The Effect of Storage Condition on Nitrite and Nitrate Content in Lettuce (*Lactuca sativa L.*)

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Abstract : Vegetables arethe main sourcesof nitrate and nitrite from food. The content of nitrate and nitrite in vegetables is affected by many factors including storage conditions. The purpose of this study was to investigate the effect of storage temperature and time on the nitrite and nitrate levels in lettuce.

The lettuce used in this study was freshly harvested, cleaned and then stored in two different conditions; storage was conducted in refrigerator at temperature of 10^0C and ambient temperature at about 28^0C and storage time was 48 hours. Nitrate and nitrite content in lettuce was analyzed periodically at every 4 hours. The assay of the nitrite and nitrate was carried out by visible spectrophotometry using N-(1-naphthyl) ethylene diamine dihidrochloride (NED) as coloring reagent and measured at maximum absorbance at wavelength of 540 nm.

The results of this study shows that during storage for 48 hours at room temperature the levels of nitrate increased from initial level of 6.07 mg/kg to 70.83 mg/kg, and nitrite levels increased from initial level of 22.63 mg/kg to 48.14 mg/kg. Levels of nitrate and nitrite at refrigerator temperature (10^0C) storage the level of nitrate increased from initial level of 3.06 mg/kg to 64.42 mg/kg, and level of nitriteincreased from 21.89 mg/kg to 40.08 mg/kg.

Based on the results of present study indicate that during storage the level of nitrate and nitrite consistently increased. The higher the temperature and the longer storage time resulted in the higher increasing levels of nitrate and nitrite in the lettuce, and the temperature of storage was found to be more influential.

Keywords: lettuce, nitrite, nitrate, storage, spectrophotometer.

Introduction

Studies on dietary intake of nitrate and nitrat from food have raised the concern of their possible adverse effects on human health. Epidemiological and clinical studies showed that high level of nitrite in vegetables contribute to the increasing the prevalence of stomach, oesophagus and bladder cancer. The main contribution of nitrite and nitrate in dietary intake is from vegetables and potable drinking water, with a lesser contribution from cured meat.[1]Vegetables estimated to contribute about 85% and 43% of daily dietary intakes of nitrate and nitrite, respectively, while the nitrate and nitrite content of fruit are usually low.[2,3,5,6]

Several processing such as washing, storage condition or freezing and refrigerating may affect the nitrite and nitrate levels in vegetables. Studies on variety of vegetables indicate that no consistent changes in their nitrate and nitrite contents during storage conditions and processes applied, but nitrite content of vegetables increase on storage, especially at higher temperatures as a result of the reduction of nitrate by plant

or bacterial enzymes.[3,4]In processed vegetables, nitrite increase may be due to the release of nitrate from vacuoles in which it is normally isolated. Therefore, the nitrite contents of processed or frozen vegetables can significantly increase compared to those of their counterparts. The other components present in vegetables such as vitamin C may also affect the changes of nitrite level.[3]Lettuce is a vegetable contains high level of nitrate (1500-1700 mg/kg),[7]which raises concern for their negative effect on health. Acceptable Daily Intake (ADI) of nitrate and nitrite has been regulated by FAO and WHO; ADI for nitrate for the individual with body weight of 60 kg is 220 mg, and nitrite is 8 mg.[5]The aim of this study was to investigate the effect of storage temperature and time on the levels of nitrite and nitrate in lettuce.

Materials and Method

Lettuce used was freshly harvested from Berastagi, and then stored at room temperature and in refrigerator at 10^0C for 0-48 hours.

Chemicals

Chemicals used were pro analysis grade product of E. Merck (Germany) including; N-(1-naftil) etylendiamin dihydrochloride (NED), sodium nitrite, sulfanilic acid, glacial acetic acid, hydrochloric acid, antipyrin, ferrous sulfate, zincum powder.

Instruments

Instruments used in this study were spectrophotometer UV-Vis (UV- 1800 *Shimadzu*), analytical balance (*Boeco Germany*), water bath, filter paper, thermometer and glass wares.

