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Potential Nitrosamine Formation and its Prevention During Biological Denitrification of Red Beet Juice

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Abstract—High nitrate intake has been shown to result in an increased risk of endogenous formation of *N*-nitroso compounds. Certain vegetables and vegetable juices contain high concentrations of nitrate. Biological denitrification using strains of *Paracoccus denitrificans* (P.d.) has been proposed as effective means to reduce nitrate contents in such vegetable juices. During this bacterial denitrification process, substantial nitrite concentrations are transiently formed. This study investigated whether *N*-nitrosation reactions might occur. The easily nitrosatable amine morpholine was added to red beet juice at high concentration (100 ppm) during denitrification 10 different batches of red beet juice served as raw material. Each batch was submitted to denitrification in the presence and absence of ascorbic acid. In the absence of ascorbic acid, formation of *N*-nitrosomorpholine (NMOR) was observed in the low ppb range (0.5–8 ppb). Addition of ascorbic acid (500 mg/litre) inhibited the formation of NMOR, except for those instances where the pH was less than 6 and/or nitrate turnover was low (< 200 mg NO_3^-/litre/hr). Under conditions leading to high rates of nitrate turnover (> 200 mg NO_3^-/litre/hr), nitrosamine formation can reliably be prevented by ascorbic acid. The results show that bacterial denitrification of red beet juice high in nitrate can be accomplished without the risk of nitrosamine formation. © 1997 *Elsevier Science Ltd*

Abbreviations: ADI = acceptable daily intake; MOR = morpholine; NMOR = *N*-nitrosomorpholine; P.d. = *Paracoccus denitrificans*; SCF = Scientific Committee for Food.

INTRODUCTION

Vegetable juices are an important source of essential nutrients for humans. However, in contrast to juices from fruits, vegetable juices often contain considerable amounts of nitrate. With an average diet, about 70–80% of the total nitrate intake of humans can be attributed to vegetables and vegetable products (Hammes and Gierschner, 1990). The Joint Expert Committee on Food Additives of the World Health Organization established an acceptable daily intake (ADI) of 5 mg sodium nitrate/kg body weight (WHO, 1962 and 1976). In 1990 the Scientific Committee for Food (SCF) of the European Commission allocated a provisional ADI for nitrate (SCF, 1992), corresponding to an intake of 255 mg nitrate ion for a 70-kg adult. The consumption of a glass of vegetable juice (150 ml) containing 2000 ppm nitrate thus would already result in an intake greater than this ADI value. The significance of nitrate to human health derives primarily from the fact that ingested nitrate is reduced to nitrite in the oral cavity at an average of about 5% (Spiegelhalder *et al.*, 1976). Basal nitrite levels increase rapidly after ingestion of nitrate-rich vegetables or vegetable juice. Nitrate ingestion and reduction to nitrite are major determinants for intragastric formation of *N*-nitroso compounds, which occurs by chemical reaction of nitrite with an amine or amide-type precursor (reviewed in Eisenbrand, 1990) and also has been found to be catalysed by bacteria (Leach *et al.*, 1987; Suzuki and Mitsuoka, 1984). Endogenous formation of *N*-nitroso compounds in the human stomach has been demonstrated by the '*N*-nitrosoproline-test' (Oshima and Bartsch, 1981).

To minimize any potential health risk while maintaining the high nutritional value of vegetables and vegetable products, it is desirable to lower the nitrate load. This aim can be achieved only to a limited extent by special cultivation. As nitrate is

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soluble in water, almost all of it is transferred into the juice. Processes for lowering the nitrate content of juices from vegetables have been described. By using polystyrene-based ion-exchange resins it is possible to remove nitrate to a great extent (Hitze, 1987). However, partial loss of colour and the delivery of other ions from the resin into the juice render this process unsuitable for the denitrification of vegetable juices.

