Direct Methylation Procedure for Converting Fatty Amides to Fatty Acid Methyl Esters in Feed and Digesta Samples†

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Two direct methylation procedures often used for the analysis of total fatty acids in biological samples were evaluated for their application to samples containing fatty amides. Methylation of 5 mg of oleamide (*cis*-9-octadecenamide) in a one-step (methanolic HCl for 2 h at 70 °C) or a two-step (sodium methoxide for 10 min at 50 °C followed by methanolic HCl for 10 min at 80 °C) procedure gave 59 and 16% conversions of oleamide to oleic acid, respectively. Oleic acid recovery from oleamide was increased to 100% when the incubation in methanolic HCl was lengthened to 16 h and increased to 103% when the incubation in methoxide was modified to 24 h at 100 °C. However, conversion of oleamide to oleic acid in an animal feed sample was incomplete for the modified (24 h) two-step procedure but complete for the modified (16 h) one-step procedure. Unsaturated fatty amides in feed and digesta samples can be converted to fatty acid methyl esters by incubation in methanolic HCl if the time of exposure to the acid catalyst is extended from 2 to 16 h.

Keywords: *Fatty acids; fatty amides; feed; digesta; methylation procedures*

Fatty acid digestibility is determined in animal metabolism trials by subtracting fatty acid excreted in feces from fatty acid consumed over a defined period of time. A preferred method of fatty acid analysis determines the total fatty acid concentration in feed and fecal samples by converting fatty acyl components in all lipid classes, such as triacylglycerols, phospholipids, and sphingolipids, to methyl esters, which are then quantified by gas chromatography (GC).

Several convenient techniques have been published for the direct methylation of all fatty acyl components in ground feed and fecal samples without the need for prior extraction. Outen et al. (*1*) described a general procedure, using 5% (w/v) methanolic HCl as the catalyst, for the direct methylation of fatty acids commonly found in the feed, digesta, and feces of sheep. Direct transesterification with 5% methanolic HCl was later shown to completely hydrolyze and esterify calcium salts of fatty acids commonly found in digesta and feces of ruminants (*2*). However, acid-catalyzed methylation was reported (*3*) to cause isomerization and loss of conjugated dienes found in milk and digesta samples. Isomerization can be avoided by methylating samples with sodium methoxide in methanol for 10 min followed by HCl in methanol for 10 min (*3*).

Fatty amides have recently been investigated as feed additives for cattle rations as a means to increase unsaturated fatty acids in plasma (*4*) and milk (*5*). Fatty amides resist hydrogenation by gut microorganisms in the forestomach of ruminants, which allows for increased delivery of unsaturated fatty acids to the small intestine for absorption. Fatty amides that reach the small intestine in rat studies were reported to undergo extensive hydrolysis by intestinal amidases, causing release and absorption of the fatty acyl component (*6*).

Direct methylation procedures commonly used for analysis of feed and digesta samples are effective for the methylation of long-chain fatty amides (R−CONH−R) present in biological lipids, such as sphingomyelin, but it is not known if they can methylate simple amides of fatty acids (R−$CONH_2$). Two direct methylation procedures were examined in this study for their abilities to convert oleamide (*cis*-9-octadecenamide) to a methyl ester. Also, several modifications of these methylation procedures were made in an attempt to improve the conversion of fatty amides to fatty acid methyl esters (FAME).

MATERIALS AND METHODS

Reagents. All solvents were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA). Sodium methoxide in methanol (0.5 M), reagent grade oleamide, acetyl chloride, and heptadecanoic acid were all purchased from Sigma (St. Louis, MO). A commercial grade of oleamide was purchased from Witco Corp. (Memphis, TN) and mixed 5% (w/w) with a sample of freeze-dried animal feed (50% corn silage and 50% concentrate, dry matter basis). Soybean oil was purchased from a local grocery store and was labeled as 100% soybean oil with no additives. A fat source high in conjugated linoleic acid (CLA) was obtained from ConLinCo, Inc. (Detroit Lakes, MN) and identified by the supplier to contain 60−65% CLA. Conjugated diene standards (*cis*-9,*trans*-11-$C_{18:2}$, *trans*-10,*cis*-12-$C_{18:2}$, and *trans*-9,*trans*-11-$C_{18:2}$) of 98+% purity were purchased from Matreya, Inc. (Pleasant Gap, PA). Methanolic HCl was prepared weekly by slowly adding acetyl chloride to methanol (1:10, by volume) with constant stirring.

