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RESEARCH ARTICLE

The influence of storage condition on nitrite, nitrate and vitamin C levels in vegetables [version 1; peer review: 1 approved with reservations, 1 not approved]

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Abstract

Vegetables are the main sources of nitrate and nitrite in food. The presence of nitrate and nitrite at a high level may cause a negative impact on health, because nitrite and nitrate when reduced to nitrite, may react with alkylamine to form carcinogenic nitrosamine. The influence of temperature and time of storage on nitrite, nitrate, and vitamin C contents in vegetables were investigated in this study. The vegetables were sweet mustard, bokchoy, spinach and lettuce obtained from a local market. Samples were stored at $\pm 25^{\circ}\text{C}$ and $\pm 5^{\circ}\text{C}$. Analysis of nitrite, nitrate, and vitamin C was conducted in fresh samples, after storage for 24 and 48 hours. Nitrite was analyzed by spectrophotometry at 540 nm. Nitrate reduced into nitrite with Zn in acidic conditions and then analyzed as nitrite. Vitamin C was analyzed by titration with 2,6-dichlorophenolindophenol. During storage, nitrite and nitrate increased, while vitamin C decreased. Nitrite and nitrate content in fresh samples were 15.22 and 22.46 mg/kg (sweet mustard), 12.57 and 6.55 mg/kg (bokchoy), 20.26 and 90.60 mg/kg (spinach), 18.77 and 32.68 mg/kg (lettuce), respectively. Vitamin C content in fresh samples was 101.15 mg/100g (mustard), 92.17 mg/100g (bokchoy), 88.95 mg/100g (spinach), 40.03 mg/100g (lettuce). After storage for 48 hours at $\pm 25^{\circ}\text{C}$, nitrite and nitrate increased 44.97% and 53.19% (mustard), 46.18% and 62.59% (bokchoy), 43.86% and 16.48% (spinach), and 41.05% and 47.09% (lettuce), respectively. Vitamin C decreased 67.57% (mustard), 24.68% (bokchoy), 81.25% (spinach), and 79.74% (lettuce). Storage at $\pm 5^{\circ}\text{C}$, showed that nitrite and nitrate increased 27.54% and 35.08% (mustard), 13.75% and 43.51% (bokchoy), 19.59% and 10.60% (spinach), 19.85% and 25.16% (lettuce), respectively. Vitamin C decreased 30.88% (mustard), 6.05% (bokchoy), 60.92% (spinach), and 74.94% (lettuce). During storage, nitrite and nitrate increased more significantly at $\pm 25^{\circ}\text{C}$ than $\pm 5^{\circ}\text{C}$ while vitamin levels C decreased and were more effective at 25°C than 5°C .

Open Peer Review

Approval Status

	1	2
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Any reports and responses or comments on the article can be found at the end of the article.

Keywords

Nitrite, Nitrate, Vitamin C, Storage Condition.

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Author roles: **Cintya H:** Conceptualization, Investigation, Methodology, Validation, Writing – Original Draft Preparation; **Silalahi J:** Conceptualization, Methodology, Supervision, Writing – Review & Editing; **De Lux Putra E:** Methodology, Supervision, Writing – Review & Editing; **Siburian R:** Conceptualization, Investigation, Supervision, Writing – Review & Editing

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Introduction

Vegetables are a major source of nitrite and nitrate intake from food. Nitrite and nitrate are also used as preservatives and coloring agents in processed meats¹⁻⁵. Nitrate and nitrite contents in vegetables vary widely from 1 to 10.000 mg/kg, and this is affected by many factors, including environmental factors such as storage condition, processing procedure, temperature and agricultural practices⁶⁻⁹.

Nitrate can be reduced into nitrite by enzyme nitrate reductase and other reducing agents, including vitamin C, which is also contained in vegetables. Nitrite may react with alkylamines to form carcinogenic nitrosamines^{10,11}. Therefore, the intake permitted (Acceptable Daily Intake = ADI) by the Food and Agriculture Organization of the United Nations/World Health Organization is 220 mg of nitrate and 8 mg of nitrite per day for adults weighing an average of 60 kg^{12,13}. Previous studies reported that the longer the storage, the higher nitrite and nitrate contents. These effects are more influential at room temperature than at refrigeration. But the effect of temperature and storage condition on vitamin C have not yet been reported to the best of our knowledge. The aim of this study was to investigate the effect of storage condition on nitrite, nitrate and vitamin C contents in vegetables.