Elimination of nitrate from vegetable juices, using bacterial strains of *Paracoccus denitrificans* (P.d.) has already been described in several publications (Hammes and Gierschner, 1990; Haug *et al.*, 1991; Kerner and Schubert, 1991; Knorr *et al.*, 1992; Mayer-Miebach and Schubert, 1991; Stetter, 1992). Basically, the vegetable juice is inoculated with the bacteria under sterile conditions at 25–35°C. The bacteria reduce nitrate to nitrogen in the course of an assimilatory nitrate reduction (Mayer-Miebach and Schubert, 1991). By this approach, juices can be obtained that contain less than 50 mg/litre of nitrate without significantly altering other components or flavour properties (Stetter, 1992). The bacteria can be added to the juice as free cells or immobilized in calcium alginate or carragenane and in membrane reactors (Kerner and Schubert, 1991). For drinking water this process is used already on a pilot scale. In addition to *Paracoccus*, other genera such as *Pseudomonas*, *Bacillus*, *Halomonas* and *Lactobacillus* have been used (Hammes and Gierschner, 1990; Stetter, 1992).

The denitrification by P.d. is a multistep anaerobic process involving several potential intermediates, including nitrite. With regard to possible intermediates of toxicological relevance, a patent reports that red beet juice obtained by this process did not contain aflatoxins, bacterial inhibitory substances, nitrosamines or nitrite (Stetter, 1992). Other toxicological studies relating to this process have so far not been reported.

During bacterial denitrification of juices of carrot, celery and red beet (native pH $\leqslant$ 6) nitrite was found to accumulate for several hours. With red beet juice of high nitrate content ($>$ 2000 ppm), an increase in pH values up to pH 7.5 was also observed during denitrification. However, after nitrate concentrations of less than 50 mg/litre had been reached, nitrite was undetectable in all cases. These results are in accordance with the findings of Knorr *et al.* (1992). The intermediate accumulation of nitrite is believed to result from higher turnover rates of nitrate reductase at pH 6, compared with the other enzymes of the denitrifying system. The optimum value for overall denitrification to the end-product nitrogen is pH 7–8 (Mayer-Miebach and Schubert, 1991). As this intermediate accumulation of nitrite during bacterial denitrification cannot be excluded, nitrosation of amine precursors (amines, amide-type compounds) might occur, resulting in formation of potentially carcinogenic *N*-nitroso compounds (Calmels *et al.*, 1987 and 1988). Vegetable juices are complex mixtures of ingredients in which the occurrence of nitrosatable structures can hardly be excluded.

To study this question, the easily nitrosatable precursor morpholine (MOR) was added in high concentration (100 ppm) to red beet juice as a nitrosation marker. After biological denitrification, *N*-nitrosomorpholine (NMOR) was analysed.

10 batches of red beet juice from different raw materials were included in the study. In parallel, juice from the same raw materials was denitrified without and with addition of the nitrosation inhibitor ascorbic acid. From the results, measures appropriate for the prevention of *N*-nitrosation during denitrification of vegetable juices can be inferred.

MATERIALS AND METHODS

Raw materials, juicing and treatment of juice

Red beets obtained directly from farms during one harvest period were used as raw material. Nine batches were of the variety 'Rote Kugel', commonly used for juice production in Germany, one batch belonged to the variety 'Formanora'. Four batches had been cultivated under so-called controlled biological conditions, the others were obtained by conventional cultivation. The juice from five batches was obtained on a pilot scale after washing and milling of the roots using a baling press (Bucher, Switzerland). The amount of juice obtained from each batch was 40–50 litres. The juice from the remaining five batches was prepared on a production scale through use of a horizontal basket press (Bucher, Switzerland), about 3000 litres juice being obtained from 4500 kg red beets.

Immediately after production the juice was heated for at least 60 sec (120–125°C) in a tubular heater. After cooling, it was stored in sterile plastic containers (No. 55310; Sieger plastic, Germany) at 4°C until fermentation. Analytical data of the juice batches prior to denitrification are given in Table 1.

