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A note on comparison of two extraction methods used to quantify C18 fatty acids in feed and digesta of ruminants*

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ABSTRACT

The aim of the present study was to compare two methods of fatty acid determination and quantification, differing in the kind of solvent used. Our hypothesis stated that diethyl ether can replace dichloromethane for the extraction of fatty acids, facilitate the analysis, and reduce health hazards. The analyses were conducted on feed and duodenal digesta samples from an experiment investigating the effects of feeding increasing amounts of either rape seed or linseed oils to sheep. Based on the obtained results, we conclude that the diethyl ether method seems to be more efficient for extraction of fatty acids from hydrolysed feed and digesta. Further investigations are needed to determine whether diethyl ether can replace dichloromethane for the extraction of fatty acids from other matrices.

KEY WORDS: C18 fatty acids, dichloromethane, diethyl ether, extraction method, gas chromatography, feed, digesta

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INTRODUCTION

There is much interest in fatty acid determination in different biological materials because of the health-promoting properties of essential fatty acids and conjugated isomers of linoleic acid. Fatty acids present in the diet of ruminants undergo transformations in the rumen that determine the composition of the fatty acids absorbable in the small intestine (Givens et al., 2001). The fatty acid metabolism in the rumen, therefore, has a major impact on the fatty acid composition of ruminant meat and milk (Jenkins et al., 2008). Fatty acids entering the rumen, after lipolysis, undergo biohydrogenation, which is the unique process of converting unsaturated fatty acids to more saturated end products by rumen microorganisms (Mosley et al., 2002). The end product of lipolysis and biohydrogenation occurring in the rumen is stearic acid. However under certain conditions, e.g., high polyunsaturated fatty acids supply, the extent of biohydrogenation may be reduced and various intermediate *cis* and *trans* fatty acid isomers will enter the duodenum. In order to better understand the dietary effects of the fatty acid composition of animal products, e.g., milk and meat, determination and quantification of all fatty acids at each step of their metabolism are important. Thus, especially in the field of animal nutrition, fatty acid analyses are very common. Because of the toxicity of traditionally used chlorinated solvents, their environmental hazards, and tedious extraction procedures, the purpose of the present investigation was to determine if dichloromethane can be replaced by diethyl ether. Our hypothesis stated that diethyl ether can replace dichloromethane for the extraction of fatty acids, facilitates the analysis, and reduces health hazards.

MATERIAL AND METHODS

Origin and production of samples

Feed and duodenal digesta samples from previous experiments were used as experimental material. The aim of these experiments was to determine the effect of feeding increasing amounts of either rape seed or linseed oils to sheep. Three ewes (50±4 kg) fitted with duodenal cannulas were used in two subsequent 3 × 3 Latin square experiments with 16 d experimental periods. The basal diets were supplemented with either 0, 3.5, or 7% (in dietary DM) of rape seed oil (Experiment 1) or linseed oil (Experiment 2). Samples of feed and duodenal digesta were collected during the last two days of each experimental period. Afterwards the representative samples were frozen, lyophilized and stored until used for further analysis.

Hydrolysis and extraction of samples

Hydrolysis was carried out in screw-cap Teflon-stoppered tubes (Pyrex, 15 ml) after adding 3 ml of 2M NaOH to 150 mg of feed (n=6 per feed) or duodenal digesta (n=8). Afterwards, samples were incubated in a block heater at 90°C for 40 min. After cooling to room temperature 1.7 ml of 4 M HCl were added to lower pH to below 2. The two different solvents were then used to extract free fatty acids:

Dichloromethane. The extraction was performed according to Czauderna and Kowalczyk (2001). Dichloromethane extraction was used as a reference method because of its similarity with the traditional extraction protocol (chloroform and methanol 2:1; Cequier-Sánchez et al., 2008). After hydrolysis, 1 ml of which was freshly prepared internal standard (IS, Triundecanin, TAG C11:0 in dichloromethane, Sigma) and 3.5 ml of dichloromethane were added and the tubes shaken vigorously using a Vortex-Genie 2. The same solvents for IS and extraction, without any side-effects, were used. A closed system of hydrolysis was used, assuming that no loss of fatty acids occurred. Furthermore IS is easily accessible and soluble. This procedure allowed the extraction to be strictly monitored. The lower organic phase was passed over a short column of Na_2SO_4. The extraction procedure was repeated twice. The solvent was then evaporated at 30°C for 10 min under a flux of nitrogen. The remaining residue was used for derivatization.