Methods

Materials

Chemicals used were analysis grade products from Merck KGaA (Germany): N-(1-naphthyl) ethylenediamine dihydrochloride (NED), sodium nitrite, sulfanilic acid, glacial acetic acid, hydrochloric acid, antipyrine, ferrous sulfate, zinc powder, sodium nitrite, ascorbic acid, metaphosphoric acid, and 2,6-dichlorophenol-indophenol.

Samples

The vegetables analyzed in this study were sweet mustard (*Brassica rapa chinensis*), bokchoy (*Brassica rapa* L.), spinach (*Amaranthus tricolor* L), and lettuce (*Lactuca sativa* L). These vegetables were obtained from a local market in Medan, Indonesia. Samples were stored for 0, 24, until 48 hours at room temperature ($\pm 25^{\circ}\text{C}$) and in a refrigerator ($\pm 5^{\circ}\text{C}$).

Determination of absorbance curve of nitrite standard solution

In total, 4 ml of standard solution of nitrite ($C=10.0\text{ }\mu\text{g/ml}$) was transferred into 50 ml volumetric flask, added 2.5 ml sulfanilic acid solution and shaken. After 5 min, 2.5 ml NED reagent was added and made to volume with distilled water and homogenized ($C=0.8\text{ }\mu\text{g/ml}$). Absorbance was measured at wave length of 400–800 nm. Then, absorbance and wave length was plotted to construct absorbance curve. Wave length of maximum absorbance was determined from the absorbance curve¹⁰.

Absorbance stability of derivatized nitrite to determine working time

In total, 4 ml of standard solution of nitrite ($C=10.0\text{ }\mu\text{g/ml}$) was transferred into volumetric flask of 50 ml, to which 2.5 ml of sulfanilic acid and stirred. After 5 min, 2.5 ml NED reagent was added and distilled water was added to make 50 ml. Absorbance

was measured at wave-length of maximum absorbance obtained from absorbance curve (540 nm), and stability of absorbance was determined by observing absorbance at every minute for 1 hr. The absorbance was found to be relatively stable within 6 min in 7–12 min¹⁰.

Determination of calibration curve

Standard solution of nitrite ($C=10.0\text{ }\mu\text{g/ml}$) of different volume (0.5, 1, 2, 3, 4 dan 5 ml) were transferred into separated volumetric flasks of 25 ml, then 2.5 ml sulfanilic acid reagent added and stirred to homogenize. After 5 min, 2.5 ml NED reagent was added, then distilled water was added to make volume of 25 ml and homogenized. The series of concentration of prepared solutions were of 0.1 $\mu\text{g/ml}$, 0.2 $\mu\text{g/ml}$, 0.4 $\mu\text{g/ml}$, 0.8 $\mu\text{g/ml}$, 1.0 $\mu\text{g/ml}$. Absorbance of each solution was measured at wave-length of 540 nm within 7 min. Calibration curve was made by plotting absorbance versus concentration of each solution. From the graph obtained, then linearity of regression equation and correlation coefficient were calculated ($Y=aX+b$)¹⁰.

Identification of nitrite and nitrate in vegetables

About 10 g ground sample, using blender, was transferred into a glass beaker. Distilled water was added to about 150 ml, heated in a waterbath (80°C) and shaken for 5 minute then cooled and filtered. The supernatant was transferred into a test tube, then 2.5 ml sulfanilic acid reagent was added and stirred. After 5 minutes, 2.5 ml reagent NED was added. Nitrite was identified using sulfanilic acid and NED solution, and the appearance of a violet color indicated the presence of nitrite. Nitrate was identified by adding several drops ferrous sulfate solution and then slowly adding a few drops of concentrated sulfuric acid. The formation of chocolate ring indicates the presence of nitrate¹⁰.

