Risk Assessment Studies

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Chemical Hazard Evaluation

Changes in Nitrate and Nitrite Levels of Cooked Vegetables during Storage

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Centre for Food Safety

Food and Environmental Hygiene Department

The Government of the Hong Kong Special Administrative Region

Correspondence:

Risk Assessment Section

Centre for Food Safety

Food and Environmental Hygiene Department

43/F, Queensway Government Offices,

66 Queensway, Hong Kong.

Email: enquiries@fehd.gov.hk

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EXECUTIVE SUMMARY

Vegetables are good sources of dietary fibres, vitamins and minerals, and the consumption of vegetables is linked to a lower incidence of cardiovascular disease and obesity. Although vegetables have a positive impact on health, some people are concerned about the alleged increase in the nitrite levels of cooked vegetables that have been stored overnight in a refrigerator.

2. Fresh vegetables have a relatively high nitrate content but a low nitrite level. Human exposure to nitrate is mainly through the consumption of vegetables while exposure to nitrite is mainly from the conversion of nitrate in the body. In the body, nitrite can oxidise haemoglobin in blood and cause methaemoglobinaemia. It may also react with certain amines and amides in the body and produce nitrosamines which are potentially cancer-causing in experimental animals. In 2010, the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) concluded that ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans (i.e. Group 2A agent). However, evidence concerning whether nitrate or nitrite per se in food can cause cancer in humans is inadequate or limited.

3. Nitrate is a major form of nitrogen absorbed by plants. It can be reduced to nitrite by nitrate reductase, an enzyme present in plant cells, or by

nitrate-reducing bacteria in the environment. Some studies found that the contents of nitrate and nitrite in fresh leafy vegetables during storage were affected by the storage time and temperature. As regards cooked vegetables, one study showed that the nitrite content in cooked Pak-choi stored at room temperature for 48 hours could be up to 3 times of those kept refrigerated. Nonetheless, relevant scientific studies of the effects of storage time and temperature of cooked vegetables on the levels of nitrate and nitrite are limited. Recently, there have been local media reports, arousing public attention, on the possibility of a significant increase in the nitrite level of cooked vegetables that have been stored overnight in a refrigerator.

4. This study (i) examined the changes in the levels of nitrate and nitrite of cooked vegetables stored at room temperature and at refrigerated temperature for up to 78 hours after cooking; and (ii) offered advice on proper ways of handling leftover cooked vegetables.

Methods

5. Vegetable samples (amaranth, Pak-choi, flowering white cabbage, Chinese lettuce and zucchini) were collected between June and August 2021 from wet market stalls and a supermarket. They were thoroughly washed and then cooked by (a) boiling in distilled water and (b) stir-frying. Before cooking, their nitrate and nitrite contents were determined. After cooking, each type of vegetable was divided into two groups, and stored separately at room and refrigerated temperature. Their nitrate and nitrite levels were determined at 0, 6, 12, 24, 36, 48, 72 and 78 hours after cooking. In addition,

the nitrate and nitrite levels of a vegetable soup (prepared by boiling beetroot, carrot and celery in distilled water) were also determined at 0, 6, 12, 24, 36, 48, 72 and 78 hours after cooking.

Results

6. Before and immediately after cooking, no nitrite was detected in all vegetable samples and soup.

7. At room temperature, nitrite started to increase (with a corresponding decrease in nitrate) in some cooked vegetables and soup after storing them for 12 hours. In contrast, at refrigerated temperature, low levels of nitrite were only detected in some samples after storing them for 72 hours.

Discussion

8. Nitrate in vegetables can be converted to nitrite by nitrate reductase in plant cells or by nitrate-reducing bacteria present in the environment. Cooking denatures nitrate reductase and hence, the detection of nitrite in cooked vegetables after storage for a period of time is expected due to the activities of nitrate-reducing bacteria in the environment.

9. When handling cooked vegetables, bacterial contamination from the air and utensils is inevitable even though good hygienic practices are followed. Subsequent storage temperature of cooked vegetables has a great effect on the growth and activity of the bacteria. If the storage temperature is low (e.g. 0°C

– 4°C), some bacteria are unable to grow while others may grow and multiply slowly.

10. In this study, nitrite began to be detected in some vegetable samples when the samples had been stored at room temperature for 12 hours after cooking, indicating that nitrate in the cooked vegetables was converted to nitrite by nitrate-reducing bacteria. When the cooked vegetables were stored at refrigerated temperature, the low temperature reduced the growth of bacteria and their activities to convert nitrate to nitrite. Hence, the formation of nitrite was delayed, and low levels of nitrite started to be detected in some cooked vegetables after storing for 72 hours.

11. It is worthwhile to note that storage temperature, instead of the types of vegetables, plays a significant role in the conversion of nitrate to nitrite because it directly affects the growth and activities of bacteria.

Conclusion and recommendations

12. This study revealed that storage temperature has a significant effect on the conversion of nitrate to nitrite in cooked vegetables. When cooked vegetables were stored overnight in a refrigerator, their nitrite contents did not increase. In fact, under refrigeration, low levels of nitrite were only detected in some samples after storing them for 72 hours.

13. From a food safety perspective, overseas authorities including WHO recommend that leftover food should be cooled in clean and shallow containers,

and refrigerated in covered containers within 2 hours after cooking. Leftovers should not be stored in the refrigerator for longer than 3 days because spoilage bacteria can still grow at refrigerated temperature even though most harmful bacteria cannot.

14. At room temperature, the total time that cooked vegetables can be kept should be limited to 4 hours because cooked vegetables are considered potentially hazardous foods which may contain harmful bacteria and will normally support their growth and formation of toxins.

Advice to Public

15. Members of the public are recommended to eat a variety of vegetables with a daily intake of at least 3 servings of vegetables, and 2 servings of fruit i.e. at least 400 grams of fruits and vegetables (about 5 servings) for the prevention of chronic diseases.

16. The public should follow the following guidelines to keep leftovers (such as cooked vegetables) safe:

- Prepare food in suitable amounts to reduce the amount of leftovers.
- Leftovers should be cooled in clean and shallow containers, and refrigerated in covered containers within 2 hours after cooking. Leftovers should be consumed as soon as possible and should not be stored in the refrigerator for longer than 3 days.
- Before consumption, leftovers should be reheated thoroughly until the core temperature reaches 75^{o}C, and should not be reheated more than once.

- Do not consume leftovers if they have been held at room temperature for more than 4 hours.

Risk Assessment Studies –

Changes in Nitrate and Nitrite Levels of Cooked Vegetables during Storage

OBJECTIVES

There is concern from time to time about the alleged increase in the nitrite levels of cooked vegetables stored overnight at refrigerated temperature. This study (i) examined the changes in the levels of nitrate and nitrite of cooked vegetables stored at room temperature and refrigerated temperature for up to 78 hours after cooking; and (ii) offered advice on proper ways of handling leftover cooked vegetables.

BACKGROUND

2. Vegetables are an essential component of a healthy diet and are good sources of dietary fibres, vitamins and minerals. The Centre for Health Protection recommends a daily intake of at least 3 servings of vegetables, and 2 servings of fruit, which is in line with the recommendation of the World Health Organization (WHO) of a minimum of 400 g of fruit and vegetables per day for the prevention of chronic diseases such as heart disease, cancer, diabetes and obesity[1,2].

3. Inside the human body, nitrite can oxidise haemoglobin in blood, making the blood unable to carry oxygen, and resulting in a medical condition called methaemoglobinaemia. Nitrite may also react with certain amines and amides in the body and produce nitrosamines. Nitrosamines are potentially cancer-causing in experimental animals[3-5]. Human exposure to nitrate is mainly through the consumption of vegetables while exposure to nitrite is mainly from endogenous nitrate conversion (i.e. nitrate is converted to nitrite in the human body), and to a lesser extent other foods[3,4,6,7].

