Effect of storage on the fatty acids of dried ryegrass

By J. W. CZERKAWSKI

Hannah Dairy Research Institute, Ayr

1. The compositions of the fatty acids in ryegrass were determined in fresh samples, and in samples dried at room temperature for 26 h, at 50° for 18 h or at 100° for 12 h. The drying of grass resulted in a small increase in palmitic acid and a decrease in linolenic acid in the total fatty acids.

2. Samples of grass dried at 50° and 100° were stored at three relative humidities (rh < 3 %, 47 % and 80 %) for 13 months.

3. There were marked changes in the samples stored at 80 % rh, with an onset of mould growth and a loss of dry matter. The samples stored at lower humidities had no mould.

4. There were two types of change in the fatty acids during storage. The deterioration brought about by mould was accompanied by a decrease in the concentration of linolenic acid and an increase in the concentrations of oleic and linoleic acids. The other type of change observed at the lower humidities resulted in a decrease in the content of linolenic and an increase in the content of palmitic, and did not affect the amounts of oleic and linoleic cells.

5. There was little difference between the changes that occurred in the composition of the total fatty acids of the grass dried at 50° and of that dried at 100°. The changes that were at all significant appeared to occur less rapidly, particularly in the early months of storage, in the grass dried at the higher temperature for the shorter time.

The successful preservation of dried forages in an intact form is of economic importance, and various aspects of this problem were studied intermittently at this Institute and elsewhere, particularly during the Second World War and immediately afterwards. On long storage in this country feeding-stuffs can be affected by moulding, and this aspect of the problem has received much attention (cf. Wright, 1941). The onset of moulding is related to the moisture content of the feeding-stuff and the latter was shown to be a direct function of the relative humidity (rh) of the atmosphere (Snow, Crichton & Wright, 1944*a*, *b*), but the emphasis was usually on prevention of moulding rather than on the changes that occurred in the composition of the material studied. The problem was reinvestigated by Waite (1949), who pointed out that the development of moulding depended on the storage temperature and that the critical humidity derived from laboratory experiments did not necessarily apply under practical conditions where the temperature was normally lower. Changes in the content of some components of the feeding-stuffs were investigated by Snow, Smith & Wright (1945) and by Snow & Wright (1945), and deterioration caused by agents other than mould was also considered.

Lipid is a minor but important constituent of roughages (Garton, 1960), but any serious investigation of changes in this constituent during storage had to await the development of the new chromatographic techniques. Collins & Shorland (1944–5) studied the change that occurred in the total amounts of fatty acids and phospholipids in grass during drying and storage periods of relatively short duration. Much later Lea (1957) drew attention to deteriorative reactions involving phospholipids in stored

foods. Couchman (1959) studied the effect of the temperature of storage on the content of fat extracted with two different solvents from hay and found that the amount of crude fat decreased. Tanhuanpää & Lindberg (1965) found that the fat content of grain decreased during storage for 2 years and that there was a small but consistent increase in linolenic acid and a decrease in linoleic acid in the total fatty acids. On the other hand, Thafvelin & Oksanen (1966) found that during the slow drying and storage of hay there was a consistent decrease in the percentage of linolenic acid in the total fatty acids. It was noted during studies of the metabolism of fatty acids in the rumen of sheep (Czerkawski, Blaxter & Wainman, 1966) that, in spite of lack of any obvious signs of deterioration, the composition of the fatty acids in a good-quality dried grass tended to change during storage. There was a decrease in the percentage of linolenic acid in the total fatty acids, but no significant change in the content of total fatty acids in the dry matter of the grass. In view of the dramatic effect of linolenic acid on the metabolism in the rumen (Czerkawski *et al.* 1966), and in view of the large proportion of this acid in the total fatty acids of grass (Lima, Richardson & Stahmann, 1965), it was desirable to investigate this decrease in linolenic acid in greater detail.

Ryegrass (*Lolium perenne*) was chosen for the present experiment because it is so widely grown. The object was to dry samples of freshly cut grass in such a way that they would contain 10% moisture, and to store the dried samples at room temperature at different humidities. An atmosphere with a rh of 80% would simulate conditions often met with outside in this country, and this level of humidity at room temperature would ensure speedy and prolific mould growth. A rh of about 50% would effectively eliminate mould growth and would maintain the existing moisture content of the dried grass. In addition the effects of storage under very dry conditions (rh < 3%) were investigated.

