ORIGINAL ARTICLE

The Effect of Home Cooking Method and Refrigeration Processes on the Level of Nitrate and Nitrite In Spinach

Mitra Keshavarz[1], MSc student; Seyed Mohammad Mazloomi[2]*, PhD; Siavash Babajafari[1], PhD

[1]Department of Nutrition, School of Nutrition and Food Sciences, Shiraz University of Medical Sciences, Shiraz, Iran;
[2]Department of Food Hygiene and Quality Control, School of Nutrition and Food Sciences, Nutrition and Food Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Correspondence:
Seyed Mohammad Mazloomi, PhD; Nutrition and Food Sciences Research Center, Department of Food Hygiene and Quality Control, School of Nutrition and Food Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
Tel: +98 71 37251001
Fax: +98 71 37260225
Email: mazloomi@sums.ac.ir
Received: 3 May 2015
Revised: 23 May 2015
Accepted: 20 June 2015

Abstract

Background: Spinach is one of the vegetables with a high concentration of nitrate which is affected by various processes such as cooking and refrigeration before consumption. The present study aimed to investigate the effect of home cooking method, which has not been studied before, on the level of nitrate and nitrite in spinach. The effect of refrigeration was studied, as well.

Methods: After removing non-edible parts of spinach, it was cut into 4-7 cm pieces. Then, it was washed with deionized water thrice and drained. A part of raw spinach was set aside to measure the nitrate and nitrite and the other part was heated in a closed container without adding water, until almost all the water of the spinach was evaporated and then refrigerated. HPLC method was used to measure the nitrite and nitrate levels. The collected data were analyzed using Wilcoxon test by SPSS.

Results: The mean concentrations of nitrate and nitrite in the raw spinach were 336.54±182.2 and 26.49±10.07 mg/kg, respectively. Cooking process caused a significant increase in the level of nitrate, while refrigeration process led to a significant decrease in this regard. Besides, a slight reduction was observed in the nitrite level after cooking, but the change was not statistically significant. Refrigeration also had no significant effects on the nitrite level of spinach.

Conclusion: Considering the non-significant changes in nitrite levels, it seems that home cooking without adding water, unlike refrigeration, is not a proper method to reduce the intake of nitrates and nitrites from spinach.

Please cite this article as: Keshavarz M, Mazloomi SM, Babajafari S. The Effect of Home Cooking Method and Refrigeration Processes on the Level of Nitrate and Nitrite In Spinach. J Health Sci Surveillance Sys. 2015;3(3):.88-93

Keywords: Nitrate; Nitrite; Cooking; Refrigeration; Spinach

Introduction

Nitrates and nitrites are the compounds naturally found in the environment which are often ingested by human via vegetables, meats and drinking water. Nitrate can convert into nitrite, which is less abundant in the environment by bacterial activity.[1] These two compounds have been known as unwanted compounds in the diet because of their carcinogenic effect,[1,2] causing some diseases such as methemoglobinemia in infants[3,4] and congenital malformations in children whose mother had drunk water with nitrate concentrations greater than 5ppm during pregnancy.[5] Furthermore, increase in the nitrate level leads to oxalate accumulation in leafy vegetables such as spinach which can cause nutrient deficiency and increase the risk of kidney stones.[6] Nevertheless, some other studies have indicated that nitrite accumulation in the blood and tissues is a biological source of NO which can reduce the blood pressure. It can enhance gastroprotection, has good effects on cardiac function, and plays a role in mitochondrial respiration.[7,8]

Nitrates are found in large amounts in vegetables, which are responsible for 70-90% of dietary nitrate intake.[9-11] Among vegetables, green leafy ones contain higher levels of nitrate.[11] Spinach is one of these vegetables which has been shown in several studies to contain high levels of nitrate, compared with other vegetables. This amount can be up to 5 grams nitrate per kg of fresh spinach.[6,12-14] Furthermore, this vegetable is affected by various processes, including cooking and refrigeration before consuming, which can affect its nitrate and nitrite levels. As many studies indicate that boiling reduces the nitrate content of spinach or other vegetables;[15-17] however, it cannot affect their nitrite level.[15,17]