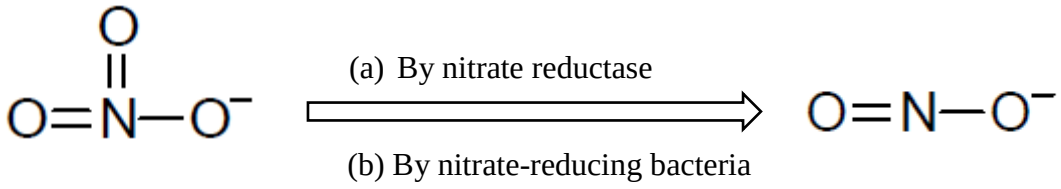
4. Previous studies suggested that the contents of nitrate and nitrite in fresh leafy vegetables during storage are affected by the storage time and temperature[3,8,9]. In general, higher temperature and longer storage time will result in a larger increase in the level of nitrite and a corresponding decrease in nitrate in fresh vegetables[3]. As regards cooked vegetables, a study revealed that the nitrite content in cooked Pak-choi stored at ambient temperature for 48 hours increased to up to 3 times of those kept refrigerated[10]. Nonetheless, relevant scientific studies of the effects of storage time and temperature of cooked vegetables on the levels of nitrate and nitrite are limited. From time to time, there have been local media reports, arousing public attention, about the possibility of a significant increase in the nitrite level of cooked vegetables that have been stored overnight in a refrigerator.

What are Nitrate and Nitrite?

5. Nitrate (NO_3^-) is the major form of nitrogen absorbed by plants for

them to produce amino acids for protein synthesis. Nitrate is the stable form of oxidised nitrogen but it can be reduced to nitrite (NO_2^-) either by the enzyme nitrate reductase present in plant cells or by nitrate-reducing bacteria present in the environment[3] (Figure 1). In contrast, nitrite ion is relatively unstable; chemical and biological processes can further reduce nitrite to various compounds (e.g. nitric oxide (NO)) or oxidise it to nitrate[4].

Figure 1. Conversion of nitrate into nitrite ion[3]



Nitrate ion Nitrite ion

6. Nitrate and nitrite are ubiquitous in the environment and occur naturally as part of the nitrogen cycle. Nitrate is a crucial nutrient for the growth and development of plants and its level varies among different vegetable types[11]. Conversely, nitrite levels are generally low in fresh undamaged vegetables[3].

7. Apart from naturally present in plants, compounds containing nitrate and nitrite can also be used as food additives in food. Sodium nitrite is used as a food preservative, especially in cured meats to inhibit the growth of *Clostridium botulinum*[3], and nitrate is sometimes also added to food to serve as a reservoir for nitrite[12].

Nitrate and Nitrite in Vegetables

8. Vegetables are the major source of dietary nitrate, accounting for about 70 – 90% of human's total estimated nitrate dietary intake[3,6,7]. Its concentration in vegetables depends on several factors including types of vegetables, season, light and temperature, method of growth and fertilisers used, etc.[3]

9. Nitrate in the soil is absorbed by the root of the plant and is transported up the plant in the xylem. The xylem carries water and nutrients (e.g. nitrate) from the roots to the leaves, whereas the phloem carries the products of photosynthesis (e.g. sucrose) from the leaves to the growth points and storage organs of the plants (i.e. stem and root tubers)[3]. This affects the distribution of nitrate in different parts of the plant. In general, the vegetable organs can be listed by decreasing nitrate content as follows: petiole > leaf > stem > root > inflorescence > tuber > bulb > fruit > seed. In lettuce and 'head chicory', inner leaves accumulate less nitrate than outer leaves and in parsley and spinach, leaf blades accumulate less nitrate than petioles[13]. As regards vegetables, leafy vegetables (e.g. cabbage and spinach) have higher nitrate concentrations, whereas root vegetables (e.g. potato and carrot) and bulb vegetables (e.g. onion and garlic) have relatively lower levels[3,13].

10. Once transported to the leaves, the majority of nitrate is stored in the vacuoles of the cells, and a small amount is converted to nitrite by nitrate reductase, an endogenous enzyme present in the cytoplasm. Nitrite is

immediately transported into the chloroplasts where it is finally combined with other photosynthetic products to synthesise amino acids for the growth of plants[14-16]. Therefore, fresh undamaged vegetables have relatively high nitrate content but a low nitrite level.

Influence of nitrogen fertilisers

11. Nitrogen fertilisation facilitates the accumulation of nitrate in plants. When taken up in excess of immediate requirement, nitrate is stored as free nitrate in the vacuole and can be remobilised subsequently when nitrogen supply is insufficient to meet the demand[13]. Applying nitrogen fertilisers increases the nitrate concentration in the xylem and the leaf, but has virtually no effect on the nitrate content in the phloem. Leafy vegetables such as lettuce or cabbage show an increased concentration of nitrate in response to nitrogen fertilisers while storage organs such as peas and beans that are fed by the phloem tend to show little effect[3].

Influence of storage

12. A great proportion of nitrate taken up by plants is stored in vacuoles inside the plant cells. Nitrate levels in raw fresh vegetables kept at room temperature decrease gradually during storage. However, ageing or damage during storage can cause loss of function of the vacuolar membrane[17]. Nitrate is then released from the vacuoles to the cytoplasm and is converted to nitrite by nitrate reductase, causing a rapid increase in nitrite level (with a corresponding rapid decrease in nitrate). Differences in plant species and the amount of bacterial contamination may also affect the levels of nitrite in leaves[3].

13. Refrigeration delays the senescence of fresh vegetables. Under refrigerated storage, nitrate levels of fresh vegetables are almost unaffected for 7 days[3,18]. Over this storage period, nitrite concentrations remain low, as under cold storage conditions endogenous nitrate reductase and bacteria are inactivated. For frozen storage, studies found that there are no significant changes in nitrate and nitrite content in vegetables for up to 12 weeks[3].

14. High levels of nitrite, however, have been reported in home-made vegetable purees even after refrigerated storage for only 12 hours. Presumably during pureeing, leaf cells and their vacuoles are broken, releasing the stored nitrate which is immediately reduced to nitrite by the enzyme nitrate reductase in the cytoplasm[3].

Influence of Food Processing

15. Since the distribution of nitrate in different parts of vegetables is not even, removal of stem and midrib will result in a decrease of nitrate content by 30 – 40% in lettuce and spinach. Peeling of potatoes, bananas, melons and beetroot is also found to decrease the nitrate content by 20 – 62%[3].

16. Nitrate is soluble in water and washing leafy vegetables can reduce nitrate levels by 10 – 15%. Different studies also showed a reduction in nitrate levels when vegetables are cooked in water. Peas, cabbage, beans, carrots, potatoes, spinach, endives and celery leaves lose about 16 – 79% of nitrate during cooking. The contents of nitrate and nitrite decrease similarly after

boiling by about 50% in carrot, parsley-root, celery and potatoes[3].

Toxicity

Kinetics and Metabolism

17. Nitrate is primarily absorbed from the upper part of the human digestive tract. Absorption is rapid and peak levels of nitrate are observed in saliva and sweat after 1 – 3 hours of nitrate intake from food or drinks. On average, about 25% of ingested nitrate is secreted in the saliva and approximately 20% of the secreted saliva nitrate is converted to nitrite by commensal bacteria on the surface of the tongue. Oral reduction of nitrate is the most important source of nitrite for humans, and accounts for approximately 70 – 80% of the human total nitrite exposure[3,12,19].

18. After nitrite is transported to the stomach, the acidic conditions will rapidly transform it to nitrous acid which spontaneously decomposes into nitrogen oxides. A low pH (i.e. pH 1 – 2) in the fasting stomach is considered too low for bacterial nitrate reduction. However, infants younger than 3 months are highly susceptible to gastric bacterial nitrate reduction because they have very little production of gastric acid. Most of the ingested nitrate is eventually excreted in the urine as nitrate, ammonia or urea while faecal excretion is negligible[3,12,19].

