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Changes in saponin content and composition during the ensilage of alfalfa (*Medicago sativa* L.)

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Two varieties of alfalfa (*Medicago sativa* L.) were ensiled under laboratory conditions after being treated with formic acid or after wilting for different times. Intact saponins, using an HPLC method, were determined in the starting material and after ensiling. The presence of two saponins were detected and measured in the experiment using the variety Hodoninka and three saponins in the second experiment using the variety Pálava. Soyasaponin I was present in both varieties, whilst the two other saponins, designated as saponin A and saponin B, had chromatographic properties consistent with glycosides, of medicagenic acid. Significant decreases in saponin concentrations were observed after ensiling varying from 12% to 100%. Soyasaponin I was found to be relatively stable, while saponin A was not detected after the first week of ensiling. Treatment with formic acid stabilised saponin B and soyasaponin I for the variety Pálava in contrast to the other treatments. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Alfalfa (*Medicago sativa* L.) has been widely used as a cultivated crop and as an animal feed both in the fresh and preserved state to ruminants and poultry in many regions, such as Central Europe and Northern America. Its ensilability has been very poor due to its low level of water-soluble carbohydrates and high content of buffering substances, especially raw protein. It cannot therefore be ensiled in the fresh state. The most common preservation methods for ruminant feeding are either field wilting to 400–450 g/kg dry matter (DM) and ensiling or medium wilting to some 300 g/kg DM and application of an effective preservative, especially formic acid, prior to ensiling. Field drying to hay has been associated with a large loss of nutrients due to physical damage to the leaves and the subsequent oxidation of carotenes. Artificial drying has been found to be too expensive but alfalfa meal has been fed to poultry, especially laying hens, as a source of proteins and carotenoids.

Saponins, which are considered to be the main antinutritional substances in alfalfa, are normally triterpenoid glycosides consisting of an aglycone (sapogenol) coupled to one or more sugar chain units. Medicagenic acid, hederagenin, zanhic acid and soyasapogenols A and B are the main aglycones of alfalfa aerial parts (Price *et al.*, 1987; Massiot *et al.*, 1988). Sugars are bound either via an ester or ether linkage normally in one or two chains, but in the case of zanhic acid with up to three chains, where they are termed mono-, bis- or tridesmosides, respectively.

Differences in biological effects of the saponins are associated with their different chemical structures (Price *et al.*, 1987). The saponins in alfalfa forage have been shown to affect palatability, intake and availability of nutrients in poultry and ruminants. Other antinutritional effects are of lower importance (Cheeke, 1983).

Very little information is available on the changes that occur in both saponin content and composition during alfalfa preservation. Szakács and Madas (1979) found a reduction of saponin hemolytic index during ensiling of leaf protein concentrate with several *Lactobacillus* species. Nevertheless, no data are available on changes in individual saponins during alfalfa ensiling. This work was therefore undertaken to address this problem.

MATERIALS AND METHODS

Silage preparations

Two ensiling laboratory experiments were carried out. Alfalfa aerial parts were either chopped in the fresh

state to 1–1.5 cm lengths and 310 g placed in preserving jars of 720 cm^3 volume, or left on swaths to wilt, being turned twice a day. Wilted forage was cut and placed in the model silos the same way as for the fresh material. The jars (two for each treatment) were closed by Omnia caps 2 h after being filled. The caps enabled fermentation gases to escape and closed the silos hermetically after the main period of fermentation. A dose of formic acid was applied as 15 ml of diluted solution per kg forage. The silos were stored in the dark at 20–22°C.

In experiment I the variety Hodonínka was used and the first cut in full bud stage was ensiled as follows:

1. Control — fresh (190 g DM/kg).
2. Preserved with formic acid (0.43% by weight) after slight wilting to 272 g DM/kg during 4 h.
3. First wilt to 350 g DM/kg after 21 h.
4. Second wilt to 642 g DM/kg after 50 h.

