Netherlands Journal of Agricultural Science 33 (1985) 17–22

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

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

Centre for Agrobiological Research (CABO) P.O. Box 14, 6700 AA Wageningen, Netherlands

Received 31 October 1984; accepted 17 January 1985

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

Summary

Fresh grass and maize samples from experimental fields with different regimes of fertilization were ensiled in the laboratory in glass preserving jars. The resulting silages were analysed for the presence of volatile N-nitrosamines with a Thermal Energy Analyzer. In the grass silages up to 4 μg N-nitrosodimethylamine (NDMA)/kg was found. In the maize silages no NDMA was found above the limit of detection (0.2 μg/kg).

Introduction

The possible presence of volatile N-nitrosamines — well-known mutagenic and carcinogenic substances — in silages has been subject of several studies. We reported on the formation of volatile N-nitrosamines in grass silages (van Broekhoven, 1980). Silages prepared from pre-wilted grass with different nitrate contents were analysed. No volatile N-nitrosamines were detected in silages with dry matter contents ranging from 500 to 600 g/kg. Silages with a dry matter content of about 300 g/kg contained N-nitrosodimethylamine (NDMA) in levels ranging from 0 to 5 μg/kg fresh material. Van Broekhoven & Davies (1982) investigated the first ten days of the ensilage of nitrate-containing grass, which was pre-wilted to a dry matter content of about 300 g/kg. The formation of nitrite was accompanied by the formation of NDMA and N-nitrosodiethylamine (NDEA) in concentrations up to about 2 μg/kg fresh material. After stabilization of the silage the concentrations of NDMA and NDEA dropped to about 0.6 μg/kg fresh material. In grass silages from experiments on a small field scale and in maize silages from local farms no NDMA or only low levels (< 1 μg/kg) were found (van Broekhoven & Davies, 1980).

Möhler & El-Refai (1981) were able to detect volatile N-nitrosamines in 60 % of 180 samples of maize silages. In general they found concentrations of about 10 μg/kg. Sometimes, however, they found more than 100 μg/kg. Dam Koffoed et al. (1981) found only traces of NDMA and N-nitrosopiperidine (less than 0.6 μg/kg) in grass silages.

Terplan et al. (1978) reported the presence of NDEA (3–24 μg/kg) and N-nitroso-di-n-propylamine (3–33 μg/kg) in silages from turnip leaves, maize and grass. Until now we have only analysed silages from pre-wilted grass. In the present paper we report on the results of silages made with fresh grass from fields, which were in different ways amended with fertilizer ans slurry. Maize samples from fields amended with fertilizer and/or manure were also ensiled.

Material and methods

The grass was taken from different experimental fields. A part of the grass fields was amended with fertilizer nitrogen only, another part was treated with cattle slurry. Details of the treatment and the composition of the grass are given in Table 1. The maize was obtained from experimental fields which were dressed with varying amounts of fertilizer nitrogen and manure. Details of the treatment and composition of the maize are given in Table 2. The grass and maize samples were mixed and chopped. The fresh material was pressed tightly in glass preserving jars of 2 l. After closing them hermetically the jars were stored in the dark at 30 °C according to Wieringa (1957). All the treatments were ensiled at least in duplicate.

The material was sampled after 20 days of fermentation. The jars were opened and the contents stored in plastic bags at –20 °C for analysis.

The analysis of the volatile N-nitrosamines was performed with a specific nitrosa-

Table 1. Fertilizing treatment and composition of the grass used in the ensiling experiments.