EXPERIMENTAL

Material. Ryegrass, free from clover and cut on 12 July 1965, was obtained from the Grass and Dairy Husbandry Department of this Institute. It contained 16·9% dry matter.

Drying procedure. The freshly cut grass (5 kg) was divided into three portions. One portion (2 kg) was dried in a forced-air oven (Birmingham and Blackburn Construction Co. Ltd) for 12 h at 100°, and a similar amount was dried for 18 h at 50°. The remainder was dried at room temperature (23°) for 26 h. The duration of drying at any given temperature was determined by the extent of removal of water, the desired final dry-matter content being about 90% in the samples dried at 50° and 100° and 75% in the samples dried at room temperature.

The dried grass in the various preparations was cut into lengths of 1–2 cm and mixed well. The samples dried at 50° and 100° were divided into three equal portions and placed in shallow polythene bags, which in turn were placed (open) in large glass containers provided with ground-glass lids. The humidity of the driest storage vessel was kept at less than 3% by inclusion of open jars filled with concentrated sulphuric acid. A solution of sulphuric acid with sp.gr. 1·35 gave an atmosphere with a rh of

47 %, and one of sp.gr. 1·20 provided the high rh of 80 %. Thus samples of grass dried at 50° and at 100° were stored at three different humidities.

The grass dried at room temperature was placed in Kilner jars which were autoclaved at 20 lb/in.^{2} for 15 min and then sealed. These jars were stored at room temperature and their contents analysed as required.

Analyses. The storage experiment began in the middle of July 1964 and the initial samples were withdrawn 1 month later, it being assumed that no microbial or chemical changes would take place during that time. Samples were subsequently withdrawn for analysis at 3-monthly intervals.

The dry-matter content of the samples was determined by drying for 16 h at 105°, and their nitrogen content was determined by the Kjeldahl method.

Preliminary experiments showed that extraction of the lipids with methanol–chloroform (Folch, Lees & Stanley, 1957) followed by saponification gave a somewhat smaller yield of fatty acids than direct saponification of the grass, although the composition of the total fatty acids was the same by both these methods. Consequently, the total fatty acids were isolated by direct saponification.

The samples of grass (9 g) were weighed in round-bottom flasks (250 ml), and refluxed for 1 h with 100 ml of ethanol–water (90 %, v/v) containing 1 % KOH. The contents were cooled to about 50° and the extracts decanted through muslin. The residues were suspended in 100 ml of warm water (50°) and shaken intermittently. The supernatant fluids were decanted through muslin and the residues shaken with two portions of 50 ml ethanol. The residues were finally shaken with two portions of 100 ml light petroleum (b.p. 40°–60°). All the extracts were pooled and separated into aqueous and organic phases in large separating funnels. The aqueous phase was extracted twice with portions of 100 ml light petroleum (b.p. 40°–60°). These light-petroleum extracts were pooled, washed with 50 ml of 1·0 % NaHCO$_3$ and then discarded. The original aqueous phase and the washings from the light-petroleum extracts were pooled, acidified with 10 N-H$_2$SO$_4$ to about pH 1·5 and extracted at once with an equal volume of diethyl ether. The aqueous phase was extracted with diethyl ether twice more and the ether extracts were pooled. The ether solutions of the fatty acids were washed with about 20 ml distilled water and the ether was removed on a rotary film evaporator. The residues were weighed.

The methyl esters of the fatty acids were prepared by the method of Kates (1964) and analysed by gas–liquid chromatography (Pye Argon Gas Chromatograph) on columns of Chromosorb W-polyethylene glycol adipate (9:1, w/w) at 197°.

The samples remaining after the final analysis of the fatty acids (13 months) were analysed for organic matter by ashing at 550°, for lignin (Czerkawski, 1967) and cellulose (Crampton & Maynard, 1938). Portions of this material were also used for the determination of the in vitro digestibility of the organic matter (Alexander & McGowan, 1966).

