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NITRATE, NITRITE AND UREA LEVELS IN SOME FRESH, COOKED AND REFRIGERATOR STORED VEGETABLES: CHEMICAL AND MICROBIOLOGICAL STUDIES

El-Okazy, A.M.; S. I. Saleh; M. M. Shabayek and S. M. Daoud
Central Lab. for Food and Feed, Agricultural Research Center, Alexandria.

ABSTRACT

Nitrate, nitrite and urea were determined in 11 fresh vegetables (eggplant, white potatoes, squash, okra, spinach, green French bean, Jew's mallow, yellow carrot, onion, garlic and tomato) collected from five Alexandrian markets. The highest nitrate level was recorded in spinach and squash, and the lowest was that of tomato. The highest nitrite content was recorded in Jew's mallow and okra while the lowest nitrite value was recorded in tomato. Garlic was found to contain the highest urea level while spinach showed the lowest. Cooking significantly reduced nitrate levels in the studied vegetables. The overall mean nitrate content showed a 77.66 % decrease on cooking. The mean nitrite content of the studied items showed increases on cooking of eggplant, white potatoes and squash. While okra, spinach and Jew's mallow nitrite levels decreased with cooking. The mean urea level of six of the studied vegetables showed increases after cooking. The highest rise in urea took place in eggplant (250.5%) while the lowest increase was recorded in squash (12.8%). Jew's mallow showed 16.3% reduction in the mean urea level after cooking. Nitrate level Significantly increased after two days of storage. Then a significant drop off occurred after 4 and 6 days of storage. Nitrite concentration increased in cooked vegetables on cold storage. It was also recognized that there was a significant variation in the effect of refrigerator storage on different vegetables under investigation. Cooked Jew' s Mallow and white potatoes showed the highest increases in concentrations of nitrite (622 % and 556 %) after six days of refrigerator storage. On the other hand, cooked squash showed the least rise in nitrite contents (129.6%) after the same period of storage under the same conditions. Urea level decreased through storage period. This reduction in urea level was directly proportional to the storage time. The total bacterial counts of the studied items showed a highly significant increase with storage under refrigerator temperature. Cooked white potato and Jew's Mallow were highly susceptible to contain higher microbial counts during storage at refrigerator temperature than other kinds of cooked vegetables. A highly significant decrease was recorded in spore counts of the studied items with storage under refrigerator temperature. *Coliform* bacteria counts increased significantly with the storage duration in *Coliform*- positive samples, okra, spinach and jew´ s mallow. The other studied types were negative for *Coliform*. All cooked items were positive for *Staphylococcus aureuos* and *Citrobacter sp.* and negative for *Salmonella sp.* along the storage period. *Shigella sp.* was detected in cooked eggplant, French bean and vegetable soup, while others were negative for it. *Enterobacter sp.* was detected in eggplant and vegetable soup. A significant negative correlation between nitrite and urea levels and a highly significant positive correlation between nitrite and bacterial total counts were found in cooked vegetables throughout the experiment. Nitrite also showed a highly significant positive correlation with *Coliform* count. Urea levels showed insignificant positive correlations with nitrate and nitrite and insignificant negative ones with bacterial, spores and *Coliform* counts during the storage period of the studied cooked vegetables.

These findings indicated that storage of cooked vegetables in the refrigerator did not sufficiently inhibit bacterial growth in the studied foods. Certain species of food-born bacteria assimilate proteins and nitrate of the cooked stuffs for the ultimate

synthesis of their own proteins resulting in increasing nitrite level. Such Increase may represent a health hazard for the consumer.

Keywords: Vegetables, Nitrate, Nitrite, Urea, Cooking, Storage, Contamination, and Microbiology.

INTRODUCTION

Humans are exposed to nitrate and nitrite primarily through the ingestion of food and water, Frank and Albert (1982). The main source of human exposure to nitrate and nitrite have been documented to be vegetables and fruits, Jakson *et al.* (1967), Brown and smith (1967), Zaldivar and Wetterstand (1975), Hunt and Turner (1994), Beijaars *et al.* (1994), Borwasska *et al.* (1996), Petersen and Stoltze (1999) and Ze-YI Z. *et al.*.(2000). According to the Food Balance Sheet of Egypt (1998), The mean percaput consumption of vegetables and fruits were estimated to be 448.3 and 261.9 g / day respectively. There are many factors affecting the accumulation of nitrate in plants. Soil type (Raikova and Petkove, 1996) and nitrogen fertilizers application (Greenwood and Hunt ,1986) are critical factors in determining nitrate level in plants. Inproper postharvest handling, processing and storage before consumption cause many serious changes in levels of nitrate and nitrite in vegetables, Philips (1968) and Poulson *et al.* (1995). In a 240- lady random sample in Alexandria, It was reported that 63.3 and 10 % of working and non-working ladies respectively used to cook twice aweek and keep cooked vegetables in the refregirator for the next three days ,Samir *et al.*. (1996). On the other hand deteriortion that may occure from microbial actions or from the action of enzymes such as nitrate reductase which is found in many plant tissues is an important factor that affect nitrate and nitrite levels in fresh and cooked vegetables , Hewitt (1975). Conversion of ingested nitrate to nitrite provides a source for in-vivo nitrosation of secondary amines in the diet to form carcinogenic nitrosamines, Walter and Smith (1981). Although nitrosamines are mostly produced from the nitrosation of amines, there are many reports on their formation from quaternary ammonium compounds and urea, Michael (1982). It also reacts with primary amines and amides to form the corresponding N-nitroso derivatives, many of which are carcinogenic, Collins – Thompson *et al.* (1972) and Van Maanen *et al.* (1998). Passmore and Eastwood (1986) reported that there is evidence consistent with the view that ingested nitrate could be a contributing cause of carcinoma of the stomach. Unlike nitrate, which is relatively inert chemically, nitrite is very reactive especially at low gastric pH in its protonated form nitrous acid which is an oxidizing and nitrosating agent, Michael (1982). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) (1995) has also established Acceptable Daily Intakes of nitrate and nitrite as 3.7 and 0.06 mg / kg body weight respectively. On the other hand, the European Commission? s EC) cientific ommittee or ood SCF) 1997) stablished an Acceptable Daily Intake (ADI) for nitrate ion of 3.65 mg / kg body weight.