Objects	Fertilizer (kg N/ha)	Slurry** (tonnes/ha)	Dry matter (g/kg fresh weight)	N-total (g/kg DM)	NO_3-N (g/kg DM)
1*	1000	–	136	36.0	3.65
2	100	–	159	24.3	0.48
3	0	–	229	16.5	0.05
4	150	–	210	16.8	0.02
5	300	–	184	18.9	0.03
6	450	–	151	25.3	1.02
7	600	–	163	27.0	1.87
8	1000	–	149	34.0	5.93
9	0	20i	268	21.5	0.02
10	200	20i	164	27.2	0.70
11	600	20i	198	40.8	4.78
12	0	80i	155	36.5	3.81
13	200	80i	202	34.5	4.30
14	600	80i	214	37.3	4.68
15	0	10b	234	22.6	0.26
16	200	10b	200	25.2	0.60
17	600	10b	234	38.1	4.72
18	0	40b	265	23.4	0.39
19	200	40b	239	27.6	0.33
20	600	40b	254	38.0	6.06

* Grasses from three experiments: 1–3, 5–8 and 9–20.
** Way of application: i, slurry injected in the sward; b, broadcast slurry application.

Table 2. Fertilizing treatment and composition of the maize used in the ensiling experiments.

Objects	Fertilizer (kg N/ha)	Manure (tonnes/ha)	Dry matter (g/kg fresh weight)	N-total (g/kg DM)	NO_3-N (g/kg DM)
1	0	–	255	8.9	0.05
2	150	–	291	13.0	0.09
3	250	–	260	12.1	0.18
4	0	200	268	13.4	0.11
5	150	200	261	13.7	0.61
6	250	200	279	14.7	0.34

mine analyser (Thermal Energy Analyzer 502 LC, Thermo Electron Co, Waltham, USA) as described previously (van Broekhoven & Davies, 1982). The results were corrected for recovery. Blanks were performed throughout the experiment. It is well-known that rubber products may contain volatile N-nitrosamines (Spiegelhalder & Preussmann, 1982). Although there is only a small contact area of the rubber closing rings with the content of the jars, some contamination of the silages with the volatile N-nitrosamines present in the rings is possible. The formation and release of volatile N-nitrosamines from the rings during ensilage was studied. In jars filled with a solution of nitrite at pH 4 less than 0.1 μg NDMA/l was found after incubation at 30 °C for one month.

The analysis of triplicate samples at different times revealed no significant change in the concentration of volatile N-nitrosamines during prolonged storage at -20 °C.

The other analyses were performed with standard methods (van Broekhoven, 1980).

Results and discussion

The results of the analyses of the grass silages are presented in Table 3. Because the dry matter and the total nitrogen contents of the silages were not significantly different from those of the corresponding grasses (Table 1), the results are not shown in Table 3.

In order to determine the quality of the silages (Wieringa, 1966) the pH, the butyric acid content and the ammonia fraction were measured. The silages from the high-nitrate grass show a high pH and a high ammonia fraction caused by the reduction of nitrate. These are indications for a poor quality. Almost no butyric acid was found. The values of the pH and ammonia fraction are in good agreement with the results obtained by Wieringa (1966) when he studied the influence of nitrate in grass on the quality of the silage. The butyric acid contents he found were higher than the ones in the present study. A comparison of the nitrate contents of the silages with those of the corresponding fresh grasses (Table 1) shows considerable disappearance of the original nitrate.

No correlation could be found between this nitrate reduction and N-nitrosamine

Table 3. Quality standard and contents of nitrate and dimethylnitrosamine of the grass silages.