RESULTS

The moisture, nitrogen and total fatty acid contents of the
grass during storage

Variations in the moisture content of the samples of grass dried at 50° and 100° and stored at three humidities ($<$ 3, 47 and 80% rh) are shown in Fig. 1 a, b. In both samples the moisture content decreased a little at low humidity, remained practically constant at a rh of 47% and increased rapidly at high humidity. The
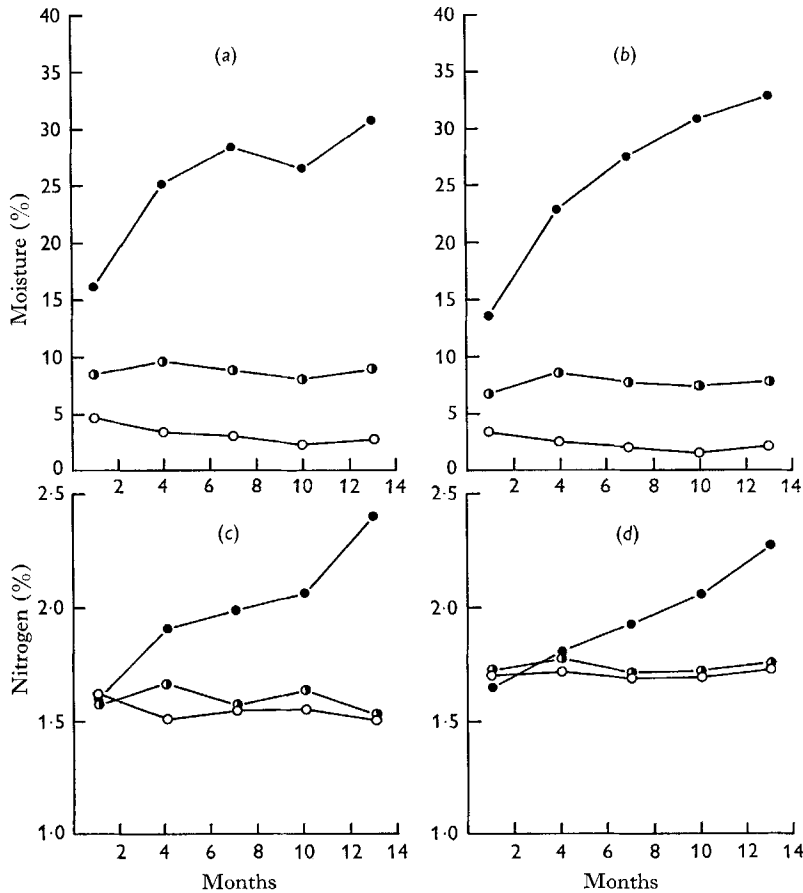

Fig. 1. Changes in the moisture content (a, b) and nitrogen content (c, d), during storage of grass which was dried at 50° (a, c) and 100° (b, d). O—O, stored in an atmosphere with $<$ 3 % rh; ◑—◑, stored in an atmosphere with 47 % rh; ●—●, stored in an atmosphere with 80 % rh. rh, relative humidity.

nitrogen content in the grass dry matter behaved similarly, showing little change except in grass stored at 80 % rh, where the nitrogen content expressed as a percentage of the dry matter increased during storage (Fig. 1 c, d).

Over the same period the moisture and nitrogen contents of the samples that were

dried at room temperature and autoclaved remained constant at $25·9 \pm 0·1$ and $1·58 \pm 0·01 \%$ respectively.

The fatty acid content of the grass did not increase or decrease regularly during storage at the three different humidities, but at 80 % rh the spread of values was much greater than at the two lower humidities and some abnormally high values were obtained. The results after 1–13 months' storage, given as means of the five determinations in each series, together with the corresponding standard errors, are shown in Table 1. The content of fatty acids increased slightly though not always significantly with increase in humidity. The concentration of fatty acids was greater when the samples were dried for a short time at 100° than when they were dried for a longer time at 50°. This difference was statistically significant when the samples were stored at low or medium humidity, but was not significant at high humidity.