Toxicity

19. The acute oral toxicity of nitrate in laboratory animals is low to moderate. It is observed that the oral lethal dose of nitrate in human adults is around 330 mg/kg body weight (b.w.). Nitrate has to be converted to nitrite before it can cause acute toxicity. Nitrate can be converted to nitrite naturally and the process is accelerated in the presence of bacteria. Nitrite is approximately ten-fold more toxic than nitrate. Various oral lethal doses of nitrite in human adults have been reported ranging from 33 to 250 mg/kg b.w. [3,12].

20. A toxicological endpoint of concern for nitrate and nitrite is nitrosamine formation. However, when nitrate is consumed in a normal diet containing vegetables, other bioactive substances such as vitamin C that are concomitantly consumed may inhibit the endogenous formation of nitrosamine[3].

Methaemoglobinaemia

21. The major acute toxic effect of nitrite poisoning is methaemoglobinaemia. Blood contains haemoglobin (Hb) which carries oxygen. When nitrite is present, Hb will be converted to methaemoglobin (metHb), making the red blood cells less efficiently transporting oxygen. MetHb in blood is maintained at a stable level by an enzyme that continually convert metHb back to Hb[3].

22. The normal metHb level in humans is less than 2%. When metHb concentrations reach 10% of normal Hb or above, symptoms of cyanosis (a bluish colour of skin and lips) usually appear. At higher concentrations,

asphyxia (unable to breathe) may occur. Population subgroups such as infants younger than 3 months of age and the elderly are more susceptible to the above condition[3,4,20]. In infants, methaemoglobinaemia is also commonly known as the blue baby syndrome.

23. At present, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has not set any acute reference doses for nitrate and nitrite that cause methaemoglobinaemia.

Genotoxicity

24. Sodium nitrate was not mutagenic in *in vitro* test. For nitrite, it was mutagenic in *in vitro* test but gave negative results on mutagenic activities in *in vivo* test[3,20,21].

Carcinogenicity

25. The epidemiological studies reviewed by JECFA in 2002 and European Food Safety Association (EFSA) in 2008 did not provide evidence that nitrate and nitrite are carcinogenic in humans[3,12,20].

26. Nitrite was shown to react with certain amines and amides in the human acid stomach environment to form N-nitroso compounds. Many N-nitroso compounds have been found carcinogenic in the animal species tested[11]. In 2010, IARC concluded that ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans (i.e. Group 2A

agent)[4]. However, evidence concerning whether nitrate or nitrite per se in food can cause cancer in humans is inadequate or limited[4].

Health-based Guidance Value

27. JECFA has established an acceptable daily intake (ADI) of 0 – 3.7 mg/kg b.w. for nitrate ion, and an ADI of 0 – 0.07 mg/kg b.w. for nitrite ion. However, in view of the benefits of vegetables and the lack of data on the possible effect of vegetable matrices on the bio-availability of nitrate, JECFA considered that it was inappropriate to compare exposure to nitrate from vegetables directly with the ADI. JECFA also noted that a high intake of certain vegetables seemed to be associated with a lower risk of gastric cancer and considered that this may be due to protective factors which are simultaneously present in vegetables[20,22].

SCOPE OF STUDY

28. This study examined the changes in the levels of nitrate and nitrite of (i) cooked leafy and fruiting vegetables, and (ii) vegetable soup during storage at room and refrigerated temperatures for up to 78 hours.

29. To investigate whether there were differences in the changes in the levels of nitrate and nitrite among different vegetables, different types of vegetables were selected for the study. The following were taken into consideration in deciding the types of vegetables to be included:

a) Leafy vegetables containing high levels of nitrate[11];

b) Vegetables commonly consumed by the local population[23];

c) A fruiting vegetable that contains a high level of nitrate and is commonly used for packing lunch boxes.

30. Five types of vegetables, namely, amaranth, Pak-choi, flowering white cabbage, Chinese lettuce and zucchini[11] were included in the study (Annex I).

31. Vegetable soup, with ingredients (i.e. beetroot, celery and carrot) of relatively high nitrate levels[11], was also included in the study (Annex I).

METHODOLOGY AND LABORATORY ANALYSIS

Sampling and Preparation

32. Vegetable samples were collected between June and August 2021 from wet market stalls and a supermarket.

33. Before cooking, the vegetable samples were prepared as follows:

- Leafy vegetables: dirt, soil and damaged areas of vegetables were removed. The vegetables were washed thoroughly and then drained. Root ends were removed.
- Zucchini: both ends were removed and the samples were thoroughly washed. After draining, the samples were cut into slices of 1/4 inch thick.
- Beetroot and carrot: for the beetroot samples, both ends were removed and the sample was peeled and washed thoroughly. After draining, they were

cut into small chunks. The chunks were divided into two equal halves and separately put into 2 soup bags. The carrot samples were treated similarly.

- Celery: tough strings and root ends were removed from 2 celery stalks and the stocks were then thoroughly washed. After draining, the celery stalks were cut into chunks of 5 cm long. The chunks were divided into two equal halves and separately put into 2 soup bags.

Cooking

34. In order to examine the effect of cooking methods on the changes in nitrate and nitrite of cooked vegetables during storage, two common methods (i.e. boiling and stir-frying) were used for cooking raw vegetable samples. Referring to the food preparation instructions for vegetable items in the First Hong Kong Total Diet Study[24], the samples were cooked as follows:

- Boiling: each type of leafy vegetable and zucchini were put in boiling distilled water and boiled for 3 minutes[25], and then drained.
- Stir-frying: each type of leafy vegetable and zucchini were stir-fried (without cooking oil) in a frying pan over medium heat, and then covered for 1 minute. After one minute, 1 tablespoon of distilled water for every 420g of vegetables/zucchini was added to prevent vegetables/zucchini from sticking to the pan[26]. The vegetable was covered and heated for another 3 minutes, and then drained.

35. To prepare the vegetable soup, the 6 soup bags were boiled in distilled water of 2 litres over low heat for 1 hour.

Storage

36. After cooking, the cooked vegetables and vegetable soup (with its ingredients) were then divided into 2 groups. One group was stored at room temperature (20°C – 25°C) and the other was stored in a refrigerator (0°C – 4°C) for 78 hours.

Analysis of nitrate and nitrite

37. The nitrate and nitrite content of different vegetables were tested before cooking. After cooking, samples of cooked vegetables and soup (equal weight of water and each ingredient) stored at room and refrigerated temperature were taken for analysis of nitrate and nitrite contents in the following time intervals (Annex II):

- immediately after cooking; and
- 6, 12, 24, 36, 48, 72 and 78 hours after cooking.

Laboratory Analysis

38. Laboratory analysis was conducted by the Food Research Laboratory of the Centre for Food Safety (CFS). The nitrate and nitrite levels in cooked vegetables were analysed by ion chromatography (IC) with UV detection

according to BS EN 12014-2:2017 “Foodstuffs - Determination of nitrate and/or nitrite content - Part 2: HPLC/IC method for the determination of nitrate content of vegetables and vegetable products”. Sample extraction was performed with hot water in a boiling water bath and then filtered through a filter paper. The filtrate was then passed through a membrane filter before instrumental analysis. Identification was confirmed by comparing the retention time with those of the nitrate and nitrite reference standards. The limits of detection (LODs) were 0.80 mg/kg for both nitrate and nitrite.

RESULTS

39. Nitrite was not detected in all vegetable types before (i.e. in the raw state) and immediately after cooking. Nitrite was also not detected in vegetable soup immediately after cooking. Nitrate contents of different vegetable types and vegetable soup immediately after cooking are shown in Table 1.