The aim of the present study is to monitor levels of nitrate, nitrite and urea in some fresh vegetables available in the Alexandrian markets, and to investigate the effect of cooking and cold refrigerator storage on these levels and on their microbial contents.

MATERIALS AND METHODS

1- Sampling:

A total of 275 samples of fresh vegetables were collected from five markets of Alexandria Governorate autumn 2001. Five samples - one-kilogram each - of eggplant, white potatoes, squash, okra, spinach, green French bean, mollokheia (Jew's Mallow), yellow carrot, onion, garlic and tomato were collected randomly from each market of five vegetable markets in Alexandria which are Backus, Zananniery, El-Hadara, El-Mansheia and El-Gomrok. Samples were transported in an icebox to the Central Laboratory for Food and Feed, Agricultural Research Center, Alexandria immediately after purchasing to minimize post harvest and transportation loses and / or contamination.

2- Cooking processes:

One kilogram of each kind of vegetable was washed, cleaned of soil and non-edible parts and prepared for cooking the mean way used in conventional Egyptian kitchen According to the Egyptian Nutrition Institute, Composite Dishes and Food Composition Tables For Egypt (1996). For vegetable soup a mixture of equal weights of French bean, white potato, carrot and onion were used. In all cases, the mixture was boiled until tender. Cooked stuffs were kept in covered glass beakers into the lab refrigerator (4 ^{o} C) for 2, 4 and 6 days.

3- Chemical Analysis:

Samples of both fresh and cooked and refrigerator-stored vegetables were homogenized and prepared for the determination of nitrate, nitrite and urea. Nitrate was determined according to Harold *et al.* (1981) using an Orion 9307 BN nitrate ion-selective electrode after the removal of chloride ion with silver sulfate and destruction of nitrite with sulphamic acid. An Orion 900200 double junction reference electrode was used. Nitrate was measured at the Department of Environmental Studies, Institute of Graduate Studies and Research, Alexandria. Nitrate calibration curve was made up using a series of concentrations of a standard nitrate solution Orion 920706.

Nitrite was extracted and assayed using the modified Griess- Illosvay reaction described by Harold *et al.* (1981) which is based on sulfanilamide di-azo die reaction after the removal of proteins, fat and other interfering substances by di-sodium tetraborate, potassium ferrocyanide, zinc acetate solutions and activated vegetable char cool. The pH was adjusted to 8.3. The Optical Density of the die formed was measured at 538 nm against reagent blank using a *Spectronic 21-D UV-VIS* spectrophotometer. A standard curve was drown by measuring absorbances of a series of concentrations of standard sodium nitrite solution.

Urea was extracted and measured according to the method of AOAC (1990) using alcoholic 2,4-dimethyl aminobenzaldehyde solution, which develops a yellow color with urea. The color intensity - which is directly proportional to urea level in the aquas extract – was measured at 420 nm by *Spectronic 21-D UV-VIS* spectrophotometer. Urea standard curve was drown by measuring the absorbance of a series of concentrations of standard urea solution.

All reagents were of analytical grade. For analytical quality assurance, a recovery test was performed with each assay by measuring nitrate, nitrite and urea in prepared samples of known concentrations subjected to experimental conditions. This was to exclude or re-assay samples showed low recovery due to losses or high recovery due to interferences.

4- Microbiological Examination:

25 g of each homogenized cooked vegetable samples were aseptically weighed on sterilized greaseproof papers and homogenized in a Stomacher blender *Seward* in 225 ml of sterile 0.1 % pepton. The aerobic plate counts were determined using Standard Method Agar (SMA) technique according to AOAC (1990) in which pour plates were incubated at 22° C for four days (Speck, 1984). The most probable number (MPN) technique was used for estimating *Coliform bacilli* according to Collins and Zyne (1976). Counts of *Coliform sp.* and *E. coli* were determined using MacConkey broth. The inoculated media were incubated at 37 ° C and 43° C for 24, 48 h consequently. *Staphylococcus aureus* was determined using Baird-Parker medium (Baird –Parker, 1962). The streak plates were incubated at 37° C for 24 - 48 h. The Standard Operating Procedure (SOP) describes a method for the detection of Salmonella *sp.* in the presence of other (competing) microorganisms (Becckers *et al.*., 1985), Anonymous (1981). Spore forming bacteria (*Bacillus sp* and *Clostridium sp)* were determined using the procedures of Ockerman (1972).