Although the effects of freezing on the nitrate and nitrite in vegetables have been examined in many studies,[15-19] few investigations have been conducted on the effect of refrigeration on the nitrate and nitrite content. At present, there are conflicting results. As Chung and colleagues showed, storing vegetables at 5±1 °C, in spite of keeping them in ambient temperature, has no effect on nitrate and nitrite levels over 7 days.[20] But Chew and colleagues indicated that refrigeration at 4 °C, after blanching at 100 °C water for 1 minute, leads to reduced nitrate and increased nitrite levels in two types of leafy vegetables called *Amaranthus gangeticus* and *Amaranthus paniculatus*.[21]

The Acceptable Daily Intake (ADI) for nitrate is 3.7 mg/kg/day, so the nitrate intake of a 60 kg adult should not be more than 222 mg nitrate per day [3,11,22] and the ADI for nitrite is 0–0.07 mg/kg/day.[23] Therefore, nitrate and nitrite intake, especially for those who consume lots of vegetables in their diet, must be reduced to a minimum level.[22]

Most studies that examined the effect of cooking on nitrate and nitrite level of vegetables have investigated on the boiling method. The present study aimed to investigate the effect of a cooking method on spinach, as one of the high nitrate vegetables. This method, which is common in Iran, has not been investigated in other studies before. The effect of refrigeration on nitrate and nitrite level of the cooked spinach, which has been investigated in a few studies, was studied, as well.

Materials and Methods

Instruments

Samples were analyzed using Waters HPLC (High Performance Liquid Chromatography) system, which was equipped with a UV Waters 2487 Dual λ Absorbance Detector, Waters 1525 Binary HPLC Pump and C18 Waters Spherisorb ® 5 µm ODs2 (250×4.6) column. For degassing the solutions, YL9100 HPLC System Vacuum Degasser was utilized. pH measurements were determined using a Metrohm827 pH meter. Sigma Laboratory Centrifuges (3k30) was utilized to centrifuge the samples before injection, and Elmasonic S60H ultrasonic system was used for preparation of samples.

Chemicals: All reagents were purchased from Merck Company.

Preparation of Spinach

Spinach was bought from green groceries in Shiraz, Fars, Iran in 2014. After removing their visible soil and non-edible parts, they were cut into 4-7 cm pieces. Then, they were washed with deionized water for 3 times and drained. A part of raw spinach was set aside to measure the nitrate and nitrite levels and the other parts were heated in a closed container until almost all water of the spinach was evaporated (about 40 min without adding water). A part of the cooked spinach was used to measure the nitrate and nitrite levels and the others were kept in 4 ºC refrigerator for 5 days.

Sample Preparation for Analysis

After the initial preparation, spinach samples were prepared for HPLC injection based on the Chou and colleagues' study.[24] All glassware were soaked in NaOH solution and then washed with deionized water for several times.

Preparation of Mobile Phase and Standard Solutions

Two aqueous methanol concentrations (40, 30% v/v) and different pH values (5.5-7.5) were tested. Different amounts of Octylamine (0.010, 0.0125, 0.015, and 0.020 M) were added to the solutions. Finally, the solutions were passed from 0.22µm filter. Eventually, the optimal condition of the mobile phase (30% methanol, pH 6.5 with 0.015 M Octylamine) was used in the experiment.

Some standard solutions containing 3.12, 6.25, 12.5, 25 and 50 µg/mL nitrite and nitrate were prepared on the day of experiment and injected to HPLC system.

HPLC Analysis

Several flow rates were tested (0.5, 0.8, 1 mL/min) and the optimal one was 0.8 mL/min. The detected UV wavelength and the injection volume were 213 nm and 5 µL, respectively. HPLC column was refreshed by passing a mixture solution of water and methanol (50:50 v/v) at the end of the analysis.

Statistical Analysis

The collected data were analyzed using SPSS, version 18. Descriptive statistics were used to report the mean and standard deviation. The levels of nitrite and nitrate in spinach were compared before and after the cooking process and also before and after refrigeration storage, using Wilcoxon test following the test of the normality of the parameters. The significance level was P<0.05.

Results

After examining various conditions to set up the HPLC system, the optimum mobile phase for detection and separation of two peaks of nitrate and nitrite was 30% (v/v) aqueous methanol with addition of 0.015 M Octylamine which was adjusted in 6.5pH value. The flow rate was 0.8 mL/min.

