Are nitrate concentrations in leafy vegetables within safe limits?

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Leafy vegetables are an important source of nutrition in the human diet. Estimation of nitrate concentration in samples of leafy vegetables collected from the local markets of Delhi has revealed that a significant number of spinach and chenopodium samples contained nitrate in concentrations higher than the Acceptable Daily Intake (ADI) limit for an average 60 kg person (if consumed @ 100 g/day). However, nitrate concentration in fenugreek, coriander and sowa samples was well

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within the safe limits for consumption. On the basis of our findings with market samples, extensive studies were conducted in nine genotypes of spinach (*Spinacia oleracea* L.) grown under greenhouse conditions. The genotypes varied markedly in their nitrate concentration as well as nitrate reductase activity. At the three-week stage of plant growth, one genotype, and at the six-week stage, six of the nine genotypes under investigation exceeded the ADI limit. Petioles possessed several times higher level of nitrate than the leaf laminae in market samples as well as in the genotypes. All the genotypes showed diurnal variation in nitrate accumulation with minimum concentration at noon.

These findings warrant thorough investigation of nitrate levels in other leafy vegetables consumed regularly and the ways and means to control them.

Keywords: Human health, leafy vegetables, nitrate toxicity, nitrate reductase, spinach.

EXCESSIVE use of nitrogenous fertilizers is (often wrongly) considered by farmers as a reasonable insurance against yield losses. However, apart from environmental contamination due to leaching, volatilization, denitrification, surface run-off, etc. it leads to accumulation of nitrate in leafy vegetables beyond safe limits. Although current epidemiological data provide conflicting evidence regarding the potential long-term health risks of nitrate levels encountered in the diet, it is widely accepted that a reduction in dietary nitrate is a desirable preventive measure[1]. The toxic effects of nitrate are due to its endogenous conversion to nitrite, which is implicated in the occurrence of methaemoglobinaemia, gastric cancer and many other diseases[2]. Incidence of methaemoglobinaemia, earlier believed to have been confined to infants only, has been reported in all age groups with high nitrate ingestion, with infants and above 45 years age groups being most susceptible to nitrate toxicity[3]. The effect of nitrate consumption on human health has been studied in detail by Gupta *et al.*[4–8].

Use of sewage water for irrigation of crops, which is prevalent throughout India, may lead to nitrate contamination of groundwater[2]. Although some attention has been paid to the level of nitrate in drinking water and groundwater bodies in India[2,9–11], there is a gap in our knowledge regarding the dietary nitrates being consumed by human beings. Approximately 70% of total intake of dietary nitrate comes from vegetables. Ysart *et al.*[12] estimated total nitrate intake of 93 mg day^{-1} for adult humans, comprising the following proportions: potatoes (33%), green vegetables (21%), other vegetables (15%), beverages (8.5%), meat products (4.2%), fresh fruit (3.5%), dairy (3.1%), milk (2.9%), miscellaneous cereals (2.1%), bread (1.6%) and others (5.1%). The European Commission's (EC) Scientific Committee on Food (SCF)[13] prescribed in 1995 the Acceptable Daily Intake (ADI) of nitrate ion as 3.65 mg kg^{-1} body wt day^{-1} (equivalent to 219 mg day^{-1} for a 60 kg person). The SCF also advised to reduce exposure to nitrate via food and urged that good agricultural practices be adopted to ensure nitrate levels as low as reasonably achievable[13].

In the present study, nitrate concentrations were estimated in various leafy vegetables available in the market and evaluated for their fitness for consumption with reference to ADI. On the basis of the findings in the market samples, extensive studies were conducted in different genotypes of spinach (*Spinacia oleracea* L.), one of the most widely consumed leafy vegetables, under greenhouse conditions.

Fifteen samples each of spinach, chenopodium, fenugreek, coriander and sowa were collected from three main vegetable markets of Delhi, namely Ghazipur, Okhla and Azadpur, and analysed for nitrate concentration in fresh plant material.

A pot culture experiment was carried out at the Herbal Garden of Jamia Hamdard, New Delhi during winter season of 2004–05 under greenhouse conditions. Prior to sowing, pots were lined with polyethylene bags and filled with 8 kg of loamy sand (83.6% sand, 6.8% silt and 9.6% clay, pH 7.1). The available nitrogen (128 mg kg^{-1} of soil) and phosphorus (4 mg kg^{-1}) were low, whereas the available potassium was 158 mg kg^{-1} of soil.

