Technical note: Common analytical errors yielding inaccurate results during analysis of fatty acids in feed and digesta samples¹

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ABSTRACT

The basic rules governing the proper fatty acid analysis of feed and digesta samples are sometimes overlooked, leading to potential errors in reporting the fatty acid content or composition of feed and digesta samples. The direct transesterification procedure of Sukhija and Palmquist (1988, J. Agric. Food Chem. 36:1202–1206) has become popular in analyzing fatty acids in feed and digesta samples obtained from animal feeding trials. One shortcoming of the Sukhija and Palmquist transesterification procedure is inaccurate analysis of fatty acids with conjugated double bonds. Digesta and milk samples from ruminant species typically contain a multitude of conjugated linoleic acid (CLA) isomers that easily undergo isomerization and epimerization following prolonged exposure to methanolic HCl. Modifications to the Sukhija and Palmquist procedure are given in this paper that allow successful determination of CLA isomers. Errors in fatty acid analysis also occur from misuse of internal standards; use of an internal standard is recommended in the Sukhija and Palmquist procedure as the preferred method to quantify total fatty acid content. The choice of internal standard may sometimes be important for obtaining accurate results. As an example, applying the direct transesterification procedure to a fat supplement high in saturated fatty acids yielded 613 mg/g of total fatty acids when C17 was used as the internal standard compared with 930 mg/g total fatty acids when C19 was used as the internal standard. Fatty acid content further increased to 952 mg/g when a unique unsaturated fatty acid (C13:1) was used as the internal standard.

Key words: fatty acid, method, methylation, internal standard

Analyses of feed and intestinal tract digesta samples are common in animal research trials aimed at studying

intake, digestion, and excretion of total and individual fatty acids. Fatty acid content and composition of feed and digesta samples are often done by gas chromatography that requires conversion of fatty acids to derivatives having reduced boiling points suitable for vaporization in a heated GC injector. Fatty acids are most often derivatized to methyl esters using a variety of acid- or base-catalyzed methods. Base catalysts do not esterify free fatty acids but generally avoid migration and isomerization of double bonds. Acid catalysts esterify all complex and simple forms of fatty acids but may cause isomerization of conjugated double bonds. Combinations of acid and base catalysts for shorter incubation times minimize epimerization and isomerization of conjugated double bonds, yielding acceptable results for conjugated linoleic acid (CLA) isomers.

One-step transesterification methods are popular in many laboratories because they hydrolyze fatty acids from complex lipid fractions and methylate released free fatty acids in a single step without the need for prior extraction. A common approach to one-step transesterification of fatty acids in feed and digesta was summarized by Sukhija and Palmquist (1988). Their procedure added a strong organic solvent (CHCl₃, benzene, toluene) containing an internal standard to a known quantity of dried sample with 10 to 50 mg of fatty acid. After addition of 10% methanolic HCl, the mixture was heated at 90°C for 2 h and neutralized with 6% K₂CO₃ after cooling. Fatty acid methyl esters were extracted in hexane and then separated and quantified by gas-liquid chromatography. See Palmquist and Jenkins (2003) for a more recent description of this direct transesterification procedure.

Reports of erroneous results from this and similar direct transesterification procedures can often be traced to improper application of the procedure to the specific sample being tested. Variation in sample physical and chemical properties may require adjustments in the procedure to obtain reliable results. Among the most common adjustments needed to the Sukhija and Palmquist (1988) procedure are alterations in methylating conditions when samples contain CLA and careful consideration given to selection of an appropriate internal standard suitable for the sample being tested.

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Table 1. Effect of the standard methylation procedure of Sukhija and Palmquist (1988) or a modified methylation procedure on fatty acid composition (g/100 g of total fatty acids) of a commercial conjugated linoleic acid (CLA) fat source and a commercial high-linoleic acid oil

Item	CLA			Linoleic acid		
	Standard	Modified	SEM	Standard	Modified	SEM
Samples (n)	5	5		5	5	
Fatty acid						
14:0	ND	0.012	0.008	0.020	0.010	0.016
16:0	6.31	5.86	0.167	2.50	2.75	0.19
16:1	ND	0.012	0.008	0.188	0.164	0.033
18:0	3.41	3.22	0.121	0.97	1.36	0.17
<i>trans</i> 18:1	0.214	0.016	0.115	0.264	0.232	0.046
18:1	25.53*	23.53	0.37	28.84	28.86	0.16
18:2	8.80	8.16	0.31	61.26	60.67	0.22
18:3	ND	0.014	0.010	5.88	5.66	0.077
<i>cis</i> -9 <i>trans</i> -11	13.14	21.29*	2.12	ND ¹	ND	
<i>trans</i> -10 <i>cis</i> -12	9.59	16.11*	1.77	ND	ND	
<i>trans</i> -9 <i>trans</i> -11	27.19*	11.96	4.15	ND	ND	
Other	5.82	9.81*	0.90	ND	ND	

¹ND = none determined.*Standard and modified methods differed ($P < 0.05$) within sample type (CLA or high-linoleic oil).

The purpose of this paper is to consider a couple of examples in which error occurs when basic procedural adjustments are not taken into account for certain types of samples, and to offer possible solutions.