Object	pH	Butyric acid (g/kg fresh weight)	Ammonia fraction (g N/kg total N)	NO_3-N (g/kg DM)	NDMA (μg/kg fresh weight)
1[1]	5.0(0.5)[2]	1.0(1.0)	220(40)	0.50(0.50)	1.9(1.1)[3]
2	4.1(0.1)	<0.1[4]	100(20)	0.06(0.04)	3.5(1.2)
3	4.4(0.1)	<0.1	70(10)	<0.01[4]	<0.2[4]
4	4.4(0.1)	0.2(0.2)	70(10)	<0.01	<0.2
5	4.4(0.1)	<0.1	110(10)	<0.01	<0.2
6	4.4(0.1)	<0.1	140(20)	<0.01	0.3(0.1)
7	4.7(0.2)	<0.1	190(20)	0.17(0.17)	0.7(0)
8	5.6(0.1)	<0.1	290(20)	<0.01	0.7(0.2)
9	4.6(0.1)	0.9(0.6)	90(10)	<0.01	0.2(0)
10	4.4(0)	<0.1	100(10)	<0.01	0.6(0.2)
11	5.7(0.8)	<0.1	170(20)	0.19(0.06)	2.9(0.9)
12	4.9(0)	<0.1	170(20)	0.04(0.01)	1.5(0)
13	5.0(0.1)	<0.1	180(0)	0.24(0.10)	1.4(0.4)
14	5.5(0.1)	<0.1	150(30)	1.38(1.38)	4.0(0.9)
15	4.7(0.1)	1.0(0.3)	80(10)	<0.01	1.1(1.3)
16	4.4(0.1)	<0.1	90(0)	0.05(0.01)	0.6(0.2)
17	5.2(0)	<0.1	180(0)	0.48(0.03)	1.7(0)
18	4.8(0)	1.2(0.6)	90(0)	<0.01	0.2(0.1)
19	4.7(0.1)	<0.1	110(10)	0.02(0.01)	0.7(0.4)
20	5.7(0.2)	2.1(0.2)	230(10)	0.05(0)	0.7(0.4)

[1] Objects 1–8: average of 5 samples; Objects 9–20: average of 2 samples.
[2] In parentheses: the standard deviation of the mean.
[3] The dimethylnitrosamine contents were corrected for recovery (70%).
[4] Detection limit.

formation. As was concluded earlier from experiments with silages from pre-wilted grass (van Broekhoven, 1980; van Broekhoven & Davies, 1982) the decrease in nitrate indicates only a temporary presence of nitrate and a possible formation of N-nitrosamines. The ultimate formation is influenced by many factors and therefore rather unpredictable. NDMA was the only volatile N-nitrosamine that could be detected. The amounts are comparable with those of earlier experiments and agree with the earlier conclusion, also drawn from results from practical field silages, that under normal conditions only low concentrations of volatile N-nitrosamines will be found in grass silages. The standard deviations found for nitrate and NDMA contents indicate a wide variation between the duplicates. A possible explanation for this phenomenon might be the differences in microbial activity in the different jars.

The grasses used were grown under different fertilizer regimes. The use of cattle slurry both by injection in the sward and broadcast application had no effect on the silage quality and on the formation of volatile N-nitrosamines during ensilage.

The maize silages were analysed in the same way. The results are presented in Table 4. Compared with the original maize (Table 2) the nitrate contents had degraded for the greater part. In order to prevent degradation of volatile N-nitrosamines during storage, these samples were analysed directly after collection. In none

Table 4. Quality standard and contents of nitrate and dimethylnitrosamine of the maize silages.

Object	pH	Butyric acid (g/kg fresh weight)	Ammonia fraction (g N/kg total N)	NO_3-N (g/kg DM)	NDMA (μg/kg fresh weight)
Maize					
1	3.9(0.1)*	ND	73(1)	<0.01**	<0.2**
2	3.9(0.0)	ND	73(2)	<0.01	<0.2
3	3.9(0.0)	ND	66(3)	0.01(0.01)	<0.2
4	3.9(0.0)	ND	75(3)	<0.01	<0.2
5	3.9(0.0)	ND	68(5)	0.23(0.13)	<0.2
6	3.9(0.0)	ND	65(4)	0.10(0.01)	<0.2

* The results are the mean of three samples. The standard deviation of the mean is given in parenthesis.
** < 0.01 and < 0.2: below the detection limit.
ND, not determined.

of the samples a measurable amount of volatile N-nitrosamines was found. The maize samples used for ensilage were obtained from fields which were treated with fertilizer and manure. But even in silages from fields amended with a high dressing of manure no volatile N-nitrosamines could be found. So, we did not succeed in reproducing on a laboratory scale maize silages containing concentrations of volatile nitrosamines comparable with the concentrations found be Möhler & El-Refai (1981) in practical-scale maize silages. The results were, however, in good agreement with the analyses, which were done earlier in practical-scale silages (van Broekhoven & Davies, 1980).