Samples for analysis were taken after 7 and 90 days.

In experiment II, the variety Pálava was used and the second cut in the flowering stage was ensiled as follows:

1. Control — fresh (213 g DM/kg).
2. Preserved with formic acid (0.40% by weight).
3. First wilt to 276 g DM/kg after 6 h.
4. Second wilt to 382 g DM/kg after 24 h.

Samples for analysis were taken after 7 days. Weather conditions for both experiments were considered to be adequate from past experience to produce good quality silage.

Sample preparation for saponin analyses

Only leaves (blades and petioles) were used for saponin determination, since the level of saponins is normally low in stems. The leaves were immediately frozen and stored at –18°C for 7–14 days and then freeze-dried. Dry samples were stored in hermetically sealed polythene bags at ambient temperatures.

Saponin isolation and derivatization

Powdered dry samples, 3–4 g accurately weighed in duplicate, were continually extracted by chloroform using a soxhlet extractor for at least 16 h to remove lipids and most of the chlorophyll. Saponins were extracted into methanol from the defatted air-dried sample for at least 30 h. The extract, after vacuum evaporation of methanol, was dissolved in 5 ml water and 2×0.5 ml portions were purified on TechElut SPE C18 columns (HPLC Technology, Macclesfield, UK) by sequential elution with water (3 ml) and methanol (6 ml). The methanolic extracts were evaporated to dryness under vacuum. One ml 0.05% $KHCO_3$ was added twice to the extract and the solution sonicated to produce potassium salts of the saponins present. The sample was then freeze-dried and the saponin salts derivatized with 2 ml of reagent containing 3.5 g 4-bromophenacyl bromide (Sigma, Poole, UK) and 0.68 g 18-crown-6 (Sigma) in 100 ml acetonitrile. The solution was heated at 100°C for 90 min and then purified on a silica gel Sep-Pak cartridge (Waters Assoc., Milford, MA, USA) by eluting with 10 ml dichloromethane followed with a 10 ml mixture of chloroform with methanol (1:1). The latter solvent mixture was evaporated under vacuum and the residue dissolved in 2 ml methanol. The sample for chromatographic analysis was prepared from 0.95 ml of the latter solution and 0.05 ml phenanthrene (0.5 mg/ml in methanol) as the internal standard. These samples were stored at –20°C prior to analysis. All the chemicals used were of AnalaR grade (BDH, Poole, UK).

Soyasaponin I (2.5 mg) was used as the external standard. The derivatization procedure was the same as that described for the alfalfa samples.

Saponin determination using HPLC

HPLC analyses, in duplicate, were performed on a PU 4100 liquid chromatograph (Pye Unicam, Cambridge, UK) with an autosampler (Gilson, Anachem, Luton, UK) using a Spherisorb 5 μm ODS2 column (250×4.6 mm ID) with an injection volume of 20 μl at a flow rate of 1 ml/min, and detection wavelength with a UV detector (PU 4025) set at a wavelength of 260 nm. Water and acetonitrile were used as solvents A and B. The solvents were continually degassed with helium. The following gradient was used: 58% A for 1 min, decreasing from 58 to 40% A during 15 min, with a further decrease from 40 to 10% A during 10 min and, 10% A for 10 min. A re-equilibrium period of 10 min was used between runs.

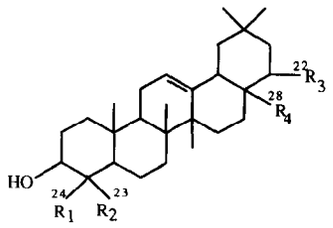
Determination of sapogenols using GC and GC/MS

A chloroform extract of alfalfa, obtained as previously described, was rotary evaporated and redissolved in 6 ml chloroform. One-quarter of the solution was dried at 33°C under nitrogen and the solids were left in a desiccator over P_2O_5 overnight. The residue was then dissolved in 300 μl pyridine and derivatized with 200 μl *N,O*-bis(trimethylsilyl)trifluoracetamide (BTSFA; Pierce, Rockford, MA, USA) at 50°C for 10 min in a closed vial.