5- Statistical Analysis:

Statistical analysis of the data was carried out using the analysis of variance (ANOVA) and the correlation coefficient according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

1- Levels of nitrate, nitrite and urea in selected fresh vegetables:

Table 1 shows values of nitrate, nitrite and urea in selected fresh vegetables. It was found that the highest nitrate level was recorded in spinach and squash, and the lowest nitrate level was that of tomato. The highest nitrite content was recorded in Jew's Mallow and okra while the lowest nitrite value was recorded in tomato. On the other hand, garlic was found to contain the highest urea level while spinach showed the lowest urea content. It is worth to mention that Joint FAO / WHO, FOOD Additives and Contaminants JECFA (1995) stated that ingestion of only 100 g of vegetables with a nitrate concentration of 2500 mg / kg fresh weight exceeds the acceptable daily intake for nitrate by 13%. On the other hand, an exposure of 1 mg nitrite will be about triple the acceptable daily intake for nitrite for a 60 kg person. It was noted that there were a significant variations between values recorded for the same kind which was appeared as high standard deviations in many cases. This was due to variations in the time between harvesting and purchasing, and variation in transportation and storage conditions. Muramoto (1999) studied the effect of storage and transportation on nitrate content of fresh leafy vegetables in California USA. It was recorded that the period from unloading to purchasing was 0.9 day (0 – 4 days) in supermarkets while it was 2.2 days (0–5 days) at natural food stores. Refrigeration temperatures varied between 1.7 and 10° C. The display rack temperatures ranged from 0.6 to 11° C. These factors and others are the causes of wide range of concentrations of nitrate, nitrite and urea in the studied vegetables.

Table 1: Levels of nitrate, nitrite, and urea (mg / kg) in selected fresh vegetables in Alexandria:

Type	Nitrate		Nitrite		Urea	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
1-Egg plant	910.46± 1317.4	310.6 – 3495.8	2.12 ± 2.35	5.5.31	0.47 ± 431.09	215.88 – 1149.26
2-White potatoes	419.17 ± 662.66	86.96 – 1413.7	0.84 ± 0.47	0.47 – 1.65	1569.22 ± 169.8	1396.89 – 1733.47
3-Squash	1435.13 ± 1140.7	434.8 – 1879.73	1.98 ± 2.47	0.75 – 6.39	955.7 ± 277.35	967.15 – 1403.24
4-Fresh okra	411.52 ± 367.98	211.24 – 963.1	12.27 ± 2.13	10.58 – 14.9	890.74 ± 575.71	317.48 – 1639.01
5-Spinach	2280.59 ± 706.74	1667.1 – 2893.26	5.0 ± 3.56	0.53 – 9.41	214.69 ± 117.38	129.11 – 381.71
6-French bean	588.21 ± 311.3	230.7 – 1129.4	0.41 ± 0.84	0.0 – 1.91	935.13 ± 252.21	679.4 – 1193.71
Jew's Mallow	831.06 ± 700.57	282.3 – 1858.91	14.51 ± 11.43	2.05 – 32.1	883.6 ± 401.74	619.09 – 1473.97
8- Carrot	60.78 ± 72.64	0.0 – 144.50	1.3 ± 2.02	0.0 - 4.77	537.32 ± 143.71	384.14 – 711.14
9- Onion	165.08 ± 134.53	23.4 – 306.9	0.17 ± 0.17	0.0 - 0.35	813.82 ± 205.78	622.25 – 1028.62
10- Garlic	430.24 ± 234.38	102.4 – 621.2	0.23 ± 0.28	0.0 - 0.71	3923 ± 166.33	3739.86 – 4143.05
11- Tomato	12.35 ± 14.65	0.0 - 28.8	0.09 ± 0.10	0.0 – 0.29	1130.85 ± 134.64	1022.27 – 1327.05

2- Effect of cooking vegetables on their nitrate, nitrite and urea contents:

Table 2 represents the effect of cooking on vegetables nitrate, nitrite and urea content. It was revealed that cooking process lowered nitrate contents in cooked vegetables than the raw ones shown in Table 1. The overall mean nitrate content of the studied cooked items showed a 77.66 % decrease on cooking. Similar results were obtained by Markowska *et al.*. (1995) who found that heat processing induced more than 50 % decline in nitrate contents of cooked vegetables. Ezeagu and Fafunso (1995) investigated the effect of cooking of seven varieties of vegetables and reported that cooking cause similar meaningful declines in nitrate contents.