The effect of internal standard selection was examined by analyzing 3 samples (milk fat, feces, and a commercial prilled fat) with 2 commonly used internal standards (C17 and C19). The commercial prilled fat (EnergyBooster 100, MSC Inc., Dundee, IL) was chosen to study the influence of internal standard selection because of repeated personal communications that fatty acid values were significantly lower than theoretical levels when analyzed by the transesterification procedure of Sukhija and Palmquist (1988). A total of 6 prilled fatty acid samples (EnergyBooster 100, supplied by MSC Inc.) were analyzed in duplicate using

both the C17 and C19 internal standards. The prilled samples were then reanalyzed using a unique C13:1 internal standard. Milk ($n = 4$) and feces ($n = 3$) were analyzed in triplicate to compare the effects of the 2 internal standards (C17 and C19) on fatty acid profiles of samples obtained from animal trials.

Modifications to the Sukhija and Palmquist (1988) procedure were made to prevent isomerization of CLA isomers. The modifications were based on work done by Kramer et al. (1997) that compared the effects of acid and base catalysts on CLA isomerization during methylation. Samples ($n = 5$) of a commercial high-linoleic acid oil (Continental Industrial Chemicals, Charlotte, NC) or a commercial CLA supplement (CLA-60, Natural Lipids, Hovdebygda, Norway) were methylated either as described by Sukhija and Palmquist (1988)

Table 2. Effect of internal standard (IS) on fatty acid content (mg/g of sample) and composition (g/100 g of total fatty acids) of a commercial prilled fat supplement using the modified method

Item	IS17:0	IS19:0	IS13:1	SEM	P-value
Samples (n)	6	6	6		
Content (mg/g of sample)	61.2	93.02	95.19	0.39	<0.01
Composition (g/100 g of total)					
14:0	3.6	3.53	3.53	0.064	
15:0	0.62	0.60	0.53	0.0059	0.04
16:0	42.76	41.97	41.13	0.089	<0.01
16:1	0.39	0.37	0.45	0.023	
17:0		1.98	1.92	0.0029	<0.01
18:0	36.48	35.58	36.90	0.14	<0.01
<i>trans</i> 18:1	2.89	2.81	2.61	0.013	<0.01
18:1	7.00	6.83	6.17	0.035	<0.01
18:2	0.70	0.72	0.65	0.037	
19:0	0.15		0.16	0.00069	
20:0	0.37	0.36	0.45	0.0016	<0.01
Other	5.02	5.20	5.51	0.21	

or by using a modified transmethylation procedure. The modified procedure involved replacing the sample incubation in 3 mL of 5% methanolic HCl for 2 h in the Sukhija and Palmquist (1988) method with the following:

1. Add 2 mL of sodium methoxide (0.5 M in methanol). Cap tightly and vortex lightly to mix.
2. Incubate in a 50°C water bath for 10 min.
3. Remove from water bath and cool for 5 min.
4. Uncap and add 3 mL of 5% methanolic HCl. Recap tightly and vortex.
5. Incubate in an 80°C water bath for 10 min.
6. Remove from water bath and cool for 7 min.

The standard versus modified procedures were then applied to samples ($n = 4$) of ruminal contents incubated for 10 d (12%/h liquid turnover rate) in continuous fermenters fed 0, 1, 2, and 3% added corn oil. Increasing the dosage of corn oil was expected to elevate CLA concentration and allow assessment of the effect of the standard versus modified methylation procedures on CLA content of digesta samples.

Fatty acid methyl esters were analyzed on an HP 5890A GC (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector, a model 7673A autosampler, and Chemstation chromatography software. Separations were performed on an SP 2380 fused-silica capillary column (100 m $\times$ 0.25 mm i.d., 0.2- μ m film thickness) obtained from Supelco (Bellefonte, PA). The column oven was programmed to increase from 140°C (held for 4 min) to 160°C (held for 44 min) at 13°C/min, and then increased to 220°C (held for 20 min) at a rate of 4°C/min. Helium was used as the carrier (20 cm/s) and make-up gas. Temperatures of the injector and detector were 250°C. The methyl ester peaks were identified based on comparison of their retention times to those of pure standards.

Data on fatty acid contents and compositions of all samples were analyzed as a completely randomized design using the general linear models procedure of SAS (SAS Institute, 2008). Means were considered different at $P < 0.05$.

Notable differences were observed between fatty acid composition of the CLA fat source when methylated by the standard Sukhija and Palmquist (1988) procedure versus the modified procedure described in this paper (Table 1). According to the supplier, the fatty acid profile contained 50 to 60% CLA consisting mainly of the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers and <10% of the *trans*-9,*trans*-11 isomer. The fatty acid profile from the modified transmethylation procedure matched these specifications. The CLA profile obtained by the standard procedure was much different than that stated

Figure 1. Comparison of the standard versus modified direct transesterification methods on concentration of conjugated linoleic acid isomers (g/100 g of total fatty acids; *c* = *cis*, *t* = *trans*) in ruminal contents obtained from continuous fermenters fed an increasing dosage of corn oil (% of diet). Color version available in the online PDF.

by the supplier. The standard Sukhija and Palmquist (1988) methylation procedure yielded about the same total CLA content (55.6%) but showed a major shift in isomer composition. *Trans*-9,*trans*-11 was the primary isomer at the expense of both the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers. Differences in other fatty acids between the standard and modified procedures were much smaller.

