

Changes in Nitrate and Nitrite Content, and Search for Nitrosamines in Storage-Abused Spinach and Beets

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Conditions favoring formation of nitrosamines—simultaneous presence of nitrite and secondary amines at acid pH—can occur during abusive storage of comminuted fresh spinach or beets. Shredded spinach, shredded or ground fresh beets, or beet juice rapidly produced nitrite. One beet sample contained 1000 ppm of nitrite after 2 days at room temperature and still appeared edible. Shredded fresh spinach samples were considered inedible when nitrite levels of 300–500 ppm were reached. Whole fresh and all forms of processed spinach and beets accumulated little ni-

trite even though containing 1500–2000 ppm of nitrate originally. Selections from these tests, cooked and uncooked, and thawed, frozen, and thawed spinach were analyzed by a glc-mass spectral method for six nitrosamines (methyl-, ethyl-, and methylethylnitrosamine, nitrosopyrrolidine, nitrosomorpholine, and nitrosopiperidine). Results were negative, indicating no detectable formation of these six nitrosamines when fresh or processed spinach or beets are stored even beyond the point of edibility.

The possible occurrence in foods of nitrates and nitrites, and especially of nitrosamines, has recently become a matter of great concern because of the toxic or carcinogenic nature of some of these compounds (Aune, 1972; IFT, 1972; Wolff and Wasserman, 1972). Some vegetables, such as spinach and beets, can accumulate high levels of nitrate, which may be reduced to nitrite during storage after preparation. Earlier concern was with the relation of nitrate and nitrite in the diet to infant methemoglobinemia (Phillips, 1969, 1971; Sinios and Wodsak, 1965; Achtzehn and Hawat, 1970). Numerous instances of this disorder have been related to excessive levels of these ions in well water; in Europe 15 cases of infant methemoglobinemia were traced to feeding with improperly stored home-prepared spinach purees. Processed vegetable products have been shown not to accumulate significant amounts of nitrite during normal storage (Phillips, 1969, 1971; Sinios and Wodsak, 1965).

Since nitrites are known to react with secondary or tertiary amines to form nitrosamines, we felt it prudent to check these vegetables for the presence of the latter com-

pounds. Keybets *et al.* (1970), using methods sensitive to 500 $\mu\text{g}/\text{kg}$ of dimethylnitrosamines and 100 $\mu\text{g}/\text{kg}$ of diethylnitrosamines, could not detect these nitrosamines in high-nitrite spinach. However, since many nitrosamines are highly potent carcinogens, and since their carcinogenic threshold values in humans are not known, it is important that more sensitive and specific analytical techniques be used.

The purpose of the present study was to examine the development of nitrite in beets and spinach subjected to storage abuse and to analyze high-nitrite samples for several nitrosamines by a method which is both specific and highly sensitive.

EXPERIMENTAL SECTION

Beet and spinach samples, both fresh and processed, were obtained from local retail markets and were subjected to storage at room temperature (25°) and at refrigeration temperature (5°). The lengths of time were from a few days at room temperature to as long as 32 days under refrigeration.

The following types of beet samples were studied: fresh beets, whole and shredded; beet juice; canned beets, sliced, whole, and ground; strained beets (baby food); and borscht. Spinach samples studied were: fresh spinach, whole and shredded; spinach juice; frozen spinach, whole

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and chopped; canned spinach; and strained creamed spinach (baby food).

Fresh vegetables were shredded by use of a juicerator (Acme Supreme Juicerator, Model 6001). The juice and shredded pulp were recombined by thorough mixing. Canned vegetables were opened and the contents transferred to beakers covered by plastic film. Contents were well stirred at the time of sampling during storage.

Nitrite and nitrate content and pH were monitored at frequent intervals during the storage period, and, generally, samples high in nitrite content were subjected to nitrosamine assay, although for control purposes some normal samples were also assayed for nitrosamines.

Samples for nitrite and nitrate analysis were prepared by grinding in a blender a weighed amount of the vegetable with a measured quantity of distilled water. The slurry was further stirred for 0.5 hr, using a magnetic stirrer, and then filtered through Whatman no. 1 paper. An aliquot of the filtrate was used for the nitrate analysis. For the nitrite analysis, an aliquot of the filtrate was decolorized using a suspension of aluminum hydroxide (American Public Health Association, 1965).