The contents of nitrate in raw and cooked spinach are given in Table 1. As shown in the table, fresh spinach had the lowest contents of nitrate that significantly increased as much as 410.16 ppm (121%) after cooking (P=0.012), but a significant decrease (246.20 ppm (33%)) was observed after refrigeration storage of the cooked spinach (P=0.012).

Table 2 shows the contents of nitrite in the raw and cooked spinach. Cooking reduces the nitrite level of spinach by 13% (3.5 ppm), but this change was not significant. No significant change was observed in the nitrite content after refrigeration, as well.

Discussion

The aim of this study was to evaluate the effect of the cooking and refrigeration processes on the level of nitrite and nitrate in spinach. As shown in the Tables, concentration of nitrate and nitrite in spinach samples varied widely. This can occur because of the plant variety, rainfall, light, amount of fertilizer, and other growth conditions.[13,25] The mean nitrate concentrations in raw spinach was 336.54±182.2 mg/kg, comparable to the content given by Gajda and colleagues [26] and Petersen and colleagues;[27] they found that the level of nitrates in spinach varied from 29 to 6757 mg/kg and 48 to 5630 mg/kg, respectively.

The mean nitrite concentration in raw spinach was 26.49±10.07 mg/kg. Based on the usual content of nitrite reported in vegetables, the raw spinach was characterized by a high content of nitrite, because the nitrite level of vegetables is low, usually below 2 mg/kg.[28,29] However, depending on the type of vegetables, light, the amount and type of the fertilizer used and other conditions, this amount can be increased. As Chou and colleagues found out, the nitrite concentration of vegetables is more than the amount reported, which was 122/0±8.5 mg/kg in spinach.[24] The results are in agreement with those reported by Petersen and colleagues too, indicating that the nitrite level of spinach varied from 0 to 162 mg/kg with a mean amount of 11±30 mg/kg.[27] Nitrite concentration of fresh spinach is reported 9.5-197.5 mg/kg by Iammarino and colleagues.[30] Nevertheless, some studies determined the nitrite concentration of spinach about 0 mg/kg by HPLC method [14] and about 1 mg/kg by spectrophotometry method.[15]

The other reason for the high nitrite concentration is poor storage conditions of vegetables.[31] In fact, improper storage conditions may increase the activity of the nitrate-reducing bacteria which results in nitrate reduction and nitrite enhancement.[27,28] Studies that reported a higher nitrite level of spinach than its usual concentrations in vegetables; due to improper storage conditions Phillips, Heisler and colleagues and Aworth and colleagues determined a nitrite concentration of 10-22 mg/kg, 140 mg/kg and 4-24 mg/kg in spinach, respectively.[31] Though in this study, spinach samples were kept in a good condition after purchase, but chopping spinach in the preparatory phase could lead to this condition. Moreover, since spinach used in this study was purchased from the green groceries in the city, it is possible that storage conditions were poor during harvesting and transporting them from farms

Table 1: Contents of nitrate in raw and cooked spinach

Groups	n	Nitrate (ppm)		
		Mean	S.D	Range
Raw spinach (1st day)	8	336.54[a]	182.20	74.71-587.82
Cooked spinach (1st day)	8	746.70[b]	594.54	112.92-1706.02
Cooked spinach after refrigeration (5th day)	8	482.50[c]	409.43	94.96-1178.13

Different letters in the same column indicate statistically significant differences (P<0.05)

Table 2: Contents of nitrite in raw and cooked spinach

Groups	n	Nitrite (ppm)		
		Mean	S.D	Range
Raw spinach (1st day)	8	26.49[a]	10.07	14.27-41.91
Cooked spinach (1st day)	8	22.99[a]	10.49	14.99-39.07
Cooked spinach after refrigeration (5th day)	8	22.08[a]	10.44	14.30-36.76

Different letters in the same column indicate statistically significant differences (P<0.05) to vegetable field and then to green groceries in the city, which usually takes about 1 day. According to Chung and colleagues, storing vegetables at ambient temperature (22±1 °C) can reduce nitrate and increase nitrite after 2-3 days.[20] Tamme and colleagues also concluded that storing vegetable juices at ambient temperature causes a reduction in nitrates and increase in the nitrite during the 2 days.[29]

