of the yoghurt meal where, on average, 161.5 mg of calcium was supplied from the yoghurt and the silver beet, an excess of calcium was supplied, which could bind to the free oxalate ion ($C_2O_4^{2-}$) in the test meals (Table 8). It was interesting to note that all meals contained soluble oxalates

which, in theory, should have been bound to the excess calcium available in each of the test meals. Insoluble oxalates are not degraded by bacteria and are excreted in faeces due to their low ability to be absorbed. An experiment to confirm this would involve the analysis of intestinal contents and faeces, which was beyond the scope of this experiment.

5.3 Urine analysis

Considerable variation in individual urine volume and urinary oxalate was observed between the participants in this study. The mean reference blank values for the females and males were determined to be 5.07 mg/6 hours and 5.57 mg/6 hours, respectively. A trend indicated that males excreted more soluble oxalates in the urine than females. Overall, the urinary excretion of oxalate increased following the consumption of 115 g of stir-fried silver beet leaves and the urinary oxalate output decreased highly significantly when low fat yoghurt was added. There was a small different response between the outputs of oxalate in the urine after ingestion of the three test meals. The results of this study also confirmed that the dietary oxalate intake affected the excretion of oxalate in the urine, the reference blank ranged from 2.8-18.9 mg/6 hours (mean 6.35 ± 1.24 mg/6 hours) and increased to a mean of 10.89 ± 0.96 mg/6 hours after ingestions of stir-fried silver beet leaves. The mean urinary output of total oxalates six hours after consumption of stir-fried silver beet leaves was low when compared to the mean urinary output reported for grilled spinach (14.0 ± 3.7 mg/6 hours) (Brogren and Savage, 2003). The absorption of soluble oxalates decreased when yoghurt was added to the food and the results were supported by a study made by Brogren and Savage (2003) feeding spinach and calcium sources as test meals. A low urinary volume increased the concentration of oxalates in the urine and therefore it was important to maintain an efficient water supply during the experimental period.

5.4 Oxalate content and its bioavailability in the test meals

The total oxalate content (209.7 ± 0.1 mg/meal) was found in lower levels than observed for boiled silver beet leaves by Simpson *et al* (2009). However, this study supported the theory that addition of calcium was an efficient way of decreasing soluble oxalates in green leafy vegetables (Brogren and Savage, 2003). In this study, the proportion of soluble oxalates in stir-fried silver beet leaves decreased effectively when yoghurt was added to the test meals.

There are several possible explanations for the lowering of soluble oxalates in the yoghurt test

meals compared to the meal with stir-fried silver beet leaves. Addition of calcium is known to have a significant effect on lowering soluble oxalate in oxalate containing foods (Marshall *et al.,* 1972; Savage *et al.,* 2000). The low fat yoghurt meal contained, both theoretically and practically, higher levels of calcium than the standard yoghurt meal. When the test meal was made more acid by addition of yogurt the fractions of the free oxalate ion ($C_2O_4^{2-}$) increased, and, by dilution, the amount of oxalate decreased in the yoghurt test meals. The proportions of soluble and insoluble oxalate varied due to the pH of the test meals and the expected fractions of oxalate species were calculated based on a pH speciation diagram (Figure 1) presented for oxalic acid (Simpson *et al.,* 2009).

Previous studies have shown that not all soluble oxalates may be bound to calcium, as in this study, even though the meal contained an excess of soluble calcium. A possible explanation for this might be the compartmentalization of oxalates in the leaves (Radek and Savage, 2008; Simpson *et al.,* 2009).

The bioavailability of oxalates in stir-fried silver beet leaves were higher than found in previous studies, where the mean absorption of oxalate after ingestion of stir-fried silver beet leaves was determined to 2.41% over a 6-hour period. Brogren and Savage (2003) determined the mean bioavailability of soluble oxalates in grilled spinach at 0.75 ± 0.48%, while Albihn and Savage (2001) determined the bioavailability oxalate from oca at 1.44 ± 1.31%, both over a 6-hour period after intake. Addition of standard yoghurt to silver beet leaves decreased the absorption of oxalates to 1.10%, and to 0.89% after the addition of low fat yoghurt; both were equally effective at lowering soluble oxalates. The insoluble oxalates formed lacked the ability to be absorbed in the body; instead they were either degraded by bacteria or excreted in faeces.

