EFFECT OF BLANCHING TIME ON SELECTIVE MINERAL ELEMENTS EXTRACTION FROM THE SPINACH SUBSTITUTE *(Tetragonia expansa)* COMMONLY USED IN BRAZIL[1]

Luciane M. KAWASHIMA[2], Lucia M.VALENTE SOARES[2,*]

SUMMARY

The true spinach *(Spinacia oleracea)* does not grow well in warm climates and for that reason is not commercialized in Brazil. Instead, a spinach substitute *(Tetragonia expansa)*, originally from New Zealand, is widely used in the country. There is scant information on the mineral profile and none on the soluble mineral fraction of this vegetable. The solubility of a mineral is one of the important factors for its absorption. For this reason, the calcium, magnesium, iron, manganese, copper, zinc, potassium, and sodium soluble fractions in the raw spinach substitute were determined and the effect of blanching times on the solubility of these minerals was investigated. Blanching times of 1, 5, and 15 minutes were employed. The magnesium, manganese, potassium, and sodium soluble fractions increased sizably with shorter blanching time. Longer blanching time (15 minutes) caused large losses of minerals. The soluble mineral fractions can contribute poorly to diet in terms of potassium, magnesium, manganese, and zinc. The spinach substitute cannot be considered a dietary source of calcium, iron and copper due to the insolubility of these minerals in the vegetable, possibly caused by the large oxalate content.

Keywords: spinach substitute; effect of blanching; mineral elements.

RESUMO

EFEITO DO TEMPO DE BRANQUEAMENTO NA EXTRAÇÃO SELETIVA DE ELEMENTOS MINERAIS DO SUBSTITUTO DE ESPINAFRE *(Tetragonia expansa)* COMUMENTE EMPREGADO NO BRASIL. O verdadeiro espinafre *(Spinacia oleracea)* não se desenvolve bem em climas quentes e por esta razão não é comercializado no Brasil. Em seu lugar, um substituto de espinafre *(Tetragonia expansa)*, originário da Nova Zelândia, é amplamente utilizado. A informação sobre o perfil mineral é resumida e inexistente sobre a fração solúvel de minerais deste vegetal. A solubilidade de um mineral é um dos fatores importantes para sua absorção. Por esta razão, as frações solúveis de cálcio, magnésio, ferro, manganês, cobre, zinco, potássio e sódio foram determinadas no substituto de espinafre cru, e o efeito dos tempos de branqueamento na solubilidade destes minerais foi investigado. Tempos de branqueamento de 1, 5, e 15 minutos foram empregados. As frações solúveis de magnésio, manganês, potássio, e sódio, aumentaram consideravelmente com o menor tempo de branqueamento. Um período mais prolongado de branqueamento (15 minutos) causou perdas sensíveis de minerais. As frações solúveis dos minerais podem ter pequena contribuição para a dieta em termos de potássio, magnésio, manganês, e zinco. O substituto de espinafre não pode ser considerado como uma fonte de cálcio, ferro e cobre para a dieta devido à insolubilidade destes minerais no vegetal, possivelmente causada pelo elevado teor de oxalato.

Palavras-chave: substituto de espinafre; efeito de branqueamento; elementos minerais.

1 - INTRODUCTION

In Brazil, a spinach substitute *(Tetragonia expansa)* is used for salads and cooking. It originated in New Zealand and is a succulent belonging to the Aizoaceae family. It grows well in tropical climates and for that reason it well adapted in this country. The true spinach *(Spinacia oleracea)*, on the other hand, a leafy vegetable of the Chenopodiaceae family, is commonly consumed in the temperate regions of the globe [5] and does not grow well in warm climates. The true spinach is not commercialized in Brazil. The concentrations of a few nutritionally important minerals and their absorbability have been studied for the true spinach *(Spinacia oleracea)* [2,8,14]. Recently, the mineral composition for the New Zealand spinach substitute used in Brazil and of other leafy vegetables has been published [9].