Methods previously used in this laboratory for nitrate and nitrite analyses (Heisler *et al.*, 1973) were used. These were the diazotization method using 1-naphthylamine and sulfanilic acid (Nelson *et al.*, 1954) for the nitrite analysis, and for the nitrate determination the nitroxyleneol method (Holler and Huch, 1949; Lipp and Dolberg, 1964) in which 3,4-dimethylphenol is nitrated and the resulting nitro-3,4-xyleneol is separated by steam distillation.

Spinach and beets were extracted and analyzed for six volatile nitrosamines: dimethyl-, methylethyl-, and diethylnitrosamines, and nitrosopiperidine, -pyrrolidine, and -morpholine. Procedures for preparation and cleanup of extracts were similar to those of Fazio *et al.* (1972) with the following modifications.

(1) **Preparation of Alkaline Digests.** One hundred grams of vegetable were comminuted in a Waring Blender with 100 ml of CH₃OH, and the resulting slurry was transferred to a boiling flask with the aid of a second 100-ml portion of CH₃OH. Subsequent digestion with KOH, liquid-liquid extraction of the digests, distillation of the extracts from alkali, and extraction of the distillates with CH₂Cl₂ were as described by Fazio.

(2) **Two-Stage Concentration of Extracts.** The CH₂Cl₂ extracts of the distillates were first concentrated to 4 ml by heating the Kuderna-Danish evaporative concentrator in a water bath at 80–85°. The concentrator tube was then attached to a trap-type adapter (Kontes Glass Co., Catalogue No. K-275090), an air condenser (K-569251) was placed on top, and the extract was concentrated further at 60°. This arrangement obviated losses from bumping or from upward-moving bubble films. After each stage, the apparatus was allowed to drain 20 min in a cold room (10°) before the concentrator tube was disconnected.

(3) **Column Chromatographic Cleanup of Extracts.** This was found to be unnecessary for these vegetables and was omitted.

The concentrated extracts were analyzed by gas-liquid chromatography (glc). A Varian Aerograph Model 1740-1, with the standard flame ionization detector modified to an alkali flame ionization detector with a KCl-coated coil (Howard *et al.*, 1970), was used. The column (10 ft × 1/8 in. o.d., stainless steel) was packed with 13% Carbowax 20M-TPA on 60–80 mesh Gas Chrom P. Column temperature was programmed from 105 to 200° at 4°/min.

Samples of the alkaline digests were run in duplicate in order to determine recoveries. Of these, one was spiked with 1 ml of standard mixture containing 0.5 µg/ml of each nitrosamine (equivalent to 20 µg of each nitrosamine per kilogram of vegetable).

Recovery values were calculated by comparing glc peak

heights of nitrosamines in the spiked vegetable extract with those obtained from the standard mixture of nitrosamines. Average values were: for dimethylnitrosamine, 57%; for methylethylnitrosamine, 69%; for diethylnitrosamine, 69%; for nitrosopiperidine, 76%; for nitrosopyrrolidine, 70%; and for nitrosomorpholine, 57%.

Unspiked extracts which yielded glc peaks with elution volumes close to those of the standard nitrosamines were selected for further analysis by a combined glc-mass spectrometer provided that the peak heights indicated that sufficient material was present for confirmation by the latter apparatus, which required approximately 7–10 µg of nitrosamine/kg of vegetable.

The glc-mass spectrometer consisted of a Varian Aerograph Model 1740-1 gas chromatograph equipped with a 5 ft × 1/8 in. o.d. stainless steel column packed with 3% SE-30 on 100–120 mesh Varaport 30 and connected to a DuPont Model 21-492 mass spectrometer. The column temperature was programmed from 100 to 170° at 6°/min. The column effluent was split, with one-half going into a flame ionization detector and the other passing *via* a line heated at 190° into the mass spectrometer. This was operated in the peak matching mode adjusted to a resolution of 1 in 12,000, as described by Dooley *et al.* (1973). Nitrosamines were identified by their parent peaks. The NO⁺ ion was not sought because of its low relative peak intensity.