It was also found that the mean nitrite content of the studied items showed increases after cooking of eggplant, white potatoes and squash. Whereas nitrite levels of okra, spinach and Jew's mallow decreased with cooking. Ezeagu and Fafunso (1995) reported an increase in nitrite content in vegetables on cooking. While Markowska *et al.* (1995) reported that thermal processing of vegetables causes loss of nitrite reached 100 % in many cases.

Table 2: Effect of cooking on selected vegetables nitrate, nitrite and urea contents:

Type	Nitrate		Nitrite		Urea	
	Mean ± SD	% Increase or reduction	Mean ± SD	% Increase or reduction	Mean ± SD	% Increase or reduction
1-Egg plant	95.75 ± 23.67	- 89.5%	0.84 ± 0.26	+ 60.4 %	1999.34 ± 592.3	+ 250.5 %
2-White potato	86.04 ± 8.08	- 79.4 %	0.91 ± 0.53	+ 8.3 %	1906.45 ± 404.2	+ 21.5 %
3-Squash	118.14 ± 18.74	- 91.8 %	1.69 ± 0.89	+ 14.6 %	1077.81 ± 8.52	+ 12.8 %
4-Okra	85.52 ± 2.77	- 79.2 %	1.67 ± 0.9	- 86.4 %	1749.1 ± 69.3	+ 96.4 %
5-Spinach	150.0 ± 35.12	- 93.4 %	3.4 ± 0.23	- 32.0 %	1540.75 ± 57.7	+ 617.7 %
6-French bean	62.95 ± 10.48	- 89.3 %	1.59 ± 0.52	+ 74.2 %	1095.13 ± 67.3	+ 17.1 %
7-Jew's Mallow	365.73 ± 133.13	- 56.0%	1.18 ± 0.68	- 91.9 %	739.69 ± 82.27	- 16.3 %

The mean urea level of six of the studied vegetables showed increases after cooking. The highest rise in urea took place in eggplant (250.5%) while the lowest increase was recorded in squash (12.8%). Jew's Mallow showed 16.3% reduction in the mean urea level after cooking.

3- Effect of storage at refrigerator temperature on cooked vegetables nitrate, nitrite ad urea contents:

Table 3 represents the effect of cold storage of cooked vegetables at refrigerator temperature on their nitrate content. It was noted that there is a statistically significant increase ($p < 0.05$) in nitrate level after two days of storage. Then a significant drop off occurred through the fourth and sixth days of storage. There were no significant differences in the effect of storage on nitrate levels ($p > 0.05$) among the selected items.

Table3: The effect of storage at refrigerator temperature on cooked vegetables nitrate content:

Type	Storage periods** at 4 ° C (days)				
	0^c	2^a	4^b	6^b	*LSD between types at 0.05 level of significance =380.81608*
Egg plant	95.75 ± 23.67	555.15 ± 263.9	439.47 ± 194.28	337.53 ± 134.23	
White potatoes	86.04 ± 8.08	1314.33 ± 692.97	278.47 ± 111.14	284.8 ± 153.58	
Squash	118.14 ± 18.74	1636.67 ± 796.92	498.13 ± 124	472.2 ± 75.09	
Fresh okra	85.52 ± 2.77	1783.73 ± 929.55	233.9 ± 90.76	121.92 ± 40.46	
Spinach	150.0 ± 35.12	890 ± 173.44	856.9 ± 234.93	1299.98 ± 57.74	
French bean	62.95 ± 10.48	614.91 ± 290.42	460.58 ± 202.35	216.58 ± 60.64	
Vegetable soup	213.1 ± 72.97	862.23 ± 375.48	463.85 ± 184.85	405.07 ± 138.65	
Jew's Mallow	365.73 ± 133.13	365.7 ± 133.13	447.93 ± 179.15	685.33 ± 300.27	
LSD between storage periods at 0.05 level of significance = 269.27763					

Values represent mean of three-replica ± SD

*** = Significant difference at 0.05 level of significance.**

**** = Highly significant difference at 0.01 level of significance.**

- Differences between means of the same superscript litters (a, b, c and d) are statistically insignificant.

Table 4 represents the influence of storage at refrigerator temperature on nitrite concentration in cooked vegetables. It is noted that a highly significant ($p < 0.01$) increase in nitrite content was recorded in cooked vegetables on storage. This rise was directly proportional to the storage duration through the experimental period. It was also recognized that there was a significant variation ($p<0.05$) in the effect of refrigerator storage on different vegetables under investigation. Cooked Jew' s Mallow and white potatoes showed the highest increases in concentrations of nitrite (622 % and 556 %) after six days of refrigerator storage. On the other hand cooked squash showed the least rise in nitrite contents (129.6%) after the same period of storage under the same conditions.