Table 1. Nitrate contents of vegetables and soup before and immediately after cooking

	Level of nitrate (mg/kg)					
	Amaranth	Pak-choi	Flowering white cabbage	Chinese lettuce	Zucchini	Soup
Before cooking	5700	5000	2400	1100	650	NA
After boiling	3000	3000	2000	760	390	955
After stir-frying	3700	3800	2350	1300	680	NA

NA = not applicable

Cooked leafy vegetables stored at room temperature

40. At room temperature, nitrite was not detected in all cooked leafy vegetables until after storing for about 20 hours. Afterward, the nitrite levels increased to a peak and then decreased gradually (Figure 2).

41. As regards nitrate, the levels of the cooked leafy vegetables decreased gradually during the first 12 to 36 hours, decreased more rapidly in the subsequent 12 to 36 hours and then the decrease slowed down again. The

patterns of changes in nitrate and nitrite levels for both cooking methods (i.e. boiling and stir-frying) were similar (Figure 2). The levels of nitrate and nitrite in cooked leafy vegetables stored at room temperature were listed in Annex III.

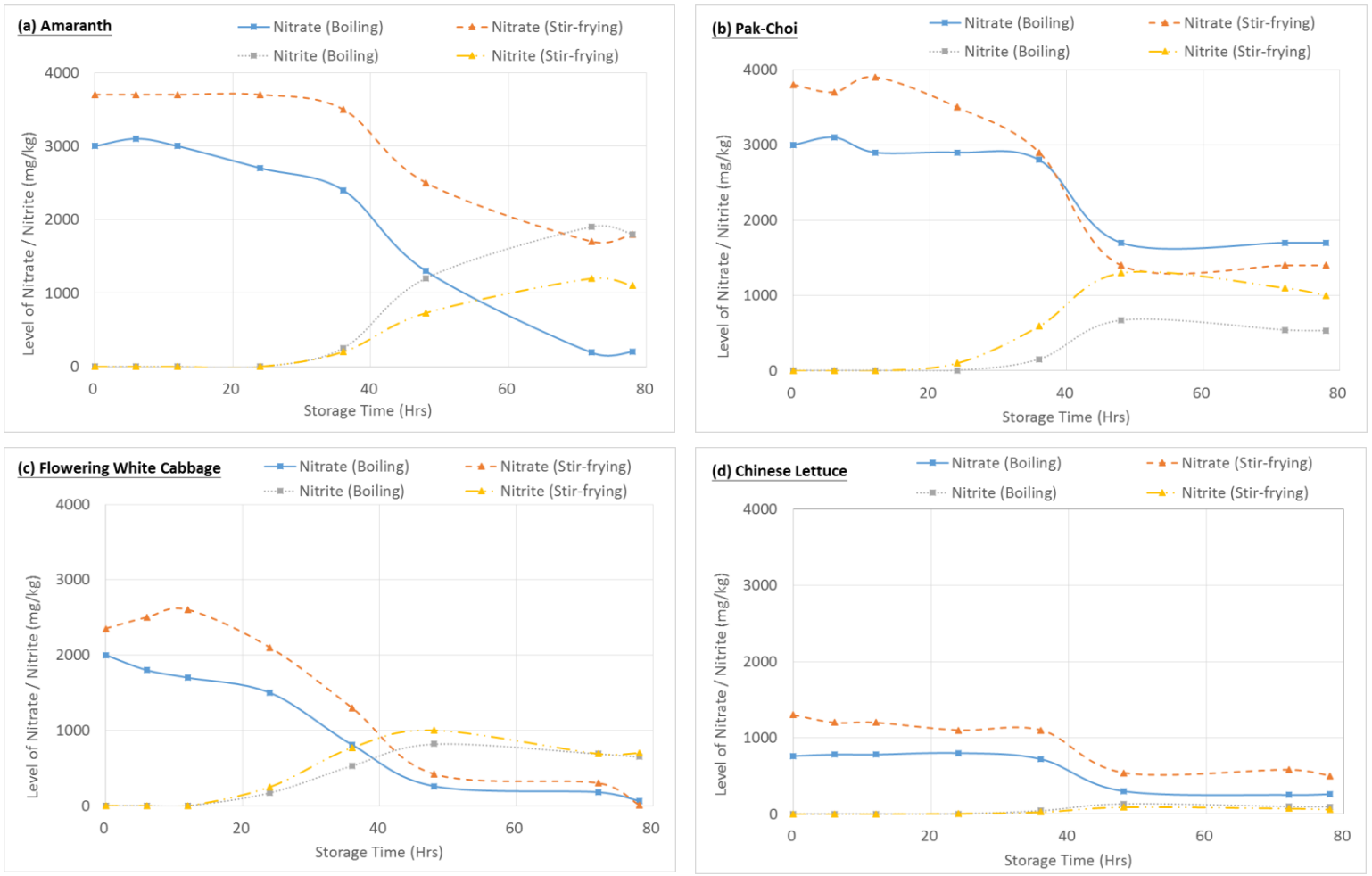


Figure 2. Nitrate and nitrite contents in boiled and stir-fried leafy vegetables stored at room temperature: (a) Amaranth; (b) Pak-choi; (c) Flowering white cabbage; and (d) Chinese lettuce.

Cooked leafy vegetables stored under refrigeration

42. At refrigerated temperature, nitrite was not detected in all cooked leafy vegetables during the whole 78-hour storage except in cooked flowering white

cabbage and stir-fried Pak-choi in which nitrite started to be detected at relatively low levels after storing for 72 hours (Figure 3).

Figure 3. Nitrate and nitrite contents of boiled and stir-fried leafy vegetables stored at refrigerated temperature: (a) Amaranth; (b) Pak-choi; (c) Flowering white cabbage; and (d) Chinese lettuce.

43. The nitrate contents of cooked leafy vegetables stored under refrigeration decreased much slower than that stored at room temperature during the whole storage period (Figure 3). The levels of nitrate and nitrite in cooked leafy vegetables stored at refrigerated temperature were listed in Annex IV.

Depending on the types of vegetables, the reduction of nitrate at the end of refrigerated storage was:

- for boiled vegetables, from 6.7% to 15% (compared with 43.3% to 96.8% under room temperature);
- for stir-fried vegetables, from 5.3% to 15.4% (compared with 51.4% to 99.6% under room temperature).

Cooked zucchini and soup stored at room temperature

44. At room temperature, nitrite was detected in cooked zucchini and vegetable soup after storing for 12 hours. Regarding nitrate, the levels in cooked zucchini and soup started to decrease after storing for 12 hours, and the decreases continued to below 6 mg/kg in zucchini and 160 mg/kg in soup (Figure 4). The levels of nitrate and nitrite in cooked zucchini and soup stored at room temperature were listed in Annex V and Annex VI.