After the research described in this paper was completed, it was learned that α -naphthylamine had been classified as a carcinogen under the "Emergency Temporary Standard on Certain Carcinogens" (Stender, 1973). We have discontinued use of this procedure and for subsequent research are using, for nitrite analysis, the method described by Schall and Hatcher (1968). This procedure uses sulfanilamide with *N*-(1-naphthyl)ethylenediamine for the coupling reagent, as earlier described for analysis of NO₂ in air by Jacobs and Hochheiser (1958). Attention of laboratories using α -naphthylamine is directed to the recommended precautions for laboratory workers handling carcinogenic aromatic amines published by the Chester Beatty Research Institute (1966).

RESULTS AND DISCUSSION

Nitrates and Nitrites. Beets. Results of analyses of stored samples of spinach and beets are shown in Figure 1. Generally, under moderate to extreme storage conditions there was no increase of nitrite content in the whole fresh beets, the sliced and whole canned beets, the strained beets, and the borscht. In all these samples, the nitrite content was below 8.0 mg/kg. Fresh beets had a nitrate content of approximately 4000 mg/kg. The canned beets and strained beets contained 1500–2000 mg/kg of nitrate and the borscht contained 325 mg/kg.

The shredded fresh beets, beet juice, and ground canned beet samples did exhibit an increase in nitrite content and a decrease in nitrate content under moderate to extreme storage conditions. The highest nitrite content, approximately 1000 mg/kg, was obtained when shredded fresh beets were stored 2 days at 25°. There was a corresponding sharp decrease in nitrate content. Figure 1A is a plot of the nitrate, nitrite, and pH values against time stored. The curves shown are the average of nine storage runs. Refrigerated storage of shredded fresh beets resulted in a nitrite content build-up to approximately 200 mg/kg at 19 days storage (Figure 1B). The nitrite content of beet juice increased to approximately 540 mg/kg after 1-day storage at room temperature (Figure 1C) and ground canned beets exhibited a nitrite content of approximately 90 mg/kg after 2 days at 25° (Figure 1D). In every case, the pH of the samples was taken during the storage studies and it can be stated that the pH generally decreased as the nitrite increased.