Table 4: The effect of storage at refrigerator temperature on cooked vegetables nitrite content:

Type*	Storage periods** at 4 ° C (days)				LSD between types at 0.05 level of significance =0.77656
	0 [c]	2 [b]	4 [b]	6 [a]	
Egg plant [bc]	0.84 ± 0.26	2.58 ± 1.05	2.48 ± 0.06	3.12 ± 0.58	
White potatoes [ab]	0.91 ± 0.53	3.09 ± 1.78	2.13 ± 1.05	5.06 ± 0.58	
Squash [c]	1.69 ± 0.89	1.84 ± 0.46	1.97 ± 0.12	2.19 ± 0.55	
Fresh okra [c]	1.67 ± 0.9	1.17 ± 0.02	2.11 ± 0.44	3.03 ± 0.58	
Spinach [bc]	3.4 ± 0.23	1.71 ± 0.4	1.91 ± 0.29	2.15 ± 0.09	
French bean [bc]	1.59 ± 0.52	1.4 ± 0.76	3.55 ± 0.93	3.45 ± 0.15	
Vegetable soup [bc]	1.55 ± 0.74	3.76 ± 0.98	1.55 ± 0.21	3.87 ± 0.06	
Jew's Mallow [a]	1.18 ± 0.68	2.92 ± 0.12	1.88 ± 0.25	7.35 ± 0.2	
LSD between storage periods at 0.05 level of significance = 0.549114					

- **Values represent mean of three replica ± SD**
- *** = Significant difference at 0.05 level of significance.**
- **** = Highly significant difference at 0.01 level of significance.**
- **Differences between means of the same superscript litters (a, b, c and d) are statistically insignificant.**

Table 5: The effect of storage at refrigerator temperature on cooked vegetables urea content:

Type**	Storage periods** at 4 ° C (days)				LSD between types at 0.05 level of significance
	0 [a]	2 [b]	4 [c]	6 [c]	
Egg plant [b]	1999.34 ± 592.3	879.33 ± 69.3	460.91 ± 121.25	507.94 ± 115.74	
White potatoes [a]	1906.45 ± 404.2	1503.26 ± 288.7	907.29 ± 58.19	865.57 ± 14.45	
Squash [b]	1077.81 ± 8.52	1129.93 ± 133.21	757.67 ± 178.7	840.45 ± 202.1	
Fresh okra [a]	1749.1 ± 69.3	1529.02 ± 237.3	903.06 ± 20.3	898.15 ± 38.68	
Spinach [a]	1540.75 ± 57.7	1391.01 ± 57.75	1030.03 ± 17.32	1103.1 ± 51.64	
French bean [b]	1095.13 ± 67.3	1045.136 ± 116.05	654.87 ± 158.77	759.6 ± 186.93	
Vegetable soup [b]	1432.05 ± 157.4	1326.62 ± 3.75	277.46 ± 194.72	680.85 ± 5.84	
Jew's Mallow [c]	739.69 ± 82.27	596.7 ± 107.5	438.04 ± 95.26	488.97 ± 106.23	
LSD between storage periods at 0.05 level of significance = 174.311871					

Values represent mean of three replica ± SD
*** = Significant difference at 0.05 level of significance.**
**** = Highly significant difference at 0.01 level of significance.**
- **Differences between means of the same superscript litters (a, b, c and d) are statistically insignificant.**

Table 5 illustrates the effect of storage on cooked vegetables urea contents. It was obtained that urea level decreased in a highly significant manner ($p<0.01$) through storage period. This reduction in urea level was directly proportional to the storage time. A highly significant difference ($p<0.01$) was also found among the mean urea content of different types of cooked vegetables along the experimental period. Potatoes, okra and spinach contained higher levels of urea and showed 45.4 %, 48.7 % and 28.4

% reduction in urea levels respectively after six days of cold storage. Eggplant, squash, French bean come second and exhibited 49.3%, 22 % and 30.64 % respectively reduction in urea after six days of cold storage. Jew's Mallow showed the least mean urea content along the experimental period and proved a 33.9 % reduction in urea content after six days of cold storage.

Reduction in nitrate and increase in nitrite through out the storage period were due to some microorganisms, which convert nitrate to ammonia or amino acids for ultimate synthesis of proteins. The bacterial enzyme involved in the first step of this process reduces nitrate to nitrite and is called assimilatory nitrate reductase. While other organisms use nitrate as a terminal electron acceptor instead of oxygen under anaerobic or partially anaerobic conditions. The enzyme involved in this process reduces nitrate to nitrite and is called dissimilatory nitrate reductase. Certain microorganisms can also make nitrite by oxidation of ammonia in a process known as nitrosification, Michael (1982).

4- Microbiological examinations:

i- Effect of storage at refrigerator temperature on cooked vegetables microbial content:

Table 6 illustrates changes took place in total bacterial count of cooked vegetables. A highly significant increase ($p < 0.01$) was recorded in the total bacterial counts of the studied items with storage under refrigerator temperature. It was also found that there was statistically highly significant ($p<0.01$) variation among the bacterial counts of various stored types after the experimental storage period. Cooked white potato and Jew's Mallow were highly susceptible to contain higher microbial counts during storage at refrigerator temperature than other kinds of cooked vegetables. Cooked spinach comes second but showed also a great tendency to contain higher bacterial counts with storage. Then come the vegetable soup and cooked okra. There were no significant differences among the mean aerobic plate counts of cooked eggplant, squash and French bean. They exhibited the lower increases in bacterial counts with storage. On the other hand, it should be mentioned that cooked eggplant showed the least increase in bacterial count throughout the storage period. Harrigan and Park (1991) reported that microbial contents of cooked vegetables are related to insufficient cooking temperature permitting survival of mesophilic microorganisms. Storing warm, cooling slowly after cooking or the improper handlings permit growth of various microorganisms. Joint FAO/WHO Food Standard Program (1997) referred to refrigerator storage of foodstuffs as high-risk process. This is because temperature abuse during processing, storage and handling by the consumer may allow the growth of pathogenic microorganism unless additional hurdles are used to prevent microbial growth. It was also reported that many factors control microbial growth and the shelf life of the refrigerator-stored foodstuffs. Ingredients, nutrients, water activity, pH, gas atmosphere, redox potential, storage temperature and storage time are important limiting factors to control microbial growth.

