

The formation of volatile N-nitrosamines in grass and maize silages

L. W. van Broekhoven and J. A. R. Davies

Centre for Agrobiological Research (CABO), P.O. Box 14, Wageningen, the Netherlands

Accepted: 4 September 1980

Key words: N-nitrosamines, silages, grass, maize

Summary

Under normal conditions no high levels of volatile N-nitrosamines could be detected in grass and maize silages. Only under special conditions about 5 μg N-nitrosodimethylamine/kg fresh was found in maize silages. In addition, four other peaks were detected. All tests indicate that these compounds are nitrosamines.

Introduction

N-nitrosamines, well-known carcinogenic substances (Fishbein, 1979), have been found in many common materials. The levels normally found in various foods and animal feeds are usually in the range of 1-10 $\mu\text{g}/\text{kg}$. They are readily formed by the reaction between secondary amines and nitrites and many factors affect this formation under several conditions.

Because of the presence of nitrate in silages, and its conversion via nitrite to ammonia the question arose whether N-nitrosamines are actually formed in these silages.

Tate & Alexander (1975) investigated the presence of N-nitrosamines in maize silages produced on laboratory scale, but were unable to detect any. Terplan et al. (1978) found N-nitrosodiethylamine and N-nitrosodipropylamine in silages.

In our first experiments (van Broekhoven, 1980) we studied the formation of N-nitrosamines in silages made on laboratory scale with grasses having different nitrate contents and pre-wilted to different dry matter contents.

It was demonstrated that no volatile N-nitrosamines were formed in silages with dry matter contents of 500 and 600 g/kg, but N-nitrosodimethylamine could be detected at levels ranging from 0-5 $\mu\text{g}/\text{kg}$ when the dry matter content was 300 g/kg. The highest nitrosamine concentrations were found in silages in which a greater proportion of the nitrate was reduced. In silages with a relatively small decrease in ni-

trate (less than 2 g/kg DM) less than 1 μg N-nitrosodimethylamine/kg was formed.

Our aim was to study whether the results found in these laboratory-scale experiments were transferable to a field scale. Therefore, comparable small field-scale grass silages were sampled and analysed. In addition, some silages from maize grown on heavily manured land were analysed.

Materials and methods

The grass silages came from experiments on small field scale. The grass silages were produced from grass with an initial nitrate content ranging from 6 to 13 g/kg DM. The grass was pre-wilted to a certain extent before ensiling.

The maize silages came from local farms.

The samples were stored in plastic bags at $-20\text{ }^{\circ}\text{C}$ for analysis. Chemicals of analytical grade from several suppliers were used without further purification. Dichloromethane (Baker) was distilled before use.

Nitrate was determined with an ion-specific nitrate electrode (Orion 93-07), a double junction reference electrode (Orion 90-02) and a pH meter (Orion 801A).

For the determination of the volatile N-nitrosamines a Thermal Energy Analyser (TEA, model 502LC, Thermo Electron Corp.) was used interfaced to a gas chromatograph (Packard Becker 427). It had the following characteristics: GC: column of stainless steel (length 3 m, 3.2 mm o.d.) packed with 10 % FFAP on Chromosorb WHP (80/100 mesh); oven: temperature-programmed, initially at $140\text{ }^{\circ}\text{C}$ (4 min), $5\text{ }^{\circ}\text{C}$ rise/min, finally at $200\text{ }^{\circ}\text{C}$ (10 min); carrier gas argon; inlet pressure 0.3 MPa; TEA: furnace temperature $450\text{ }^{\circ}\text{C}$; reaction chamber pressure 133 Pa, cold trap $-160\text{ }^{\circ}\text{C}$.

For confirmation studies the TEA was also coupled to a high-performance liquid chromatograph (HPLC) constructed from a high-pressure pump (Waters, model 6000A), an injector (Waters, model U6K) and a column ($\mu\text{Porasil}$, length 30 cm, 3.9 mm i.d., Waters). Mixtures of hexane and acetone were used (normally 95/5) and the system was operated isocratically. The flow was 1 ml/min. TEA: furnace temperature $550\text{ }^{\circ}\text{C}$; reaction chamber pressure 133 Pa, cold traps -80 and $-100\text{ }^{\circ}\text{C}$.