Diethyl ether. The extraction was performed as described above, but with diethyl ether instead of dichloromethane. Due to the lower density of diethyl ether, the organic phase separated above the aqueous layer, which facilitates handling. The organic phase was transferred into a reaction tube with a Pasteur pipette and the drying step was omitted. The extraction procedure was repeated twice per sample. The solvent was evaporated as described above and the remaining residue was used for derivatization.

Derivatization procedure

The extracted fatty acids were esterified using 0.5 M NaOH in methanol and subsequently converted to fatty acid methyl esters (FAME) using borontrifluoride (Fluka) as described by IUPAC (1987). A 0.34 M NaCl solution and hexane were added and shaken vigorously. The organic phase containing the FAME was used for gas chromatographic analyses.

Gas chromatographic procedure

The long-chain fatty acids in feed and duodenal digesta were quantified by GC (Hewlett-Packard model 6890 equipped with a flame ionization detector). A Chrompac CP-Sil 88 column (100 m, 0.25 mm, 0.2 μm film thickness, Varian) was used. Hy-

drogen was used as a carrier gas at a constant flow of 30.0 ml/min and a split ratio of 1:10. The oven temperature was programmed as follows: initially 175°C for 25 min, then increasing at 5°C/min to 235°C. The fatty acid peaks were identified by comparison with the retention times of known standards (FAME Mix, Supelco, Belfonte, USA) and according to Collomb and Bühler (2002). Fatty acid standards (Supelco, Belfonte, USA) were used to create calibration curves for the quantification of C18:0, C18:1 *trans* 9, C18:1 *cis* 9 and C18:2. The derivatization procedure for oil and fatty acid standards was the same as for the feed and digesta samples.

The mean values were compared using the pair-wise Student t-test.

RESULTS AND DISCUSSION

Biological samples containing fatty acids like feed or duodenal digesta can be analysed to determine their fatty-acid profile. In this study, we compared two solvents for fatty acid extraction: dichloromethane (M) and diethyl ether (D). Though dichloromethane is less toxic than, e.g., chloroform ($CHCl_3$) that is often used for lipid and fatty acid extraction, it has also been suspected of being toxic for humans (Cequier-Sanchez et al., 2008). Dichloromethane is carcinogenic and is assigned to class 3. Diethyl ether is not only safer but is also more convenient to work with, because the organic layer formed lies above the aqueous phase and therefore can be easily obtained.

In the present study, the total amount of identified fatty acids, C18, C18:0, C18:1, C18:2 and C18:3 (mg/g sample) in the analysed concentrate was numerically higher when solvent D was applied in comparison with solvent M (Table 1).

Using duodenal digesta samples (Table 2) of ewes fed diets supplemented with linseed oil total C18 (mg/g sample) was significantly higher when extracted by diethyl ether. However, no statistical difference in total C18 (mg/g sample) was noticed between the solvents when duodenal digesta from ewes fed diets supplemented with rape seed oil was analysed. The total identified fatty acids (mg/g sample) extracted using diethyl ether or dichloromethane in both examined duodenal digesta did not differ significantly. According to Palmquist and Jenkins (2003) diethyl ether extracts significant amounts of non-nutritive, nonsaponifiable lipids from forages (waxes, chlorophyll, etc.), and often incompletely extracts lipids of nutritional value, especially fatty acids present as salts of divalent cations. Preextraction hydrolysis of the tested material with 2 M NaOH should influence the extraction of microorganism membrane lipids that are present in the analysed digesta. This resulted in similar or even higher amounts of fatty acids as methyl esters. Significantly higher amounts of C18:0, C18:1 *trans* 9, C18:1 *cis* 9 and C18:2 *cis* 9, *cis* 12 (mg/g sample) were found in duodenal digesta of sheep fed diets supplemented with linseed oil when solvent D was applied. A significantly higher