Table 6: Effect of storage at refrigerator temperature on cooked vegetables microbial content:

Type**	Storage periods** at 4 ° C (days) 0 ^{b}	2 ^{b}	4 ^{b}	6 ^{a}	LSD between types at 0.05 level of significance =7292.941566
Egg plant ^{d}	0.5 ± 0.05	3.5 ± 0.44	3.0 ± 0.3	5.0 ± 1.1	
White potatoes ^{a}	2.7 ± 0.15	132 ±8.0	2000.0 ± 13.23	270000 ± 48218	
Squash ^{d}	1.0 ± 0.1	30.0 ± 5.0	100.0 ± 17.44	300.0 ± 40.0	
okra ^{cd}	0.95 ± 0.05	7.1 ± 1.05	95.0 ± 6.2	17400 ± 2116.7	
Spinach ^{b}	2.9 ± 0.0	73.67± 8.74	1010.0 ± 7.64	129000 ± 8453	
French bean ^{d}	0.35 ± 0.05	127.7 ± 25.17	102.0 ± 6.08	96.0 ± 10.15	
Vegetable soup ^{c}	1.5 ± 0.1	5.0 ± 0.1	900.0 ± 70.0	30000 ± 3000	
Jew's Mallow ^{a}	2.5 ± 0.1	64.0 ± 11	900.0 ± 10.0	250000 ±13000	
LSD between storage periods at 0.05 level of significance = 5156.888436					

Values represent mean x 10^3 of three replica ± SD
*** = Significant difference at 0.05 level of significance.**
**** = Highly significant difference at 0.01 level of significance.**
- Differences between means of the same superscript litters (a, b, c and d) are statistically insignificant.

Table 7: The effect of storage at refrigerator temperature on cooked vegetables spore content:

Type**	Storage periods** at 4 ° C (days) 0 ^{a}	2 ^{a}	4 ^{b}	6 ^{c}	LSD between types at 0.05 level of significance =0.7106771942
Egg plant ^{d}	1.3 ± 0.1	1.0 ± 0.087	1.1 ± 0.13	0.75 ± 0.2	
White potatoes ^{d}	1.2 ± 0.0	1.0 ± 0.087	1.1 ± 0.13	0.75 ± 0.2	
Squash ^{c}	2.9 ± 0.1	3.0 ± 0.2	1.48 ± 0.34	1.05 ± 0.13	
Fresh okra ^{cd}	2.0 ± 0.1	2.0 ± 0.3	1.2 ± 0.07	0.5 ± 0.03	
Spinach ^{b}	10.1 ± 0.1	10.4 ± 0.7	8.57 ± 0.4	3.13 ± 0.32	
French bean ^{a}	13.8 ± 1.1	13.6 ± 4.3	10.0 ± 1.32	1.0 ± 0.53	
Vegetable soup ^{c}	3 ± 0.0	2.5 ± 0.03	1.5 ± 0.26	0.48 ± 0.02	
Jew's Mallow ^{b}	10.5 ± 0.2	10.0 ± 0.5	8.0 ± 1.5	2.0 ±0.4	
LSD between storage periods at 0.05 level of significance = 0.5025246632					

Values represent mean x 10^3 of three replica ± SD
*** = Significant difference at 0.05 level of significance.**
**** = Highly significant difference at 0.01 level of significance.**
Differences between means of the same superscript litters (a, b, c and d) are statistically insignificant.

Table 8: The effect of storage at refrigerator temperature on cooked vegetables *Coliform sp.* content:

Type**	Storage periods** at 4 ° C (days) 0 ^{c}	2 ^{c}	4 ^{b}	6 ^{a}	LSD between types at 0.05 level of significance =1.494231943
Egg plant ^{d}	0.0	0.0	0.0	0.0	
White potatoes ^{d}	0.0	0.0	0.0	0.0	
Squash ^{d}	0.0	0.0	0.0	0.0	
okra ^{c}	0.3 ± 0.05	0.4 ± 0.02	6.5 ± 0.2	15 ± 1	
Spinach ^{b}	4 ± 0.2	7.5 ± 0.15	15 ± 1.2	100 ± 10	
French bean ^{d}	0.0	0.0	0.0	0.0	
Vegetable soup ^{d}	0.0	0.0	0.0	0.0	
Jew's Mallow ^{a}	4.5 ± 0.5	7.5 ± 0.0	20 ± 1.5	110 ± 0.04	
LSD between storage periods at 0.05 level of significance = 1.056581539					

Values represent mean x 10^3 of three replica ± SD
*** = Significant difference at 0.05 level of significance.**
**** = Highly significant difference at 0.01 level of significance.**
Differences between means of the same superscript litters (a, b, c and d) are statistically insignificant.