Overall, eating silver beet leaves increased the urinary oxalate level by approximately 58% compared to the reference blank value for each person. Addition of calcium from standard yoghurt and low fat yoghurt decreased the absorption of oxalate. The low fat yoghurt seemed to be slightly more efficient in lowering soluble oxalates in stir-fried silver beet leaves. This was probably due to the higher calcium content and lower fat content in the low fat yoghurt meal. The differences in pH seemed to have a very small effect on the bioavailability of oxalates. The pH changed the proportion of oxalate species present; however, calcium seemed to have a greater impact on the bioavailability of soluble oxalates than pH changes.

6. Conclusions

The total and soluble oxalate of stir-fried silver beet leaves in this study were approximately 75% lower than previously reported values for boiled silver beet leaves (Savage *et al.,* 2004). This indicates that the cooking procedure, used in this study, was effective in lowering the amount of total and soluble oxalate in silver beet leaves. Stir-frying usually included cooking in fat, which contributed to the changes in oxalate content. Further studies need to be carried out to clarify the effect of fat to distinguish it from the effect of addition of calcium. This study confirmed that addition of calcium to foods rich in oxalates decreases the bioavailability of soluble oxalate. Addition of yoghurt to green leafy vegetables is an efficient way to reduce soluble oxalates. Consuming a combination of stir-fried silver beet leaves with yoghurt is unusual but, in this case, the combination was used to utilise the available calcium in the yoghurt. At a more acid pH the proportion of the semi-dehydro-oxalic acid in the mix increased; this was then more likely to combine with calcium. The combination of stir-fried silver beet leaves and yoghurt was a little challenge for some of the participants in the feeding trial. If this experiment is repeated then another combination of silver beet and calcium containing food may need to be used.

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Appendix

Appendix 1a - Raw data for chemical analysis on a dry matter basis

Chemical composition (g/100 g DM)

Meal (g)	Residual DM	Ash	Fat	Protein	Acid detergent fibre	Neutral detergent fibre
	92.5	9.4	45.3	20.2	5.2	9.7
Stir-fried silver beet leaves	92.4	9.4	47.5	19.7	5.5	9.9
Stir-fried silver beet leaves	92.5	9.4	45.0	20.2	5.1	9.7
Stir-fried silver beet leaves	**92.5**	**9.4**	**45.9**	**20.0**	**5.3**	**9.8**
	0.0	**0.0**	**0.8**	**0.2**	**0.1**	**0.1**
	92.0	5.8	37.1	25.6	4.1	9.7
Standard yoghurt meal	92.1	5.8	37.1	25.6	4.0	10.2
Standard yoghurt meal	92.1	5.8	36.8	25.6	4.0	9.9
Standard yoghurt meal	**92.1**	**5.8**	**37.0**	**25.6**	**4.0**	**9.9**
	0.0	**0.0**	**0.1**	**0.0**	**0.0**	**0.1**
	90.6	6.5	29.5	29.5	2.6	6.4
Low fat yoghurt meal	90.5	6.5	29.7	29.5	2.7	6.1
Low fat yoghurt meal	90.6	6.5	29.6	29.1	2.7	5.4
Low fat yoghurt meal	**90.6**	**6.5**	**29.6**	**29.4**	**2.7**	**6.0**
	0.0	**0.0**	**0.1**	**0.1**	**0.0**	**0.3**

Appendix 1b – Total oxalates and pH values for the three test meals

Test meal	Total oxalate (mg/meal FW ± SE)	pH
Stir-fried silver beet leaves	209.7 ± 0.1	5.67 ± 0.01
Stir-fried silver beet leaves and standard yoghurt meal	209.1 ± 0.2	4.08 ± 0.02
Stir-fried silver beet leaves and low fat yoghurt meal	209.7 ± 0.2	4.25 ± 0.03

Appendix 2 Guidelines for feeding experiment

Green leafy vegetables such as silver beets contain high amount of oxalates which can cause health problems like kidney stone formation or decreasing the absorption of minerals. Kidney stones are formed due to free soluble oxalates ending up in the kidney and binding to calcium where they form insoluble calcium oxalate. Absorption of oxalates can be reduced by avoiding food containing high amounts of oxalates, such as spinach, silver beet leaves, rhubarb and tea. Studies have also shown that the levels of oxalates also can be reduced by eating the food together with a high calcium containing food. Less oxalate will then be absorbed due to binding between oxalates and free calcium in the gut, which makes oxalates insoluble and thereby passes through the intestinal tract without absorption.