Spinach. Generally, under moderate to extreme storage

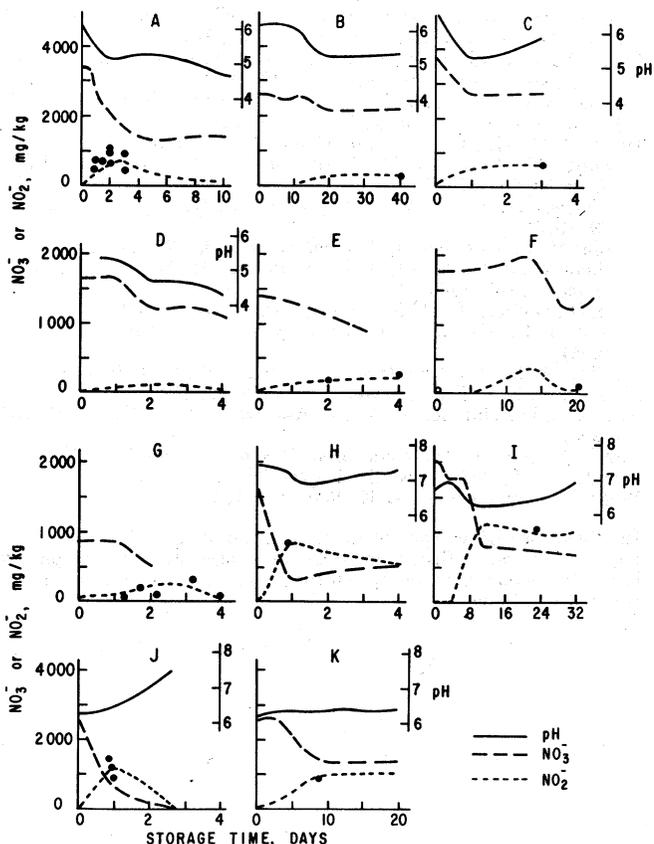


Figure 1. Changes in nitrate, nitrite, and pH value in storage of beet and spinach samples: (A) fresh beets, shredded, stored at 25°; curves represent average of nine runs; (B) fresh beets, shredded, stored at 5°; (C) beet juice (from fresh beets); juice stored at 25°; (D) whole canned beets, ground, stored at 25°; (E) fresh spinach, stored at 25°; curves represent average of four runs; (F) fresh spinach, stored at 5°; average of five runs; (G) frozen spinach, thawed and stored at 25°; average of five runs; (H) spinach juice (from fresh spinach), stored at 25°; (I) spinach juice (from fresh spinach), stored at 5°; (J) fresh spinach, shredded, stored at 25°; average of two runs; (K) fresh spinach, shredded, stored at 5°. Solid circles indicate samples selected for nitrosamine analysis.

conditions, there was no increase in nitrite content in canned spinach or strained creamed spinach; in all the samples studied, the nitrite content was below 10 mg/kg during 29 days at 5° or 2 days at 25°. The nitrate content of canned spinach was approximately 575 mg/kg and that of the strained creamed spinach was approximately 1250 mg/kg.

There was a moderate increase in nitrite content on storage of fresh spinach leaf and frozen spinach. In fresh spinach, the nitrite content was initially under 5 mg/kg. The nitrate content ranged initially from 1300 to 1400 mg/kg. Under room temperature storage, fresh spinach is very perishable, the limit of storage being approximately 3 days, at which time the nitrite reached approximately 140 mg/kg. The nitrate exhibits a generally declining pattern. Under refrigeration, there was an increase in nitrite content to approximately 300 mg/kg after a relatively long storage period of 14 days. Again, the nitrate generally declined (Figures 1E and 1F). Frozen spinach containing initially approximately 900 mg/kg of nitrate was allowed to thaw for periods of up to 4 days and the nitrite and nitrate contents were determined at intervals. The nitrite content generally increased during the first 2 or 3 days and then decreased. A high of 370 mg/kg was reached in one case. The nitrate showed a corresponding sharp decrease; see Figure 1G.

Spinach juice was also stored at room temperature and

under refrigeration. Here again, the nitrite content increased to the relatively high level of from 900 to 1000 mg/kg. At room temperature, it reached a maximum in 21 hr; under refrigeration it took approximately 11 days (Figures 1H and 1I).

The nitrite content reached a much higher level when fresh spinach leaf was shredded and stored. Stored spinach juice also exhibited a high nitrite content. Considering the shredded fresh spinach, the nitrite content reached approximately 1500 mg/kg after 1-day storage at room temperature. Under refrigeration, the nitrite content reached a maximum of 1020 mg/kg after 17 days of storage. In both cases the nitrate decreased in a corresponding manner (Figures 1J and 1K). None of these high-nitrite products was considered edible.

Nitrosamines. Stored spinach and beet samples were withdrawn and analyzed for nitrosamines at the points indicated by solid circles in Figure 1. Five samples of processed beets representing initial and extreme storage conditions were assayed for nitrosamine content. In most cases the raw samples were taken for the nitrosamine assay, but in two instances high nitrite beet samples containing 423 and 207 mg/kg were cooked by immersing a covered beaker containing the 100.0-g sample of vegetable tissue in a boiling water bath for 45 min prior to work-up for nitrosamine assay. Of the four stored fresh spinach samples (see solid circles in Figures 1H and 1I) assayed for nitrosamine content, two of them containing 1022 and 872 mg/kg of nitrite were cooked as previously described prior to work-up for the nitrosamine assay. Four samples of processed spinach were assayed for nitrosamine content: two of canned spinach, and two of strained creamed spinach, representing initial and extreme storage states. In addition, a sample of frozen spinach was assayed for nitrosamine content. It was treated as follows: sample was thawed 0.5 hr, cooked as described above, drained, and an aliquot assayed for nitrosamine content. The rest of the sample was refrozen and then thawed 3 hr and again sampled and assayed for nitrosamine content. In both cases the nitrite content remained under 10 mg/kg.