Table 1. *Mean concentrations of total fatty acids and their standard errors in grass stored for 1, 4, 7, 10 and 13 months (expressed as % dry matter)*

Temperature of drying (°C)	Storage conditions (relative humidity %)		
	< 3	47	80
50	$2·14 \pm 0·11$	$2·24 \pm 0·12$	$2·64 \pm 0·20$
100	$2·84 \pm 0·10$	$2·96 \pm 0·23$	$2·98 \pm 0·28$
Difference	0·70**	0·72*	0·34†

* $P < 0·05$ (t test).
** $P < 0·01$ (t test).
† The samples stored for 7 months gave an abnormally high yield of fatty acids (3·3 and 3·8 % respectively). The difference was not significant.

Table 2. *Composition (%) of the fatty acids in ryegrass immediately before and after drying under different conditions*

Fatty acid	Fresh grass	Grass dried at 23° and autoclaved	Grass dried at 50°	Grass dried at 100°
Palmitic	11·8	13·2	16·2	15·0
Stearic	1·5	1·3	1·6	1·8
Oleic	2·2	2·0	1·6	2·5
Linoleic	12·7	13·5	12·8	12·4
Linolenic	70·0	66·9	62·7	62·8

Small amounts of C_{12}, C_{14} and unidentified acids are not included in this table.

The composition of fatty acids in grass after drying and during storage

The conditions of drying had only a small effect on the composition of the fatty acids in grass (Table 2). There was a small increase in the percentage of palmitic acid and a decrease in the percentage of linolenic acid when fresh grass was dried at room temperature. These changes were somewhat greater when the grass was dried at higher temperatures, but there was no difference between the fatty acid composition of grass dried at 50° and of that dried at 100°.

The variation in the composition of the unsaturated C_{18} acids during storage is shown in Fig. 2. Linolenic acid decreased rapidly when the samples dried at 50° were

stored at 80% rh and there was an increase followed by a net decrease of this acid in the samples stored at the lower humidities. The changes were similar in samples dried at 100°, but were generally less extensive (Fig. 2a, b). With the grass stored at low and medium humidities, the percentage of linoleic acid did not change in samples
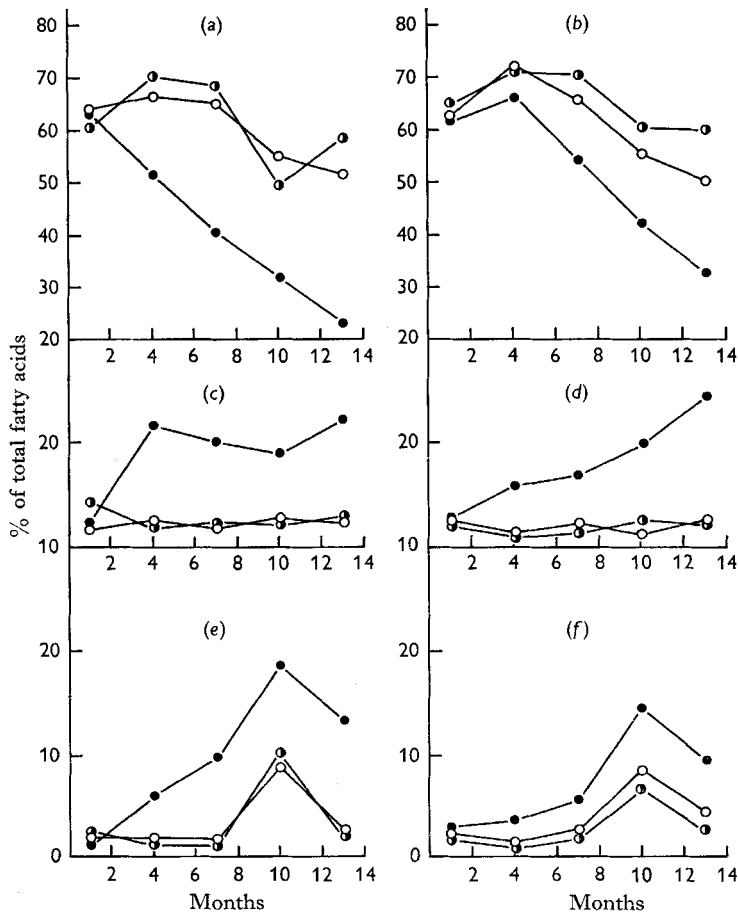

Fig. 2. Changes in composition of unsaturated C_{18} fatty acids (% of total fatty acids) during storage of grass which was dried at 50° (a, c, e) and 100° (b, d, f). a, b, linolenic acid; c, d, linoleic acid; e, f, oleic acid; O—O, stored at < 3 % rh; ◑—◑, stored at 47 % rh; ●—●, stored at 80 % rh. rh, relative humidity.

dried at either 50° or 100°, but at high humidities the concentration of this acid increased rapidly in samples dried at 50° and more slowly in samples dried at 100° (Fig. 2c, d). There was a marked increase in the proportion of oleic acid in grass stored at 80% rh, the change in the first 4 months being more rapid in the material that was dried at 50°.