The presence of a nutrient in the food does not mean availability. The absorption of mineral nutrients is dependent upon their chemical form and the presence of enhancers or inhibitors within the food as well as the nutritional status and requirements of the individual ingesting the food [3,4,6]. *In vitro* and *in vivo* tests have been used to measure mineral availability. *In vitro* methods involve a simulation of the digestive process and the information supplied is partial because it does not include the utilization of the mineral by the human organism. *In vivo* studies measure either radioactive or stable isotopes in human subjects or animals [1,3,12]. They are closer to the natural absorption process although lengthier and more expensive to be conducted than *in vitro* methods. Estimates of availability through mathematical models that take into account the concentrations of minerals and those of absorption inhibitors and enhancers have also been proposed [20].

Solubility of the mineral is one of the conditions for absorption by the digestive tract and its knowledge provides initial information about its potentiality for absorption [2,10]. Solubility measurements are simpler and less costly to be conducted than *in vivo* and *in vitro* studies. In this line TESSIER, CAMPBELL & BISSON [18] proposed an interesting protocol first used for the study of river sediments. It consists of a simplified method by extracting selectively minerals under soluble form,

bound to ligands with $pK \geq 5$, bound to ligands with $pK \geq 2$, bound to organic insoluble ligands, and residual. This approach has been applied to experimental animal diets and gastrointestinal contents in the study of iron digestion and absorption in rats [17]. REYKDAL & LEE [14] followed another approach and measured dialyzable calcium in milk and true spinach in samples submitted to HCl and pepsin *in vitro* digestion and non-digested samples. The dialyzable calcium content was more than twice as large in digested samples when compared with non-digested samples. SCHMIDT, MACDONALD & KELLY [16], by their turn, measured iron, calcium, and magnesium in water extracts and in extracts submitted to *in vitro* peptic digestion obtained from raw and cooked amaranth and collard leaves. The results from both procedures were close for the raw samples, but in the case of the cooked samples the results were twice as large in the peptic digested extracts when compared with the water extracts.

Oxalates are an important inhibitor to mineral absorption because they form insoluble salts with calcium, iron, and magnesium. The presence of oxalates in foods is of concern, especially for calcium, as they reduce the absorbability of this mineral [19]. Calcium absorbability from true spinach, a high oxalate food, has been shown to be low for human subjects [8]. The amount of oxalate present in the raw New Zealand spinach substitute, also used in Brazil, has been determined and it was approximately 5 times higher ($1764.7 \pm 95.7mg/100g$) than that of the true spinach ($329.6 \pm 0.8mg/100g$) [15]. Note that the standard deviation indicates the variability encountered in nature and not the uncertainty of the determination.

The present research aimed at (a) fractionating six nutritionally important minerals (Ca, Mg, Mn, Fe, Zn and Cu) and two electrolytes (K and Na) in the spinach substitute used in Brazil in order to verify the distribution profile of the minerals in the soluble fraction (extractable at pH 7.0), in the fraction bound to insoluble ligands (capable of solubilization by a mild peroxide and acid treatment), and in the residual fraction (resistant to mild peroxide and acid treatment) and (b) the effect of increasing blanching times on the mineral profile of these fractions and on possible losses by leaching.

2 - MATERIAL AND METHODS

2.1 - Sample preparation

Three bunches of spinach substitute *(Tetragonia expansia)* were acquired in markets in the city of Campinas, state of São Paulo, five times during the period of six months. The bunches of leaves were washed under running water to remove sandy particles and then washed with de-ionized water, dried in a hand spinning kitchen vegetable drier, and patted dry with paper towels. Hard stems and wilted leaves were removed. The leaves and tender stems were comminuted, well mixed by hand and about 250g were removed and further homogenized with the help of a food processor. The food processor parts in contact with samples were treated with a hot aqueous solution of 2% EDTA and 2% citric acid [13] in order to remove any trace of metals, washed with de-ionized water and air dried before use.