Table 9: Values of correlation coefficient (r) between measured parameters

	Nitrate	Nitrite	Urea	Bactrial count	Spores	*Coliform*
Nitrate	1	0.00924	0.080995	0.106065	-0.03627	0.254832*
Nitrite		1	-0.3912**	0.701227**	-0.15101	0.460886**
Urea			1	-0.18906	-0.03927	-0.17716
Bactrial count				1	-0.16147	0.645494**
Spores					1	-0.00583
Coliform						1

* Significant correlation at 0.05 level of significance.
** Highly significant correlation at 0.01 level of significance

ii- Effect of storage at refrigerator temperature on cooked vegetables spore count:

Table 7 illustrates variations in spore count of cooked vegetables stored at refrigerator temperature for six days. A highly significant decrease ($p < 0.01$) was recorded in spore counts of the studied items with storage under refrigerator temperature. Decreases in spore count may indicate that storage under refrigerator conditions is suitable for spore-forming microorganisms to germinate and reproduce, Lisa McGowan (1997). It was also found that there was a statistically highly significant ($p<0.01$) variation among spore counts of various stored types after the experimental storage period. Cooked French bean contained the highest spore count followed by spinach and Jew's Mallow. There were no significant differences among the mean spore counts of cooked eggplant, white potatoes, squash, okra and vegetable soup. Soil is the primary source of Bacillus and Clostridium species. These spores survive most conventional cooking; they grow very slowly at 10º C and slower with decreasing the chilled storage temperature, Betty and Diane (1993).

iii- The effect of storage at refrigerator temperature on cooked vegetables *Coliform sp.* content:

Coliforms are widespread and can be detected in many types of food products. They have been employed as indicators of possible fecal contamination of foods. The effect of storage duration at refrigerator temperature on total *Coliform* count in different types of cooked vegetables is shown in Table 8. It was noticed that *Coliform* bacteria counts increased significantly with the storage duration in *Coliform*- positive samples, okra, spinach and jew´ s mallow. On the other hand, the other studied types were negative for *Coliform*. *Coliform Sp* may reach vegetables through contaminated soils or irrigation water. EA-ESTI (1997) Detected the total *Coliform count* in many locations at the downstream of the fresh water Nubareia Canal south west of Alexandria as 1200 MPN / 100ml.

d- Examination of other microorganisms in cooked vegetables stored at refrigerator temperature:

It was found that all cooked items were positive for *Staphylococcus aureuos* and *Citrobacter sp.* and negative for *Salmonella sp.* along the storage period. *Shigella sp.* was detected in cooked eggplant, French bean and vegetable soup, while others were negative for *Shigella sp.* *Enterobacter sp.* was detected in eggplant and vegetable soup. The presence of

Staphylococcus aureus is considered as an indicator of potential health hazard due to staphylococcal enterotoxin, as well as questionable sanitation, Bartram (1967). Also the presence of Citrobacter, Shigella and Enterobacter are useful indicators of contamination. They are found in soil, human sewage waste. A considerable amounts of vegetables consumed in Alexandria were cultivated at Abees area, east of the city through which passes the major sewage waste water drain (Kalaa Drain). This may be a significant source of contamination of irrigation canals and soils with many hazardous microorganisms, EA-ESTI (1997), Joint FAO/WHO Food Standard Program (1998).

5- Correlations between chemical and microbiological determinants:

Table 8 presents correlation matrix between chemical and microbiological parameters measured. It was noted that there is a significant negative correlation (r = -0.39116**) between nitrite and urea levels in cooked vegetables throughout the experiment. Also, there is a highly significant positive correlation (r = 0.701227**) between nitrite content and bacterial total counts of the stored cooked vegetables. Nitrite also showed a highly significant positive correlation (r= 0.460886 **) with Coliform count. It should be mentioned that Urea levels showed insignificant positive correlation with nitrate and nitrite and insignificant negative correlation with bacterial, spores and Coliform counts during the storage period of the studied cooked vegetables.

These findings indicate that storage in the refrigerator did not sufficiently inhibit bacterial growth in the studied foods. Certain species of food-born bacteria assimilate proteins of the cooked stuffs resulting in increasing nitrate and urea levels. Such raise in nitrate and urea levels appeared insignificant because bacteria broken them down into nitrite which showed a highly significant positive correlation with bacterial count and a highly significant negative one with urea levels

CONCLUSIONS

Finding revealed that storage under refrigerator temperature increases nitrite levels in cooked vegetables, which may increase the opportunity of endogenous formation of the hazardous nitrosamines and N-nitrosocompounds.

Findings rejected the hypothesis that cold storage increases the risk of ingesting a combination of nitrite and urea where urea as well as nitrates was utilized by microorganisms throughout storage period and most probably converted to nitrite.

Vegetables should be cultivated in clean soils and irrigated with clean water because contamination of soil and water with sewage or untreated sludge increase the risk of microbial contamination.