Gas chromatographic peaks large enough to permit further analysis by mass spectrometry occurred in positions coincident with those of three nitrosamines: methylethyl-nitrosamine, nitrosopyrrolidine, and nitrosomorpholine. In all instances, mass spectral analyses showed these compounds to be absent. Only one of these peaks occurred in more than one sample. This peak, which occurred in several beet samples, had an elution volume identical or almost identical with that of nitrosomorpholine. Four samples were subjected to mass spectrometry. Two, which averaged 980 mg/kg of nitrite, were from fresh beets that had been ground and stored 2 days at room temperature. The third, ground fresh beets stored 3 days at room temperature, was past the point of maximum nitrite concentration and contained 867 mg/kg of nitrite. The fourth (fresh beets, ground, stored 2 days at room temperature, then cooked) contained 423 mg/kg of nitrite; it also contained another peak coincident with nitrosopyrrolidine. Mass spectrometry showed that the peaks obtained from these samples were not nitrosomorpholine or nitrosopyrrolidine.

Almost half the beet samples showed a very small peak at the position of dimethylnitrosamine, but the maximum value which appeared in only one sample was 5 μ g/kg. This concentration was too low for confirmation by mass spectrometry.

A few spinach samples also had a peak coincident with nitrosopyrrolidine, but the maximum concentration was 5 μ g/kg and was reached by only two samples. In one sample (fresh spinach, shredded, containing 885 mg/kg of nitrite after 9 days refrigeration) an 8- μ g/kg peak near methylethyl-nitrosamine occurred, but mass spectrometry showed that this nitrosamine was not present.

The data further confirm that storage of comminuted high-nitrate fresh spinach and beets will eventually result in accumulation of large amounts of nitrite at the expense of nitrate. Since the six nitrosamines sought by highly specific and sensitive methods were not found, even in materials stored beyond the limits of edibility, the principal public health concern with these high-nitrite vegetables remains the relation of nitrite to methemoglobinemia in infants.

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LITERATURE CITED

- Achtzehn, M. K., Hawat, H., *Nahrung* 14, 383 (1970).
American Public Health Association, "Standard Methods for the Examination of Water and Wastewater," 12th ed, New York, N.Y., 1965.
Aune, T., *Nord. Veterinaermed.* 24, 356 (1972).
Chester Beatty Research Institute, London, "Precautions for Workers who Handle Carcinogenic Aromatic Amines," Institute of Cancer Research, Royal Cancer Hospital, London, 1966.
Dooley, C. J., Osman, S. F., Martin, G. P., Fiddler, W., presented at the 21st Annual Conference on Mass Spectrometry and Allied Topics, San Francisco, Calif., May 1973.
Fazio, T., Howard, J. W., White, R., *N-Nitroso Compounds Anal. Form. Proc. Work. Conf.* 1971 3, 16 (1972).
Heisler, E. G., Siciliano, J., Krulick, S., Porter, W. L., White, J. W., Jr., *J. Agr. Food Chem.* 21, 970 (1973).
Holler, A. C., Huch, R. V., *Anal. Chem.* 21, 1385 (1949).
Howard, J. W., Fazio, T., Watts, J. O., *J. Ass. Offic. Anal. Chem.* 53, 269 (1970).
IFT Expert Panel on Food Safety and Nutrition, *J. Food Sci.* 37, 989 (1972).
Jacobs, M. B., Hochheiser, S., *Anal. Chem.* 30, 426 (1958).
Keybets, M. J. H., Groot, E. H., Keller, G. H. M., *Food Cosmet. Toxicol.* 8, 167 (1970).
Lipp, G., Dolberg, U., *Beitr. Tabakforsch.* 2, 345 (1964).
Nelson, J. L., Kurtz, L. T., Bray, R. H., *Anal. Chem.* 26, 1081 (1954).
Phillips, W. E. J., *J. Can. Inst. Food Technol.* 2, 160 (1969).
Phillips, W. E. J., *Food Cosmet. Toxicol.* 9, 219 (1971).
Schall, E. D., Hatcher, D. W., *J. Ass. Offic. Anal. Chem.* 51, 763 (1968).
Sinios, A., Wodsak, W., *Deut. Med. Wochenschr.* 90, 1856 (1965).
Stender, J. H., *Fed. Regist.* 38, 10929 (1973).
Wolff, I. A., Wasserman, A. E., *Science* 177, 15 (1972).