Quantification of nitrite and nitrate in vegetables

Nitrite. Determination of nitrite was carried out with procedure previously described¹⁰. Around ten (10) gram grounded sample transferred into 250 ml beaker glass to which hot distilled water ($\pm 80^{\circ}\text{C}$) was added about 150 ml. This mixture was homogenized by stirring and heated on waterbath for 15 minute while stirring. Allowed to cool and then transferred quantitatively into 250 ml volumetric flask, distilled water added to volume, then filtered. Ten (10 ml) of filtrate transferred into a volumetric flask of 50 ml, then 2.5 ml sulfanilic acid reagent was added and stirred. After 5 minutes, 2.5 ml reagent NED was added, then distilled water added to make 50 ml, and then homogenized. Absorbance was measured at wavelength of 540 nm after period of 7 to 12 minutes time.

Nitrate. In total, 10 g grounded sample transferred into 250 ml beaker glass to which hot distilled water ($\pm 80^{\circ}\text{C}$) was added to about 150 ml. This mixture was homogenized by stirring and heated on waterbath for 15 minute while stirring. Allowed to cool and then transferred quantitatively into 250 ml volumetric flask, distilled water added to volume, then filtered. 10 ml of filtrate transferred into a volumetric flask of 50 ml, then 0.1 g Zn powder and 1 ml HCl 1 N added and allowed to stand for 10 minutes to reduce nitrate to nitrite, then 2.5 ml sulfanilic

acid reagent was added and stirred. After 5 minutes, 2.5 ml reagent NED was added, then distilled water added to make 50 ml, and then homogenized. Absorbance was measured at wavelength of 540 nm after period of 7 to 12 minutes time.

Nitrite concentration from reduction of nitrate into nitrite was calculated:

Nitrite concentration from nitrate reduction = Total nitrite content - Initial nitrite concentration in samples

Nitrate content was calculated:

Nitrite concentration from nitrate reduction = Concentration of nitrate $\times \frac{\text{Molecule Weight nitrate}}{\text{Molecule Weight nitrite}}$

Identification of vitamin C in vegetables

10 gram grounded sample using blender. About 0.5 ml of sample solution in a test tube was neutralized to a pH of 6–8 with NH_4OH 1 N, three drops of 3% FeCl_3 was added – a purple color indicates the presence of vitamin C.

Analysis of vitamin C in vegetables

10 g grounded sample using blender was transferred into 100 ml volumetric flask, then acetic metaphosphoric acid 3% added to make 100 ml, then homogenized and filtered. Two (2) ml of filtrate was transferred into an erlenmeyer, and then added 5 ml of acetic metaphosphoric acid, then titrated with 2,6-dichlorophenol indophenol solution 0.025% until pink steadily¹¹. The levels of vitamin C was calculated.

$$\text{Vitamin C (mg/g)} = \frac{(Vt - Vb) \times \text{Equivalence} \times VL}{Vp \times Bs}$$

Vb = The volume of blank (ml); VL = The volume of volumetric flask (100 ml); Vp = The volume of pipetted sample solution(ml); Bs = Sample weight (g)

Results

Nitrite and nitrate

It is found that samples contain nitrite indicated by the appearance of violet color to prove that all samples contained nitrite. The reaction with antipyrine in dilute hydrochloric resulted in the formation of green color to prove the present of nitrite. Nitrate in samples was identified using ferrous sulfate and concentrated sulfuric acid produced brown ring¹⁰.

Vitamin C

Identification using FeCl_3 3% reagent generated violet color to prove that all samples contained vitamin C¹¹.

The wave-length of maximum absorption of nitrite derivative

The absorbance curve of the nitrite derivative solution (10 $\mu\text{g/ml}$) is presented in Figure 1. From Figure 1 it is shown that the maximum absorption was at 540 nm, which is similar to the value previously reported^{2,7}, which was used to determine the analysis of nitrite and nitrate in samples.

Working time for measurement

Working time for nitrite and nitrate analysis was determined to know the period of time within which the absorbance of solution still remains stable. Absorbance of nitrite derivative with Griess reagent presented in Figure 2. Figure 2 shows that absorbance was stable within minute 7 to minute 12 then used in the analysis procedure^{2,7}.

Calibration curve of nitrite derivative

Calibration curve made by plotting absorbance versus concentration of each solution, then linearity of regression equation was determined. The calibration curve presented in Figure 3. Regression equation obtained is $Y = 0.58064X + 0.0015$ with coefficient correlation (r) of 0.99977 (where $r > 0.999$).

Figure 1. Absorbance curve of nitrite derivative.

Figure 2. Absorbance of nitrite derivative with time.