Methylation Procedures. Transesterification reactions were done in 16 × 125 mm glass culture tubes according to a one-step procedure (methanolic HCl for 2 h at 70 °C) as described by Sukhija and Palmquist (*2*) or a two-step procedure (sodium methoxide for 10 min at 50 °C followed by methanolic HCl for 10 min at 80 °C) as described by Kramer et al. (*3*). Each culture tube contained 1.0 mL of internal standard solution (2 mg of heptadecanoic acid/mL of benzene).

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Oleamide was added to the culture tubes by pipeting 0.5 mL of a solution containing 10 mg of reagent grade oleamide/mL of benzene. Soybean oil and CLA were weighed (15−25 mg) into culture tubes to the nearest 0.1 mg. Approximately 500 mg of a freeze-dried sample of dairy feed containing 5% commercial oleamide was weighed into culture tubes to the nearest 0.1 mg.

Care was taken that the opening of each tube was not chipped or cracked and that they were sealed during incubations by wrapping the threads with Teflon tape and tightly closing with screw caps having Teflon liners.

The one-step procedure of Sukhija and Palmquist (*2*) was modified by increasing the length of incubation in methanolic HCl at 70 °C from 2 h to either 8 or 16 h. The two-step procedure of Kramer et al. (*3*) was modified by increasing the time and temperature of the initial incubation in sodium methoxide without any modifications to the final incubation in methanolic HCl for 10 min at 80 °C. The methoxide incubation at 50 °C was increased from 10 min to either 8 or 16 h. Further modifications included 16 h of incubation in methoxide at either 80, 90, or 100 °C. All incubations were done in a water bath except for the 100 °C incubations that were done in a gravity oven.

Tubes were taken from the water bath or oven after the appropriate incubation time and allowed to cool to room temperature. After cooling, 5 mL of 6% K_2CO_3 was added to each tube, and the contents were then vortexed. Then 2 mL of hexane was added to each tube, and the contents were vortexed and centrifuged (5 min at 1000*g* at room temperature). The upper organic phase was removed by a Pasteur pipet and analyzed directly by GC.

GC Procedures. Fatty acid methyl esters and amide 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 (30 m × 0.25 mm i.d., 0.2 μm film thickness) obtained from Supelco (Bellefonte, PA). The column oven was temperature programmed to increase from 140 °C (held for 3 min) to 220 °C (held for 20 min) at a rate of 3 °C/min. Helium was used as the carrier (20 cm/s) and makeup gas. Temperatures of the injector and detector were 250 °C. The FAME and oleamide peaks were identified on the basis of comparison of their retention times to those of pure standards.

Calculations. The percentage of oleamide recovered as free oleic acid (oleic acid methyl ester × 0.94) was determined by dividing the milligrams of oleic acid analyzed per tube by 4.7 mg of oleic acid added per tube (from 5 mg of oleamide) and then multiplied by 100. Percent recovery of amide was determined by dividing milligrams of amide analyzed by GC divided by 5 mg of oleamide added per tube multiplied by 100.

All recoveries were based on four observations for each time and temperature combination of the one- and two-step procedures. Mean recoveries and their standard deviations were calculated as described by Snedecor and Cochran (*7*). Data on the fatty acid composition of the animal feed containing 5% oleamide were analyzed as a completely randomized design using the general linear models procedure of SAS (*8*). Means were separated by LSD and considered different at $P < 0.05$.