Preparation of Reagents

Acetic solution 15% was prepared by dissolving 75 ml glacial acetic acid and diluted with distilled water to make 500 ml. NED solution was made by dissolving 0.350 g N-(1-naftil) etylendiamin dihydrochloridein 250 ml acetic acid solution 15%, filtered and stored in brown bottle. Sulfanilic acid solution was made by dissolving 0.850 g sulfanilic acid in 250 ml acetic solution 15%, filtered and stored in brown bottle.[8]

Preparation of nitrite standard stock solution

The amount of 100 mg sodium nitrite was transferred into 100 ml volumetric flask and dissolved in distilled water, and then made to 100 ml (C = 1000μg/ml) (Solution I). From this solution 1 ml was pipetted into 100 ml volumetric flask and added distilled water to make 100 ml (C = 10 μg/ml) (Solution II).

Identification of nitrite andnitrate

Some amount of grounded sample of each treatment was transferred in to beaker glass, added distilled water, heated on water bath and shaking for a little while, then cooled and supernatant was taken and placed in a test tube for nitrite and nitrate identification. Presence of nitrite was identified with sulfanilic acid and NED solution and allowed for a little while. The appearance of violet color is to indicate the presence of nitrite. The presence of nitrate was conducted by adding several drops ferrous sulfate solution and then added slowly few drops of concentrated sulfuric acid through the inner wall of the test tube. The presence of nitrate was indicated by the formation of chocolate ring.[9,10]

Determination of absorbance curve of nitrite standard solution

Four (4) mlof stock standard solution II was transferred into 50 ml volumetric flask, added 2.5 ml sulfanilicacid solution and shaken, after 5 minutes 2.5 ml NED reagent was added and made to volume with distilled water and homogenized (C = 0.8 μg/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 curve.

Absorbance stability of derivatized nitrite to determine working time

Four (4) mL stock solution II transferred into volumetric flask of 50 ml, to which 2.5 ml of sulfanilic acid and stirred. After 5 minutes, 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 (540 nm), and stability of absorbance was determined by observing absorbance at every minute for 1 hour. The absorbance was found to be relatively stable within four minutes at minute 4 to 8.

Determination of calibration curve

Standard stock solution II (10 µg/ml) of different volume (1 ml; 1.5 ml; 2 ml; 2.5 ml, and 3 ml) were transferred into different volumetric flasks of 25 mL, then 2.5 mL sulfanilic acid reagent added and stirred to homogenize. After 5 minutes, 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 0.4µg/ml; 0.6 µg/ml; 0.8 µg/ml; 1.0 µg/ml; 1.2 µg/ml. Absorbance of each solution was measured at wave-length of 540 nm within 5 minutes. 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.

Determination of Nitriteand Nitratein Lettuce Samples

Determination of Nitrite and Nitrate levels invegetables was conducted according to the procedure described in *Association Official Methods Of Analytical Chemists* (AOAC)XVII edition.[8]Around ten (10) gram grounded sample transferred into 250 mL beaker glass. Then, hot distilled water (± 80ºC) was added to make volume of 150 ml. Homogenized by stirring and heated on water-bath for 2 hours while stirring. Allowed to cool and then transferred quantitatively into volumetric flask of 250 mL. Added distilled water to make volume of 250 mL, then filtered. About 10 mL first filtrate was discarded. Ten (10 mL) of filtrate and transferred into volumetric flask of 50 mL, added Zn (1 g) allowed to stand for 10 minutes (to reduce nitrate to nitrite for nitrate quantification), then 2.5 mL sulfanilic acid reagent was added and stirred. After 5 minutes, 2.5 mL reagent NED was added, then distilled water was added to make 50 mL, then homogenized. Absorbance was measured at wave-length of 540 nm within 5 minutes time. Level of nitrite was calculated using from the regression equation:

$Y = aX + b.$
Nitrite concentration was calculated:

$$C = \frac{X \times V \times Fp}{Sample\ weigth\ (g)}$$

Notes:
Y = Absorbance
C = concentration of nitritein sample(µg/g)
X = Nitrite concentration in diluted sample solution calculated from regression equation(µg/ml)
V = volume of sample solution before dilution (ml)
Fp = dilution factor

Concentration of nitrite from the reduction of nitrate into nitrite = concentration of total nitrite after reduction – concentration of nitrite before reduction.

$$Nitrate\ concentration = nitrite\ concentration\ after\ reduction \times \frac{MW\ nitrate}{MW\ nitrite}$$

Results and Discussion

Identification of Nitritein Lettuce

From the qualitative determination using sulfanilic acid and NED reagents indicated by the appearance of violet color to prove that all samples contained nitrite. The identification reaction was also confirmed with antipyrine in dilute hydrochloric resulted in the formation of green color. Based on the color produced it was indicated that the levels of nitrite in samples increased with time of storage shown by increasing intensity of

color produced. Identification of Nitrate in samples was conducted using ferrous sulfate and concentrated sulfuric acid which resulted in brown ring indicating the presence of nitrate in samples. It was also shown the nitrate levels increased during the storage time indicated by the increasing intensity of the chocolate color produced.

Absorption Curve of Nitrite and Determination of Working Time

The wave-length at which maximum absorption occurred was determined by measuring absorption of a solution with concentration of 10 μg/mL. Absorption was measured at a range of wave length from 400 nm through 800 nm of wave-length. Measurement of nitritewas using solution with concentration of10 μg/mL, and for nitrate was measured after reducing nitrate standard solution with Zn to convert nitrate to nitrite. Maximum absorption was found to be 540 nm. This finding is similar to that value previously reported.[8]Maximum absorption observed was then used to determine working time for analysis of nitrite and nitrate in samples.

Working time of measurement

Working time of measurement for nitriteand nitratewas determined to know the period of time on which the absorbance of solution still remains stable. Absorbance of 0.8 ug/mL solution was measured at wave-length of 540 nm for 60 minutes.It was found that the absorbance was stable within minute 4 to minute 8. This working time was used in analysis procedure.

Calibration Curve

From calibration curve was known that there is linearity between concentration and absorbance. Regression equation obtained is Y= 0.60739X + 0.00308 with coefficient correlation (r) of0.99949 where r> 0.99. This is to indicate thatcorrelation is high.[11]

The Effect of Storage Condition on Nitrite and Nitrate Levels in Lettuce

The effect of storage condition on nitrite and nitrate levels in lettuce was presented in Table1, Figure 1 and Figure 2.

Tabel 1.The Effect of Storage Condition on Nitrite and Nitrate Levels in Lettuce

No.	Storage Condition	Time of Storage (hours)	Nitrite concentration (mg/kg) ±SD	Nitrate concentration (mg/kg) ±SD
1.	Ambient temperature (25^0C)	0	22.636± 0.437	6.078 ± 3.249
		6	24.608± 0.479	9.574 ± 1.451
		12	27.308 ± 0.770	22.037 ± 6.139
		18	28.317 ± 1.863	25.105 ± 7.117
		24	33.495 ± 2.557	26.668 ± 4.614
		30	35.958± 10.295	30.261 ± 8.029
		36	36.133 ± 1.568	40.073 ± 15.557
		42	37.781 ± 1.894	40.728 ± 12.765
		48	48.140 ± 8.565	70.838 ± 35.987
2.	Refrigerated (10^0C)	0	21.891 ± 0.938	3.063 ± 3.151
		6	22.974 ± 1.728	4.999 ± 5.224
		12	24.167 ± 0.249	11.182± 3.099
		18	26.266 ± 8.594	14.215 ± 6.613
		24	27.558 ± 4.166	21.824 ± 4.685
		30	29.344 ± 4.166	26.788 ± 9.566
		36	32.925 ± 3.237	26.362 ± 18.509
		42	36.917 ± 2.245	43.122 ± 14.952
		48	40.088 ± 3.825	64.427 ± 12.079