Figure 3. Calibration curve of nitrite derivative.

Figure 3 shows that the correlation coefficient was high ($r=0.999$) indicated linearity between concentration and absorbance^{2,7}.

Influence of storage condition

The levels of nitrite, nitrate, and vitamin C during storage at $\pm 25^{\circ}\text{C}$ and $\pm 5^{\circ}\text{C}$ can be seen in Table 1 and Table 2.

Nitrite and nitrate levels in fresh samples were 15.22 and 22.46 mg/kg (sweet mustard), 12.57 and 6.55 mg/kg (bokchoy), 20.26 and 90.60 mg/kg (spinach), and 18.77 and 32.68 mg/kg (lettuce), respectively. Vitamin C levels in fresh samples were 101.15 mg/100g (sweet mustard), 92.17 mg/100g (bokchoy), 88.95 mg/100g (spinach), and 40.03 mg/100g (lettuce).

From Table 1 can be seen that the levels of nitrite and nitrate also increased with storage time. After storage for 48 hours at $\pm 25^{\circ}\text{C}$, nitrite and nitrate levels increased 44.97% and 53.19% (sweet mustard), 46.18% and 62.59% (bokchoy), 43.86% and 16.48% (spinach), and 41.05% and 47.09% (lettuce), respectively. While, vitamin C decreased 67.57% (sweet mustard), 24.68% (bokchoy), 81.25% (spinach), and 79.74% (lettuce).

Table 2 shows that storage at $\pm 5^{\circ}\text{C}$, nitrite and nitrate levels increased 27.54% and 35.08% (sweet mustard), 13.75% and 43.51% (bokchoy), 19.59% and 10.60% (spinach), 19.85% and

25.16% (lettuce), respectively. Vitamin C levels decreased 30.88% (sweet mustard), 6.05% (bokchoy), 60.92% (spinach), 74.94% (lettuce), respectively.

Dataset 1. Raw data including working time and calibration curve data; nitrite, nitrate and vitamin C levels 25°C and 5°C and 0, 24 and 48 hours storage for 6 replicates for mustard, bokchoy, spinach and lettuce

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Discussion

From Table 1 and Table 2 it can be seen that nitrite levels are generally relatively low in fresh vegetables compared to nitrate levels, except in bokchoy. This value is similar with those reported by researchers that nitrate is usually higher than nitrite¹². The nitrate in plant also changes with age of the plant. The differences in nitrate content may be due to the fertilization, harvesting time, and storage time⁸⁻¹².

The results indicate that storage temperature and time affect nitrite, nitrate, and vitamin C levels in vegetables. The longer the storage time the higher nitrite and nitrate levels and the lower vitamin C levels. These effects are more influential at room temperature than at refrigeration, as has previously been reported^{3,12}.

Table 1. Influence of storage at $\pm 25^{\circ}\text{C}$ on nitrite, nitrate and vitamin C content in vegetables.

Sample	Storage Time (hour)	Nitrite Content		Nitrate Content		Vitamin C Content	
		Content ($\mu\text{g/g}$)	Increase (%)	Content ($\mu\text{g/g}$)	Increase (%)	Content (mg/100g)	Decrease (%)
Mustard	0	15.2253 ± 0.1821	0	22.4560 ± 0.2437	0	101.1469 ± 0.4003	0
	24	25.3392 ± 0.0330	66.41	35.1203 ± 0.0441	47.49	66.0211 ± 0.8542	34.73
	48	27.6690 ± 0.0160	9.19	47.9751 ± 0.0510	36.60	34.8247 ± 0.9050	47.25
Bokchoy	0	12.5689 ± 0.2222	0	6.5524 ± 0.2776	0	92.1662 ± 0.1893	0
	24	15.5065 ± 0.0222	23.37	11.6594 ± 0.3833	77.94	79.9839 ± 1.3634	13.21
	48	23.3534 ± 0.03615	50.60	17.5143 ± 0.0208	50.21	69.4170 ± 0.9639	13.22
Spinach	0	20.2602 ± 0.0871	0	90.6008 ± 0.3526	0	88.9529 ± 0.6847	0
	24	31.9694 ± 0.4653	57.79	101.0392 ± 0.8337	11.52	33.3578 ± 0.6244	62.49
	48	36.0902 ± 0.0318	12.89	108.4851 ± 1.2673	7.37	16.6800 ± 0.8632	49.99
Lettuce	0	18.7662 ± 0.0120	0	32.6785 ± 0.1543	0	40.0353 ± 0.8969	0
	24	28.8374 ± 0.1236	53.67	61.4563 ± 0.1436	88.06	20.7612 ± 0.5996	48.14
	48	31.8365 ± 0.0998	10.40	61.7739 ± 0.1445	0.5	8.1104 ± 0.5536	60.93