Table 1. Fatty acid composition in feed - comparison of two solvents

Feed	Fatty acids mg/g sample	Type of solvent		P-value
		D	M	
		n=6		
Hay	Total identified fatty acids	2.91 ± 0.17	3.51 ± 0.15	0.230
	total C18	1.70 ± 0.04	1.68 ± 0.05	0.961
	C18:0	0.57 ± 0.01	0.56 ± 0.03	0.545
	C18:1	0.29 ± 0.02	0.29 ± 0.02	0.767
	C18:2	0.37 ± 0.01	0.37 ± 0.03	0.845
	C18:3	0.47 ± 0.01	0.46 ± 0.01	0.784
Concentrate R	Total identified fatty acids	16.85 ± 11.46	12.05 ± 6.85	0.123
	total C18	12.58 ± 8.33	8.21 ± 4.27	0.090
	C18:0	1.76 ± 1.26	1.42 ± 0.88	0.128
	C18:1	7.17 ± 5.68	4.31 ± 3.01	0.102
	C18:2	2.77 ± 0.75	1.89 ± 0.37	0.057
	C18:3	0.88 ± 0.53	0.59 ± 0.39	0.076
Concentrate L	Total identified fatty acids	30.22 ± 25.07	26.15 ± 16.77	0.598
	total C18	22.48 ± 15.80	17.55 ± 11.44	0.410
	C18:0	1.78 ± 1.21	1.72 ± 0.99	0.869
	C18:1	15.85 ± 15.44	11.95 ± 9.15	0.396
	C18:2	4.27 ± 2.28	3.43 ± 1.11	0.348
	C18:3	0.58 ± 0.36	0.45 ± 0.19	0.337

data are presented as means ± SD; D - diethyl ether; M - dichloromethane; concentrate R - concentrate supplemented with rapeseed oil; concentrate L - concentrate supplemented with linseed oil

Table 2. Fatty acids composition in duodenal digesta - comparison of two solvents

Fatty acids mg/g sample	Experiment	Type of solvent		P-value
		D	M	
		n=8		
Total identified fatty acids	R	70.81 ± 47.73	73.56 ± 49.15	0.083
	L	63.44 ± 29.90	59.89 ± 31.32	0.086
Total C18	R	52.88 ± 35.59	53.44 ± 36.36	0.231
	L	44.41 ± 24.08	38.82 ± 23.06	0.002
C18:0	R	36.36 ± 24.95	37.09 ± 25.59	0.132
	L	27.38 ± 14.07	25.05 ± 14.56	0.001
C18:1 *trans* 9	R	6.57 ± 5.36	6.50 ± 5.49	0.370
	L	7.22 ± 7.88	5.84 ± 6.30	0.042
C18:1 *cis* 9	R	7.68 ± 5.35	7.53 ± 5.21	0.074
	L	7.34 ± 3.81	6.08 ± 3.53	0.003
Total C18:1	R	14.25 ± 10.61	14.03 ± 10.61	0.046
	L	14.56 ± 11.14	11.92 ± 9.30	0.001
C18:2 *cis* 9, *cis* 12	R	1.69 ± 0.33	1.67 ± 0.35	0.243
	L	1.50 ± 0.31	1.12 ± 0.29	0.000
C18:2 *cis* 9, *trans* 11	R	0.09 ± 0.04	0.08 ± 0.04	0.001
	L	0.15 ± 0.14	0.10 ± 0.10	0.013
C18:3 *cis* 9, *cis* 12, *cis* 15	R	0.48 ± 0.12	0.46 ± 0.11	0.013
	L	0.83 ± 0.50	0.63 ± 0.37	0.013

data are presented as means ± SD; D - diethyl ether; M - dichloromethane; R - samples from Experiment 1 using rapeseed oil; L - samples from Experiment 2 with linseed oil

extraction efficiency of diethyl ether in comparison with dichloromethane was also noticed in total C18:1, as the values reached higher levels in the linseed oil as well as in the rape seed oil groups. Statistically significant differences between the two solvents for both digesta samples (rape seed and linseed oil) were also found for C18:2 *cis* 9, *trans* 11 and C18:3 *cis* 9, *cis* 12, *cis* 15, which may suggest that the diethyl ether extraction method was more efficient.

The higher extraction efficiency may be explained by the chemical affinity of this solvent. Electron-deficient double bonds of unsaturated fatty acids lead to the formation of ether oxygen and fatty acid double bond complexes. The extraction efficiency of the tested solvents may, however, depend on the number of unsaturated bonds.

CONCLUSIONS

The present results show that at this stage of knowledge, dichloromethane may be replaced by diethyl ether in fatty acid extraction procedures for feed and duodenal digesta. However, although the diethyl ether method may be more efficient in terms of a higher yield of some fatty acids, full acceptation of diethyl ether as a common solvent for fatty acid extraction requires further investigation. A new extraction solvent is much needed. The diethyl ether method would provide a simpler and more rapid way of preparing free fatty acid extracts and at the same time is less toxic than dichloromethane.

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