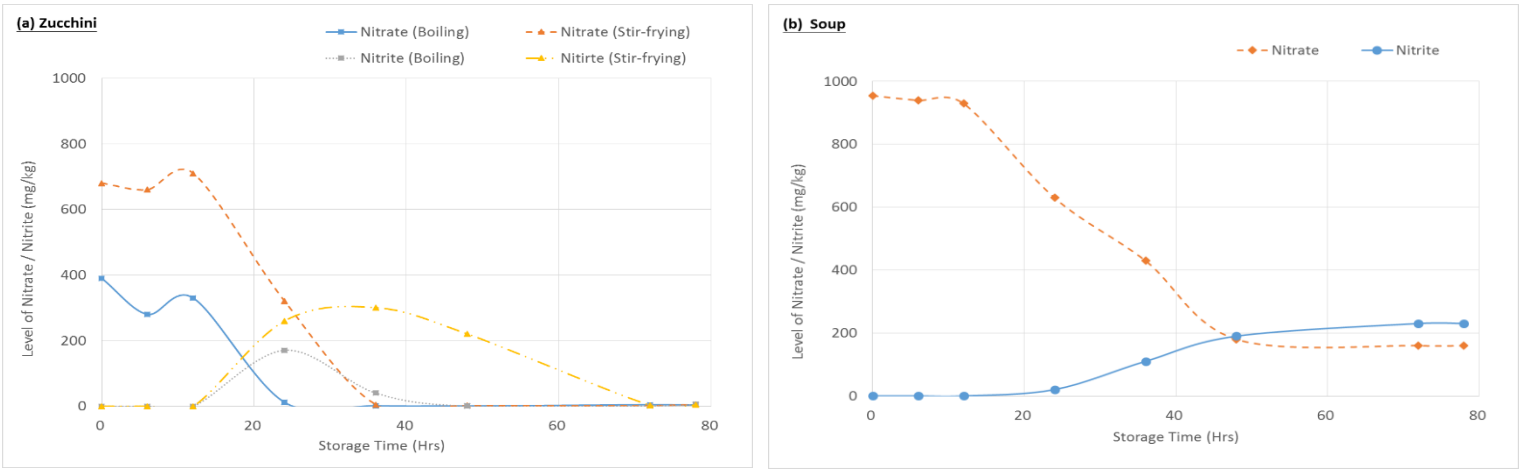


Figure 4. Nitrate and nitrite contents in (a) cooked zucchini and (b) soup stored at room temperature.

Cooked zucchini and soup stored at refrigerated temperature

45. At refrigerated temperature, no nitrite was detected in cooked zucchini and vegetable soup during the whole 78-hour storage, and the nitrate contents remained relatively stable (Figure 5). The levels of nitrate and nitrite in cooked zucchini and soup stored at refrigerated temperature were listed in Annex V and Annex VI.

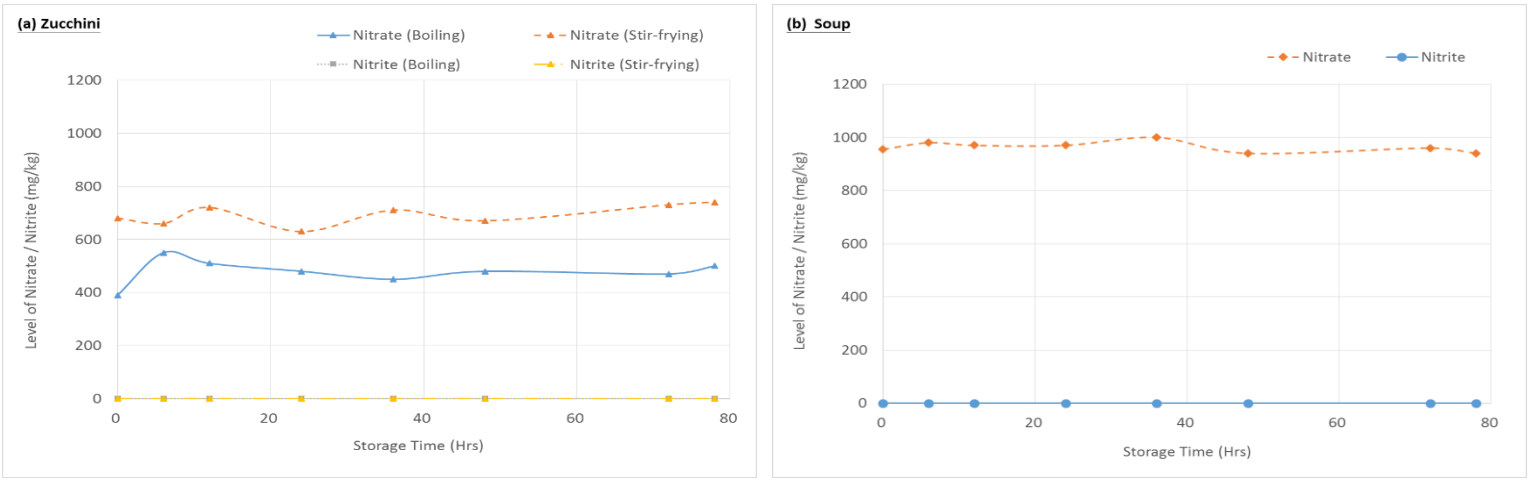


Figure 5. Nitrate and nitrite contents (a) cooked zucchini and (b) soup stored at refrigerated temperature.

DISCUSSION

46. Nitrate in vegetables can be converted to nitrite by two means: by the enzyme nitrate reductase present in plant cells or by nitrate-reducing bacteria present in the environment[3]. In this study, nitrite was not detected in all vegetable samples (i.e. leafy vegetables, zucchini and vegetable soup) immediately after cooking. During cooking, nitrate reductase in plant cells was denatured and lost the ability to convert nitrate to nitrite. Therefore, the

formation of nitrite in cooked vegetables after storing them for a period of time is expected due to the activities of nitrate-reducing bacteria in the environment.

47. Bacteria are ubiquitous in the environment. When handling cooked vegetables in the kitchen, bacterial contamination (including nitrate-reducing bacteria) from the air and utensils such as spoons and bowls is inevitable even though good hygiene practices are observed. When bacteria are introduced into a new environment (e.g. cooked vegetables in this study), they have to go through a period of time (i.e. a lag phase[a]) to adapt themselves to the new growth conditions before the start of rapid growth[27]. During the lag phase, little or no bacterial growth occurs. Temperature has a significant effect on both the lag phase and the generation time[b] of bacteria[27,28]. Lag phase is shorter at the optimum temperature and is prolonged as temperature is lowered[29]. If the temperature is low (e.g. 0°C - 4°C), some bacteria (i.e. mesophiles) are unable to grow while others (i.e. psychrotrophs) may take a long period to adapt to the new conditions before they can grow and multiply[27] (Table 2). For example, *Pseudomonas fluorescens*, a spoilage and nitrate-reducing bacteria, is commonly found in the environment contaminating foods, and is able to grow slowly at refrigerated temperature (i.e. a psychrotroph)[30-32]. A study showed that in a new medium the lag phase of *Pseudomonas fluorescens* increased from 1 day at 20°C to 3 days at 5°C, to 4 days at 0°C, and to 6 days at –3°C[33].

[a] Lag phase is the initial period in the life of a bacterial population when cells are adjusting to a new environment before starting exponential growth.
[b] Generation time is the time it takes for a population of bacteria to double in number.

Table 2. Growth temperature ranges for bacteria commonly found in food

Group of bacteria	Temperature (°C)		
	Minimum	Optimum	Maximum
(a) Mesophiles *(bacteria that cannot grow at refrigerated temperature)*	5 to 15	30 to 45	35 to 47
(b) Psychrotrophs *(bacteria that can grow slowly at refrigerated temperature)*	–5 to +5	25 to 30	30 to 35

48. In this study, nitrite began to be detected in some vegetable samples when they had been stored at room temperature for 12 hours after cooking, indicating that nitrate-reducing bacteria were present, and nitrate in the cooked vegetables was converted to nitrite by the bacteria. This finding is consistent with a recent study which showed that the nitrite contents of boiled spinach and stir-fried cabbage did not change during the first 12 hours of storage at room temperature[34].

49. When cooked vegetables were stored at refrigerated temperature, the low temperature lengthened the lag phase, and reduced the growth of nitrate-reducing bacteria as well as their activities to convert nitrate to nitrite. Hence, the formation of nitrite was delayed, and in this study, only low levels of nitrite were detected in some vegetable samples after storing for 72 hours after cooking. Other studies, involving the storage of cooked vegetables at refrigerated temperature for 24 hours reported by relevant authorities in the Mainland, also found that the levels in nitrite of cooked vegetables remained low at the end of the 24-hour refrigerated storage[35-39].

50. It is worthwhile to note that storage temperature, instead of the types of vegetables, plays a significant role in the conversion of nitrate to nitrite because it directly affects the growth and activities of bacteria.