It has also been suggested that oils and fats in foods can affect the way oxalates are absorbed in the digestive tract. Until recently the two effects have been confused together since milk products contain both calcium and milk fat.

The purpose of this study is to set up a test meal feeding experiment serving high fat yoghurt and low fat yoghurt separately with a standard diet of silver beet leaves, containing moderate to high levels of soluble oxalates. The effect of the diets consumed is measured by measuring the output of soluble oxalates in the urine. A reduction of output in soluble oxalates indicates that soluble oxalates are bound to calcium and, thereby, passing through the intestinal tract without absorption.

Twice a week, you are asked to come to **Food preparation room, 4th floor (Hilgendorf building)** between **8 am and 9 am** to follow the eating instructions accordingly. After consumption of the test meal you will be given a container to collect urine for six hours *exactly from the time when you consume the test meal*. Please return the bottle to me (room 429) before 4.30 pm the same day. The feeding experiment will take place on the following days (if it is not possible for you to participate during any of the dates, place contact me so we can arrange another time for you):

Thursday 17th of Sept. Control (only urine collection)

Tuesday 22nd of Sept. Test meal 1 + urine collection

Thursday 24st of Sept. Test meal 2 + urine collection

Tuesday 29th of Sept Test meal 3 + urine collection

During the day and the night before, please **do not eat foods like silver beet, spinach, rhubarb, orange juice or tea,** which are rich in oxalates, and also try to avoid consumption of **peanuts, chocolate and strawberries**, which contain moderate amounts of oxalates. Please also avoid food rich in calcium, such as **milk products**, the night before and during the test period. Do not eat breakfast on the test morning; do drink at least half cup of water every hour during the test day.

The results from the experiment will be presented at the end of December.

Do not hesitate to contact me if you have any further questions.

Thanks for your participation!

Appendix 3 - Enzymic analysis of oxalate

Trinity biotech

OXALATE
(Procedure No. 591)

INTENDED USE

Trinity Biotech Oxalate reagents are for the quantitative, enzymatic determination of oxalate in urine at 590 nm.

SUMMARY

Oxalate was confirmed as a normal constituent of urine in 1951, but only recently has the significance of calcium oxalate crystalluria and its relationship to urinary tract stone formation been fully recognized. Formation of the sparingly soluble calcium salt of oxalate in the urinary tract is considered the major factor in urolithiasis.[2] Oxalate in urine may arise either as an end-product of intermediary metabolism or from dietary sources. A decreased excretion of oxalate in the urine is associated with hyperglycinemia and hyperglycinuria.[3] An increased excretion of oxalate can be attributed to increases in ingestion of oxalate precursors or oxalate rich foods, formation of oxalate due to metabolic defects such as in primary hyperoxaluria, and absorption of oxalate in a number of gastrointestinal disorders that produce severe fat malabsorption. This latter group includes patients with inflammatory bowel disease, ileal resection, biliary diversion, pancreatic insufficiency, sprue, small intestinal stasis with bacterial overgrowth, and following jejunoileal by[illegible]or resection for the treatment of obesity.[4-12]

Currently, urinary oxalate determination is performed by procedures based on isotope dilution, gas and ion chromatography, as well as coupled enzyme reactions.[1,13] These procedures are very time consuming and may require equipment not readily available in the clinical laboratory. The enzymatic method described below is based on the oxidation of oxalate by oxalate oxidase followed by measurement of hydrogen peroxide (H_2O_2) produced during the reaction by a peroxidase-catalyzed reaction.[14] The procedure is specific for oxalate. It requires no special equipment and is easily adaptable for use on clinical automated analyzers.

PRINCIPLE

The enzymatic reactions involved in the assay procedure are as follows:

$$\text{Oxalate} + O_2 \xrightarrow{\text{Oxalate Oxidase}} 2CO_2 + H_2O_2$$

$$H_2O_2 + \text{MBTH} + \text{DMAB} \xrightarrow{\text{Peroxidase}} \text{Indamine Dye} + H_2O$$

Oxalate is oxidized to carbon dioxide and hydrogen peroxide by oxalate oxidase. The hydrogen peroxide reacts with 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino) benzoic acid (DMAB) in the presence of peroxidase to yield an indamine dye which has an absorbance maximum at 590 nm. The intensity of the color produced is directly proportional to the concentration of oxalate in the sample.