RESULTS AND DISCUSSION

Conversion of the oleamide standard to oleic acid methyl ester was incomplete whether the sample was methylated by using the one-step procedure of Sukhija and Palmquist (*2*) or by using the two-step procedure of Kramer et al. (*3*). Of the 5 mg of oleamide methylated with the one-step procedure, 59% was recovered as oleic acid and 31% was recovered as oleamide (Table 1). The two-step procedure was less effective in converting oleamide to FAME with 16% of the oleamide standard recovered as oleic acid and 72% accounted for as the amide.

Table 1. Effects of Extended Time and Temperature of Two Methylation Procedures on Amide and Oleic Acid Recovery from Oleamide

time	temp (°C)	amide recovered[a] mg	amide recovered[a] %	oleic acid recovered[b] mg	oleic acid recovered[b] %
Two-Step Procedure					
10 min[c]	50	3.61 ± 0.02	72.3 ± 0.3	0.75 ± 0.01	16.0 ± 0.2
8 h	50	3.37 ± 0.04	67.3 ± 0.8	0.92 ± 0.03	19.5 ± 0.6
16 h	50	3.12 ± 0.02	62.5 ± 0.3	1.22 ± 0.03	26.0 ± 0.6
16 h	80	1.85 ± 0.19	37.0 ± 3.9	2.84 ± 0.17	60.5 ± 3.5
16 h	90	0.96 ± 0.01	19.2 ± 0.4	3.93 ± 0.05	83.7 ± 1.0
16 h	100	0.19 ± 0.02	3.7 ± 0.4	4.63 ± 0.12	98.4 ± 2.5
24 h	100	ND[d]	ND	4.82 ± 0.04	102.7 ± 0.8
One-Step Procedure					
2 h[e]	70	1.55 ± 0.01	31.1 ± 0.2	2.77 ± 0.02	58.9 ± 0.5
8 h	70	0.06 ± 0.01	1.1 ± 0.1	4.60 ± 0.14	97.8 ± 3.0
16 h	70	ND	ND	4.71 ± 0.08	100.2 ± 1.8

[a] Based on 5 mg of oleamide added per tube. Values are means ± standard deviations of four observations. [b] Based on 4.7 mg of oleic acid contained in the initial 5 mg of oleamide added per tube. Values are means ± standard deviations of four observations. [c] Time and temperature of the first methylation step (sodium methoxide in methanol) of the two-step procedure as described by Kramer et al. (*3*). Only the times and temperatures for the first step were modified with the second step (methanolic HCl) being held constant at 80 °C for 10 min. [d] Not detected. [e] Time and temperature of the one-step procedure as described by Sukhija and Palmquist (*2*).

Time of incubation was lengthened in the one-step procedure from 2 h to 8 and 16 h in an attempt to increase the recovery of oleic acid from the amide. Conversion of amide to FAME improved as the time of incubation in methanolic HCl increased to 8 and 16 h (Table 1). Only a small amide peak remained after 8 h of incubation in methanolic HCl, and oleic acid recovery increased to 98%. Extending the incubation time of the one-step procedure to 16 h caused complete disappearance of the amide peak with recovery of oleic acid averaging 100%.

Modification of the two-step procedure also proved to be successful in improving the conversion of oleamide to FAME. All changes to the two-step procedure involved only the initial methoxide incubation. The second incubation in methanolic HCl for 10 min at 80 °C was maintained to minimize stereomutation and double-bond migration. Extending the incubation time of oleamide in methoxide to 16 h while holding the temperature constant (50 °C) recovered only 26% of the oleamide as oleic acid. When the time of incubation in methoxide was held constant at 16 h and the temperature was increased from 50 to 100 °C, there was a substantial increase in the recovery of oleic acid. A minimum of 16 h at 100 °C in methoxide was necessary to reduce recovery of amide to <4% and increase oleic acid recovery to >98%. Extending the incubation time in sodium methoxide to 24 h at 100 °C followed by incubation in methanolic HCl for 10 min at 80 °C caused complete disappearance of the amide peak and 103% recovery of oleic acid.

