A Comparison among Analytical Methods to Assess Fatty Acids and Conjugated Linoleic Acids (CLA) Content and Repeatability of Ruminant Faeces

Giacomo CESARO (✉)
Luca GRIGOLETTO
Giovanni BITTANTE
Stefano SCHIAVON

Summary

Methods to determine fatty acids (FAs) and CLA contents of faeces should limit isomerisation, provide a good repeatability of the measures, avoid the use of harmful substances. Three methods of FAs extraction from faeces for GC analysis were compared: Est-DF_{tol}, based on extraction and esterification of FAs contained in dry faeces using Na-methoxide, methanolic-HCl and toluene as solvent; Est-EE_{tol}, based on acid-base extraction and esterification of FAs on the faecal ether extract (EE), using toluene as solvent; and AEst-$EE_{hept,}$ based on an acid catalyzed esterification of FAs contained in EE, using *n*-heptane as solvent. Faeces were collected from bulls receiving 0, 8 and 80 g/d of rumen protected CLA (rpCLA). The faeces of 9 bulls (3 for each dose) were analysed in triplicates by each method. Methods were compared by linear regression. The measurements performed with Est-EE_{tol} and AEst-EE_{hept} regressed against those of Est-DF_{tol}, evidenced, in particularly for CLA isomers and their sum, positive intercepts and slopes significantly lower than the unity. The proportions of c18:2,*t*9,*t*11 found with Est-DF_{tol} and AEst-EE_{hept} were correlated to the dose of rpCLA (R = 0.87 and 0.51, respectively), whereas those found with Est-EE_{tol} did not (R = 0.17). The Est-DF_{tol} method is recommended because it minimizes the isomerisation of the polyunsaturated fatty acids and yields a more accurate measurement of the FAs profile.

Key words

CLA, faeces, fatty acid, gas chromatography, repeatability

University of Padova, Department of Animal Science,
Viale dell'università 16, 35020 Legnaro (Pd), Italy
✉ e-mail: giacomo.cesaro@studenti.unipd.it

Received: May 31, 2011 | Accepted: July 20, 2011

ACKNOWLEDGEMENTS
The authors greatly appreciate the technical assistance of all the people of the laboratory staff. Thanks to the Autonomous Province of Trento for founding and also to SILA s.r.l. (Noale, VE, Italy) for providing the rumen protected CLA used in this trial.

Aim

The analysis of FAs and CLA contents in the faeces of ruminants is useful in studies on digestibility and metabolism of individual FAs. This study was aimed to compare three analytical methods for measuring the faecal FAs profile and their CLA contents, considering their effects on CLA isomerisation and the repeatability of their profile measurements.

Materials and methods

This study is part of a research program carried out at the experimental farm of the University of Padova aimed to evaluate the effects of diets supplemented with rumen protected CLA (rpCLA) (Sila s.r.l., Noale, Italy) on ruminants (Dal Maso et al., 2008, 2009; Schiavon et al., 2010). Fifty-four crossbred young bulls and heifers were fed a total mixed ration supplemented with 0, 8 or 80 g/d of rpCLA from about 5 to 16 months of age. The rpCLA supplement consisted of methyl esters of CLA bound to a silica matrix and coated with hydrogenated soybean oil. The lipid-coated rpCLA was composed of 800, 178, and 22 g/kg of lipid, ash, and moisture, respectively. The lipid portion contained 456 g/kg of palmitic and stearic acids, 79.2 and 76.8 g/kg of *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA isomers, respectively, and 91 g/kg of other FAs (Schiavon et al., 2011).

Faecal grab samples were collected from 9 bulls (3 for each rpCLA dose) after 180 days on trial. The faeces were oven dried (55°C), finely ground (1 mm screen) and stored at 4°C till the analysis. Before the GC analysis, the faecal samples were processed in triplication according to the following methods:

I. Acid-base esterification of FAs performed directly on the dry faecal samples (Est-DF_{tol}). The procedures described by Sukhija and Palmquist (1988) and later modified by Jenkins (2010) were applied using toluene as non-polar solvent and methyl 12-tridecenoate (2 mg/mL in toluene) as internal standard.

II. Acid-base esterification of FAs performed on the EE recovered from faeces (Est-EE_{tol}).The esterification procedure described by Sukhija and Palmquist (1988) and later modified by Jenkins (2010) was applied to the EE recovered from the faeces as described by Sanderson (1986).