REAGENTS

OXALATE REAGENT A

DMAB	3.2 mmol/l
MBTH	0.22 mmol/l
Buffer	pH 3.1 ± 0.1
Nonreactive ingredients and stabilizers	

OXALATE REAGENT B

Oxalate Oxidase (Barley)	3000 u/l
Peroxidase (Horseradish)	100,000 u/l

SAMPLE DILUENT

EDTA	10 mmol/l
Buffer	pH 7.6 ± 0.1

SAMPLE PURIFIER TUBES

Activated Charcoal

PRECAUTIONS:

The reagents are for "in vitro diagnostic use". Normal precautions exercised in handling laboratory reagents should be followed. Dispose of waste observing all local, state and federal laws.

Oxalate Reagent A is an IRRITANT. Irritating to eyes respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing.

CAUTION: Avoid contact and inhalation of Oxalate Reagent B.

Refer to Material Safety Data Sheets for any updated risk, hazard or safety information.

PREPARATION:

Reconstitute Oxalate Reagent A with volume of deionized water indicated on vial label. If reagent is to be used in a discrete analyzer, please refer to the respective application procedure for reagent preparation instructions. After the addition of water, stopper the vial and mix until it is completely dissolved.

Reconstitute Oxalate Reagent B with volume of deionized water indicated on vial label. Stopper the vial and immediately mix by gentle inversion. DO NOT SHAKE.

Sample Diluent is prepared as follows. Remove a sample diluent label from the kit and affix onto a clean dry container of appropriate size. Transfer the entire powder from a vial into the newly labeled container and add volume of deionized water indicated on the vial label. After addition of water, cap the container and immediately mix several times by inversion.

STORAGE AND STABILITY:

Store the dry reagents refrigerated (2-8°C). Reagents are stable until the expiration date indicated on the labels. Store the sample purifier tubes at room temperature (18-26°C)

Reconstituted Oxalate Reagent A is stable for 1 day at ambient temperature (18 - 26°C) and 1 month when stored refrigerated (2-8°C).

Reconstituted Oxalate Reagent B should be used immediately upon reconstitution. Alternatively it can be stored for 2 days at 2-8°C or aliquoted and stored at -20°C for 28 days. Each aliquot to be used once and not refrozen.

Reconstituted Sample Diluent is stable for 1 week at ambient temperature (18 - 26°C) and 3 months when stored refrigerated (2-8°C).

NOTE: Warm Oxalate Reagent B to approximately 37°C in order to dissolve any crystalline material which may form during storage in the refrigerator.

DETERIORATION:

Do not use dry Oxalate Reagent A, Oxalate Reagent B, or Sample Diluent if they indicate any moisture penetration.

Reconstituted Oxalate Reagent A is not suitable for use if the initial absorbance of the freshly reconstituted reagent measured in a 1 cm lightpath at 590 nm vs water as reference is greater than 0.2.

The reconstituted reagents should be clear and free of particulate matter. If the reagents develop haziness due to bacterial contamination, they should be discarded.

DISCRETE ANALYZER APPLICATIONS

Application procedures using Trinity Biotech Oxalate reagents are available for various automated instruments. Please contact Trinity Biotech Technical Services Department for more information.

SPECIMEN COLLECTION AND PREPARATION

A 24-hour urine specimen is collected in a glass or plastic bottle containing 10 ml concentrated hydrochloric acid. Record the volume in litres. Oxalate in acidified urine is stable for 7 days when stored refrigerated or frozen. Ascorbic acid (vitamin C) at a very high concentration (exceeding 16 mmol/l) can interfere. It is recommended that patients refrain from taking excessive amounts of vitamin C or vitamin C rich food for at least 48 hours prior to urine collection. Prior to assay, dilute urine with equal volume of Sample Diluent. Please refer to sample preparation instructions given under "Manual Procedure" section for details.

INTERFERING SUBSTANCES:

Excessive amount of vitamin C in urine (exceeding 16 mmol/l) may affect the test results.