The cooking process mentioned before, (heating for 40-45 min without adding any water, until almost all water of the spinach was evaporated) increased the level of nitrate of spinach as much as 410.16 ppm. However, Jaworska showed that boiling reduces the nitrate level in spinach and New Zealand spinach.[15] Prasad and colleagues in their effort to study the effects of cooking and freezing on nitrate level of leafy vegetables also concluded that boiling for 10 minutes reduced the nitrate content by 47-56% in leafy vegetables.[16] Moreover, Leszczynska and colleagues observed that boiling for 10-15 minutes led to a reduction in the nitrate level in vegetables, too.[32] Although other studies indicated that cooking can reduce the nitrate level of vegetables, it should be pointed out that cooking method of those studies is different from that used in this study; in others, some water had been added to vegetables first, and after boiling, they were drained and the water was discarded.[15,16,32] But no water was added to vegetables in this study and cooking process continued until the tissue of spinach became soft and almost all its water was evaporated. As a result, no water was discarded. So, it is likely that nitrate, which is a water-soluble compound, remained in spinach and was condensed during cooking. Other studies have also confirmed that, depending on the method of boiling and duration of cooking and the variety of vegetables, changes in nitrite and nitrate levels are different.[32]

A slight reduction (3.5 ppm) was observed in the nitrite level after the cooking process, but the change was not statistically significant. Although cooking method of this study was different from previous studies, the result is similar to that of Jaworska[15] and Huarte-Mendicoa and colleagues,[17] investigating the effect of cooking on nitrate and nitrite of spinach, New Zealand spinach,[15] and broccoli.[17] On the other hand, Leszczynska and colleagues could not observe a clear effect of boiling on nitrite in cruciferous vegetables; they observed that boiling had no significant effect on the nitrite level of some types of cruciferous vegetables (curly kale, broccoli and green cauliflower), but it caused a significant decrease in white cauliflower and significant increase in Brussels sprouts. Cieslik observed a reduction in the nitrite level after boiling of potato, too. These results also show that, depending on the duration of cooking and the variety of vegetables, changes in nitrite and nitrate are different.[32]

Refrigeration at 4°C for 5 days reduced the nitrate level of spinach about 264.2 ppm, but it had no significant effect on the nitrite concentration. The results of previous research in this field are inconsistent. Bosch and colleagues determined the concentration of nitrite of frozen spinach after boiling and refrigerating at 2-5 °C. They observed that the concentration of nitrite and nitrate became higher and lower, respectively, as the spinach was stored longer in the refrigerator.[33] Chew and colleagues also observed reduction in nitrate and enhancement in nitrite after blanching *Amaranthus gangeticus* and *Amaranthus paniculatus*, two types of leafy vegetables, in boiling water (100 °C) for 1 minute and refrigerating them at 4 °C.[21] However, Chung showed that storing vegetables at 5±1 °C, unlike storing them at ambient temperature, had no effect on the nitrate and nitrite content during 7 days. It is worth mentioning that the measurement of nitrite and nitrate levels in Chung's study was performed on raw vegetables;[20] however, Bosch and Chew's studies were performed on processed vegetables. This point and the difference in processing could be the reason of these contradictions. In the current study, spinach was cooked through different methods before refrigeration, so the reduction in nitrate concentration was similar to Bosch and colleagues[33] and Chew and colleagues' studies,[21] but the result obtained about nitrite was different. This can be due to differences in the previous process of refrigeration, which may have a greater role in conversion of nitrite to other nitrogen derivatives. Because, in addition to enzymatic mechanisms, whose probability is low here, nitrite can convert into NO by non-enzymatic mechanisms, e.g. via chemical reduction.[34] Indeed, nitrate and nitrite are parts of nitrogen cycle; in addition to converting into each other, they can also convert into other nitrogenous derivatives. Spoelstra, in determination of nitrite level in silage, revealed that nitrate converts to ammonia and nitrous oxide by the action of bacteria eventually, and nitrite and nitric acid are intermediate products of these transformations.[35]

One of the strong points of this study was investigation of the effect of a common method of cooking of spinach in Iran which has not been studied before. This study showed that this type of cooking is not a proper method to reduce nitrate and nitrite intake from spinach. Cooked spinach is usually kept in refrigerator for some days for other uses; this study revealed how nitrate and nitrite levels of spinach change after refrigeration for 5 days. However, as previously mentioned, it seems that differences in the previous process of refrigeration are effective in the obtained result. In this study, it was found, though it wasn't a part of the objectives of the study, that the amount of nitrite in raw spinach was high comparable to the usual content of nitrite reported in vegetables. So investigation of the causes of this issue in agricultural conditions in Iran, including management of agricultural water and fertilizers and other effective conditions, is necessary.