III. Acid catalyzed methylation of FAs performed on EE extracted from faeces (AEst-EE_{hept}).The method proposed by Christie (1993) was performed on the EE recovered from the faeces (Sanderson, 1986) as done for Est-EE_{tol}, but using *n*-heptane as organic solvent and methyl 12-tridecenoate as internal standard (0.6 mg/mL in *n*-heptane).

Gas chromatography analysis. The samples obtained with the 3 different procedures were analysed for their FA profile using a double column GC (Agilent Technologies 7890 A, CA, United States) equipped with a modulator (Agilent G3486A CFT, CA, USA), an automatic sampler (Agilent 7693, CA, USA), a FID detector connected with a chromatography data system software (Agilent ChemStation, CA, USA). The operative conditions of the GC apparatus were: first column of 60 m × 180 µm (i.d.) × 0.2 µm (film thickness) (Agilent custom HP88, CA, USA) flow of 0.2 mL/min increased to 0.3mL/min at a rate of 0.003 mL/min; second column of 3 m × 250 µm (i.d.) × 0.25 µm (film thickness) (Agilent HP-50$^+$, CA, USA) flow of 24 mL/min held for 58 min then increased to 25 mL/min at a rate of 0.1 mL/min. Planned oven temperature variation: increase from 120°C (held for 5 min) to 150°C (held for 20 min) at 8°C/min and then increased to 240°C (held for 20 min) at 2°C/min. Valves: modulation delay, 1 min; modulation period, 3 sec; sample time, 2.85 sec. Gas flows: hydrogen, 20 mL/min; air, 450 mL/min. Sample injection: 0.8 µL (pulsed split mode, injection pressure 1.724 bar × 0.3 min, split ratio 150:1). The resulting three-dimensional chromatograms were analysed with the comprensive GC × GC software (Zoex Corp., TX, USA) to evaluate the volumes of each fatty acid peak. Fatty acids were identified by comparison of the peaks position in the samples with peaks position of fatty acids presents in a GC reference standard (674 nu-chek prep, inc. MN, USA), which was a mixture of 52 pure FAs, and in *c*9*t*11 CLA and *t*10*c*12 CLA standards (Nu-Chek Prep, Inc. MN, USA). The proportion of single FA was expressed as proportion of single FA peak volume in comparison to total FAs volume.

Statistical analysis. The FAs composition of the faeces obtained for each method were analysed using the Proc MIXED, and the REML procedure, of SAS 9.2 (SAS Institute Inc., NC, USA) with a hierarchic model which considered the dose of rpCLA as fixed effect, the animal within dose as random effect, and the residual. The dose of CLA was tested using animal as error line. The results were expressed as root of the variances (standard deviations) of the effects of rpCLA dose, animal and the residual (RSD). It was found, using Bartlett's test (Bartlett, 1937) in SAS 9.2 (SAS Institute Inc., NC, USA), that the variances associated to the various methods were not homoscedastic, and so use of ANOVA models was not applicable to compare effects of methods. Thus, the various methods were compared by linear regression PROC REG of SAS 9.2 (SAS Institute Inc., NC, USA) using the means obtained from the three replications available for each animal (n = 9) and testing the differences of intercepts and slopes from zero and the unity, respectively.

Results and discussion

Lipid reaching the intestines of ruminant species is similar in quantity but dramatically different in structure compared with lipid consumed (Jenkins and Lee, 2007). An accurate and precise FAs profile quantification of the faeces is important for nutritional studies aimed to evaluate the effects of animals and of diets, especially in the case of rpCLA integration. The acid catalysis AEst-EE_{hept} method applied to the faecal acid hydrolysed EE provides strong condition and prolonged high temperature reactions. The acid-base esterification of FAs with the Est-EE_{tol} method acts with milder conditions and shorter incubation periods compared to the previous method, but includes the same acid hydrolysis and ether extraction of fat from the faeces. The Est-DF_{tol} procedure provides mild conditions for the extraction of FAs and was applied directly to the dry faecal samples.

The three methods differed for RSD, a measure of repeatability. Overall, the AEst-EE_{hept} method proposed in the present paper, showed, for almost all FAs and their sums, the lower RSD compared to Est-EE_{tol}, while the RSD obtained for Est-DF_{tol} was intermediate (Table 1). It is also interesting to note that the effect of rpCLA on the proportions of C18:2,*t*10,*c*12, of C18:2,*t*9,*t*11 and of CLA sum was highly significant (P<0.01) only with the Est-DF_{tol} method, whereas with the other two methods the effect of the rpCLA was much smaller for C18:2,*t*10,*c*12 and not significant for C18:2,*t*9,*t*11 and CLA sum.