MANUAL PROCEDURE

MATERIALS PROVIDED:

Oxalate Reagent A
Oxalate Reagent B
Sample Diluent
Sample Purifier Tubes

MATERIALS REQUIRED, BUT NOT PROVIDED:

Spectrophotometer, with temperature controlled cuvette compartment, capable of accurately measuring absorbance at 590 nm
Pipeting devices for the accurate delivery of volumes required for the assay
Timer
Plastic or glass container
Oxalate Standard (0.5 mmol/l), Catalogue No. 591-3
Centrifuge or Whatman filter paper

PROCEDURE:

Sample Preparation:

1. Prepare Sample Diluent according to instructions.
2. Set up a series of labeled tubes for urine sample and Controls.
3. Pipette 5 ml or any suitable volume of urine Samples and Controls into appropriately labeled tubes.
4. Add equal volume (as in Step 3) of Sample Diluent into each tube and mix.
5. Check the pH. It should be between 5.0 and 7.0. If not, adjust the pH using 1 N hydrochloric acid or 1 N sodium hydroxide.
6. Set up a series of sample purifier tubes for urine Samples and Controls.
7. Pipette 2 ml each of diluted urine Samples and Controls to appropriately labeled sample purifier tubes and mix for approximately 5 minutes by intermittent mixing. A rotator mixer is recommended for mixing.
8. Centrifuge the tubes for 5 minutes at 2000 rpm (1500 x g) or filter using Whatman filter paper.

Determine the oxalate concentration in the supernatants as described below.

Determination of Oxalate:

1. Warm oxalate reagents to assay temperature (any temperature between ambient and 37°C).
2. Label tubes for Reagent Blank, Standard, urine Control and urine Sample.
3. Pipette 1 ml Oxalate Reagent A into each tube.
4. Pipette 50 µl of Supernatants or Filtrates ("Sample Preparation" section, Step 9), to respective tubes. Add 50 µl deionized water to Reagent Blank tube and 50 µl standard to tube labeled Standard.
5. Pipette 0.1 ml of Oxalate Reagent B into each tube and immediately mix by gentle inversion.
6. Incubate the tubes at desired temperature (18 - 37°C) for 5 minutes.
7. Read absorbances (A) of Blank, Standard, Control and urine Sample at 590 nm.
8. Determine the corrected absorbances (ΔA) of Standard, Control and Sample by subtracting Reagent Blank absorbance from the absorbance readings of Standard, Control and urine Sample.
9. To determine oxalate concentration in urine Sample, refer to "Calculations" section.

CALIBRATION:

The procedure is calibrated using aqueous Oxalate Standard, Catalog No. 591-3. The concentration of oxalate in the sample is determined by comparing absorbance of the sample to that of the Oxalate Standard. Alternatively, the concentration of oxalate in unknown sample can also be extrapolated from a standard curve prepared using multi-level Oxalate Standard Set, Catalogue No. 591-11.

QUALITY CONTROL:

The reliability of test results should be monitored by routine use of urine controls of known oxalate concentrations such as Trinity Biotech Oxalate Urine Control-E (elevated) and Control-N (normal), Catalogue Nos. O 6502 and O 6627, respectively. The oxalate concentration determined by this procedure should fall within the stated range of the controls.

CALCULATIONS

Determine oxalate concentration in sample as follows:

$$\text{Oxalate (mmol/l)} = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Standard}} \times 0.5 \times 2$$

Where:

0.5 = Concentration (mmol/l) of oxalate in standard
2 = Dilution factor

Quantity of Oxalate Excreted During 24-Hour Period = Oxalate (mmol/l) x Volume of Urine Voided during 24 hours (l)

EXAMPLE:

Volume of Urine Voided during 24 hours = 1.43 l

A_{BLANK} = 0.042
$A_{STANDARD}$ = 0.751
A_{SAMPLE} = 0.172
ΔA_{SAMPLE} = 0.172 - 0.042 = 0.130
$\Delta A_{STANDARD}$ = 0.751 - 0.042 = 0.709

Urine Oxalate (mmol/24 hr) =

$$\frac{0.130}{0.709} \times 0.5 \times 2 \times 1.43 = 0.262 \text{ mmol/24 hours}$$

Multiply concentration in mmol by 90 to obtain oxalate excretion in mg/24 hr.

LIMITATIONS

The reagents can measure urinary oxalate concentration up to 2 mmol/l without further diluting the sample. If oxalate concentration in urine exceeds the upper limit of linear range, dilute 1 part sample with 1 part deionized water and reassay. Multiply the result by 2 to compensate for the dilution.