Nine genotypes of spinach (Table 1) obtained from National Bureau of Plant Genetic Resources (NBPGR), IARI, New Delhi were grown in triplicates. The applied levels of nitrogen (N), phosphorus (P) and potassium (K) were 120, 30 and 80 mg kg^{-1} of soil respectively, based on soil test recommendations. Sources of N, P and K were NH_4NO_3, NaH_2PO_4 and KCl respectively. All the plant samples were analysed simultaneously for nitrate concentration and nitrate reductase activity.

Extraction and estimation of nitrate were done by the methods of Grover *et al.*[14] and Downes[15] respectively. *In vivo* nitrate reductase assay was done by the method of Jaworski[16].

Nitrate concentrations in fresh leafy vegetables procured from local markets are summarized in Table 2. They range from 71 to 4293, 204 to 4451, 288 to 524, 289 to 769 and 684 to 1071 mg kg^{-1} fresh wt of sample for spinach, chenopodium, fenugreek, coriander and sowa respecti-

Table 1. Accession numbers and codes used in the study for *Spinacia oleracea* L. genotypes obtained from NBPGR

Sl. no.	Code no.	Accession no.
1	S1	EC 1915010
2	S2	IC 143920
3	S3	IC 143921
4	S4	IC 284910
5	S5	IC 326869
6	S6	IC 374686
7	S7	IC 374705
8	S8	IC 381477
9	S9	IC 382264

Table 2. Nitrate concentration in leafy vegetables collected from different markets in Delhi

Market	Vegetable	No. of samples	Range (mg kg^{-1} fresh wt of samples)†
Ghazipur	Spinach	15	**71**–1685
	Fenugreek	15	**288–524**
	Chenopodium	15	2794–**4451***
	Coriander	15	314–518
	Sowa	15	785–**1071**
	Petiole of spinach	15	462–887
Okhla	Spinach	15	1117–**4293***
	Fenugreek	15	208–331
	Chenopodium	15	**204**–510
	Coriander	15	**289**–486
	Sowa	15	**684**–847
	Petiole of spinach	15	5989–6269*
Azadpur	Spinach	15	2112–2892*
	Fenugreek	15	302–360
	Chenopodium	15	1420–2685*
	Coriander	15	388–**769**
	Sowa	15	866–934
	Petiole of spinach	15	2097–3568*

†Values are means of 15 samples.
*Samples exceeding the ADI for an average 60 kg person (if consumed 100 g day^{-1}).
Values in bold represent minimum and maximum concentrations in a given vegetable.

Table 3. Acceptable Daily Intake (ADI) for nitrate ion as recommended by the European Commission's Scientific Committee for Food in 1995 according to the body weight of a person

Body weight (kg)	ADI (mg day^{-1})
30	109.5
40	146.0
50	182.5
60	219.0
70	255.5
80	292.0

vely. A significant number of samples of spinach (34%) and chenopodium (45%) exceeded the ADI limit for an average 60 kg person (if consumed @ 100 g day^{-1}). Nitrate concentration in the petiole of spinach was much higher (462–6269 mg kg^{-1} fresh wt) than in the leaf lamina. On the other hand, nitrate levels in fenugreek, coriander and sowa were well within the safe limits for human consumption. Since the SCF has set ADI for nitrate ion on the basis of body weight of the consumer, i.e. 3.65 mg kg^{-1} body wt day^{-1}, it was important to work out the ADI for different people having different body weight. The ADI of nitrate was calculated for people ranging in body weight from 30 to 80 kg (Table 3). As expected, the percentage of samples of spinach and chenopodium with nitrate levels exceeding ADI was higher for people having lesser body weight than for those having more body weight (Figure 1).

Since nitrate levels in a significant number of market samples of spinach and chenopodium exceeded the ADI limit for an average 60 kg person, *S. oleracea* L. was selected for an extensive screening of nitrate accumulation.

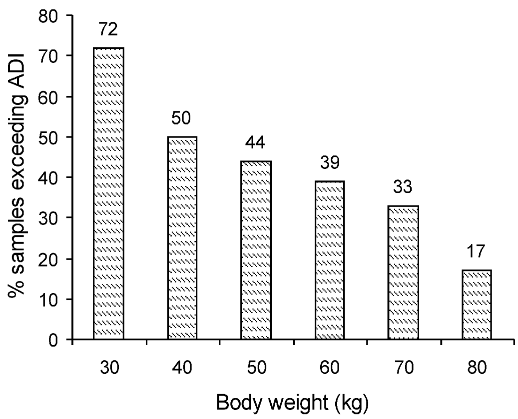

Figure 1. Percentage of market samples (spinach and chenopodium) exceeding ADI on the basis of body weight of consumers (if consumed 100 g day^{-1}).