Cultivation of P.d.

P.d. biomass was maintained anaerobically sterile on nutrient broth 2 (25 g/litre; Oxoid, Germany), containing 2000 mg/litre potassium nitrate. Prior to inoculation of the juice, the broth was separated from the biomass by centrifugation. The biomass was washed twice in physiological sodium chloride solution and finally with sterile water. The wet biomass (10–15 g, viable cells 10^{11}/ml) was then suspended under sterile conditions in 500 ml red beet juice.

Denitrification

Denitrification was performed in a 10-litre pilot fermenter (LF 14, Chemap, Switzerland) under sterile conditions. To start up, the red beet juice containing the biomass in suspension was pumped aseptically

into a fermenter that had been flushed for 5 min with nitrogen. The volume was aseptically made up to 10 litres with red beet juice. This suspension was stirred during fermentation (100 rpm, 28°C) under monitoring of pH, nitrate and nitrite and checking for sterility. As a result of differences in the initial nitrate content and nitrate turnover rate of the biomass, fermentation times varied from 6 to 12 hr.

To test for NMOR formation, sterile samples (2 × 250 ml) were taken and nitrite values (ranging from 50 to 310 ppm) determined. MOR (100 ppm) was then added, and samples were kept sterile (28°C) with monitoring of pH, nitrate and nitrite. After 12 hr, the incubation was stopped by adjusting to pH 12 with sodium hydroxide. In control experiments, authentic juices without addition of MOR were analysed for NMOR before and after denitrification. Juices fortified with MOR (100 ppm) and nitrite (150 ppm) and incubated at different pH values served as positive nitrosation controls.

Each 10-litre fermentation was run also in the presence of sterile ascorbic acid solution (ascorbic acid concentration in juice 500 mg/litre). One sample (no. 2) inadvertently had only 5 mg/litre added. All fermentations with ascorbic acid (except for no. 4) were started by leaving 2 litres of denitrified red beet juice as a preculture in the fermenter and adding fresh red beet juice from the same batch; this ensured suitable starting pH values and better adaptation of the bacteria to the red beet juice. MOR was added as described. For batch no. 4 the procedure was reversed: fermentation in the presence of ascorbic acid was performed without preculture; fermentation without ascorbic acid was performed with preculture.

At the beginning of fermentation viable cell counts ranged from 10^8 to $10^{8.5}$/ml. During fermentation, cell counts increased to a maximum of $10^{9.5}$/ml. Average nitrate reduction rates, expressed as mg NO_3^-/litre/hr, were calculated from NO_3^- values and the time interval needed for denitrification.

Analytical determinations

Nitrate and nitrite were quantified by HPLC (Gromes *et al.*, 1991), or analysed by test strips (Merckoquant no. 10020 and 10007). The detection limit for nitrate and nitrite was 3 mg/litre in each case.

MOR was estimated by conversion into a fluorescent DANSYL derivative and subsequent thin-layer chromatography, using MOR standard solutions for semiquantitative fluorescence assay (Askar *et al.*, 1972).

To determine NMOR, juices were adjusted to pH 12 immediately after sampling and stored at − 20°C prior to analysis. According to a standard procedure (Eisenbrand *et al.*, 1983), an aliquot (20 ml) was placed on an Extrelut column for solid phase extraction. After equilibration, NMOR was extracted into methylene chloride. The extract was concentrated to 1 ml and submitted to gas chromatographic/thermal energy analysis (column: Carbowax 20 M terephthalic acid 10%). Under these conditions, detection and determination limits for NMOR were 0.5 and 1 μg/litre, respectively.

RESULTS

In control experiments with original juice NMOR was not detected before or after denitrification (n = 3). Even when nitrite (150 ppm) was added and the pH was adjusted to 3 (the optimum for chemical nitrosation), NMOR formation was not observed (12 hr incubation at 28°C). This suggests that the precursor MOR is not present in sufficient amounts in original juice to generate NMOR in detectable concentrations.