A large amount of oleic acid was present in the samples after 10 months of storage at all three humidities but no explanation for this can be offered (Fig. 2e, f).

Stearic acid (Fig. 3c, d) followed trends similar to those shown by linoleic acid. The

increases at 80% rh during the first 6 months were more pronounced when the material was dried at 50°. The differences in humidities of storage did not significantly influence the concentration of palmitic acid (Fig. 3 *a*, *b*) in the grass dried at 100°. All the curves relating the concentration of palmitic acid to time of storage showed a minimum at 4–7 months.

The results of analysis of the samples that were dried at 23° and autoclaved before storage are summarized in Table 3. The amounts of linoleic, oleic and stearic acids
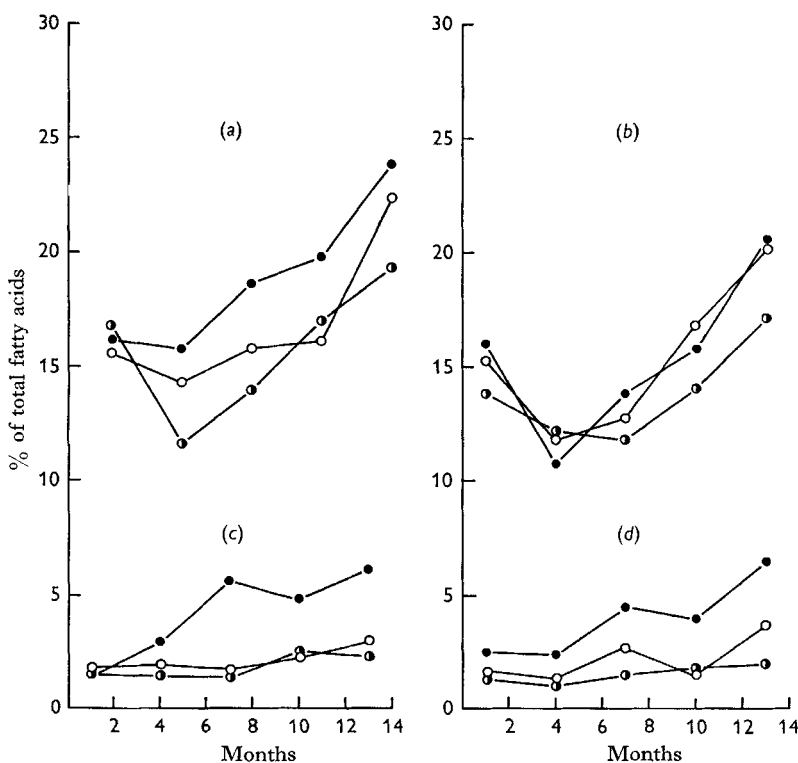

Fig. 3. Changes in the composition of the major saturated fatty acids (% of total fatty acids) during storage of grass which was dried at 50° (*a*, *c*) and at 100° (*b*, *d*). *a*, *b*, palmitic acid; *c*, *d*, stearic acid; O—O, stored at < 3 % rh; ◑—◑, stored at 47 % rh; ●—●, stored at 80% rh. rh, relative humidity.

did not change appreciably. There was a significant decrease in the concentration of linolenic acid and an increase in the concentration of palmitic acid.

The samples also contained small amounts of lauric and myristic acids, traces of fatty acids containing odd numbers of carbon atoms and about 2% of an unidentified component. The methyl ester of the unidentified material had the retention volume relative to methyl stearate equal to 0·62 on a polythene glycol adipate column at 197°. The concentration of this component had no tendency to change during storage.