Gas chromatographic and mass spectrometric conditions were as described previously (Muzquiz *et al.*, 1993).

RESULTS AND DISCUSSION

Samples for saponin determination were taken from the fresh alfalfa and also after 7 and 90 days of ensiling in experiment I, and after 7 days in experiment II. Silage quality, characterized from field experience and by a combination of pH value, levels of fermentation acids, ammonia and sensory evaluation was judged to be good for the processes involving wilting and formic acid, whereas the control samples gave silage of poor quality which demonstrates the need for the pretreatments to ensiling.



Aglycone	R_1	R_2	R_3	R_4
Soyasapogenol B	$-CH_2OH$	$-CH_3$	-OH	$-CH_3$
Medicagenic acid	$-CH_3$	-COOH	-H	-COOH

Fig. 1. Structure of saponins.

Three saponins were separated and quantified in this study: two present in variety Hodonínka (experiment I) and a third in the variety Pálava (experiment II). However, it should be noted that the derivatization procedure used here would not produce a derivative of the zarkic acid tridesmoside reported in aerial parts of alfalfa (Oleszek *et al.*, 1992), since this saponin lacks a free carboxylic acid group. Thus, at least one further saponin could be present in alfalfa aerial parts and not detected using this procedure.

From their elution sequence and comparison with standard compounds, two of the saponins were identified as saponin A, 3-*O*-glucuronyl, 28-*O*-arabinosyl-rhamnosylxylosyl-medicagenate ($R_t = 16.1$ min), and saponin B, 3-*O*-glucuronyl, 28-*O*-arabinosyl-rhamnosyl-medicagenate ($R_t = 17.1$ min). These two saponins have already been shown to be the major medicagenates present in alfalfa leaf (Oleszek *et al.*, 1992) together with soyasaponin I ($R_t = 17.8$ min) which was confirmed as the third saponin present (saponin C) (see Fig. 1).

Large differences in the saponin composition of the fresh material, presumably due to the two varieties used or the differing stages of maturity of these two samples, were evident as shown in Table 1. The variety Pálava contained 43% more saponin than Hodonínka, whilst the difference in composition was shown by the ratios of saponins A:B:C of 6.3:1.0:1.7 and 0:1.5:1.0, respectively.

Table 1. Saponin content of fresh leaf material for each variety

Saponin	Saponin content (mg 100 g^{-1} dry wt) Hodonínka	Pálava
A	nd	169
B	101	27
C	67	45
Total	168	241

Means of duplicate extraction +/– 10%.
Means of duplicate analysis +/– 5%.

For the variety Paláva used in experiment II (Table 2), saponin A was found to be completely unstable under all the conditions employed, including the control conditions which would indicate that this particular saponin would be rapidly degraded under most ensiling conditions regardless of pretreatments. In contrast, saponin B was found to be partially stabilised in the presence of formic acid with only 12% loss as compared to the control (43%). The first and second wilting processes resulted in slightly more degradation of the saponin as compared to the control with 55 and 68% losses, respectively. Saponin C behaved in a similar manner to saponin B in that it was stabilised in the presence of formic acid, 18% loss compared to the control of 38%. The first wilting process similarly increased the degradation of the saponin up to a 26% loss, although the second stage of wilting gave no increase; wilting also appeared to stabilise saponin C in the following ensilage when compared to the control sample with losses of 25 and 38%, respectively.