If MOR (100 ppm) and nitrite (150 ppm) were added to the juice and the pH was adjusted to 3, substantial NMOR formation (290 and 390 ppb) was observed. If incubation was performed at pH 5.3, the

Table 1. Nitrate, pH and extract of red beet juices prior to fermentation, weight of ascorbic acid added, and residual nitrate

Batch no.	Nitrate (mg/litre)	pH value	Extract (g/100 g)	Ascorbic acid dose (mg/litre)	Residual nitrate (mg/litre)
1	1970	6.2	14.5	-	< 10
				500	230
2	4700	6	7.5	-	< 10
				5	< 10
3	2300	6.1	14	-	< 10
				500	120
4	2800	6.3	11.5	-	30
				500	20
5	2920	6.3	11	-	30
				500	< 10
6	1230	5.7	11.5	-	< 10
				500	< 10
7	1300	5.8	12	-	< 10
				500	< 10
8	1490	5.7	11	-	< 10
				500	< 10
9	1750	5.7	10.5	-	10
				500	< 10
10	1750	5.7	11	-	< 10
				500	< 10

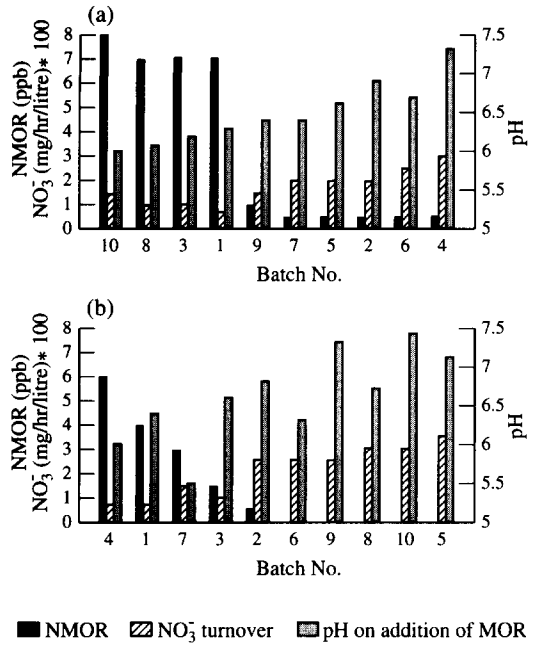

Fig. 1. NMOR, pH and nitrate turnover (a) without and (b) with addition of ascorbic acid (samples listed according to NMOR concentration).

lowest pH observed by us in red beet juice, NMOR formation (45 ppb) was drastically reduced.

MOR was not found to be degraded during fermentation and NMOR also remained stable during an incubation period of 12 hr.

After denitrification of red beet juice, residual nitrate concentrations were between 10 and 50 mg/litre in four trials (see Table 1). In two further trials, 120 and 230 mg/litre were found. In the latter two cases the fermentations had obviously been stopped too early. In all other trials nitrate was below 10 mg/litre. Residual nitrite was not detected in any of the denitrified samples.

In the absence of ascorbic acid, formation of NMOR in the lower ppb range was observed (Fig. 1a). The results suggest that the rate of MOR nitrosation depends on nitrate turnover rate of the biomass and on the pH on addition of MOR.

Thus, at high nitrate turnover (> 200 mg NO_3^-/litre/hr) and pH $\geqslant$ 6.3, NMOR was formed in only trace amounts (< 1 ppb).

In the presence of ascorbic acid (Fig. 1b) NMOR formation was effectively prevented in five of the 10 trials. These fermentations are characterized by high nitrate turnover ($\geqslant$ 200 mg NO_3^-/litre/hr) and rather high pH values (> 6.3). In contrast, all positive samples showed low nitrate turnover (< 200 mg NO_3^-/litre/hr) and/or low pH (< 6.3). Obviously, under the latter conditions ascorbic acid could not fully prevent NMOR formation. If ascorbic acid was added only in small amounts (5 mg/litre, batch no. 2) NMOR was formed despite high nitrate turnover and pH > 6.