Note: data is the mean of six replicates

Table 2. Influence of storage at $\pm 5^{\circ}\text{C}$ on nitrite, nitrate and vitamin C content in vegetables.

Sample	Storage Time (hour)	Nitrite Content		Nitrate Content		Vitamin C Content	
		Content ($\mu\text{g/g}$)	Increase Nitrite (%)	Content ($\mu\text{g/g}$)	Increase Nitrate (%)	Content (mg/100 g)	Content ($\mu\text{g/g}$)
Mustard	0	15.2253 ± 0.1821	0	22.4560 ± 0.2437	0	101.1469 ± 0.4003	0
	24	20.0538 ± 0.3264	31.71	27.4262 ± 0.4394	22.13	83.9400 ± 0.5162	17.01
	48	21.0116 ± 0.0159	4.78	34.5940 ± 0.0505	26.13	69.9127 ± 0.8541	16.71
Bokchoy	0	12.5689 ± 0.2222	0	6.5524 ± 0.2776	0	92.1662 ± 0.1893	0
	24	12.9326 ± 0.3512	2.89	9.4563 ± 0.4730	44.31	89.2655 ± 0.6481	3.15
	48	14.5726 ± 0.0158	12.68	11.5997 ± 0.0503	22.67	86.5874 ± 0.7859	3.0
Spinach	0	20.2602 ± 0.0871	0	90.6008 ± 0.3526	0	88.9529 ± 0.6847	0
	24	23.4830 ± 0.7816	15.91	98.1230 ± 1.1258	8.30	58.4130 ± 0.6865	34.33
	48	25.1991 ± 0.2102	7.3	101.3455 ± 0.2744	3.28	34.7609 ± 0.5144	40.49
Lettuce	0	18.7662 ± 0.0120	0	32.6785 ± 0.1543	0	40.0353 ± 0.8969	0
	24	21.4087 ± 0.0927	14.08	43.0090 ± 0.1151	31.61	26.6342 ± 0.8295	33.47
	48	23.4131 ± 0.1003	9.36	43.6641 ± 0.2161	1.5	10.0336 ± 0.6035	62.32

Note: data is the mean of six replicates

Table 1 and Table 2, suggest that the vitamin C and other anti-oxidant content in vegetables may reduce nitrate into nitrite, and then nitrite may react with amine compounds, especially secondary amines to form a carcinogenic nitrosamine. On the other hand, ascorbic acid available in fresh vegetables may prevent the formation of nitrosamine^{8–12}.

Conclusion

Storage condition affects nitrite, nitrate and vitamin C content in vegetables. The higher the temperature and the longer the time of storage, the higher nitrite and nitrate levels, and the lower vitamin C levels. This effect is more influential at 25°C than at 5°C.

Data availability

F1000Research: Dataset 1. Raw data including working time and calibration curve data; nitrite, nitrate and vitamin C levels

25°C and 5°C and 0, 24 and 48 hours storage for 6 replicates for mustard, bokchoy, spinach and lettuce., <https://doi.org/10.5256/f1000research.16853.d227224>¹⁴

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Barbora Piknova

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Vegetables and processed meat are the main dietary sources of inorganic nitrate. Nitrate itself has no known direct physiological effects. However, the mammalian body can convert nitrate into nitrite and nitric oxide (NO) in a two-step process either by mammalian nitrate and nitrite reductases or by the help of the oral and gut microbiome. It had been well documented over the past decade that NO and nitrite have a wide array of beneficial effects on cardiovascular health, such as lowering blood pressure, and improves the ability to exercise by decreasing the oxygen cost of exercise and/or by improving the blood flow into contracting muscle - for a review about the effect of nitrate and nitrite on exercise performance see Jones *et al.*, 2018¹.