The working-up procedure was performed as described previously (van Broekhoven, 1980).

For the confirmation studies the methods described by Krull et al. (1979) were used. After the various treatments the mixtures were measured with GC/TEA and HPLC/TEA. To prevent damage to the GC column the incubations with acetic acid/HBr and acetic acid were diluted with water, poured on a Preptube cartridge (Thermo Electron Corp.) and extracted with dichloromethane. In this way the greater part of the acid was removed.

Results and discussion

Table 1 gives the results of the analysis of the grass silages for NO_3 , N_t , soluble carbohydrates and N-nitrosodimethylamine (NDMA). Just as in the previous experi-

Table 1. Analysis of grass silages with respect to DM, nitrate before and after ensiling, N_t, soluble carbohydrates and NDMA content.

DM (g/kg fresh)	NO ₃ before ensiling (g/kg DM)	NO ₃ after ensiling (g/kg DM)	N _t (g/kg DM)	Soluble carbohydrates (g/kg DM)	NDMA* (µg/kg fresh)
330	13.5	1.1	28.9	7.3	0.78
480	13.5	7.3	35.5	23.3	0.83
560	13.5	13.5	33.5	47.7	0.53
420	10.7	1.9	30.9	7.4	0.23
490	10.7	4.8	32.7	20.6	0.10
400	6.2	1.4	24.2	7.0	0.13
420	6.2	1.2	25.7	10.7	0.01
480	12.6	9.7	34.9	80.0	0.10
480	12.6	13.5	36.6	69.4	0.15
600	12.6	14.8	37.0	76.9	0.07
350	11.6	9.9	33.2	6.2	0.21
290	11.6	7.7	32.0	9.2	0.69
550	9.3	7.3	36.0	74.6	0.17
600	9.3	8.5	33.9	73.9	0.03

* NDMA = N-nitrosodimethylamine. Corrected for recovery (70 %). Detection limit 0.01 µg/kg. The values <0.2 µg/kg are difficult to interpret because of possible artifact formation.

ments on laboratory scale (van Broekhoven, 1980), in almost every sample a small amount of NDMA was found. In most cases no other N-nitrosamine could be detected. Mostly only very low levels of NDMA were found (less than 0.5 µg and never more than 1 µg/kg fresh). These results agree very well with those obtained earlier. From these results it could be concluded that under normal conditions (decrease in NO₃ not more than 10 g/kg DM and a DM content above 300 g/kg) no high levels of NDMA will be formed (< 1 µg/kg fresh).

Van Dijk & Willemsen (1975) investigated the nitrate contents of fresh and ensiled maize grown on fields with heavy dressings of organic manure. They found an ave-

Table 2. Analysis of maize silages with respect to DM, nitrate, N_t, soluble carbohydrates and NDMA content.

DM (g/kg fresh)	N _t (g/kg DM)	NO ₃ (g/kg DM)	Soluble carbohydrates (g/kg DM)	NDMA* (µg/kg fresh)
249	15.8	5.8	8.17	ND
224	15.6	3.9	6.16	ND
286	16.1	3.0	4.68	ND
257	15.8	2.4	5.93	ND
240	16.0	5.0	6.15	ND
279	14.1	2.8	5.61	ND
224	14.5	4.2	4.42	ND
258	14.9	3.6	7.30	ND

* ND = not detected, detection limit 0.01 µg/kg.

rage of 6.4 g NO₃/kg in the ensiled maize and only a loss of some 10 % only of the originally present nitrate. A lower DM content could increase the breakdown of nitrate. We analysed maize silages comparable with those described by van Dijk & Willemsen. The results are presented in Table 2. No volatile N-nitrosamines could be detected here.