LIMITATIONS

51. Other food ingredients such as meat, oil and condiments (e.g. salt, pepper, sugar, garlic, etc.) that are commonly used in the domestic food preparation practices may affect the physical characteristics (e.g. salinity, pH, water activity, etc.) of the dishes which in turn affect the duration of the lag phase and the growth of bacteria. Also, this study was conducted under laboratory conditions which may be different from home kitchen conditions with respect to sources and levels of bacterial contamination. Nonetheless, studies have shown that for foods with a high level of water activity and a pH value near to neutrality, temperature is the most important environmental factor affecting the duration of lag phase, and the lag phase is little affected by the bacterial load. Under the same storage temperature, suboptimal conditions (e.g. high concentrations of sodium chloride) lengthen the lag phase which may be affected by the bacterial load[40,41].

CONCLUSIONS AND RECOMMENDATIONS

52. This study revealed that storage temperature has a significant effect on the conversion of nitrate to nitrite in cooked vegetables. When cooked vegetables are stored overnight in a refrigerator, their nitrite contents do not

increase. In fact, under refrigeration, low levels of nitrite were only detected in some samples after storing them for 72 hours.

53. From a food safety perspective, overseas authorities including WHO recommend that leftover food should be cooled in clean and shallow containers, and refrigerated in covered containers within 2 hours after cooking. Leftovers should not be stored in the refrigerator for longer than 3 days[42-48] because spoilage bacteria can still grow at refrigerated temperature even though most harmful bacteria cannot[27].

54. At room temperature, the total time that cooked vegetables can be kept should be limited to 4 hours because cooked vegetables are considered potentially hazardous foods which may contain harmful bacteria and will normally support their growth and formation of toxins[49,50].

Advice to Public

Members of the public are recommended to eat a variety of vegetables with a daily intake of at least 3 servings of vegetables, and 2 servings of fruit i.e. at least 400 grams of fruits and vegetables (about 5 servings) for the prevention of chronic diseases.

55. The public should also follow the following guidelines to keep leftovers (including cooked vegetables) safe:

- Prepare food in suitable amounts to reduce the amount of leftovers.

- Leftovers should be cooled in clean and shallow containers, and refrigerated in covered containers within 2 hours after cooking. Leftovers should be consumed as soon as possible and should not be stored in the refrigerator for longer than 3 days.
- Before consumption, leftovers should be reheated thoroughly until the core temperature reaches 75°C, and should not be reheated more than once.
- Do not consume leftovers if they have been held at room temperature for more than 4 hours.

REFERENCE

1 WHO. Promoting fruit and vegetable consumption. Available from URL: https://www.euro.who.int/en/health-topics/disease-prevention/nutrition/activities/technical-support-to-member-states/promoting-fruit-and-vegetable-consumption

2 Centre for Health Protection. Department of Health. Dietary Recommendations for Fruit and Vegetable Intake. Available from URL: https://www.chp.gov.hk/en/static/90022.html

3 EFSA. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission to perform a scientific risk assessment on nitrate in vegetables, *The EFSA Journal* (2008) Journal number, 689, 1-79. Available from URL: https://www.efsa.europa.eu/en/efsajournal/pub/689

4 IARC. Ingested nitrate and nitrite, and cyanobacterial peptide toxins. IARC monographs on the evaluation of carcinogenic risks to human volume 94. 2010. Available from URL: https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Ingested-Nitrate-And-Nitrite-And-Cyanobacterial-Peptide-Toxins-2010

5 EFSA. Nitrites and nitrates added to food. June 2017. Available from URL: https://www.efsa.europa.eu/sites/default/files/corporate_publications/files/nitrates-nitrites-170614.pdf

6 WHO. Nitrate and nitrite – intake assessment. In: Safety evaluation of certain food additives (Food additives Series 50). Geneva: WHO; 2003. Available from URL: http://www.inchem.org/documents/jecfa/jecmono/v50je07.htm

7 US EPA. Intergrated Risk Information System – Nitrate; CASRN 14797-55-8. 1991. Available from URL: https://iris.epa.gov/static/pdfs/0076_summary.pdf

8 魏穎, 孫雅文, 厲玉婷, 劉國紅, 赫英英, 於紅. 不同加工方式與存放條件對蔬菜中亞硝酸鹽含量的影響. 食品與藥品. 2015 年第 17 卷第 6 期.

9 梁平. 探究剩菜中亞硝酸鹽含量的變化. 生物學通報. 2010 年第 45 卷第 10 期.

10 蘇州大學公共衛生學院；蘇州工業園區疾病防治中心. 隔夜菜中亞硝酸鹽含量的測定及分析. 2017 年 5 月

11 Centre for Food Safety. Food and Environmental Hygiene Department. Nitrate and Nitrite in Vegetables Available in Hong Kong. 2010. Available from URL: https://www.cfs.gov.hk/english/programme/programme_rafs/programme_rafs_fc_01_23_Nitrate_Nitrite_Vegetables.html

12 WHO. Nitrate and nitrite in drinking water. Background document for development of WHO guidelines for drinking-water quality. 2003. Available from URL: https://apps.who.int/iris/bitstream/handle/10665/75380/WHO_SDE_WSH_04.03_56_eng.pdf?sequence=1

13 Non Renseigné, Shahid Umar, Muhammad Iqbal. Nitrate accumulation in plants, factors affecting the process, and human health implications. A review. Agronomy for Sustainable Development, Springer Verlag/EDP Sciences/INRA, 2007, 27 (1), pp.45-57. ffhal-00886336f. Available from URL: https://hal.archives-ouvertes.fr/hal-00886336

14 R. TISCHNER. Nitrate uptake and reduction in higher and lower plants Plant, Cell and Environment (2000) 23, 1005–1024. Available from URL: https://www.semanticscholar.org/paper/Nitrate-uptake-and-reduction-in-higher-and-lower-Tischner/20ff45d4a83b275753d84e4160a60ef2a420fe49

15 Adriano Nunes-NesiAlisdair R. FernieMark Stitt. Metabolic and Signaling Aspects Underpinningthe Regulation of Plant Carbon Nitrogen Interactions. 2010. Available from URL: https://www.sciencedirect.com/science/article/pii/S1674205214605487

16 Céline Masclaux-Daubresse, Françoise Daniel-Vedele, Julie Dechorgnat, Fabien Chardon, Laure Gaufichon, and Akira Suzuki. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. 2010. Available from URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2887065/

17 Brizzolara S, Manganaris GA, Fotopoulos V, Watkins CB and Tonutti P (2020) Primary Metabolism in Fresh Fruits During Storage. Front. Plant Sci. 11:80. doi: 10.3389/fpls.2020.00080. Available from URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7042374/

18 Chung, J.C., Chou, S.S. and Hwang, D.F. 2004. Changes in nitrate and nitrite content of four vegetables during storage at refrigerated and ambient temperatures. Food Addit. Contam., 21, 317–322.