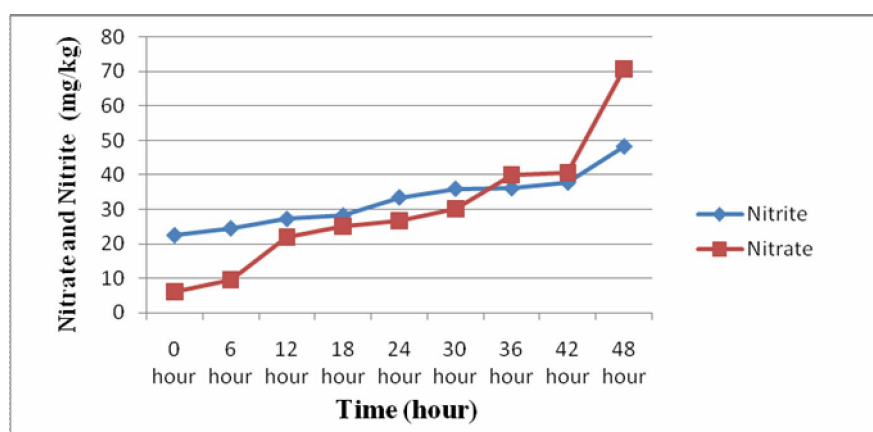*Note*: data is the mean of three replicates

Figure 1.The effect of storage time at room temperature (25^{0}C) on nitrite and nitrate levels in lettuce
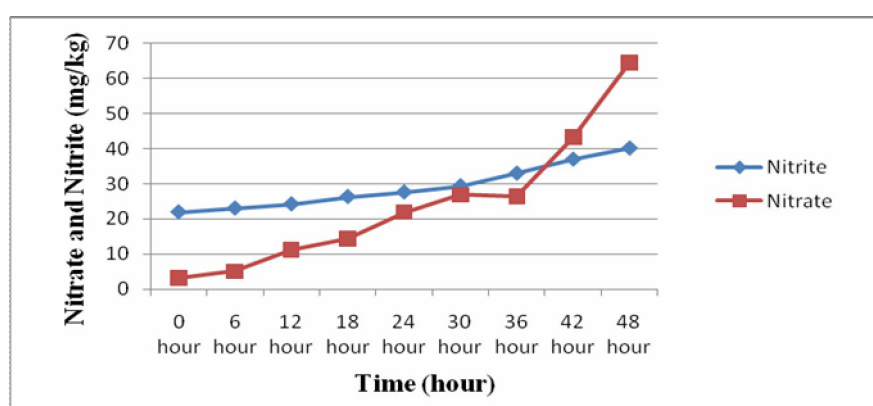

Figure 2.The effect of storage time at cold temperature (10^{0}C) on nitrite and nitrate levels in lettuce

From Table 1, Fig 1 and Fig 2, can be seen that before storage the levels of nitrite was similar but nitrate was not homogenous in the stored samples before treatment which could be affected by several factors including the location and type of soil where the vegetables grown.[12]The levels of nitrite is higher than nitrate in fresh lettuce and after storage which is different from values reported by Maynard,etal[12], who informed that nitrate is usually higher than nitrite in vegetables.Storage condition affected the levels of nitrite and nitrate in samples. Level of nitrite (22.63 mg/kg)was higher than nitrate (6.07 mg/kg) in fresh samples (before storage). This value is reverse with that previously reportedby Raczuk, et al.[7], who found that nitrite level in fresh lettuce was 25 mg/kg, but nitrate level in fresh lettuce was much higher with level of 1725 mg/kg. This difference could be due to the different samples grown on different soil, fertilization and environmental conditions affecting the nitrate and nitrite levels in the lettuce.[3,12]

The levels of nitrite and nitrate also increased with time of storage. During 48 hours of storage at room temperature nitrite levels increased from 22.63 mg/kg to 48.140 mg/kg, while nitrate increased from 6.07 mg/kg to 70.83 mg/kg. Similar trend also occurred in the samples during storage at room temperature. Nitrite levels increased from initial level (21.9 mg/kg) to the higher level (40.08 mg/kg) after 48 hours of storage. Nitrate content increased from initial level (3.06 mg/kg) to 64.42 mg/kgafter 48 hours of storage.

