Content, Pattern and Esterification of Fatty Acids in Fresh Grass in Relation to Extraction Solvents and Sample Storage Conditions

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Introduction The relatively high content of linolenic acid (C18:3 n-3) in forages has lead to research on the role of forage based feeding strategies in the production of healthier milk. In these studies, forage samples are often stored, although analysis immediately after harvest has been considered to avoid oxidative deterioration and transformation of unsaturated fatty acids (FA) (Christie, 1993; Frankel, 1998). In the current study, we evaluated the effect of different storage conditions (fresh grass samples under liquid nitrogen (liq.N_2) or in a cool box during 3h or at -20ºC, -80ºC or in the extraction solvent at -20°C, during 24h) on the total amount of FA extracted, the FA pattern and the extent of FA esterification. The effectiveness of *iso*propanol to inhibit plant enzyme activity (Hawke, 1973), which has been reported to be particularly high in plant tissues (Christie, 1993), was considered. Measures to avoid thawing losses during sample handling were also compared.

Materials and methods *Experiment 1* Fresh grass samples of *Lolium multiflorum* were harvested. Samples (5g) of the control group were immediately analysed, while the rest were stored either under liq.N_2 or in a cool box for 3h. Samples were cut into 1 cm strips prior to FA extraction or storage during 24h at -20ºC, -80ºC or in the extraction solvent at -20ºC. Alternatively, grass was stored whole for 24h and then cut into 1 cm strips. Half of the samples were extracted with chloroform/methanol (C/M, 2/1, v/v), while the other half were extracted with *iso*propanol/chloroform (I/C, 1/1, v/v). Separation of the lipid classes in the samples conserved in liq.N_2 was performed by thin layer chromatography. *Experiment 2* Fresh grass samples of *Lolium perenne* were harvested. Samples (5g) of the control group were analysed immediately. The rest, stored for 3h in liq.N_2, were freeze-dried (FD) and extracted (2.5g) with C/M (2/1, v/v). All samples were homogenised with an ultra-turrax prior to extraction and water content was adjusted to C/M/water and I/C/water ratios of 8/4/3 and 2/2/1, respectively. For FD samples 30 ml of C/M (2/1, v/v) and 20 ml of distilled water were used.

Results *Experiment 1* Higher amounts of total FA and proportions of C18:3 n-3 were obtained in samples stored for 3h either in liq.N_2 or in a cool box compared to the 24h storage (Table 1). Increased proportions of free FA (FFA) to total FA (%) are indicative of ongoing lipolysis during or after sample storage (1.3, 2.7, 6.0, 7.6, for direct analysis, 3h in liq.N_2, 24h storage at -20ºC and at -80ºC, respectively). Although the use of *iso*propanol has been recommended for FA extraction from plant tissues, significantly lower amounts of FA were extracted compared to extractions with C/M and separation in lipid classes showed proportionally more FFA to total FA (6.4 *vs*. 4.4 %). *Experiment 2* Reduced amounts of FA and lower C18:3 n-3 proportions were observed when performing FA analysis on FD samples (Table 2).

Table 1 Total FA content and C18:3 n-3 proportions in *Lolium multiflorum* samples stored under different conditions and extracted with either C/M (2/1, v/v) or I/C (1/1, v/v)

Fatty acids	Direct analysis (n=12)	3h liq.N_2 or cool box (n=4)	24h (n=8)			SEM	Mean[1]	
			-20ºC	-80ºC	Solvent			
Total FA (mg/g fresh grass)	7.28[a]	6.99[a]	5.52[b]	5.88[b]	5.57[b]	0.243	C/M I/C	7.20** 4.85
C18:3 n-3 (% of total FAME)	64.6[b]	65.8[a]	63.3[b]	63.3[b]	66.0[a]	0.34	C/M I/C	66.6*** 62.3

[1]Mean of extractions with C/M or I/C; [a,b,c] Values within a row lacking a common superscript differ significantly ($p<0.05$)

Table 2 Effect of freeze-drying grass samples before FA extraction on total FA content and proportion of C18:3 n-3 (n=3)

Fatty acids	Direct analysis	3h liq.N_2 and freeze-drying	SEM	Sign.
Total FA (mg/g DM)	38.7	19.2	1.36	***
C18:3 n-3 (% of total FAME)	65.4	60.5	0.42	***

Conclusions FA should be extracted from grass immediately after harvest or after a short storage in liquid N_2. *Iso*-propanol/chloroform does not inhibit lipolysis and less FA is extracted compared to chloroform/methanol.

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