Mould growth

There was no odour or any visible sign of mould growth in any of the samples after 1 month. After 4 months the samples dried at 50° and stored at 80% rh had a faint fruity odour. The fruity odour became more and more noticeable during further storage at high humidity in samples dried at 50° and in samples dried at 100°. After 10 months these samples became lighter in colour, and examination under a low-power microscope revealed heavy mould growth. As was expected (Wright, 1941), the samples stored at low or medium humidity showed no mould.

Table 3. *Concentrations (% of total fatty acids) of fatty acids in grass that had been dried at 23°, autoclaved and stored in Kilner jars for varying periods*

Period of storage (months)	Fatty acid				
	Palmitic	Stearic	Oleic	Linoleic	Linolenic
1	12·0	1·5	2·0	14·1	67·8
4	13·1	1·1	2·0	13·9	66·9
7	14·3	1·4	1·6	14·1	65·3
13	17·0	2·3	2·8	14·0	61·0

Table 4. *Concentration (% dry matter) of cellulose, lignin and ash at the conclusion of the experiment, given as means of values for grass dried at 50° and 100°*

Storage conditions	Cellulose	Lignin	Ash	Cellulose / Ash	Lignin / Ash
Control, sealed jars	30·7	3·26	8·46	3·6	0·39
< 3% rh	30·4	3·30	8·16	3·7	0·40
47% rh	30·6	3·09	8·22	3·7	0·38
80% rh	38·4	4·51	10·86	3·5	0·41

rh, relative humidity.

Some characteristics of the samples at the conclusion of the experiment

Irrespective of the conditions of storage the percentages of ash, lignin and cellulose in the samples of grass dried at 50° were the same as the percentages of ash, lignin and cellulose in the corresponding samples dried at 100°. On the other hand, the concentration of these resistant components in samples stored at the two lower humidities were lower than in the samples stored at 80% rh (Table 4). The ratios of the concentration of cellulose and lignin to the concentration of ash were approximately the same (3·65 ± 0·06 and 0·40 ± 0·01 respectively) whether the samples were stored at high or low humidity.

If it can be assumed that ash and lignin remain intact during storage under the conditions of this experiment, their concentrations can be used to compare changes that occurred in the other components of the grass. This has been done in Table 5, where the various values are given in arbitrary units, calculated by assuming that the combined concentration of ash and lignin was equal to unity. Expressed in this way, the content of crude protein was similar in all samples. There was a considerable loss of dry matter (24%) in the samples stored at 80% rh, which was confined to organic

matter other than lignin or cellulose, e.g. soluble carbohydrate. The loss of this material was about 50%.

The results of the in vitro determination of digestibility of the samples stored for 13 months at the different humidities are shown in Table 6. There was no difference in digestibility whether the samples were dried at 50° or 100°. The digestibility of the organic matter of the samples stored at the two low humidities was generally about 10 units higher than that of the organic matter of samples stored at 80% rh. The difference between the digestibilities of the lignin-free organic matter of samples stored at low and high humidities was almost as great (9 units). The digestibility of samples which were autoclaved at 20 lb/in.² was lower than that of the samples dried at 50° and 100° and stored at low humidity, and higher than that of the samples stored at high humidity.

Table 5. *Relative composition of grass samples stored for 13 months at different temperatures and different relative humidities (rh)*

(It has been assumed that the ash and lignin contents remained constant, and the quantities of various components were calculated in weight units per unit weight of ash + lignin. The proportion of ash to lignin was 0·7–0·3)ᐟ

	Dried at 50°			Dried at 100°		
Component	< 3 % rh	47 % rh	80 % rh	< 3 % rh	47 % rh	80 % rh
(a) Dry matter	8·47	8·79	6·43	8·29	9·07	6·66
(b) Cellulose	2·52	2·77	2·48	2·78	2·69	2·51
(c) Crude protein (N × 6·25)	0·81	0·84	0·95	0·90	0·95	0·95
(d) Moisture	0·31	0·88	2·72	0·20	0·77	3·16
(e) Soluble carbohydrate†	5·14	5·18	3·00	4·61	5·43	3·20

† $a - (b + c)$.