After washing and drying and before comminuting, 250g portions of the samples, leaves and tender stems, were separated for blanching. The leaves and stems were heated in a glass pan containing 2L of boiling de-ionized water for periods of 1, 5, and 15 minutes, respectively. The blanched vegetable was then dried in a hand spinning kitchen vegetable drier and homogenized in a food processor as described above.

2.2 - Total solids

Duplicate determinations were conducted in raw and blanched samples by drying the ground sample in an aluminum dish in an atmospheric oven at $105°C$ until constant weight. Fifteen mg/cm^2 of diatomaceous earth were added to each aluminum dish for better heat distribution. The total solids determination was used to calculate the minerals concentration on dry basis and to compare the levels of elements among the sequentially extracted fractions.

2.3 - Determination of individual minerals

Total contents of each mineral were determined in duplicates after digestion of the samples with nitric acid. After complete destruction of organic matter, the residue was dissolved with enough nitric acid and lanthanum solution in order to reach a final concentration of 1% HNO_3 + 0.5% lanthanum in a volumetric flask of appropriate size. The solution was used to determine potassium, sodium, calcium, magnesium, iron, copper, manganese, and zinc by flame atomic absorption spectrometry on a microprocessor controlled Perkin-Elmer instrument, model 5100 PC with deuterium background correction lamp. The instrument operating conditions used in determining each element can be found in *Table 1*. An air acetylene flame was used and standards for calibration were from Carlo Erba, Merck and J.T. Baker. The standards were prepared with the same concentration of nitric acid and lanthanum as the samples. De-ionized water (Milli-Q) with 18 megaohm of resistivity was used whenever water was need. Glassware and polyethylene flasks were soaked 24 hours in nitric acid 10% and then washed with de-ionized water before use. The individual concentrations of each mineral in each sample were calculated on wet and dry weight basis. The data on dry basis was used to calculate the concentration of each element in each extraction fraction as well as losses to blanching water for each individual sample.

2.4 - Sequential extraction of minerals

The soluble fractions of individual minerals and individual minerals bound to insoluble ligands were

TABLE 1 - Operating conditions used for determining each element

Element	Wavelength (nm)	Bandwidt (nm)	Burner height (mm)	Air/Acethylene (v/v)
Calcium	422.7	0.7	7-8	10.0/3.0
Magnesium	202.6	0.7	7	10.0/2.0
Iron	248.3	0.2	9	10.0/2.0
Manganese	279.5	0.2	9	10.0/2.0
Copper	324.8	0.7	7	10.0/2.0
Zinc	213.9	0.7	9	10.0/2.0
Potassium	766.5	1.4	9	10.0/2.0
Sodium	589.0	0.4	8	10.0/2.0

determined [18]. The soluble fraction was defined as the relationship between the concentrations of the element extractable at pH 7.0 and its total concentration in the spinach substitute. For the soluble fraction of minerals, three replicates were prepared for each sample. The sample was weighed (10g) into a polypropylene centrifuge tube and shaken one hour with 8mL 0.15M ammonium acetate solution (pH 7,0). After centrifuging 30 minutes at 10,000rpm the supernatant was separated and the residue in the tube washed with 10mL de-ionized water. The tube with water and residue was again centrifuged at 10,000rpm for 15 minutes. The second supernatant was collected and added to the first and this was labeled soluble fraction. Each mineral was determined in the soluble fraction by atomic absorption spectrometry as described for the total minerals content determination.

The fraction bound to insoluble ligands was defined as the relationship between the concentration of the element extractable after mild peroxide and acid treatment and its concentration in the spinach substitute. The minerals bound to insoluble ligands were determined in the residue from the previous extraction by adding 3mL of 0.02M HNO_3 and 5mL of 30% H_2O_2 adjusted to pH 2 with HNO_3. The mixture was heated to 85°C for two hours in a water bath and shaken occasionally. After that period, another 3 mL portion of 30% H_2O_2 was added. The mixture was kept at 85°C for another period of 3 hours with occasional shakings. After that the mixture was allowed to cool and 5 mL of 3.2 M ammonium acetate in 20% HNO_3 were added. The mixture was diluted to 20mL with de-ionized water and shaken for 30 minutes. The contents of the flask were allowed to settle and the supernatant was collected for the minerals determination as described above.