DISCUSSION

The above model experiments were designed to include 'worst case' conditions for the risk of *N*-nitrosation. The native pH value of red beet juice does not favour NMOR formation. The nitrosation marker MOR, which is not present in red beet juice, is very easily nitrosated and was added at high concentration.

Obviously, pH and intermediate nitrite concentrations as a consequence of the turnover rate are of major influence. At pH values of more than 6.3 and at high nitrate turnover rates (> 200 mg NO_3^-/litre/hr), NMOR formation is reliably inhibited by addition of ascorbic acid (Fig. 1b). In four batches, NMOR formation (> 1 ppb) was observed as a result of low nitrate turnover rates (< 200 mg NO_3^-/litre/hr) and low pH values (< 6.3). If such conditions cannot be reliably avoided, ascorbic acid cannot fully inhibit the nitrosation.

As a consequence of these results, we conclude that the denitrification of red beet juice by P.d. has to be carried out at high nitrate turnover rates. This condition can be obtained by high initial amounts of biomass (> 10^8 viable cells/ml) and by using a preculture technique to reach higher starting pH values. In addition to high nitrate turnover rates, the presence of sufficient ascorbic acid is necessary to avoid NMOR formation. According to our results red beet juice naturally contains less than 10 mg ascorbic acid/litre.

The low nitrate turnover rates of batches 1, 3 and 4 (Fig. 1b) resulted from low initial biomass (only 1 g/litre for batches 1 and 3 and no preculture inoculation for batch 4). As a consequence, some NMOR formation was observed despite the presence of ascorbic acid. NMOR formation in batch 7 (Fig. 1b) apparently is caused by an extremely low pH value not found in juices that are correctly prepared and stored. Starting pH values of less than 6 can be caused by bacterial contamination during standing times after juice extraction before heat treatment. Heat treatment, therefore, needs to be performed without delay after juicing to prevent contamination.

High nitrate turnover rates can reliably be established by the above described technical measures, ensuring favourable starting pH, good adaptation of bacterial enzymes and high cell counts. As an anaerobic system the denitrifying enzyme machinery of P.d. also is extremely sensitive to oxygen. Absence of oxygen therefore is important, because oxygen causes a loss of activity (Carr and Ferguson, 1990; Snyder *et al.*, 1987).

Kinetics of the reaction between nitrite and related nitrosating agents and ascorbic acid have been investigated in great detail (Kim *et al.*, 1982; Licht *et al.*, 1988; Mirvish, 1981; Mirvish *et al.*, 1972). It has been reported that the initial ratio of ascorbic acid/nitrite concentrations required to prevent nitrosation for 30 min is 2.0 and 1.0, respectively (Archer

et al., 1975; Fan and Tannenbaum, 1973). As the stoichiometric ratio in an anaerobic system would be only 0.5, this discrepancy reflects differences in the oxidative recycle of NO produced from the reaction. Moreover, since nitrite concentrations during incubation (6–12 hr) can rise to substantial values, the relatively high concentration of 500 mg/litre ascorbic acid was deemed necessary to ensure prevention of nitroso compound formation.

To summarize, our results confirm that nitrate in red beet juice can be successfully eliminated by anaerobic denitrification with P.d. During fermentation, conditions of high nitrate turnover and pH above 6.3 need to be maintained and sufficient ascorbic acid has to be present in order to preclude *N*-nitrosation reactions.

Whether red beet juice contains amine precursors that can form toxicologically relevant *N*-nitroso compounds is not known at present. The results of this 'worst case' study show, however, that under appropriate conditions nitrosamine formation during bacterial denitrification of red beet juice can be avoided reliably.

REFERENCES

Archer M. C., Tannenbaum S. R., Fan T. Y. and Weisman M. (1975) Reaction of nitrite with ascorbate and its relation to nitrosamine formation. *Journal of the National Cancer Institute* **54,** 1203–1205.