Table 6. *Digestibility in vitro of organic matter and of lignin-free organic matter in grass after storage for 13 months*

Conditions of storage	Digestibility† of organic matter (%)	Digestibility‡ of lignin-free organic matter (%)
Autoclaved, sealed jars	73·7	76·7
< 3 % rh	78·6§	81·5§
47 % rh	78·2§	80·9§
80 % rh	68·3§	72·0§

† Determined by the method of Alexander & McGowan (1966).
‡ Calculated on the assumption that lignin remained unchanged during digestion.
§ Means of values for samples dried at 50° and 100°. The pairs of values differed by less than 1 digestibility unit.

DISCUSSION

As expected, the moisture content of grass increased at high humidity and, although the nitrogen concentration increased when expressed on a dry-matter basis, its absolute amount, per unit weight of ash and lignin, remained remarkably constant.

Changes in the concentration of total fatty acids in the dry matter were too small to

be determined with accuracy, but the values obtained suggested that, unlike those of cellulose, ash and lignin, the concentrations of total fatty acids did not increase or decrease regularly during storage and, since there was a loss of about 24% organic matter in samples stored at 80% rh, it must be assumed that the fatty acids shared in this loss, although the soluble carbohydrate fraction appeared to have suffered most.

The changes in the composition of the total fatty acids were of two types. The first was associated with mould growth, which led to a marked decrease in the concentration of linolenic acid and to increases in the concentrations of linoleic and oleic acids. The second type of change, observed in samples free from mould, was exemplified by an initial increase in the concentration of linolenic acid followed by a decrease to a level that was below the level at the start of the experiment. The increases or decreases in linolenic acid were invariably compensated for by changes in the concentration of palmitic acid. The inverse relationship between the concentrations of linolenic and palmitic acids is well illustrated by comparing the analyses of the control samples which were autoclaved before storage (Tables 2 and 3). Linoleic and oleic acids did not appear to be involved in the second type of change.

The deterioration of the grass during mould growth appeared to be extensive and was accompanied by loss of organic matter.

The results of these studies confirmed the previous observation that there was a change in the composition of fatty acids during storage (Czerkawski *et al.* 1966). They also showed that ryegrass or forages rich in ryegrass should be dried as rapidly as possible at high temperature, rather than for a long time at low temperature, and should be stored at relatively low humidity.

REFERENCES

Alexander, R. H. & McGowan, M. (1966). *J. Br. Grassld Soc.* **21**, 140.
Collins, F. D. & Shorland, F. B. (1944–5). *N.Z. Jl Sci. Technol.* A, **26**, 372.
Couchman, J. F. (1959). *J. Sci. Fd Agric.* **10**, 513.
Crampton, E. W. & Maynard, L. A. (1938). *J. Nutr.* **15**, 383.
Czerkawski, J. W. (1967). *Br. J. Nutr.* **21**, 325.
Czerkawski, J. W., Blaxter, K. L. & Wainman, F. W. (1966). *Br. J. Nutr.* **20**, 349.
Folch, J., Lees, M. & Stanley, G. H. S. (1957). *J. biol. Chem.* **226**, 497.
Garton, G. A. (1960). *Nature, Lond.* **187**, 511.
Kates, M. (1964). *J. Lipid Res.* **5**, 132.
Lea, C. H. (1957). *J. Sci. Fd Agric.* **8**, 1.
Lima, I. M., Richardson, T. & Stahmann, M. A. (1965). *J. agric. Fd Chem.* **13**, 143.
Snow, D., Crichton, M. H. G. & Wright, N. C. (1944*a*). *Ann. appl. Biol.* **31**, 102.
Snow, D., Crichton, M. H. G. & Wright, N. C. (1944*b*). *Ann. appl. Biol.* **31**, 111.
Snow, D., Smith, J. A. B. & Wright, N. C. (1945). *J. agric. Sci., Camb.* **35**, 65.
Snow, D. & Wright, N. C. (1945). *J. agric. Sci., Camb.* **35**, 126.
Tanhuanpää, E. & Lindberg, P. (1965). *Acta Agric. scand.* **15**, 171.
Thafvelin, B. & Oksanen, H. E. (1966). *J. Dairy Sci.* **49**, 282.
Waite, R. (1949). *Ann. appl. Biol.* **36**, 496.
Wright, N. C. (1941). *J. agric. Sci., Camb.* **31**, 194.