I believe these 2 points need to be addressed in the present manuscript before being accepted for indexing:

1. The manuscript by Cintya *et al.* contains useful information about the amounts of nitrite/nitrate and vitamin C in various vegetables and on the effect of storage on the amount of these ions and vitamin C. However, it seems that the general message the authors try to convey in their abstract and introduction is that nitrite is a carcinogen and should be avoided. This idea had long been proven wrong and the message should be modified. Nitrate and nitrite are an integral part of a healthy diet and their importance for cardiovascular health is not questionable (Bryan *et al.*, 2012² and Lundberg and Weitzberg, 2013³); evidence for nitrate/nitrite-caused cancer is not convincing (Eichholzer and Gutzwiller, 1998⁴ and Forman *et al.*, 1985⁵). As some case studies show, nitrite poisoning is usually a result of sodium nitrite overdose when used as meat-preserving salt (Jones *et al.*, 2018¹) or when sodium nitrite is used instead of table salt (sodium chloride) by mistake (Lee *et al.*, 2017⁶).
2. While the finding that storage affects levels of nitrate/nitrite is important, there is no mention about the reasons. Even if the reasons may be as trivial as loss of water during storage, it still should be mentioned.

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Competing Interests: No competing interests were disclosed.

Reviewer Expertise: nitric oxide metabolic pathway

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 21 January 2019

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Marco Iammarino

National Reference Center for the Detection of Radioactivity in Feed and Foodstuff, Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Foggia, Italy

In this paper, the authors have reported about the influence of storage condition (5°C and 25°C) on nitrite, nitrate and ascorbic acid in vegetables.

The topic has already been investigated, so, the originality is lacking.

1. The references section is incomplete. Many papers dealing about this topic were not included.
2. The results obtained in this study cannot be considered as reliable. Indeed, the analytical techniques adopted were not validated or compared to reference parameters. Moreover, they seem obsolete. This weakness is confirmed by evaluating the results obtained for both

nitrite (many authors do not report quantifiable concentrations of nitrite in spinach and lettuce) and (specially) nitrate, since the NO₃- concentrations in spinach and lettuce reported in the bibliography are well higher.

3. The authors have not specified the number of samples analyzed and their characteristics (origin, type, etc.). Consequently the statistical analysis is not possible and the significance of these results is not high.

In view of these criticisms, I regret to not recommend this paper for indexing.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Food Safety; Food Science and Technology; Analytical Chemistry; Analytical methods validation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 06 Feb 2019

Rikson Siburian, Universitas Sumatera Utara, Medan, Indonesia

Dear Referee,

Marco Iammarino, National Reference Center for the Detection of Radioactivity in Feed and Foodstuff, Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Foggia, Italy

Thank you so much for your comments:

In this paper, the authors have reported about the influence of storage condition (5°C and 25°C) on nitrite, nitrate and ascorbic acid in vegetables.

The topic has already been investigated, so, the originality is lacking.

1. *The references section is incomplete. Many papers dealing about this topic were not included.*
2. *The results obtained in this study cannot be considered as reliable. Indeed, the analytical techniques adopted were not validated or compared to reference parameters. Moreover, they seem obsolete. This weakness is confirmed by evaluating the results obtained for both nitrite (many authors do not report quantifiable concentrations of nitrite in spinach and lettuce) and (specially) nitrate, since the NO₃- concentrations in spinach and lettuce reported in the bibliography are well higher.*
3. *The authors have not specified the number of samples analyzed and their characteristics (origin, type, etc.). Consequently the statistical analysis is not possible and the significance of these results is not high.*

We would like to respond below as follows:

1. We have revised and added the references according with previous study.

2. We have made the validation in this research accordingly as the reviewer required. After that, we have compared the result of the previous study with my research. In this research, validation was done, namely recovery. In the previous article before revision, this was not included in the article. According to the referee's comments, we have corrected and added validation in this study. The recovery shows good accuracy. The recovery percentage meets the requirement between 80%-120%.

3. We have specified the number of samples analyzed and their characteristics in the methods. About the statistical analysis, its possible because we have analyzed and have significant difference $p < 0.05$.

For detail and further revision, we also sent our revised manuscript to F1000 editors. Hopefully, it may be clear now.

Best regards,

Rikson Siburian

Competing Interests: No competing interests were disclosed.

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