Table 1. Means and standard deviations due to the dose of rumen protected CLA (rpCLA), to the animal and the residual of major fatty acids, CLA and FAs groups contents (in %) of feces assessed with three different analytical methods[1]

Method	Est-DF_{tol}[2]				Est-EE_{tol}[3]				AEst-EE_{hept}[4]			
	Mean	Standard deviation			Mean	Standard deviation			Mean	Standard deviation		
		rpCLA	Animal	Residual		rpCLA	Animal	Residual		rpCLA	Animal	Residual
Major fatty acids (FAs)												
C14:0 Myristic	1.541	0.520	0.645	0.047	1.611	0.305	0.718	0.049	1.867	0.280	0.776	0.057
C15:0 Pentadecanoic	2.484	1.514	0.706	0.055	2.033	1.221*	0.505	0.245	2.108	1.170	0.599	0.040
C16:0 Palmitic	15.535	3.706*	1.531	0.121	16.825	4.053*	1.587	0.163	17.572	3.755*	1.614	0.170
C18:0 Stearic	51.761	13.691	7.905	0.325	49.748	14.565	8.092	0.879	47.055	12.137	7.601	0.498
C18:1,*t*11 Vaccenic	4.226	0.318	0.581	0.107	3.264	0.423	0.383	0.100	3.363	0.165	0.477	0.108
C18:1,*c*9 Oleic	6.885	2.217	2.275	0.233	6.953	1.943	2.083	0.285	7.479	1.706	1.997	0.165
C18:2 Linoleic	6.218	2.734	3.866	0.218	7.095	3.009	3.885	0.223	7.283	2.619	3.632	0.162
CLA												
C18:2,*c*9,*t*11	0.273	0.211	0.108	0.055	0.192	0.018	0.062	0.017	0.200	0.025	0.066	0.009
C18:2,*t*10,*c*12	0.175	0.427**	0.089	0.021	0.112	0.140*	0.043	0.038	0.103	0.209**	0.063	0.029
C18:2,*t*9,*t*11	0.123	0.105**	0.028	0.038	0.246	0.027	0.083	0.016	0.255	0.084	0.080	0.020
CLA sum	0.554	0.762**	0.182	0.057	0.550	0.177	0.179	0.048	0.558	0.317	0.182	0.031
Groups of FAs												
SFA	80.586	4.848	6.366	0.410	80.465	5.924	6.047	0.565	79.390	4.332	5.737	0.271
MUFA	11.720	2.473	2.347	0.209	11.275	2.670	2.042	0.388	12.068	1.866	1.978	0.217
PUFA	7.693	2.397	4.111	0.220	8.260	3.245	4.089	0.252	8.542	2.472	3.861	0.171

[1] Data were on 3 bulls×3 doses of rpCLA (0, 8 and 80 g/d)×3 replications with animal nested within rpCLA. [2] Method based on acid-base extraction and esterification of fatty acids (FAs) directly performed on dried faeces using toluene as solvent (Sukhija and Palmquist, 1988, modified by Jenkins, 2010). [3] Method based on petroleum ether extraction (EE) (Sanderson, 1986), acid-base extraction and esterification of FAs contained in faecal EE using toluene as solvent (Jenkins, 2010). [4] Method based on acid extraction and esterification of FAs presents in the faecal EE (Christie, 1993) using *n*-heptane as solvent. Statistical differences are evidenced as ***= P<0.001; **= P<0.01; *= P<0.05.

Table 2. Relationships among methods[1]: intercepts, slopes of the regressions and R^2