Askar A., Rubach K. and Schormüller J. (1972) Dünnschichtchromatographische Trennung der in Bananen vorkommenden Amin-Fraktion. *Chemie Mikrobiologie Technologie der Lebensmittel* **1,** 187–190.

Calmels S., Oshima H. and Bartsch H. (1988) Nitrosamine formation by denitrifying and non-denitrifying bacteria: implication of nitrite reductase and nitrate reductase in nitrosation catalysis. *Journal of General Microbiology* **134,** 221–226.

Calmels S., Oshima H., Rosenkranz H., McCoy E. and Bartsch H. (1987) Biochemical studies on the catalysis of nitrosation by bacteria. *Carcinogenesis* **8,** 1085–1088.

Carr G. J. and Ferguson S. J. (1990) Nitric oxide formed by nitrite reductase of *Paracoccus denitrificans* is sufficiently stable to inhibit cytochrome oxidase activity and is reduced by its reductase under aerobic conditions. *Biochimica et Biophysica Acta* **1017,** 57–62.

Eisenbrand G. (1990) Endogenous nitrosation—findings and problems. In *The Significance of N-Nitrosation of Drugs*. Edited by G. Eisenbrand, G. Botzler and H. v. Nicolai. pp. 47–69. Fischer Verlag, Stuttgart.

Eisenbrand G., Ellen G., Preussmann R., Schuller P. L., Spiegelhalder B., Stephany R. W. and Webb K. S. (1983) Determination of volatile nitrosamines in food, animal feed and other biological materials by low-temperature vacuum distillation and chemiluminescence detection. In *Environmental Carcinogens; Selected Methods of Analysis, Vol. 6. N-Nitroso Compounds*. Edited by H. Egan, R. Preussmann, I. K. O'Neill, G. Eisenbrand, B. Spiegelhalder and H. Bartsch. pp. 181–203. IARC Scientific Publications No. 45. International Agency for Research on Cancer, Lyon.

Fan T. Y. and Tannenbaum S. R. (1973) Natural inhibitors of nitrosation reactions: the concept of available nitrite. *Journal of Food Science* **38,** 1067–1069.

Gromes R., Glaser D., Sattig B. and Schnellbacher B. (1991) Bestimmung des Nitratgehaltes in Lebensmitteln mit HPLC und einem neuen enzymatischen Test. *Deutsche Lebensmittelrundschau* **87,** 346–349.

Hammes W. P. and Gierschner K. (1990) Mikrobiologische Nitrat-Entfernung aus Gemüsesäften bzw. Gemüse-Flüssigprodukten. *Internationale Fruchtsaft-Union* **21,** 287–308.

Haug M., Dirks U. and Vorlop K. (1991) Denitrification of vegetable juices by immobilized cells. In *Engineering and Food, Vol. 3*. Edited by W. E. L. Spiess and H. Schubert. pp. 536–543. Elsevier, London.

Hitze W. (1987) Verfahren zur selektiven Entfernung bzw. Reduzierung von Nitrationen aus Gemüsehomogenaten. *German Patent Application 1987 DE 3728372.*

Hoglen J. and Hollocher T. (1989) Purification and some characteristics of nitric oxide reductase containing vesicles from *Paracoccus denitrificans*. *Journal of Biological Chemistry* **13,** 7556–7563.

Hotchkiss J. H. and Cassens R. G. (1987) Nitrate, nitrite, and nitroso compounds in foods. *Food Technology* **6,** 127–133.

Izumi K. (1992) Reaction of nitrite with ascorbic acid or ascorbic acid-2-derivatives. *Journal of Food Science* **57,** 1066–1067.

Kerner M. and Schubert H. (1991) Mikrobieller Nitrat-Abbau in einem Membranreaktor. *Chemie Ingenieur Technik* **63,** 361–368.