The residual fraction was defined as the relationship between the concentration of the element not extractable after mild peroxide and acid treatment and its total concentration in the spinach substitute. The residual fraction for each element was calculated subtracting the values of soluble and insoluble fractions from the total concentration of the respective element.

Losses of a mineral to the blanching water by leaching were calculated as the difference between the total amount of the element found in the raw sample and the total amount found in the blanched sample. The results were calculated for each individual sampling and averaged.

2.5 - Analytical quality control

The detection limits for each element were determined preparing two solutions of the element, one 5 times and the other 2 times the expected detection limit. Twenty readings were made of each solution in alternation with a blank. The detection limit was calculated multiplying the concentration of the standard by 3 times the standard deviation obtained. The result was divided by the mean of the determinations.

A blank and an internal reference sample were added to each batch of samples analyzed as an every day quality control check. The certified reference material 1573a Tomato Leaves (NIST, Gaithersburg, U.S.A.) was used to check the accuracy of the instrument and of the procedure employed to determine the total values of the elements being studied.

3 - RESULTS AND DISCUSSION

The limits of detection and the analytical uncertainties under the experimental conditions are presented in *Table 2*. The average standard deviation for duplicates of the same sample expresses the analytical uncertainties of the analyses and indicates the number of significant digits of each individual result.

TABLE 2 - Limits of detection and analytical uncertainty under the conditions employed during the present work

	Elements							
	Ca	Mg	Fe	Mn	Cu	Zn	K	Na
Limit of detection (mg/L)	0.04	0.14	0.02	0.02	0.012	0.012	0.03	0.012
Average SD (mg/100g) for duplicates of the same sample	0.21	0.07	0.1	0.002	0.001	0.002	1.2	0.14

Five samplings were conducted during six months and the variation was expressed as the standard deviation among samples *(Table 3)*. A great variability in the concentration of the minerals was found among the samples of the vegetable throughout the six months of study. This variability between samples is of one or two orders of magnitude greater than the analytical uncertainty *(Table 2)*, except in cases the mineral present in the fraction analyzed is at trace level (Fe, Mn, Cu and Zn). Such data gives support to the analytical results and to the conclusions to be derived. Interestingly, the total solids content varied among samples from 4.82 to 7.60g/100g, with an average and standard deviation of 6.08 ± 1.14g/100g. The variation among samples was smaller than the one found also among samples for the minerals present in higher content (Ca, Mg, K and Na).

The concentration of soluble calcium was very low *(Table 3)* in the spinach substitute. A lengthy blanching time such as 15 minutes *(Figure 1)* did not improve the solubility of this mineral. The fraction bound to insoluble ligands but capable of sulubilization by mild acidic and oxidizing conditions was also low *(Figure 2)*. The residual fraction in the raw vegetable contained 98% of the total content of calcium *(Figure 3)* and varied little with

TABLE 3 - Individual element concentrations in the fractions of the raw spinach substitute *(Tetragonia expansa)*

Fraction	Average element concentration (mg/100g, wet mass) and standard deviation*							
	Ca	Mg	Fe	Mn	Cu	Zn	K	Na
Solube	0.5±0.3	20±3	0.06±0.03	0.3±0.3	0.02±0.01	0.12±0.05	201±27	28±6
Solubilized with peroxide and acid	0.6±0.2	21±6	0.2±0.1	0.39±0.35	0.03±0.03	0.16±0.06	189±31	0.16±0.06
Residual	63±21	14±7	0.8±0.8	0.23±0.16	0.02±0.01	0.07±0.05	14±7	35±6

* Standard deviation indicates natural variability among samples.