Conclusion

Based on the usual content of nitrate and nitrite reported in spinach, the raw spinach was characterized by a low content of nitrate but a high content of nitrite. This could be due to weather conditions, amount of the fertilizers used, and other growth conditions. Moreover, it can indicate damage to the spinach tissue and activation of nitrate reductase during harvesting, transporting, or preparing the spinach.

It seems that cooking spinach without adding water for 40-45 minutes, which was used in this study, is not a proper method to reduce nitrate and nitrite intake. For this purpose, it is better to use other cooking methods such as boiling followed by removing the boiled water. Furthermore, this study showed that refrigerating spinach, which was cooked via the mentioned method, at 4 °C can significantly reduce the nitrates' concentration.

Acknowledgements

The authors wish to thank Shiraz University of Medical Sciences for financial support and Dr. Nasrin Shokrpour at Center for Development of Clinical Research of Nemazee Hospital for editorial assistance and also Ms Rousta for performing the statistical analysis. This research was extracted from the MS thesis written by Mitra Keshavarz which was funded by Shiraz University of Medical Sciences (Project No. 6849-92).

Conflict of Interest: None declared.

References

1 World Health Organization. Chemical fact sheets [internet]. In Guidelines for drinking-water quality, Volume 1: Recommendations. Third edition; 2008. p. 398-400. Available from: http://www.who.int/water_sanitation_health/dwq/gdwq3rev/en

2 Machha A, Schechter AN. Dietary nitrite and nitrate: a review of potential mechanisms of cardiovascular benefits. Eur J Nutr 2011; 50(5): 293-303.

3 Katan MB. Nitrate in foods: harmful or healthy?. The American Journal of Clinical Nutrition 2009; 90(1): 11-12.

4 Yan PM, Xue WT, Tan SS, Zhang H, Chang XH. Effect of inoculating lactic acid bacteria starter cultures on the nitrite concentration of fermenting Chinese paocai. Food Control 2008; 19(1): 50-5.

5 Sobhan Ardakani S, Shayesteh K, Afyouni M, Mahbobi Soufiani N. Nitrate Concentration in Some Plants in Isfahan. Journal of Environmental Studies 2005; 31(37): 69-76.

6 Najafi N, Parsazadeh M. Effect of Nitrogen Form and pH of Nutrient Solution on the Shoot Concentration of Phosphorus, Nitrate, and Nitrogen of Spinach in Hydroponic Culture. ejgcst 2010; 1(1): 41-56.

7 Sobko T, Marcus C, Govoni M, Kamiya S: Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers**.** Nitric Oxide 2010; 22(2): 136-40.

8 World Health Organization. Nitrate and nitrite in drinking-water, Background document for development of WHO Guidelines for Drinking-water Quality [internet]. Geneva; 2011. Available from: http://www.who.int/water_sanitation_health/dwq/chemicals/nitratenitrite2ndadd.pdf

9 Wolff IA, Wasserman AE. Nitrates, nitrites and nitrosamines. Extensive research is needed to establish how great a food hazard these nitrogenous substances present. Science 1972; 177: 15-19.

10 Hord NG, Tang Y, Bryan NS. Food sources of nitrates and nitrites: the physiologic context for potential health benefits. Am J Clin Nutr 2009; 90(1): 1-10.

11 Food Standards Agency. 2004 UK Monitoring Programme for Nitrate in Lettuce and Spinach. Food Survey Information Sheet 74/05 [internet]. London; 2005. Available from: http://www.food.gov.uk/science/surveillance

12 Tamme T, Reinik M, Roasto M, Juhkam K, Tenno T, Kiis A. Nitrates and nitrites in vegetables and vegetable-based products and their intakes by Estonian population. Food Additives and Contaminants 2006; 23(4): 355-61.

13 Shahbazzadegan S, Hashemimajd K, Shahbazi B. Determination of Nitrate Concentration of Consumed Vegetables and Fruits in Ardabil. Journal of Ardabil University of Medical Sciences 1398; 10(1): 38-47.

14 Hsu J, Arcot J, Lee NA. Nitrate and nitrite quantification from cured meat and vegetables and their estimated dietary intake in Australians. Food Chemistry 2009; 115: 334-9.