19 Nitrate (and potential endogenous formation of N-nitroso compounds). WHO food additives series: 50. 2002. Available from URL: http://www.inchem.org/documents/jecfa/jecmono/v50je06.htm#2.3.1

20 JECFA. Evaluation of certain food additives: fifty-ninth report. 2002. Available from URL: https://apps.who.int/iris/bitstream/handle/10665/42601/WHO_TRS_913.pdf?sequence=1

21 EFSA. Scientific Opinion on the re-evaluation of potassium nitrite (E 249) and sodium nitrite (E 250) as food additives. EFSA Journal 2017; 15(6):4786, 157. Available from URL: https://www.efsa.europa.eu/en/efsajournal/pub/4786

22 IPCS. Nitrate. In: Safety evaluation of certain food additives (Food additives Series 35). 1996. Available from URL: http://www.inchem.org/documents/jecfa/jecmono/v35je14.htm

23 Centre for Food Safety. Report of the Second Hong Kong Population-based Food Consumption Survey. June 2021. Available from URL: https://www.cfs.gov.hk/english/programme/programme_firm/programme_fcs_2nd_Survey.html

24 Centre for Food Safety. The first Hong Kong Total Diet Study: Methodology. December, 2011. Available from URL: https://www.cfs.gov.hk/english/programme/programme_firm/programme_tds_1st_HKTDS_report.html

25 National Center for Home Food Preservation. Blanching. Available from URL: https://nchfp.uga.edu/how/freeze/blanching.html

26 Hunt for Hope Wellness. Time Table for Perfect Stir-frying. 2015. Available from URL: https://huntforhopewellness.com/2015/02/06/time-table-for-perfect-stir-frying/

27 Institute of Food Technologists. Evaluation and Definition of Potentially Hazardous Foods. Compr. Rev. Food Sci. Food Saf. —Vol. 2 (Supplement), 2003. Available from URL: https://www.fda.gov/files/food/published/Evaluation-and-Definition-of-Potentially-Hazardous-Foods.pdf

28 Ingraham, J. L. 1958 Growth of psychrophilic bacteria. J. Bacteriol., 76, 75-80. Available from URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC290156/

29 Elliott, Paul and Michener, H.D. Micro-organisms in foods: A Review By R. Western Utilization Research and Development Division Technical Bulletin No. 1320. Agricultural Research Service, United States Department of Agriculture (USDA). Available from URL: https://ageconsearch.umn.edu/record/171228/files/tb1320.pdf

30 Meta Sterniša, Mihael Čargo1 and Sonja Smole Možina. Spoilage bacteria pseudomonas - production of hydrolytic enzymes and ability to grow at 5°C. Acta Periodica Technologica 2019 Issue 50, Pages: 278-285. Available from URL: http://www.doiserbia.nb.rs/img/doi/1450-7188/2019/1450-71881950278S.pdf

31 Harsh Kumar, Laura Franzetti, Ankur Kaushal & Dinesh Kumar. *Pseudomonas fluorescens*: a potential food spoiler and challenges and advances in its detection. Annals of Microbiology (2019) 69:873–883. https://annalsmicrobiology.biomedcentral.com/articles/10.1007/s13213-019-01501-7

32 Albrecht, Anna (2013) "Identification of Two Unknown Species of Bacteria," ESSAI: Vol. 10, Article 8. Available from URL: http://dc.cod.edu/essai/vol10/iss1/8

33 Ingraham, J. L. and Stokes, J. L. 1959 Psychrophilic bacteria. Bacteriological Reviews 23(3):97-108. Available from URL: https://journals.asm.org/doi/10.1128/br.23.3.97-108.1959

34 Wu, S.; Liu, Y.; Cui, X.; Zhang, Q.; Wang, Y.; Cao, L.; Luo, X.; Xiong, J.; Ruan, R. Assessment of Potential Nitrite Safety Risk of Leafy Vegetables after Domestic Cooking. Foods 2021, 10, 2953. Available from URL: https://doi.org/10.3390/foods10122953

35 樂山市人民政府. 常吃剩飯剩菜致癌？2016-12-06. Available from URL: https://www.leshan.gov.cn/lsswszf/yqhy/201612/bc22b3e471cd45ac8a7a656750b4e8a5.shtml

36 汕頭市衛生健康局(中醫藥局). 長期吃隔夜菜，女子患重度貧血 2021-10-15. Available from URL: https://www.shantou.gov.cn/stswsj/gkmlpt/content/1/1977/post_1977771.html#3521

37 四川省衛生健康委員會. 嚇人！隔夜菜竟會致癌？那放了多久算隔夜？ 2019-11-15. Available from URL: http://wsjkw.sc.gov.cn/scwsjkw/jkys/2019/11/15/fbfadcd752f94c309d66f0602b6ebc48.shtml

38 浦江縣人民政府. 隔夜的東西究竟哪些能吃？哪些不能吃？ 2021-12-12. Available from URL: http://www.pj.gov.cn/art/2021/12/12/art_1229197982_59088479.html

39 中央政府門戶網站. 科學生活：隔夜菜究竟能不能吃？ 2011 年 11 月 16 日. Available from URL: http://www.gov.cn.qingcdn.com/fwxx/kp/2011-11/16/content_1994724.htm

40 De Silvestri A, et al.Ferrari E, Gozzi S, Marchi F and Foschino R (2018) Determination of Temperature Dependent Growth Parameters in Psychrotrophic Pathogen Bacteria and Tentative Use of Mean Kinetic Temperature for the Microbiological Control of Food. Front. Microbiol. 9:3023. doi: 10.3389/fmicb.2018.03023. Available from URL: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03023/full

41 Tobin P. Robinson, Olosimbo O. Aboaba, Anu Kaloti, Maria J. Ocio, Jozsef Baranyi, Bernard M. Mackey. The effect of inoculum size on the lag phase of *Listeria monocytogenes*. Int. J. Food Microbiol. 70 (2001) 163–173. Available from URL: https://www.researchgate.net/publication/11597554_The_effect_of_inoculum_size_on_the_lag_phase_of_Listeria_monocytogenes

42 World Health Organization (WHO). Five Keys To Safer Food Manual. Available from URL: https://www.who.int/publications/i/item/9789241594639

43 USDA. Kitchen Companion: Your Safe Food Handbook. Revised March 2015. Available from URL: https://www.fsis.usda.gov/news-events/publications/kitchen-companion-your-safe-food-handbook

44 USDA, Food Safety and Inspection Service, Cornell University, Food Marketing Institute. FoodKeeper App. Available from URL: https://www.foodsafety.gov/keep-food-safe/foodkeeper-app

45 Centers for Disease Control and Prevention. Game Day Food Safety Tips. Available from URL: https://www.cdc.gov/foodsafety/communication/rules-of-game.html

46 National Institute on Aging (NIA), National Institutes of Health (NIH). Healthy eating: Food Safety. Available from URL: https://www.nia.nih.gov/health/food-safety

47 The Government of Canada. Safe food storage. Available from URL:

https://www.canada.ca/en/health-canada/services/general-food-safety-tips/safe-food-storage.html

48 Food Safety Authority of Ireland (FSAI). Home Cooking and Storage. Available from URL:
https://www.fsai.ie/faq/domestic.html

49 Food Standard Australia New Zealand. Safe Food Australia - A guide to the Food Safety Standards. Available from URL:
https://www.foodstandards.gov.au/publications/Pages/safefoodaustralia3rd16.aspx

50 Food and Drug Administration (FDA) (2017). Food Code. Available from URL:
https://www.fda.gov/media/110822/download

Annex I

List of Vegetables being Analysed

Name of Vegetable	Photo
Amaranth (Chinese spinach) 莧菜	
Pak–choi (Chinese white cabbage) 白菜	
Flowering white cabbage 菜心	
Chinese lettuce 唐生菜	
Zucchini 翠玉瓜	
Vegetable soup with the following ingredients: • Beetroot 紅菜頭 • Celery 西芹 • Carrot 甘筍	 