blanching. In such situation it is expected that calcium in the spinach substitute would not contribute to the diet. The high insolubility of calcium in this vegetable might be explained by the high content of insoluble oxalate as reported for the New Zealand spinach [15].
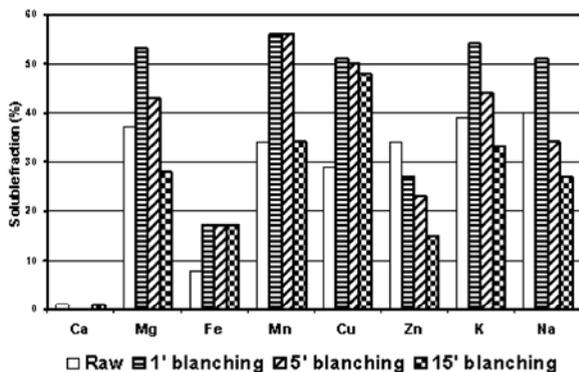

FIGURE 1 - Average changes in the soluble fraction of minerals caused by increasing blanching time. The soluble fraction (%) was defined as the ratio between the concentrations of the element extractable at pH 7.0 and its total concentration in the raw spinach substitute
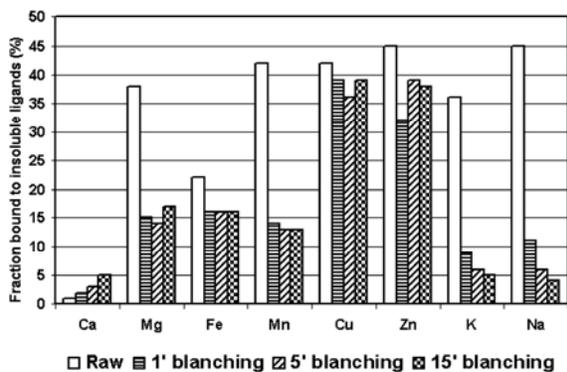

FIGURE 2 - Average changes in the fraction of minerals bound to insoluble ligands caused by increasing blanching time. The fraction bound to insoluble ligands (%) was defined as the ratio between the concentration of the element extractable after mild peroxide and acid treatment and its concentration in the raw spinach substitute
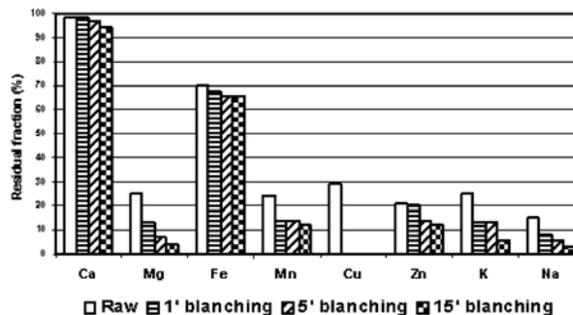

FIGURE 3 - Average changes in the residual fraction of minerals caused by increasing blanching time. The residual fraction (%) was defined as the ratio between the concentration of the element not extractable after mild peroxide and acid treatment and its total concentration in the raw spinach substitute

Magnesium was mostly in soluble form. The soluble fraction of magnesium in the raw vegetable contained 20mg/100g *(Table 2)* and represented about 37% of the total content of the mineral *(Figure 1)*. The RDA for magnesium for adults of both sexes is 4.5mg/kg of body weight [11]. The data obtained on solubility indicated that the contribution of magnesium by the spinach substitute is small. Blanching for 1 or 5 minutes increased the soluble amounts of the mineral *(Figure 1)*. But a noticeable decrease in the soluble fraction occurred with blanching for 15 minutes due to losses of over 50% to the water by leaching *(Table 4)*.

TABLE 4 - Average mineral losses (%) in the spinach substitute *(Tetragonia expansa)* to the water by leaching

Element	Blanching times		
	1'	5"	15"
Ca	Nd	Nd	Nd
Mg	20	36	55
Fe	2	5	5
Mn	14	13	42
Cu	9	14	13
Zn	21	24	35
K	19	35	53
Na	25	51	57

Nd – not detected

For iron, another mineral with solubility affected by the presence of oxalates, a concentration of 0.06mg/100g was found in the soluble fraction of the raw edible parts *(Table 2)*. The soluble fraction represented 8% of the total iron concentration in the raw

vegetable and this fraction doubled with 1 minute blanching. The soluble fraction did not exhibit further increase after 5 or 15 minutes blanching *(Figure 1)*. Losses due to leaching were low *(Table 4)* with any blanching time.