From Fig. 1 and 2, is also indicated that the increasing trend of nitrite and nitrate levels during storage is similar at different temperature of storage. Storage at room and cold temperature resulted in the similar trend on the increasing rate of nitrite and nitrate levels after 48 hours. Nitrite levels increased from intial level of 22. 6 mg/kg to 48.1 mg/kg after 48 hours during storage at room temperature; and increased from 21.9 mg/kg to 40 mg/kg at cold temperature of storage. The levels of nitrate increased from 6.0 mg/kg to 70.8 mg/kg during storage at room temperature; and increased from 3.0 mg/kg to 64.4 mg/kg at cold temperature of storage. This result is different from previous findings by Philips[13] who reported that the levels of nitrate and nitrite decreased during storage at low temperature below room temperature.

Conclusion

Results indicate that the level of nitrite in fresh lettuce is higher than nitrate. The levels of both nitrite and nitrate increased during storage. The increasing trend of nitrate levels is higher than that of nitrite during storage either in room and cold temperature; the higher the temperature of storage the higher the increasing rate of nitrite and nitrate levels. This result also suggests that fresh lettuce should not be stored too long before cooking.

References

1. Packer, PJ, and leach, SA. Human exposure, pharmacology and Metabolism of nitrate and nitrite.Cambridge: Woodhead Publishing Limited. 131-155.
2. Chew SC, K., Bao, NPY., and Ismail, A. Changes in Nitrate and Nitrite Levels of Blanched Amaranthus During Refrigeration Storage. 2011; 9(1); 29-34.
3. Hill, MJ. Nitrates and Nitrites in Food and Water. Woodhead Publishing Limited. New York. 1996; 93-105.
4. Novary, E. Penanganan dan Pengolahan Sayuran Segar. Jakarta: Penebar Swadaya. 1999; pp. 189-191.
5. Silalahi, J. Masalah Nitrit dan Nitrat dalam Makanan. *Medika: Jurnal Kedokteran Indonesia.* 2005; 31(5): 460 – 462.
6. Keshavarz, M, Maziomi, SM, Bahajavari, S. The effect of Home Cooking Method and Refrigeration Processes on the Nitrate and Nitrite in Spinach. *J Health Sci Surveilance Sys.* 2015; 3(3): 88-93.
7. Raczuk Jolanta, Wanda Wadas, Katarzyna Głozak. Nitrates And Nitrites In Selecte Vegetables Purchased at Supermarkets In Siedlce, Poland.*Rocz Panstw Zakl Hig*;2014; 65(1):15-20.
8. Hess, J. Association Official Methods of Analytical Chemists. Edisi XVII. Virginia: AOAC Inc. 2000.p. 8.
9. Vogel. Buku Teks Analisis Anorganik Kualitatif Makro dan Semimikro. *Edisi V.* Jakarta: PT. Kalman Media Pusaka. 1990;pp. 332, 356.
10. Lusiana, R. Analysis of Nitrite and Nitratein Sousages Marketed in Medan City by Visible Spectrophotometry. Thesis.Faculty of Pharmacy University of Sumatra Utara. Medan: Indonesia. 2013. p 22.
11. Syarief, W.R. Analisis Farmasi: Buku Ajar Untuk Mahasiswa Farmasi dan Praktisi Kimia Farmasi. Edisi Kedua. Jakarta: EGC. 2007; p. 126.
12. Maynard, D. N., A. V. Barker, P. L. Minotti, and N. H. Peck.. Nitrate Accumulation In Vegetables.Advances In Agronomy.28. 1976. pp. 71-118.
13. Phillips, W.E. Changes in the nitrate and nitrite contents of fresh and processed spinach during storage. *J. Agric. Food Chem*.1968. 16(1): 88-91.

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