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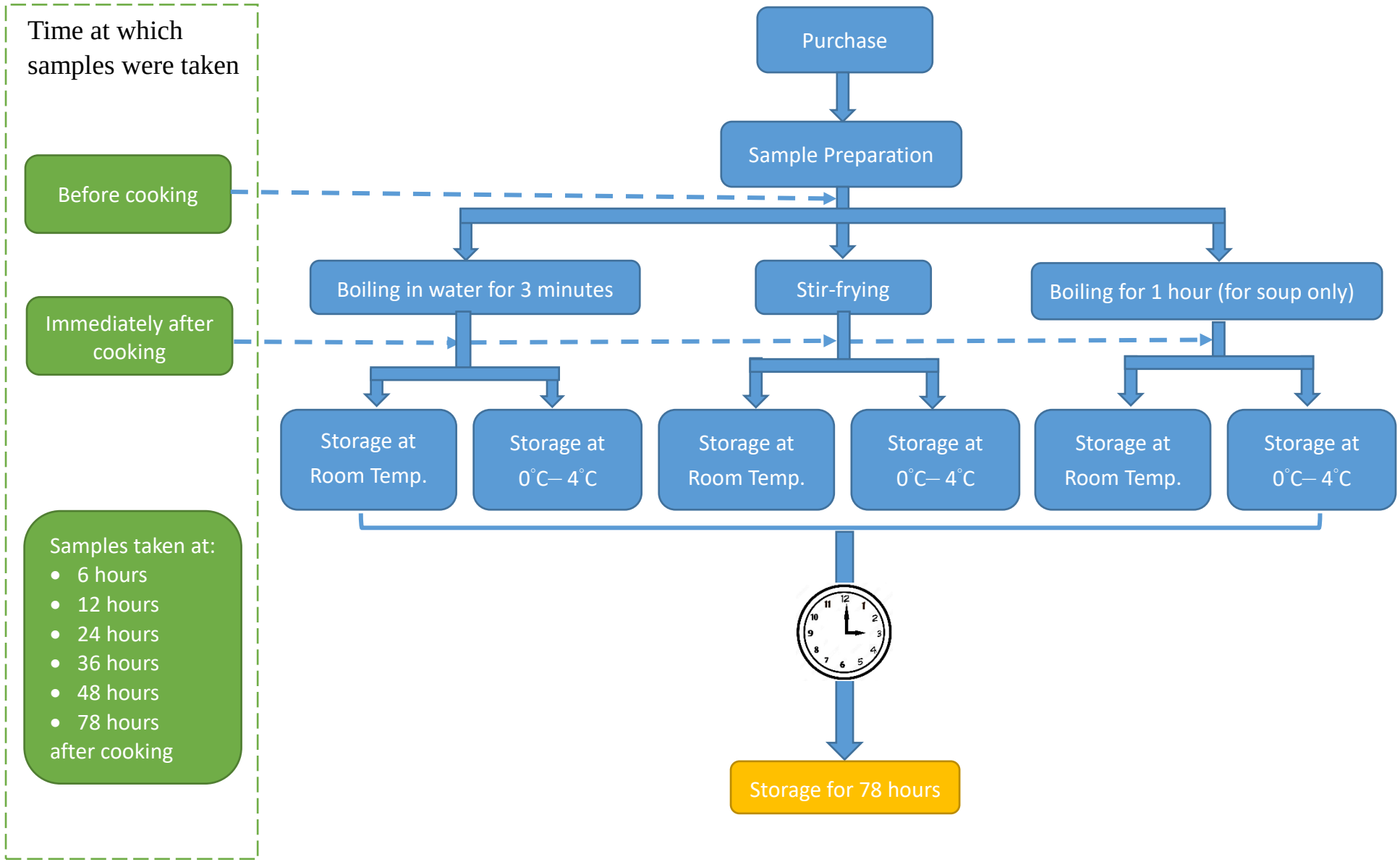
Flow diagram on handling and preparation of vegetable samples for analysis



Annex III

Nitrate and nitrite levels of boiled vegetables stored at room temperature and refrigerated temperature

Samples	Storage condition	Level (mg/kg)	Storage time (hours)							
			0	6	12	24	36	48	72	78
Amaranth	Room temperature	Nitrate	3000	3100	3000	2700	2400	1300	190	200
		Nitrite	ND	ND	ND	ND	250	1200	1900	1800
	Refrigerated temperature	Nitrate	3000	3000	2800	2600	2600	2600	2500	2600
		Nitrite	ND	ND	ND	ND	ND	ND	ND	ND
Pak-choi	Room temperature	Nitrate	3000	3100	2900	2900	2800	1700	1700	1700
		Nitrite	ND	ND	ND	2.5	150	670	540	530
	Refrigerated temperature	Nitrate	3000	2900	2800	2800	2700	2800	2800	2800
		Nitrite	ND	ND	ND	ND	ND	ND	ND	ND
Flowering white cabbage	Room temperature	Nitrate	2000	1800	1700	1500	810	260	180	64
		Nitrite	ND	ND	ND	170	530	820	690	650
	Refrigerated temperature	Nitrate	2000	1800	2000	2100	1900	1800	1700	1700
		Nitrite	ND	ND	ND	ND	ND	ND	6.4	30
Chinese lettuce	Room temperature	Nitrate	760	780	780	800	720	300	250	260
		Nitrite	ND	ND	ND	1.2	43	130	97	94
	Refrigerated temperature	Nitrate	760	750	760	760	760	690	670	700
		Nitrite	ND	ND	ND	ND	ND	ND	ND	ND

*ND = Not detected

Annex IV

Nitrate and nitrite levels of stir-fried vegetables stored at room temperature and refrigerated temperature

Samples	Storage condition	Level (mg/kg)	Storage time (hours)							
			0	6	12	24	36	48	72	78
Amaranth	Room temperature	Nitrate	3700	3700	3700	3700	3500	2500	1700	1800
		Nitrite	ND	ND	ND	ND	200	730	1200	1100
	Refrigerated temperature	Nitrate	3700	3700	3400	3700	3700	3400	3300	3500
		Nitrite	ND	ND	ND	ND	ND	ND	ND	ND
Pak-choi	Room temperature	Nitrate	3800	3700	3900	3500	2900	1400	1400	1400
		Nitrite	ND	ND	ND	100	590	1300	1100	1000
	Refrigerated temperature	Nitrate	3800	3900	3800	3500	3700	3700	3700	3600
		Nitrite	ND	ND	ND	ND	ND	ND	3.6	6.0
Flowering white cabbage	Room temperature	Nitrate	2350	2500	2600	2100	1300	420	300	10
		Nitrite	ND	ND	ND	250	770	1000	690	700
	Refrigerated temperature	Nitrate	2350	2600	2500	2700	2400	2400	2400	2200
		Nitrite	ND	ND	ND	ND	ND	ND	14	100
Chinese lettuce	Room temperature	Nitrate	1300	1200	1200	1100	1100	540	580	500
		Nitrite	ND	ND	ND	7.4	27	89	73	60
	Refrigerated temperature	Nitrate	1300	1200	1200	1200	1100	1100	1100	1100
		Nitrite	ND	ND	ND	ND	ND	ND	ND	ND

*ND = Not detected

Annex V

Nitrate and nitrite levels of boiled and stir-fried zucchini stored at room temperature and refrigerated temperature

Samples	Storage condition	Level (mg/kg)	Storage time (hours)							
			0	6	12	24	36	48	72	78
Boiled zucchini	Room temperature	Nitrate	390	280	330	12	1.4	0.88	4.3	3.4
		Nitrite	ND	ND	ND	170	40	0.82	1.0	5.3
	Refrigerated temperature	Nitrate	390	550	510	480	450	480	470	500
		Nitrite	ND	ND	ND	ND	ND	ND	ND	ND
Stir-fried zucchini	Room temperature	Nitrate	680	660	710	320	4.7	2.2	2.1	5.2
		Nitrite	ND	ND	ND	260	300	220	2.8	3.7
	Refrigerated temperature	Nitrate	680	660	720	630	710	670	730	740
		Nitrite	ND	ND	ND	ND	ND	ND	ND	ND

*ND = Not detected

Annex VI

Nitrate and nitrite levels of soup stored at room temperature and refrigerated temperature

Samples	Storage condition	Level (mg/kg)	Storage time (hours)							
			0	6	12	24	36	48	72	78
Soup	Room temperature	Nitrate	955	940	930	630	430	180	160	160
		Nitrite	ND	ND	ND	20	110	190	230	230
	Refrigerated temperature	Nitrate	955	980	970	970	1000	940	960	940
		Nitrite	ND	ND	ND	ND	ND	ND	ND	ND

*ND = Not detected