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**A note on sample preparation in the analysis of nitrate and nitrite in forage**

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**Introduction**

Nitrate content in forage is of interest as it pose a risk of poisoning for ruminants at levels above 1000 mg $NO_3$-N per kg DM (Strickland et al, 1995; Undersander et al, 1999). Analysing nitrate in forages by colorimetric methods have been questioned during decades due to interference of coloured substances in the forage (Wiseman & Jacobson, 1962; Wegner, 1972; Anderson & Case, 1999). Nevertheless, the colorimetric method using flow injection analysis (FIA) with a Cu-Cd reduction column has become a standard method in analysing nitrite and nitrate after reduction to nitrite (MacKown & Weik, 2004). The problem of diverging results in nitrate analysis has often appeared as a result of different nitrate extraction techniques, as opposed to differences due to detection methods after extraction (Anderson & Case, 1999). The sample preparation and analytical method in use at the departments of Soil and Environment, Crop Production Ecology and Animal Nutrition and Management at the Swedish University of Agricultural Science has been water extraction of dried samples followed by the nitrite and nitrate analysis according to the ISO method 13395;1996. As nitrate, and in particular nitrite, are unstable compounds, doubts have been raised concerning the influence of the sample preparation step. The present study was made with the aim to compare result of nitrite and nitrate analyses of dried or un-dried samples and extraction with boiling water or room tempered water.

**Materials and Methods**

Samples of fresh mixed grass-red clover forage were treated as in Figure 1.
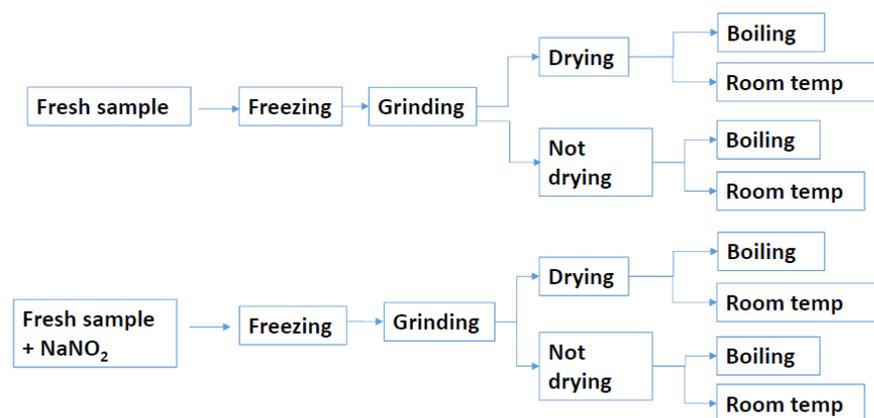


**Figure 1.** Set up of experiment to evaluate the effect of drying and extraction on the level of nitrite and nitrate content in a grass- clover forage crop.

Two kg of the fresh forage crop was wilted to 50% DM and divided in two 1 kg portions. To one portion, 3 ml Safesil® (equivalent to 30.435 mg $NO_2$-N/kg) was added in the form of an aerosol to cover the entire forage surface and mixed inside an inflated plastic bag. Both portions were frozen to -20ºC and ground frozen in a meat grinder and thoroughly blended.

These two portions, one blank and one nitrite spiked, were each of them treated as follows. One half was dried at 60ºC for 16 h and two 1-g sub-samples were mixed with 100 ml distilled water each. The other half was not dried and 2 samples, equivalent in size on DM basis to the dried samples, were mixed with 100 ml distilled water each. Extraction was performed as follows: one of each parallel water suspension was boiled for 10 minutes and one was shaken for 1 h at room temperature after which they were diluted with distilled water to 250 ml and filtered through filter paper (Whatman 602H½).

After extraction, all samples were analysed without delay according to the procedure prescribed in the ISO method 13395:1996 as follows: liquid extracts were analysed for nitrite-N by a computerized flow injection analysis (FIA) system (FIAstar™ 5000 Analyzer with the PC software 'soFIA' from FOSS, [www.foss.dk](www.foss.dk) ). The colorimetric detection was based on nitrite with sulfonilamide forming an azo dye product with N-(1-naphthyl)ethylenediamine (Griess reagent). Absorbance was determined at 540 nm. Nitrate was then reduced to nitrite in a copper-cadmium column and nitrite-N was analysed again. Nitrate was then calculated by difference. Absorbance for all samples was also determined without reagents and deducted from the gross determinations of nitrite. Finally, the nitrate- and nitrite-N were calculated to as per mg/kg DM.

Results were processed by the GLM procedure by SAS using nitrite-treatment, preparation method and extraction method as class variables (SAS ver. 9.4. SAS Institute Inc., Cary. NC. USA).