The soluble fraction of manganese *(Table 3)* contained 0.3mg/100g. The recommended daily intake for manganese for adults ranged from 2.0 to 5.0mg [11], independently of body weight, what pointed to a rather low possible contribution to diet by this spinach substitute. The soluble fraction of manganese represented 34% of this mineral in the raw vegetable. The data indicated that blanching caused losses of about 14% when the vegetable was submitted up to 5 minutes blanching and higher (42%) for 15 minutes blanching from the total amount of manganese initially present in the raw vegetable *(Table 4)*.

The levels of copper (0.02mg/100g) in the soluble fraction (45%) of the raw vegetable could only contribute poorly to diet *(Table 3)*. The recommended daily intake for copper ranges from 1.5 to 3mg daily for adults of any body weight [11] and consequently the spinach substitute does not qualify as a source for copper in the diet. The soluble fraction increased with blanching at the expense of the residual and organic bound fractions *(Figures 1, 2 and 3)*. Calculated losses to the blanching water were small and did not increase much with blanching time *(Table 4)*.

The concentration of soluble zinc in the raw vegetable was 0.12mg/100g *(Table 3)*, too low to make any sensible contribution to diet. Zinc deficiency has been a matter of concern for developing countries [7]. The recommended daily intakes for zinc are 12 and 15mg for women and men, respectively, independent of body weight [11]. Zinc was present in the soluble fraction of the raw vegetable at about 34% of the total concentration of the element. Blanching decreased the amount of the element in the soluble fraction *(Figure 1)*. The losses of zinc to the blanching water increased with longer blanching times *(Table 4)*.

The raw vegetable contained 200 mg of soluble potassium /100g *(Table 3)*. The RDA for potassium is 3500mg for adults of any body weight [11] and so the contribution of this element to diet by this spinach substitute is marginal. Surprisingly the soluble fraction contains 39% of the total amount of the mineral indicating that part of the mineral is connected to insoluble structures and is not readily accessible in the raw vegetable. Blanching for 1 minute and 5 minutes increased the concentration of potassium in the soluble fraction to 54 and 44%, respectively, of the total amount of element initially present. At the same time, the fraction bound to insoluble ligands and the residual fraction decreased *(Figures 2 and 3)*. Losses of potassium to the blanching water increased from 25 to 53% with longer blanching times.

The concentration of soluble sodium in the raw vegetable was 28mg/100g and represented 44% of the total sodium present *(Table 3)*. The concentrations of soluble sodium increased with blanching times *(Figure 1)*. Longer blanching times increased losses of sodium to the blanching water and these losses ranged from 26 to 57% *(Table 4)*. Longer blanching times reduced the residual fraction and the fraction bound to insoluble ligands was smaller in the blanched vegetable when compared with the same fraction in the raw spinach substitute, suggesting both fractions served as the source of sodium for the soluble fraction *(Figures 2 and 3)* as leaching increased due to longer blanching.

4 - CONCLUSIONS

For the New Zealand spinach substitute commonly used in Brazil, a shorter blanching time of one minute is the most beneficial in yielding higher concentrations of soluble minerals. However, the vegetable is a poor source for soluble potassium, magnesium, and manganese; an even meager source for zinc and cannot be considered a dietary source for calcium, iron, and copper.

5 - REFERENCES

[1] BARBERÁ, R.; FARRÉ, R. Biodisponibilidad de los elementos traza. **Revista Española de Ciencia y Tecnología de Alimentos**, v. 34, n. 4, p. 381-399, 1992.

[2] BENWAY, D.A.; WEAVER, C.M. Assessing chemical form of calcium in wheat, spinach, and kale. **Journal of Food Science**, v. 50, n. 3, p. 605-608, 1993.