With the variety Hodonínka, which was used in experiment I (Table 3), saponin B was found to be much less stable under all the equivalent conditions after 7 days of ensilage with at least 92% degraded in all cases. Saponin C was also significantly less stable than in experiment II with a 74% loss recorded for the control and 92% and 86% losses for the two wilting processes after 7 days ensiling, respectively. The addition of formic acid did reduce the degradation to a 61% loss when compared with both control and wilting, but this value

Table 2. Losses of saponins in experiment II

Saponin	% Loss of saponins after 7 day ensilage for experiment II Control	Formic acid	First wilt	Second wilt
A	100	98	100	100
B	43	12	55	68
C	38	18	26	25

Table 3. Losses of saponins in experiment I

Saponin	% Loss of saponins after ensilage for experiment I Control		Formic acid		First wilt		Second wilt	
	7 day	90 day	7 day	90 day	7 day	90 day	7 day	90 day
B	92	88	93	95	95	72	94	67
C	74	90	61	72	92	74	86	57

is much higher than that for the variety Pálava in experiment II. The anomalous increase in saponin content indicated by a reduction in the percentage loss found for the 90 day samples could be attributed to the loss of biomass occurring during the extended ensilage time, resulting in an apparent increase in concentration of saponin.

During both the wilting of alfalfa and the initial stage of ensiling in non- and low-acidic conditions in alfalfa, there are reports of extensive enzymatic proteolysis (McKersie, 1985) when many enzymes are deactivated. These enzymes are relatively more stable at pH values under 4.2, conditions which were found in both experiments with the formic acid treatment. The reduced losses of saponins observed with this treatment may therefore be due to reduced enzymic activity.

The observed decrease in the level of intact saponins after ensilage may be explained by cleavage of the sugar side chains. A similar conclusion was given for the decrease in hemolytic index of ensiled alfalfa leaf concentrate (Szakács and Madas, 1979). However, gas chromatography analysis did not detect either of the free sapogenols, soyasapogenol B or medicagenic acid, which would be expected from enzymolysis in either the fresh leaves for both experiments or the samples after 7 days of ensiling.

This study showed that there were large losses in total content of the saponins measured (up to 100%) and that the medicagenic acid saponins, which are generally accepted as having the highest antinutrient activity, were less stable than the soyasapogenol B saponin. The different treatments produced varying losses and changes in composition of these saponins which appeared to be largely dependent on varietal difference. Further studies are required to measure the changes in the other group of saponins in alfalfa leaf, the zarkic acid saponins, to assess their stability during ensilage, but for this to be possible further advances in the analytical methodology are necessary.

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REFERENCES

Cheeke, P. R. (1983). Biological properties and nutritional significance of legume saponins. In *Leaf Protein Concentrates*, eds L. Telek & H. D. Graham. AVI Publ. Co., CN, USA, pp. 396–414.

Massiot, G., Lavaud, C., Guillaume, D. & Le Men-Olivier, L. (1988). Reinvestigation of the sapogenins and prosapogenins from alfalfa (*Medicago sativa*). *J. Agric. Food Chem.*, **36**, 902–909.

McKersie, B. D. (1985). Effect of pH on proteolysis in ensiled legume forage. *Agron. J.*, **77**, 81–86.

Muzquiz, M., Ridout, C. L., Price, K. R. & Fenwick, G. R. (1993). The saponin content and composition of sweet and bitter lupin seed. *J. Sci. Food Agric.*, **63**, 47–52.

Oleszek, W., Jurzysta, M., Ploszynski, M., Colquhoun, L. L., Price, K. R. & Fenwick, G. R. (1992). Zahnic acid tridesmoside and other dominant saponins from alfalfa (*Medicago sativa* L.) aerial parts. *J. Agric. Food Chem.*, **40**, 191–196.

Price, K. R., Johnson, I. T. & Fenwick, G. R. (1987). The chemistry and biological significance of saponins in foods and feedingstuffs. *CRC Crit. Rev. Food Sci. Nutr.*, **26**, 27–135.

Szakács, G. Y. & Madas, E. (1979). Wet preservation of leaf protein concentrate by Lactobacilli. *Biotech. Bioeng.*, **21**, 721–723.