**Results and Discussion**

The fresh crop selected was very low in nitrate and nitrite. The values were all very close to the detection limit and are presented in Table 1. Nitrite treatment increased both nitrate and nitrite levels ($p<0.0028$). No difference could be seen between extraction methods ($p>0.8156$). However, preparation method made a difference ($p<0.0035$), as drying the samples resulted in considerably lower nitrate and nitrite levels compared to no drying. Sample drying seemed to reduce nitrate content with by on average 36% and the nitrite content by 90%.

**Table 1** Nitrate and nitrite in fresh crop samples with or without added $NO_2$, with or without drying and extraction in boiling or room temperate water (average ± SEM of two replicates)

| Sample | Extraction | $NO_3$-N, mg/kg DM | | $NO_2$-N, mg /kg DM | |
|---|---|---|---|---|---|
| | | Dried | Un-dried | Dried | Un-dried |
| Fresh crop | Boiled | 61 ± 1.0 | 61 ± 1.0 | 7 ± 0.5 | 10 ± 0.5 |
| Fresh crop | Room temp | 60 ± 1.0 | 59 ± 0.5 | 3 ± 0.0 | 4 ± 0.0 |
| Fresh crop + $NO_2$ | Boiled | 72 ± 2.0 | 181 ± 2.0 | 11 ± 0.0 | 80 ± 1.5 |
| Fresh crop + $NO_2$ | Room temp | 68 ± 0.0 | 188 ± 2.0 | 5 ± 0.0 | 85 ± 2.5 |

The low basal nitrate and nitrite content of the crop was not, or only slightly, affected by the drying process. For samples where $NaNO_2$ was added, the drying process reduced the nitrate and nitrite content ($p<0.05$).

After evaluation of the effect on fresh crop similar analyses continued with silages. As the extraction method did not affect the result of nitrate and nitrite analyses, it was decided only to evaluate the effect of sample preparation (dried or un-dried) on recovery of nitrate and nitrite in silages. The result from 4 silage samples from the Czech Republic and 7 silages from the USA are presented in Table 2.

**Table 2.** Nitrate and nitrite in silage samples prepared with or without drying. All samples were extracted in boiling water

|  | $NO_3$-N, mg/kg DM | | $NO_2$-N, mg/kg DM | |
|---|---|---|---|---|
|  | Dried | Un-dried | Dried | Un-dried |
| Silage Czech republic 1 | 1155 | 1134 | 0.7 | 0.5 |
| Silage Czech republic 2 | 892 | 908 | 0.7 | 0.5 |
| Silage Czech republic 3 | 849 | 827 | 0.7 | 0.6 |
| Silage Czech republic 4 | 123 | 95 | 0.7 | 0.6 |
| Silage USA 1 | 182 | 191 | 0.7 | 1.6 |
| Silage USA 2 | 19 | 25 | 3.9 | 5.6 |
| Silage USA 3 | 237 | 270 | 5.1 | 9.6 |
| Silage USA 4 | 253 | 246 | 5.6 | 10.1 |
| Silage USA 5 | 11 | 14 | 4.2 | 5.5 |
| Silage USA 6 | 21 | 32 | 5.9 | 8.9 |
| Silage USA 7 | 23 | 26 | 7.1 | 8.8 |

No clear difference between dried and non-dried samples in nitrate and nitrite results can be seen in the Czech Republic samples. These samples were maize silages. In the samples from USA, which were alfalfa silages, a tendency for 7% lower nitrate values of the dried compared to un-dried samples were seen. Nitrite was affected to a greater extent by drying showing a 34% reduction.

In this experiment, nitrate and nitrite analysis according to ISO 13395:1996 was performed immediately upon extraction. In a preliminary investigation, we observed that freezing to -18ºC, followed by thawing, decreased both nitrate and nitrite content in the sample. This observation, together with the main results in this paper suggest that further detailed studies are needed in order to establish complete recommendation of sample preparation for nitrate and nitrate analysis.

**Conclusions**

No differences in the nitrate and nitrite levels of fresh forage were observed if the extraction step was made by boiling the sample in water for 10 minutes or shaking in water at room temperature for 1 hour. Effects of drying the samples prior to extraction were however noticed. When $NaNO_2$ was added to the fresh crop, drying at 60ºC for 16 h resulted in nitrate loss of 36% and nitrite loss of 90%. In preparation of silage samples, the drying process resulted in a 13% loss of nitrate and 63% loss of nitrite in some maize and alfalfa silages. It is

recommended not to use drying during sample preparation for nitrate and nitrite analyses. Instead extractions of fresh or frozen samples should be made.

**References**

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