[3] COZZOLINO, S.M.F. Biodisponibilidade de minerais. **Revista de Nutrição da PUCCAMP**, v. 10, n. 2, p. 87-98, 1997.

[4] EKMEKCIOGLU, C. Intestinal bioavailability of minerals and trace elements from milk and beverage in humans. **Nahrung**, v. 44, n. 6, p. 390S-397S, 2000.

[5] ENCYCLOPAEDIA BRITANNICA, INC. **Encyclopaedia Britannica**. 15ᵗʰ edition, Chicago, v. 3, p. 375, 1979.

[6] FAIRWEATHER-TAIT, S.J. Bioavailability of dietary minerals. **Biochemical Society Transactions**, v. 24, p. 775-780, 1996.

[7] GIBSON, R.S. Zinc nutrition in developing countries. **Nutrition Research Reviews**, v. 7, p. 151-173, 1994.

[8] HEANEY, R.P.; WEAVER, C.M.; RECKER, R.R. Calcium absorbability from spinach. **American Journal of Clinical Nutrition**, v. 47, p. 707-709, 1988.

[9] KAWASHIMA, L.M.; VALENTE SOARES, L.M. Mineral profile of raw and cooked leafy vegetables consumed in Southern Brazil. **Journal of Food Composition and Analysis**, v. 16, p. 605-611, 2003.

[10] KIM, H.; ZEMEL, M.B. In vitro estimation of the potential bioavailability of calcium from sea mustard *(Undaria pinnatifida)*, milk, and spinach under simulated normal and reduced gastric acid conditions. **Journal of Food Science**, v. 51, n. 4, p. 957-959, 1986.

[11] NATIONAL RESEARCH COUNCIL. 1989. **Recommended dietary allowances**. National Academy Press, Washington, D.C. 284 p.

[12] RAO, B.S.N. Methods for the determination of bioavailability of trace metals: a critical evaluation. **Journal of Food Science and Technology**, v. 31, n. 5, p. 353-361, 1994.

[13] RAZAGUI, I.B.; BARLOW, P.J. A chemical clean-up procedure to reduce trace metal contamination from laboratory blenders. **Food Chemistry**, v. 44, n. 4, p. 309-312, 1992.

[14] REYKDAL, C.; LEE, K. Soluble, dialyzabel and ionic calcium in raw and processed skim milk, whole milk and spinach. **Journal of Food Science**, v. 56, n. 3, p. 864-866, 1991.

[15] SAVAGE, G.P.; VANHANEN, L.; MASON, S.M.; ROSS, A.B. Effect of cooking on the soluble and insoluble oxalate content of some New Zealand foods. **Journal of Food Composition and Analysis**, v. 13, p. 201-206, 2000.

[16] SCHMIDT, D.R.; MACDONALD, H.A.; KELLY, W.C. Solubility of iron, calcium and magnesium in amaranth and collard leaves. **Qualitas Plantarum, Plant Foods for Human Nutrition**, v. 4, p. 373-378, 1974.

[17] SIMPSON, R.J.; SIDHAR, S.; PETERS, T.J. Application of selective extraction to the study of iron species present in diet and rat gastrointestinal tract contents. **British Journal of Nutrition**, v. 67, p. 437- 444, 1992.

[18] TESSIER, A.; CAMPBELL, P.G.C.; BISSON, M. Sequential extraction procedure for the speciation of particulate trace metals. **Analytical Chemistry**, v. 51, n. 7, p. 844-850, 1979.

[19] WEAVER, C.M.; PLAWECKI, K.L. Dietary calcium: adequacy of a vegetarian diet. **American Journal of Clinical Nutritio**n, v. 59 (suppl), p. 1238-1241, 1994.

[20] WOLTERS, M.G.E.; DIEPENMAAT, H.B.; HERMUS, R.J.J.; VORAGEN, A.G.J. Relation between in vitro availability of minerals and food composition: a mathematical model. **Journal of Food Science**, v. 58, n. 6, p. 1349-1355, 1993.

6 - ACKNOWLEDGEMENT