EXPECTED VALUES

Expected ranges were established using an oxalate reagent similar to the described product. Twenty-four hour urine specimens from 108 clinically healthy adult males and females and 12 children from 7 - 14 years old, on unrestricted diets were assayed for oxalate by a similar method. The mean value obtained for 67 adult males was 26 mg/24 hr and for 41 adult females was 17 mg/24 hr. The mean value obtained for children was 26 mg/24 hr. Normal ranges were calculated as the mean ± 2SD (SD = 9 for males, 7 for females, and 6 for children). Normal range for children under the age of 7 has not been determined. The expected ranges for oxalate concentration for adult males, adult females and children were determined to be as follows:

	mg/24 hr	mmol/24 hr
Adult Males	7-44	0.08-0.49
Adult Females	4-31	0.04-0.32
Children	13-38	0.14-0.42

Similar normal ranges have been reported in the literature. Yriberri and Posen,[15] using an oxalate decarboxylase-formate dehydrogenase, reported a range of 18 to 47 mg oxalate excreted per 24 hours for a mixed adult population. Biggs and Watts,[16] using an isotope dilution method, reported a mean oxalate excretion rate of 33 mg per 24 hours (SD 6.5) for adult males and 35 mg per 24 hours (SD 6.7) for adult females. Similar values were reported by Hodgkinson and Williams[17] for adults, but children under age 14 excreted 30 - 50% less per 24 hours. Pik and Kerckhoffs,[18] however, reported oxalate excretion in children being very similar to that of adults with values between 10 and 45 mg per 24 hours. It is recommended that each laboratory establish its own normal range.

PERFORMANCE CHARACTERISTICS

COMPARISON:

A total of 30 urine specimens with oxalate concentrations ranging from 0.1 - 1.2 mmol/l was assayed by the described method and by a similar procedure. Comparisons of oxalate values obtained by both the procedures yielded a correlation coefficient of 0.99 and the regression equation was y = 0.94x + 0.008.

SENSITIVITY:

An absorbance change of 0.150 measured in a 1-cm lightpath corresponds to oxalate concentration of 0.1 mmol/l when a spectrophotometer typically found in a clinical laboratory is used for the measurement under the stated conditions.

PRECISION:

Within-run and run-to-run precision studies yielded the following data.

	Within-Run		
	Urine 1	Urine 2	Urine 3
Mean (mmol/l)	0.17	0.70	1.28
Standard Deviation (mmol/l)	0.00	0.01	0.01
Coefficient of Variation (%)	1.45	0.94	0.96
Number of Assays	20	20	20

	Run-to-Run		
	Urine 1	Urine 2	Urine 3
Mean (mmol/l)	0.16	0.70	1.29
Standard Deviation (mmol/l)	0.00	0.01	0.01
Coefficient of Variation (%)	2.18	1.69	1.03
Number of Assays	20	20	20

RECOVERY STUDIES:

Known amounts of oxalate were added to 59 urine specimens and the oxalate concentration in these samples was determined by this procedure to obtain percentage of oxalate recovery. The recovery of added oxalate ranged from 93-107%. The mean recovery was 100.5%.

Trinity Biotech warrants that its products confirm to the information contained in this and other Trinity Biotech publications. Purchaser must determine the suitability of the product for its particular use.

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ORDERING INFORMATION

KITS

Catalogue Number	591-C	591-D
Maximum Assays	20	100
Contents - Catalogue No.		
Oxalate Reagent A, 591-10	2x10 ml	10x10 ml
Oxalate Reagent B, 591-2	2 ml	5x2 ml
Sample Diluent, 591-4	100 ml	5x100 ml
Sample Purifier Tubes, 591-20	20 tubes	-
Sample Purifier Tubes, 591-100	-	100 tubes

INDIVIDUAL REAGENTS

Catalogue No.	Item	Quantity
591-10	OXALATE REAGENT A	10 ml
591-2	OXALATE REAGENT B	2 ml
591-4	SAMPLE DILUENT	100 ml
591-3	OXALATE STANDARD 0.50 mmol/l	25 ml
591-20	SAMPLE PURIFIER TUBES	20 Tubes
591-100		100 Tubes

REAGENT REQUIRED BUT NOT PROVIDED

Catalogue No.	Item	Quantity
591-11	OXALATE STANDARD Set, 2x25 ml each of 0.25, 0.50 and 1.0 mmol/l	6x25 ml