Although conditions during ensilage seem favourable to the formation of N-nitroso compounds, we conclude from the present study and the results obtained earlier that the formation of high concentrations of volatile N-nitrosamines in silages from grass and maize are unlikely. The concentrations found in these studies were usually not higher than 5 μg/kg and mostly lower than 1 μg/kg. From the calculations made by Juszkiewicz & Kowalski (1974) on the possible transfer of these products into the milk it can be concluded that no measurable transfer of volatile N-nitrosamines into the milk can be expected from these silages whenever they were fed to the cow.

Future research should be aimed at the presence of non-volatile N-nitroso compounds in silages. The first attempts to investigate the presence of non-volatile N-nitrosamines were made with the aid of the total N-nitroso determination. Calculated on a molecular weight of 100, about 85 mg N-nitroso compounds per kg freeze-dried sample were found (van Broekhoven & Davies, 1982). Further experiments are needed to validate these data and to optimalize the method of analysis.

Acknowledgements

Thanks are due to Mr J. H. Geurink and Ir H. G. van der Meer (Centre for Agrobiological Research) for providing the grass samples and to Dr K. Dilz (Agriculture Bureau of the Netherlands Fertilizer Industry) for the maize 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 (Eds.), The role of nitrogen in intensive grassland production, pp. 113–117. Pudoc, Wageningen.

Broekhoven, L. W. van & J. A. R. Davies, 1980. The formation of volatile N-nitrosamines in grass and maize silages. *Netherlands Journal of Agricultural Science* 28: 238–241.

Broekhoven, L. W. van & J. A. R. Davies, 1982. N-nitrosamines in grass silages. In: H. Bartsch, I. K. O'Neill, M. Castegnaro & M. Okada (Eds.), N-nitroso compounds: Occurrence and biological effects. IARC Scientific Publications No 41 IARC, Lyon, pp. 319–326.

Dam Kofoed, A., O. Nemming, K. Brunfeldt, E. Nebelin & Johs. Thomson, 1981. Investigations on the occurrence of nitrosamines in some agricultural and horticultural products. *Acta Agricultural Scandinavicae* 31: 40–48.

Juszkiewicz, T. & B. Kowalski, 1974. Passage of nitrosamines from rumen into milk in goats. In: P. Bogovksi & E. A. Walker (Eds.), N-nitroso compounds in the environment. IARC Scientific Publications No 9. IARC, Lyon, pp. 173–176.

Möhler, K. & El-Sayed Mustafa El-Refai, 1981. Das Nitrosamin Problem in der menschlichen Umwelt. Bestimmung von flüchtigen N-Nitrosaminen in Maissilage und Milch mit der Nitramin-Methode. Vergleich mit der Thermoluminescenz-Analyse. *Zeitschrift für Lebensmitteluntersuchung und Forschung* 172: 449–453.

Spiegelhalder, B., R. Preussmann, 1982. Nitrosamines and rubber. In: H. Bartsch, I. K. O'Neill, M. Castegnaro & M. Okada (Eds.), N-nitroso compounds: Occurrence and biological effects. IARC Scientific Publications No 41. IARC, Lyon, pp. 231–243.

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.

Wieringa, G. W., 1957. Verslag van enige oriënterende inkuilproeven op laboratoriumschaal. Publikatie A14, IBVL, Wageningen.

Wieringa, G. W., 1966. The influence of nitrate on silages fermentation. In: A. G. G. Hill (Ed.), Proceedings of the 10th International Grassland Crongress (Helsinki): 537–540.