Spinach genotypes belonging to different geographical regions of India varied in their tendency to accumulate nitrate. The minimum and maximum nitrate concentrations were found to be 998 and 2674 mg kg^{-1} fresh wt in S6 and S5 genotypes respectively, at the three-week stage of plant growth (Figure 2). At this stage, only one genotype (S5) exceeded SCF's ADI limit for nitrate ion for an average person weighing 60 kg (if consumed @ 100 g per day). However, in six-week-old plants, it exceeded in six of the nine genotypes under investigation. Nitrate concentration ranged from 1161 to 5364 mg kg^{-1} fresh wt of leaves (Figure 3). At this stage also, S6 and S5 genotypes possessed the minimum and maximum concentrations respectively.

In comparison to leaf blades, petioles contained several-fold higher nitrate concentration (Figure 3), with the minimum (1371 mg kg^{-1} fresh wt of petioles) in S6 and maximum (35,328 mg kg^{-1} fresh wt) in S5. The difference in nitrate concentration between leaf lamina and petiole of S5 was as high as 6.6-fold.

Genotypic variation was apparent also in the nitrate reductase activity (NRA) of spinach plants, with a maximum level (3.798 μmol NO_2^{-1} h^{-1} g^{-1} fresh wt of leaves) in the plant that had minimum nitrate concentration (S6) and vice versa (S5; Figure 4). A significant negative relationship existed between NRA and nitrate concentration in genotypes with the correlation coefficient –0.969 (Figure 5).

Diurnal variations in nitrate concentration and NRA were determined at three different times of the day. Nitrate concentrations were the lowest at 1:00 pm and highest at 5:00 pm in all the genotypes. Data for two such genotypes are shown in Figure 6. Nitrate concentration was 2025 and 2674 mg kg^{-1} in S5, and 641 and 998 mg kg^{-1} leaf material in S6 at 1:00 and 5:00 pm respectively, in three-week-old plants.

Accordingly, NRA also showed diurnal variation, with its maximum and minimum occurring at 1:00 and 5:00 pm respectively, in three-week old plants of all the genotypes. Data on two such genotypes are shown in Figure 7. These results are consistent with the negative relationship between NRA and nitrate concentration.

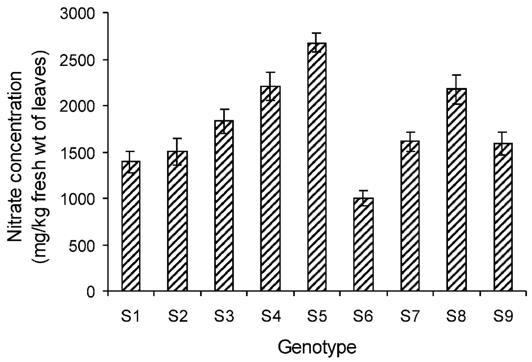

Figure 2. Genotypic variation in nitrate concentration in leaves of spinach genotypes in three-week-old plants.

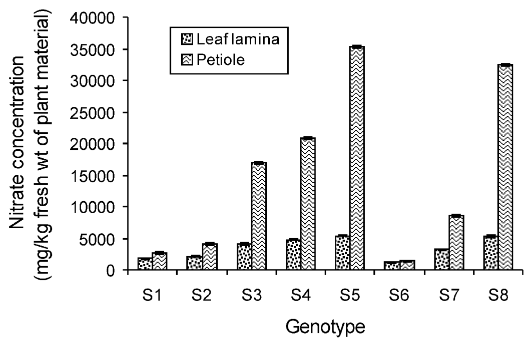

Figure 3. Nitrate concentration in leaf lamina and petiole of spinach genotypes in six-week-old plants.