15 Jaworska G. Nitrates, nitrites, and oxalates in products of spinach and New Zealand spinach Effect of technological measures and storage time on the level of nitrates, nitrites, and oxalates in frozen and canned products of spinach and New Zealand spinach. Food Chemistry 2005; 93: 395-401.

16 Prasad S, Chetty AA. Nitrate-N determination in leafy vegetables: Study of the effects of cooking and freezing. Food Chemistry 2008; 106(2):772-80.

17 Huarte-Mendicoa JC, Astiasarán I, Bello J. Nitrate and nitrite levels in fresh and frozen broccoli. Effect of freezing and cooking. Food Chemistry 1997; 58(1-2):39–42.

18 Nabrzyski M, Gajewska R. The content of nitrates and nitrites in fruits, vegetables and other foodstuffs. Rocz Panstw Zakl Hig 1994; 45(3): 167-80.

19 Abo Bakr TM, El-Iraqui SM, Huissen MH. Nitrate and

Nitrite Contents of Some Fresh and Processed Egyptian Vegetables. Food Chemistry 1986; 19: 265-75.

20 Chung JC, Chou SS and Hwang DF. Changes in nitrate and nitrite content of four vegetables during storage at refrigerated and ambient temperatures. Food Additives and Contaminants 2004; 21(4): 317-22.

21 Chew SC, Yang Bao KNP, Ismail A. Changes in nitrate and nitrite levels of blanched amaranthus during refrigeration storage. Journal Sains Kesihatan Malaysia 2011; 9(1): 29-34.

22 Pirsaheb M, Rahimian S, Pasdar Y. Nitrite and Nitrate content of fruits and vegetables in Kermanshah (2010). Journal of Kermansha University of Medical Sciences 2012; 16(1): 76-83.

23 Joint FAO/WHO Expert Committee on Food Additives [Internet]. Available from: http://apps.who.int/ipsc/database/evaluations/chemical.aspx?chemID=711

24 Chou SS, Chung JC and Hwang DF. A High Performance Liquid Chromatography Method for Determining Nitrate and Nitrite Levels in Vegetables. Journal of Food and Drug Analysis 2003; 11(3): 233-8.

25 Bednar CM, Kies C, Carlson M. Nitrate-Nitrite levels in commercially processed and home processed beets and spinach. Plant Foods for Human Nutrition 1991; 41: 261-8.

26 Gajda J, Karłowski K. The content of nitrates in vegetables and potatoes in 1987–1991. Roczniki Pan´stwowego Zakładu Higieny 1993; 44(4): 301-7.

27 Petersen A, Stoltze S. Nitrate and nitrite in vegetables on the Danish market: content and intake. Food Additives and Contaminants 1999; 16(7): 291-9.

28 Dennis M, Wilson LA. Nitrates and Nitrites. In Encyclopedia of Food Sciences and Nutrition. Second Edition; 2003. p. 4136-42.

29 Tamme T, Reinik M, Püssa T, Roasto M, Meremäe K, Kiis A. Dynamics of nitrate and nitrite content during storage of home-made and small-scale industrially produced raw vegetable juices and their dietary intake. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2010; 27(4): 487-95.

30 Iammarino M, Taranto AD, Cristino M. Endogenous levels of nitrites and nitrates in wide consumption foodstuffs: Results of five years of official controls and monitoring. Food Chemistry 2013; 140:763-71.

31 Hunt J, Turner MK. A survey of nitrite concentrations in retail fresh vegetables. Food Additives & Contaminants 1994; 11(3): 327-32.

32 Leszczynska T, Filipiak-Florkiewicz A, Cieslik E, Sikora E, Pisulewski PM. Effects of some processing methods on nitrate and nitrite changes in cruciferousvege tables. Journal of Food Composition and Analysis 2009; 22: 315-21.

33 Bosch NB, Mata MG, Pefiuela MJ, Ruiz Galfin T, Lopez Ruiz B. Determination of nitrite levels in refrigerated and frozenspinach by ion chromatograph. Journal of Chromatography A 1995; 706: 221-8.

34 Ferreira LC, Cataneo AC. Nitric oxide in plants: a brief discussion on this multifunctional molecule. Scientia Agricola 2010; 67(2): 236-43.

35 Spoelstra SF. Nitrate in silage. Grass and Forage Science 1985; 40: l-11.