Method	Est-EE_{tol}[3] *vs.* Est-DF_{tol}[2]			AEst-EE_{hept}[4] *vs.* Est-DF_{tol}[2]			AEst-EE_{hept}[4] *vs.* Est-EE_{tol}[3]		
	intercept	slope	R^2	intercept	slope	R^2	intercept	slope	R^2
Major fatty acids (FAs)									
C14:0 Myristic	0.090	1.007	0.941***	0.270	1.057	0.899***	0.148	1.067	0.987***
C15:0 Pentadecanoic	0.112	0.774*	0.932***	0.169	0.779*	0.940***	0.101	0.985	0.964***
C16:0 Palmitic	0.380	1.068	0.985***	1.993	1.013	0.969***	1.558*	0.953	0.992***
C18:0 Stearic	-4.571*	1.043	0.995***	-0.851	0.920	0.989***	3.208	0.881**	0.992***
C18:1,*t*11 Vaccenic	0.405	0.677*	0.816***	0.432	0.693	0.750**	0.345	0.922	0.747**
C18:1,*c*9 Oleic	0.693**	0.906**	0.995***	1.612***	0.848**	0.990***	0.972**	0.935	0.992***
C18:2 Linoleic	0.681*	1.014	0.991***	1.445**	0.937	0.989***	0.828**	0.922*	0.994***
CLA									
C18:2,*c*9,*t*11	0.137**	0.212***	0.323	0.130**	0.268***	0.457*	0.002	1.036	0.945***
C18:2,*t*10,*c*12	0.047**	0.379***	0.947***	0.012	0.525***	0.972***	-0.047*	1.334*	0.951***
C18:2,*t*9,*t*11	0.208**	0.339	0.117	0.169**	0.717	0.426	0.004	1.012	0.830***
CLA sum	0.373**	0.324***	0.566*	0.288**	0.490***	0.834***	-0.089	1.169	0.884***
Groups of FAs									
SFA	0.631	0.991	0.983***	7.244*	0.896*	0.991***	7.688	0.891	0.980***
MUFA	0.447	0.921	0.969***	2.456***	0.817***	0.991***	2.389*	0.858	0.957***
PUFA	0.358	1.027	0.982***	1.289**	0.942	0.986***	1.022*	0.910*	0.988***

[1] Data were on 3 bulls×3 doses of rpCLA (0, 8 and 80 g/d) averaging 3 replications performed for each bull (n = 9). [2] Method based on: acid-base extraction and esterification of fatty acids (FAs) presents in the faeces using toluene as solvent (Sukhija and Palmquist, 1988, modified by Jenkins, 2010). [3] Method based on: petroleum ether extraction of lipid (Sanderson, 1986) and acid-base extraction and esterification of FAs contained in the faecal EE using toluene as solvent (Jenkins, 2010). [4] Method performed on the EE (Sanderson, 1986) applying Christie's (1993) acid extraction and esterification of FAs using *n*-heptane as organic solvent. Statistical differences of intercept and slopes from zero and unity, respectively, and of the regression model are evidenced as ***= P<0.001; **= P<0.01; *= P<0.05.

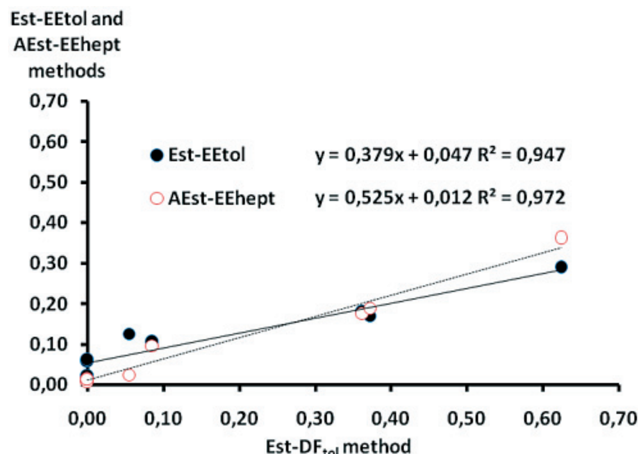

Figure 1. Relationships between the proportions of C18:2,t10,c12 (% total FA) assessed with different methods: Est-DFtol based on acid-base extraction and esterification of fatty acids (FAs) directly performed on dried faeces using toluene as solvent (Sukhija and Palmquist, 1988, modified by Jenkins, 2010); Est-EEtol based on petroleum ether extraction (EE) (Sanderson, 1986), acid-base extraction and esterification of FAs contained in faecal EE using toluene as solvent (Jenkins, 2010); and AEst-EEhept based on acid extraction and esterification of FAs presents in the faecal EE (Christie, 1993), but using n-heptane as solvent. Data were on 3 bulls×3 doses of rpCLA (0, 8 and 80 g/d) averaging 3 replications performed for each bull (n=9)