The ADI limit for nitrate (if consumed @ 100 g day^{-1} by an average 60 kg person) exceeded substantially in a significant number of market samples of spinach and chenopodium, while it was within limits for fenugreek, coriander and sowa. Excessive use of nitrogenous fertilizer may not be specific to some vegetables. Spinach and chenopodium belong to Chenopodiaceae, a family known

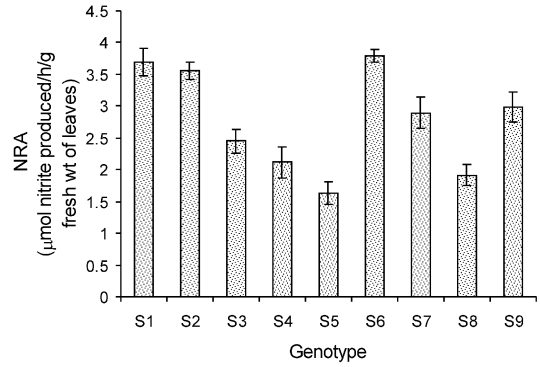

Figure 4. Genotypic variation in nitrate reductase activity in leaves of spinach genotypes in 3-week-old plants.

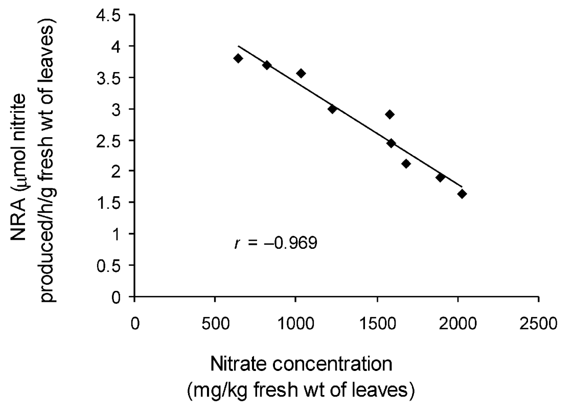

Figure 5. Relationship between nitrate concentration and nitrate reductase activity in leaves of spinach genotypes in three-week-old plants.

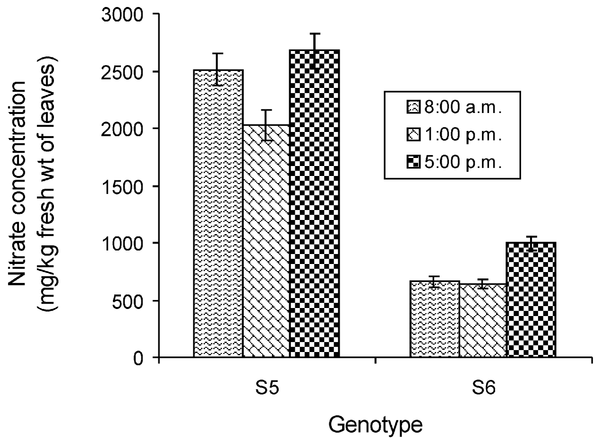

Figure 6. Diurnal variation in nitrate concentration in two genotypes of spinach in three-week-old plants.

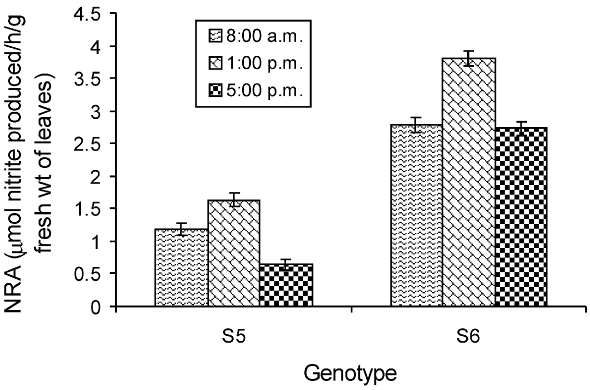

Figure 7. Diurnal variation in nitrate reductase activity in two genotypes of spinach in three-week-old plants.

to have a high preference for nitrate accumulation in the shoots and uses nitrate in vacuoles for osmoregulation[17]. Since ADI limit is less for people with low body weight, the consumption level of these vegetables by children and low-weight adults must be paid greater attention, in view of the health hazards reported by Gupta *et al.*[3–8].

Accumulation of large amounts of nitrate in certain genotypes may be due to their low NRA, as evident by the negative relationship. Other causal factors may be related to variations in the uptake and distribution of nitrate or other elements needed for NRA, differences in generation of electron donors needed in the assimilative pathway[18], in photosynthetic capacity[19], or ability to generate and translocate respiratory substrate and reducing equivalents. All these factors need further study. Variation in nitrate content between plant species and even between cultivars of the same species has been reported earlier also[20–23]. Over expression of NR genes may be a useful approach to reduce the nitrate content of plants that have a propensity to accumulate the same and to improve their quality for human consumption, although genetic manipulation of activities of nitrate assimilatory enzymes may not increase yield and/or nitrogen use efficiency of plants[24].