OPTIONAL REAGENTS

Catalogue No.	Item	Quantity
	OXALATE URINE CONTROLS	
O 6502	Elevated	6x5 ml
O 6627	Normal	6x5 ml

CE

Procedure No. 591
046-141
Revised: 2005-07

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Appendix 4 - Raw data for the bioavailability test

Person	Test meal	Urine volume (mL)	Oxalate conc. (µmol/l)	Total oxalate output (µmol/6h)	Urinary oxalate output (mg/6h)	
						Mean males/females
1	Reference	1099.0	45.0	49.5	4.5	
2	Reference	395.7	333.2	131.9	11.9	
3	Reference	240.0	128.6	30.9	2.8	
4	Reference	573.8	94.0	53.9	4.9	
5	Reference	1342.3	40.1	53.9	4.9	
6	Reference	356.8	86.4	30.8	2.8	
7	Reference	719.6	60.5	43.6	3.9	5.07
8	Reference	161.1	413.0	49.8	4.5	
9	Reference	686.2	65.6	45.0	4.1	
10	Reference	482.4	435.6	210.1	18.9	
11	Reference	260.0	314.8	81.8	7.4	
12	Reference	351.7	106.9	37.6	3.4	
13	Reference	707.9	180.4	127.7	11.5	
14	Reference	306.8	133.3	40.9	3.7	7.63
				Mean	**6.4**	
				SE	**1.2**	
1	Stir-fried silver beet leaves	584.1	190.5	111.3	10.0	
2	Stir-fried silver beet leaves	586.3	249.2	146.1	13.2	
3	Stir-fried silver beet leaves	822.3	98.1	80.7	7.3	
4	Stir-fried silver beet leaves	223.7	582.9	130.4	11.7	
5	Stir-fried silver beet leaves	1034.6	80.8	83.6	7.5	
6	Stir-fried silver beet leaves	242.1	360.0	87.2	7.9	
7	Stir-fried silver beet leaves	248.3	319.3	79.3	7.1	9.24
8	Stir-fried silver beet leaves	484.6	407.9	197.7	17.8	
9	Stir-fried silver beet leaves	713.1	208.0	148.3	13.4	
10	Stir-fried silver beet leaves	405.2	322.4	130.7	11.8	
11	Stir-fried silver beet leaves	788.9	180.3	142.2	12.8	
12	Stir-fried silver beet leaves	306.2	233.0	71.3	6.4	
13	Stir-fried silver beet leaves	559.5	329.1	184.1	16.6	
14	Stir-fried silver beet leaves	221.0	452.4	100.0	9.0	12.53
				Mean	**10.89**	
				SE	**0.96**	

1	Standard yoghurt meal	641.3	201.9	129.5	11.7	
2	Standard yoghurt meal	326.8	95.0	31.1	2.8	
3	Standard yoghurt meal	472.2	74.9	35.4	3.2	
4	Standard yoghurt meal	604.8	137.3	83.0	7.5	
5	Standard yoghurt meal	262.2	504.0	132.1	11.9	
6	Standard yoghurt meal	232.0	211.3	49.0	4.4	
7	Standard yoghurt meal	328.0	106.3	34.9	3.1	6.37
8	Standard yoghurt meal	208.2	433.9	90.3	8.1	
9	Standard yoghurt meal	400.6	196.9	78.9	7.1	
10	Standard yoghurt meal	294.9	228.8	67.5	6.1	
11	Standard yoghurt meal	434.3	204.0	88.6	8.0	
12	Standard yoghurt meal	571.6	140.4	80.2	7.2	
13	Standard yoghurt meal	959.5	106.8	102.5	9.2	
14	Standard yoghurt meal	553.2	114.9	63.5	5.7	7.35
				Mean	**6.86**	
				SE	**0.77**	
1	Low fat yoghurt meal	263.93	187.17	49.40	4.45	
2	Low fat yoghurt meal	713.45	67.55	48.19	4.34	
3	Low fat yoghurt meal	796.01	142.72	113.61	10.23	
4	Low fat yoghurt meal	436.43	106.33	46.41	4.18	
5	Low fat yoghurt meal	1020.17	58.71	59.89	5.39	
6	Low fat yoghurt meal	411.79	132.21	54.44	4.90	
7	Low fat yoghurt meal	742.93	118.39	87.96	7.92	5.92
8	Low fat yoghurt meal	264.45	478.87	126.64	11.40	
9	Low fat yoghurt meal	690.92	206.68	142.80	12.86	
10	Low fat yoghurt meal	1041.92	136.09	141.79	12.77	
11	Low fat yoghurt meal	913.04	155.76	142.22	12.80	
12	Low fat yoghurt meal	176.6	311.04	54.93	4.95	
13	Low fat yoghurt meal	875.28	109.27	95.64	8.61	
14	Low fat yoghurt meal	553.22	205.58	113.73	10.24	10.52
				Mean	**8.22**	
				SE	**0.93**	