The three methods produced comparable results in term of means of the various FAs concentrations and of their sums, with some exceptions (Table 1). In particularly, it was observed that the sum of the various CLA isomers was comparable among the three methods, being on average 0.55%, but the proportions of individual CLA isomers were not. With Est-DF_{tol} the mean proportions of C18:2,*c*9,*t*11 (0.27%) and of C18:2,*t*10,*c*12 (0.18%) were higher compared to the corresponding values obtained with Est-EE_{tol} (0.19 and 0.11%, respectively) and with AEst-EE_{hept} (0.20 and 0.10%, respectively). On the opposite, with the Est-DF_{tol} the mean proportion of the C18:2,*t*9,*t*11 (0.12%), which likely results from isomerisation due to the sample processing, was much smaller than the mean values obtained for Est-EE_{tol} (0.25%) and for AEst-EE_{hept} (0.26%). The measurements performed with Est-EE_{tol} regressed against those of Est-DF_{tol}, evidenced in many cases, but in particularly for CLA isomers and their sum, a significant positive intercept and a slope significantly lower than the unity (Table 2, Figure 1). The same was observed when the AEst-EE_{hept} measures were regressed against the Est-DF_{tol} ones. On the opposite the measurements obtained from AEst-EE_{hept} and Est-EE_{tol}, were linearly related, with some exceptions. In addition, it was found that the proportions of c18:2,*t*9,*t*11 found with Est-DF_{tol} were, as expected, correlated to the dose of rpCLA (R = 0.87), whereas those found with Est-EE_{tol} (R = 0.17) and with AEst-EE_{hept} (R = 0.51) did not. These results indicated that both the methods based on the EE have likely induced a FA isomerisation compared to the method based on the direct treatment of the dry faeces. To this regard it is confirmed, as indicated by Kramer et al. (1997), that all acid catalyzed procedures result in an increased concentration of C18:2,*t*9,*t*11. These results indicated that the Est-DF_{tol} method was more efficient to detect differences due to increasing dosage of rpCLA.

Conclusions

The results reported in this work are addressed to avoid common analytical errors yielding inaccurate results during analysis of fatty acids in feed and digesta samples and to produce a more effective measurement of lipid with nutritional values (Palmquist and Jenkins, 2003). The results show that Est-DF_{tol} is the most recommendable method for determining the CLA content of faeces, as it is least burdened with the side reactions during FAME preparation compared to the other ones. These results are useful for nutritional studies regarding the lipid components of feeds and faeces.

References

Bartlett M. S. (1937). Properties of sufficiency and statistical tests. Proceedings of the Royal Society of London, Series A, 160, 268-282.

Christie W. W. (1993). Preparation of ester derivatives of fatty acids for chromatographic analysis. In: Advances in Lipid Methodology-Two (WW Christie, ed), Oily Press, Dundee, Scotland, 69-111.

Dal Maso M., Schiavon S., Bailoni L., Tagliapietra F., Bittante G. (2008). Low doses of rumen protected conjugated linoleic acid (CLA) on dairy cows in mid lactation: effects on milk yield and quality. In: Book of abstracts of the 59th Meeting of the EAAP, vol 14. Vilnius, Lithuania. Wageningen Academic publishers, p 146.

Dal Maso M., Schiavon S., Tagliapietra F., Simonetto A., Bittante G. (2009). Growth performance and N excretion of double muscled Piemontese bulls fed low protein rations with or without the addition of rumen protected conjugated linoleic acid. Ital J Anim Sci 8(3): 175-177.

Jenkins T. C., Lee Y. J. (2007). An overview of fatty acid biotransformations in the rumen. J Anim Sci 85: Suppl 2: 127.

Jenkins T. C. (2010). Technical note: Common analytical errors yielding inaccurate results during analysis of fatty acids in feed and digesta samples. J Dairy Sci 93: 1170-1174.

Kramer J. K. G., Fellner V., Dugan M. E., Sauer F. D., Mossoba M. M., Yurawecz M. P. (1997). Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. Lipids 32: 1219-1228.

Palmquist D. L., Jenkins T. C. (2003). Challenges with fats and fatty acid methods. J Anim Sci 81: 3250-3254.

Sanderson P., (1986). A new method of analysis of feeding stuffs for the determination of crude oils and fats. In: Recent Advances in Animal Nutrition (W Haresign and DJA Cole, eds), Butterworths, London, 77-80.

Schiavon S., Tagliapietra F., Dal Maso M., Bailoni L., Bittante G. (2010). Effect of low protein diets and rumen protected conjugated linoleic acid on production and carcass traits of growing double-muscled Piemontese bulls. J Anim Sci 88: 3372-3383.

Schiavon S., De Marchi M., Tagliapietra F., Bailoni L., Cecchinato A., Bittante G. (2011). Effect of high or low protein ration combined or not with rumen protected conjugated linoleic acid (CLA) on meat CLA content and quality traits of double-muscled Piemontese bulls. Meat Sci. 89: 133-142.

Sukhija P. S., Palmquist D. L. (1988). Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J Agric Food Chem 36: 1202-1206.

acs76_69