Higher nitrate concentration in six-week-old plants in comparison to three-week-old plants may be because in fully expanded leaves with low NRA, higher nitrate contents are of limited use for nitrogen metabolism due to low nitrate mobility in the phloem[17] and therefore get accumulated. Moreover, interruption of nitrate supply to the roots at later stages may lead to a drop in both NRA in the leaves and the shoot growth rate, despite a high nitrate content in the shoot[25]. Therefore, selection among the available genotypes and consumption of spinach harvested early in the vegetative stage are likely to ensure a significantly reduced consumption of nitrate through leafy vegetables and the subsequent risk of nitrate poisoning.

Petioles contained several-fold higher nitrate concentration than the leaf laminae, presumably because translocation of nitrate from the roots to the leaf lamina takes place through the petiole which serves as a nitrate reservoir, or because of a relatively lower level of NRA in the petiole, which remains to be assessed. Removal of petiole prior to processing the leafy vegetable could help minimize the nitrate intake. According to Santamaria *et al.*[26], vegetable organs can be listed by decreasing nitrate content as: petiole > leaf > stem > root > inflorescence > tuber > bulb >fruit > seed. In certain regions of the country, even the stem portion of the spinach plant is consumed, the nitrate content of which invites caution.

Observations on diurnal variation in nitrate accumulation and NRA have revealed minimum nitrate accumulation and maximum NRA during noontime. The concentration of nitrate in leaves is affected by light in various ways. However, characterization of the effect of light on nitrate accumulation is still incomplete. Light intensity has been identified as one of the major factors that influence nitrate content in vegetables[18]. In particular, light intensity before or at harvest is known to be a critical factor in determining nitrate levels in spinach[27] and other vegetables[28,29]. Accumulation of nitrate under low light intensity may primarily be the result of a restricted NRA without a concomitant reduction in nitrate uptake[30]. Leaves of plants grown under low light intensity usually have low levels of NRA, which increase after the plants are transferred to conditions of high light intensity. The diurnal rhythm by NRA with a maximum during noontime, may be due to illumination that stimulates translation of the NIA transcript and inhibits degradation of the NIA protein[31], leading to an increase in NIA protein during the light period[32].

Given the above, nitrate consumption through vegetables can be kept low by harvesting them at the proper time. Some earlier reports have advised the harvest of spinach crop in the afternoon of a sunny day when nitrate concentration in the leaves is low[33,34]. Our findings, however, suggest that the leaf nitrate concentration is at its lowest at 1:00 pm, when the sunlight is maximum. The discrepancy may be due to differences in the prevailing environmental conditions in India and regions where the earlier studies had been conducted. Therefore, for spinach grown under Indian climatic conditions, harvesting at noontime may be recommended. Indian farmers normally harvest the crop in the early morning, a time when the concentration of nitrate in plants is appreciably high. Therefore, it is germane to educate consumers regarding the nitrate content in vegetables and its health implications and persuade farmers to harvest spinach at noon and supply it to the market by the evening so that deterioration in the quality of plants due to nitrite formation does not take place during the post-harvesting period.

Thus a careful selection of vegetable genotypes based on the relationship between nitrate and NRA, coupled with due management of the nutrition and harvest regime, help avoid nitrate accumulation and the associated health hazards. Moreover, by cooking vegetables in water (with low nitrate concentration), at least 50% of accumulated nitrate can be removed[35]. Since ascorbic acid and tocopherols inhibit the formation of N-nitrocompounds[36], an increase in the content of these vitamins and a reduction in nitrate content can attach added value to vegetable products (already rich in carotenoids, selenium, dietary fibre, plant sterols, glucosinolates and indoles, isothiocyanates, flavonoids, phenols, etc.)[1].

The market samples as well as genotypes studied showed that a significant number of samples of spinach and chenopodium contained nitrate concentration above the ADI limit. Nitrate concentration had a negative correlation with NRA. Harvesting young plants at noontime and removal of petioles could minimize the dietary intake of nitrate from spinach. Further investigations on this and other vegetables are needed to understand nitrate accumulation patterns and avoid nitrate toxicity.

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