Vegetables must be properly washed, cleaned and stewed and consumed directly after cooking to avoid microbial contamination and growth, which - in turn – adversely changes the chemical composition of cooked vegetables.

Cooked vegetables should not be stored within the temperature range 4 – 60 ° C to avoid spoilage and pathogenic microorganisms.

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مستويات النيترات و النيتريت و اليوريا في بعض الخضر الطازجة و المطبوخة و المخزنة بالثلاجة- دراسة كيميائية و ميكروبيولوجية

أحمد محمد العكازي-سناء ابراهيم صالح-ماجدة محمد شبايك – سلوى محمد داوود
المعمل المركزي للاغذية و الأعلاف – مركز البحوث الزراعية – الإسكندرية

تم تقدير النيترات و النيتريت و اليوريا في ١١ صنفاً من الخضر الطازجة و هي الباذنجان ، البطاطس، الكوسة، البامية، السبانخ، الفاصوليا الخضراء، الجزر الأصفر، البصل، الثوم و الطماطم بالإسكندرية في خريف عام ٢٠٠١ . و كان أعلى مستويات النيترات في السبانخ و الكوسة بينما احتوت الطماطم على أقل تركيز من النيترات. و أظهرت الملوخية و البامية أعلى تركيزات النيتريت بينما كان أقل تركيز للنيتريت في الطماطم. و كان أعلى مستوى لليوريا في الثوم و أقل تركيز لها في السبانخ.

و قد أثبتت الدراسة أن عملية الطبخ تقلل النيترات بصورة معنوية حيث كان المتوسط العام للإنخفاض ٧٨% بعد الطبخ. من ناحية أخرى قد أحدثت عملية الطبخ زيادة في مستوى النيتريت في كل من الباذنجان، البطاطس و الكوسة، و انخفاض في البامية و السبانخ و الملوخية. أظهرت كل الأصناف ارتفاعا في تركيز اليوريا بنسب مختلفة بعد الطبخ فيما عدا الملوخية فقد قلت اليوريا بمعدل ١٦,٣% بعد الطبخ.

وتم أيضا دراسة تأثير تخزين الخضر المطبوخة بالثلاجة على مستويات النيترات و النيتريت و اليوريا بها. و قد أظهر مستوى النيترات ارتفاعا معنويا بعد يومين من التخزين بالثلاجة ثم تبعه انخفاضا معنويا في اليوم الرابع و السادس. بينما أظهر مستوى النيتريت ارتفاعاً طردياً مع طول فترة التخزين بالثلاجة. و لوحظ أيضاً أن هناك تفاوت في نسب الإرتفاع في النيتريت مع التخزين ، فقد وصلت الى ٦٢٢% في حالة الملوخية و ٥٥٦% في حالة البطاطس، بينما ارتفع النيتريت في الكوسة ١٣٠% بعد ٦ أيام تخزين في نفس الظروف . لوحظ انخفاض مستوى اليوريا بصورة متناسبة طرديا مع فترة التخزين.

و قد أظهر العد البكيري الكلي للأصناف المطبوخة ارتفاعاً معنويا عند التخزين في درجة حرارة الثلاجة(٤ م) وتناسب هذا الإرتفاع طرديا مع طول فترة التخزين. و أظهرت كلا من البطاطس و الملوخية أعلى قابلية لإحتواء أكبر عدد من البكتيريا خلال التخزين. و قد سجل أيضا انخفاضاً عالي المعنوية في محتوى الجراثيم في الخضر المطبوخة و المخزنة بالثلاجة. و لوحظت زيادة كبيرة في عدد بكتيريا الكوليفورم مع طول فترة التخزين وذلك في العينات الموجبة (البامية و السبانخ و الملوخية) بينما كانت باقي الأصناف سالبة لبكتيريا الكوليفورم. و قد كانت كل الأصناف موجبة للإستافيلوكوكس اوريوس و سيتروباكتر، و سالبة للسالمونيلا على طول فترة التخزين. و رصدت بكتيريا الشيجيلا و الإنتيروباكتر بكل من الباذنجان المطبوخ و الفاصوليا الخضراء و التورلي بينما لم توجد في باقي الأصناف.و عند حساب معاملات الإرتباط بين التقديرات الكيماوية و الميكروبيولوجية وجد أن هناك ارتباط معنوي سالب بين قيم النيتريت و قيم اليوريا. و ارتباط موجب عالي المعنوية بين النيتريت وكلا من العد البكتيري الكلي والكوليفورم.

أوصت الدراسة بأن حفظ الخضر المطبوخة في الثلاجة لم يحد تماما من نشاط البكتيريا و التي تقوم بتحليل البروتينات و النيترات في الخضر المطبوخة الي النيتريت – الذي له تأثير سلبي على المستهلك- تزداد تركيزاته بطول فترة التخزين. وأن هناك أصناف تتحمل التخزين في الثلاجة لأيام و أخرى يجب تناولها بعد طبخها مباشرة لتفادي تأثير البكتيريا علي تركيبها الكيميائى بما يؤثر سلباً على صحة المستهلك.