Similar differences between the standard and modified procedures were seen for CLA concentration in ru-

Table 3. Effect of internal standard (IS) on fatty acid content (mg/g of sample) and composition (g/100 g of total fatty acids) of a centrifuged milk fat sample and fecal sample using the modified method

Item	Milk		Feces	
	IS17:0	IS19:0	IS17:0	IS19:0
Samples (n)	6	6	3	3
Content (mg/g of sample)	565.1	574.7	34.5	36.7
Composition (g/100 g of total)				
6:0	2.12	2.25		
8:0	1.06	1.12		
10:0	2.22	2.28		
11:0	0.199	0.206		
12:0	2.46	2.49	0.78	0.39
14:0	8.65	8.68	2.51	2.56
14:1	0.48	0.47	0.35	0.42
15:0	0.72	0.71	0.96	0.96
16:0	25.4	25.6	34.19	33.73
16:1	1.35*	0.24	0.53	0.51
17:0	ND ¹	0.105		1.32*
18:0	15.4	15.5	32.15	30.97
<i>trans</i> 18:1	4.60	4.52	3.61	3.66
18:1	21.79	21.78	8.41	8.35
18:2	3.84	3.82	4.67	4.58
19:0	0.560	ND	0.19*	
20:0	0.056	0.156	0.75	0.75
20:1	0.484	0.463		
18:3	0.045	0.021		
<i>cis</i> -9, <i>trans</i> -11 CLA ²	0.75	0.75	0.14	0.11
<i>trans</i> -10, <i>cis</i> -12 CLA	0.053	0.043		
<i>trans</i> -9, <i>trans</i> -11 CLA	0.143	0.133		
22:0	0.190	0.187	0.51	0.53
Other	7.43	8.47	9.62	10.52

¹ND = none determined.²Conjugated linoleic acid.*IS17:0 and IS19:0 fatty acids differed ($P < 0.05$) within sample type (milk or feces).

minal contents obtained from continuous culture. Corn oil addition increased ($P < 0.05$) total CLA concentration in culture contents (Figure 1), but the modified method yielded higher *cis*-9,*trans*-11 ($P = 0.025$) and lower ($P = 0.001$) *trans*-9,*trans*-11 concentrations than the standard method.

The high-linoleic acid oil was analyzed by using both the standard and modified methylation procedures to determine if the modified procedure gave equivalent results for non-CLA fat sources. No differences were seen in fatty acid profile between the standard and modified methods. From these results, the modified methylation procedure presented in this paper should be used in place of the Sukhija and Palmquist (1988) method for any samples in which CLA are of interest. This would apply to feed samples containing high-CLA lipid sources, rumen in vitro contents, and any samples from ruminant species potentially containing CLA isomers including digesta, blood, milk, and body tissues.

The selection of internal standard had major consequences on total fatty acid content of the prilled fatty acid source (Table 2). The product analyzed at only

61% total fatty acid content using C17 as the internal standard. Because the product is advertised to consist of only free fatty acids, a theoretical value of 100% fatty acid content would be expected. Additional nutritional analysis of the product revealed 2 to 3% nonlipid components consisting of water, N, and ash. Thus, theoretical fatty acid values >95% are more reasonable. When the product was analyzed by the standard Sukhija and Palmquist (1988) procedure using C19 as internal standard, the fatty acid content averaged 93%. Because the product contains 2% C17, use of C17 as an internal standard grossly underestimated the total fatty acid content but had only a minimal effect on fatty acid composition (aside from not reflecting C17 content).

The product contained a much smaller concentration of C19 (0.15%). To assess the effect of using C19 as an internal standard, the samples were analyzed a third time using a unique fatty acid (C13:1). Total fatty acid content increased to 95.2% for C13:1, again with only minimal changes on fatty acid composition. Fatty acid results were initially lower for C13:1 because of some

merging of C13:1 with adjacent fatty acid peaks. Column temperature programs were adjusted to minimize the merging and maximize fatty acid recovery.

The effect of C17 versus C19 internal standards on milk and feces samples is seen in Table 3. The milk sample selected for this study contained 0.11% C17 and 0.56% C19. The 2 internal standards did not differ in total fatty acid content or in fatty acid composition except for C16:1. The discrepancy with C16:1 might have arisen from its low value combined with its closeness to the C17:0 internal standard peak, resulting in a shift in the manner in which the baseline was drawn.

The results show the importance of selecting a unique fatty acid as an internal standard that separates well from adjacent peaks. The C17 and C19 standards are used routinely in the Sukhija and Palmquist (1988) procedure and work well for most feed and digesta samples,

as shown here for milk and feces. As demonstrated by the commercial prilled product, however, serious errors in fatty acid content can be made if the choice of internal standard is not given serious consideration when analyzing unique samples.

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