Kim Y. K., Tannenbaum S. R. and Wishnok J. S. (1982) Effect of ascorbic acid on the nitrosation of dialkyl amines. In *Ascorbic Acid: Chemistry, Metabolism, and Uses*. Edited by P. A. Seib and B. M. Tolbert. pp. 571–585. Advances in Chemistry Series 200, American Chemical Society, Washington, DC.

Knorr D., Riebe K. and Dunktel C. (1992) Mikrobieller Nitratabbau mit Paracoccus Denitrificans. *Lebensmittelteltechnik* **12,** 44–51.

Leach S. A., Thompson M. and Hill M. (1987) Bacterially catalysed *N*-nitrosation reactions and their relative importance in the human stomach. *Carcinogenesis* **8,** 1907–1912.

Licht W. R., Tannenbaum S. R. and Deen W. M. (1988) Use of ascorbic acid to inhibit nitrosation: kinetic and mass transfer considerations for an *in vitro* system. *Carcinogenesis* **9,** 365–372.

Mayer-Miebach E. and Schubert H. (1991) Biokatalytische Verringerung der Nitratkonzentration in pflanzlichen Lebensmittelrohstoffen. *BioEngineering* **2,** 34–43.

Mirvish S. S. (1981) Inhibition of the formation of carcinogenic N-Nitroso compounds by ascorbic acid and other compounds. In *Cancer Achievements, Challenges and Prospects for the 1980s*. Vol. 1. Edited by J. H. Burchenal and H. F. Oettgen. pp. 557–587. Grune and Stratton, New York.

Mirvish S. S., Wallcave L., Eagen M. and Shubik P. (1972) Ascorbate-nitrite reaction: possible means of blocking the formation of carcinogenic *N*-nitroso compounds. *Science* **177,** 65–67.

Oshima H. and Bartsch H. (1981) Quantitative estimation of endogenous nitrosation in humans by monitoring *N*-nitrosoproline excreted in the urine. *Cancer Research* **41,** 3658–3662.

SCF (1992) *Report of the Scientific Committee for Food on Nitrate and Nitrite, Opinion expressed on 19th October*, 1990, *26th Series*. Luxembourg, Commission of the European Community Scientific Committee for Food.

Snyder S. W., Bazylinki D. A. and Hollocher T. C. (1987) Loss of N_2O reductase activity as an explanation for poor growth of *Pseudomonas aeruginosa* on N_2O. *Applied and Environmental Microbiology* **53,** 2045–2049.

Snyder S. W. and Hollocher T. C. (1986) Purification and some characteristics of nitrous oxide reductase from *paracoccus denitrificans*. *Journal of Biological Chemistry* **262,** 6515–6525.

Spiegelhalder B., Eisenbrand G. and Preussmann R. (1976) Influence of dietary nitrate on nitrite content of human saliva: possible relevance to *in vivo* formation of *N*-nitroso compounds. *Food and Cosmetics Toxicology* **14,** 545–548.

Stetter K. (1992) Verfahren zur Verminderung des Nitratgehaltes von pflanzlichen Lebensmitteln. *European Patent* 0 244 663.

Suzuki K. and Mitsuoka T. (1984) Nitrosation by intestinal bacteria. In *Relevance of N-Nitroso Compounds to Human Cancer: Exposures and Mechanisms*. Edited by H. Bartsch, O. K. O'Neill and R. Schulte-Hermann. pp. 292–296. IARC Scientific Publications No. 84. International Agency for Research on Cancer, Lyon.

WHO (1962) Evaluation of the toxicity of a number of antimicrobials and antioxidants. *Sixth report of the Joint FAO/WHO Expert Committee on Food Additives, WHO Technical Report Series* **228**, 69–75.

WHO (1976) Toxicological evaluation of some food additives including anticking agents, antimicrobials, antibiotics, emulsifiers and thickening agents. *WHO Food Additives Series* **5**, 92–106.