Some of the maize silages showed an orange coloured edge. The reason for this colouration is unknown. Therefore, this edge was also sampled and analysed. In this sample 5 µg NDMA/kg was detected. In addition four unknown peaks were observed in the GC/TEA chromatograms. They are shown in Fig. 1, together with the place of seven known nitrosamines. The peak near N-nitrosodibutylamine coincides with the peak that was sometimes observed in grass silages both with GC and HPLC. In order to confirm the nature of the unknown peaks the method as presented by Krull et al. (1979) was used. During GC/TEA analysis always a cold trap of -160 °C was used, so that most of the organics were frozen out. The TEA response remained unchanged after placing a Tenax-GC column (length 10 cm, 6 mm o.d.) between the cold trap and the reaction chamber. After treatment with hydrobromic acid in glacial acetic acid the four unknown peaks disappeared completely. The TEA-response remained unchanged after treatment of sample with glacial acetic acid only. The four peaks disappeared after irradiation with UV light. The results indicate with reasonable certainty that the unknown compounds have N-nitroso structures. Efforts will be made to isolate and purify the compounds further in order to enable an identification with mass spectrometry. The peak heights suggest

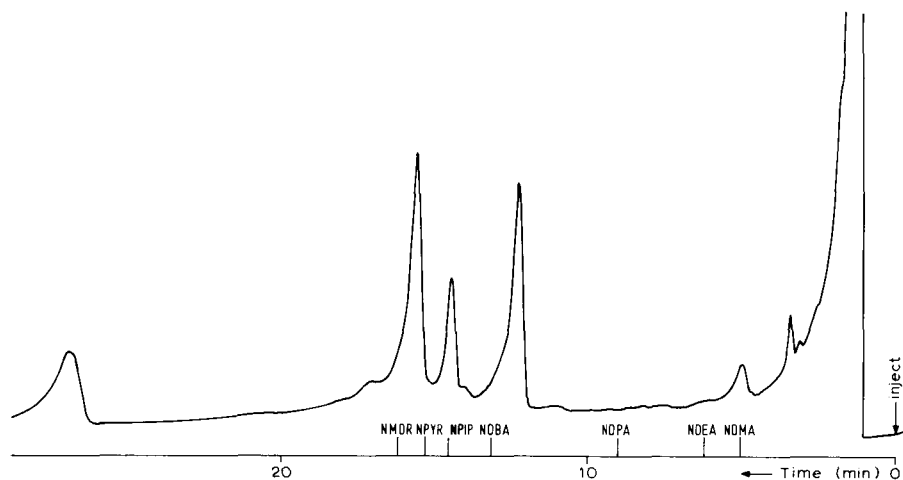


Fig. 1. GC/TEA chromatogram of sample extract of orange coloured maize silage. Injection: 2 µl Att = 16. For GC/TEA conditions see text. The places of the following volatile N-nitrosamines are indicated N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodi-n-propylamine (NDPA), N-nitrosodi-n-butylamine (NDBA), N-nitrosopiperidine (NPIP), N-nitrosopyrrolidine (NPYR) and N-nitrosomorpholine (NMOR).

that the concentrations of the unknown compounds must be several tens of $\mu\text{g}/\text{kg}$ fresh.

Further investigations are needed to explain and evaluate the occurrence of these compounds in the coloured edge of maize silages. The general conclusion of this study is that only minute amounts of NDMA (less than $1 \mu\text{g}/\text{kg}$ fresh) were found in the grass silages and no detectable amounts in the maize silages. It may be assumed that no N-nitrosamines in detectable amounts from this food after ingestion by a cow are transferred to the milk or to the blood.

Acknowledgement

Thanks are due to Mr Schukking and Mr Hengeveld for providing the silage samples.

References

- Broekhoven, L. W. van, 1980. Formation of volatile N-nitrosamines during the fermentation of grass silages. In: W. H. Prins & G. H. Arnold (Ed.), *The role of nitrogen in intensive grassland production*, pp. 113-117. Pudoc, Wageningen.
- Fishbein, L., 1979. Overview of some aspects of occurrence, formation and analysis of nitrosamines. *Science Total Envir.* 13: 157-188.
- Krull, I. S., E. U. Goff, G. G. Hoffman & D. H. Fine, 1979. Confirmatory methods for the thermal energy determination of N-nitroso compounds at trace levels. *Analyt. Chem.* 51: 1706-1709.
- Tate III, R. L. & M. Alexander, 1975. N-nitrosamines: absence from sauerkraut and silage. *J. Agric. Food Chem.* 23: 896-897.
- Terplan, G., E. Hallermayer, W. Kalbfuss, P. Unsinn, C. D. Gärtner & C. Heerdegen, 1978. Untersuchungen zum Vorkommen von Nitrosaminen in Futtermitteln, Milch und Milchprodukten. *Milchwissenschaft* 33: 142-145.