Appendix 5 - Correlation between selected cations, K_{sp}-value and concentration (mg/100 g DM ± SE) in stir-fried silver beet leaves and yoghurt test meal

Mineral	K_{sp} (Budavari *et al,. 1989)*	Stir-fried silver beet leaves	Standard yoghurt meal	Low fat yoghurt meal
Ca	$2.7 \cdot 10^{-9}$	360.9 ± 11.3	520.3 ± 13.7	580.8 ± 25.5
Zn	$1.7 \cdot 10^{-9}$	5.0 ± 0.1	3.9 ± 0.1	4.1 ± 0.2
Cu	$1.7 \cdot 10^{-7}$	1.5 ± 0.0	0.7 ± 0.0	0.5 ± 0.0
Fe	$1.5 \cdot 10^{-6}$	10.4 ± 0.1	4.6 ± 0.1	3.1 ± 0.2
Mg	$2.2 \cdot 10^{-5}$	232.5 ± 10.7	143.4 ± 6.4	147.6 ± 4.6
Mn	$3.4 \cdot 10^{-5}$	9.8 ± 0.4	3.9 ± 0.3	2.8 ± 0.3

Appendix 6 - Calcium content of the soy sauce in stir-fried silver beet leaves

	% DM	Calcium (mg/100 g)	Calcium (mg/meal)
Soy sauce	33.2	12.35	1.79
Soy sauce	32.6	12.06	1.83
Soy sauce	33.0	11.75	1.70
Mean	**32.9**	**12.05**	**1.80**
SE	**0.2**	**1.7**	**0.04**

Appendix 7 - Mineral analysis for the test meals and the yoghurt mixes

Six selected cations (mg/100 g DM ± SE) in the three test meals and the two yoghurt mixes

Sample/mineral	Ca	Mg	Fe	Mn	Zn	Cu
Stir-fried silver beet leaves	360.9 ± 11.3	232.5 ± 10.7	10.4 ± 0.1	9.8 ± 0.4	5.0 ± 0.1	1.5 ± 0.0
Standard yoghurt meal	520.3 ± 13.7	143.4 ± 6.4	4.6 ± 0.1	3.9 ± 0.3	3.9 ± 0.1	0.7 ± 0.0
Low fat yoghurt meal	580.8 ± 25.5	147.6 ± 4.6	3.1 ± 0.4	2.8 ± 0.3	4.1 ± 0.2	0.5 ± 0.0
Yoghurt mix*	109.4 ± 9.6	13.6 ± 1.1	0.5 ± 0.1	0.01 ± 0.0	0.5 ± 0.0	n.d.
Low fat yoghurt mix	183.3 ± 29.5	17.9 ± 3.5	0.01 ± 0.0	0.02 ± 0.0	0.5 ± 0.1	n.d.

* = only two determinations, n.d. = not detected

Appendix 8 – Values from manufacturer and determined calcium value (mg/100 g FW) in the three test meals

Sample/mineral	Manufacturer´s value[1]	Determined value
Stir-fried silver beet leaves	-	42.8 ± 0.01
Standard yoghurt	81.4 ± 0.1	64.6 ± 0.01
Low fat yoghurt	92.7 ± 0.1	75.9 ± 0.10

[1] Value given by manufacturer on the package (EasiYo Products Ltd., NZ).

Appendix 9 - Calculation of K_{sp}-value for selected cations

$CaC_2O_4 \leftrightarrow Ca^{2+} + C_2O_4^{2-}$

$K_{sp} = [Ca^{2+}] + [C_2O_4^{2-}]$ (saturated solution)

Solubility of calcium oxalate at 20°C = 0.0067g/L = 0.000052 M

Molar ratio of calcium to oxalate = 1:1

$K_{sp} = (0.000052)^2$

$= 2.7 \cdot 10^{-9}$

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