Both modified procedures were then examined for their abilities to handle conjugated dienes. Conjugated dienes are common in digesta and tissue samples obtained from ruminant species and arise from the incomplete hydrogenation of dietary fatty acids by ruminal microbes (*9*). They undergo extensive isomerization and conversion to allylic methoxy artifacts upon exposure to methanolic HCl for as little as 1 h (*3*). Kramer et al. (*3*) proposed the two-step procedure for the analysis of samples containing conjugated dienes which

Table 2. Effect of the Modified Two-Step Methylation Procedure on the Composition of Three Diene Peaks in a Fat Source High in Conjugated Linoleic Acid

time	temp (°C)	diene isomers[a] (%) *cis*-9,*trans*-11	*trans*-10,*cis*-12	*trans*-9,*trans*-11
		Two-Step Procedure[b]		
10 min	50	19.5 ± 0.1	15.9 ± 0.1	5.7 ± 0.2
24 h	100	20.2 ± 0.2	16.8 ± 0.2	5.6 ± 0.04
		One-Step Procedure		
2 h[c]	70	12.5 ± 0.05	10.0 ± 0.05	16.4 ± 0.13

[a] Values are means ± standard deviations of four observations with each isomer expressed as a percentage of the total fatty acids on a weight basis. [b] Time and temperature of the first methylation step (sodium methoxide in methanol) of the two-step procedure as described by Kramer et al. (*3*). The modified two-step procedure consisted of increased time (24 h) and temperature (100 °C) of the first step with the second step (methanolic HCl) being held constant at 80 °C for 10 min. [c] Time and temperature of the one step procedure as described by Sukhija and Palmquist (*2*).

Table 3. Effect of the Modified One-Step (Methanolic HCl for 16 h at 70 °C) versus the Modified Two-Step (Sodium Methoxide for 24 h at 100 °C Followed by Methanolic HCl for 10 min at 80 °C) Procedures on Fatty Acid Content of an Animal Feed Spiked with 5% Oleamide

fatty acid	theoretical[a] (mg/g of feed)	modified one-step (mg/g of feed)	modified two-step (mg/g of feed)
$C_{14:0}$	1.5 ± 0.03	1.4 ± 0.04	0.58 ± 0.03[b]
$C_{16:0}$	6.2 ± 0.13	6.5 ± 0.16	4.4 ± 0.14[b]
$C_{16:1}$	2.4 ± 0.01	2.2 ± 0.08	0.85 ± 0.09[b]
$C_{18:0}$	2.4 ± 0.12	2.4 ± 0.08	1.7 ± 0.06[b]
$C_{18:1}$*trans*	2.0 ± 0.01	2.0 ± 0.09	0.90 ± 0.05[b]
$C_{18:1}$*cis*	32.3 ± 0.22	31.1 ± 1.2	13.8 ± 0.91[b]
$C_{18:2}$	7.9 ± 0.29	8.4 ± 0.18[b]	7.4 ± 0.25
$C_{18:3}$	1.5 ± 0.04	1.4 ± 0.04	1.3 ± 0.10[b]
total	56.9 ± 0.78	56.2 ± 1.7	45.1 ± 1.9[b]
amide	50.0	0.13 ± 0.16	14.1 ± 0.95[b]

[a] Based on separate fatty acid analysis of four samples of feed and four samples of oleamide, which were then combined (95:5 on a weight basis, respectively) to arrive at the theoretical content of each fatty acid. [b] Differs from theoretical ($P < 0.05$).

utilized sodium methoxide in methanol for 10 min at 50 °C followed by incubation in methanolic HCl for 10 min at 80 °C.

A CLA-rich fat source contained 19.5% *cis*-9,*trans*-11-$C_{18:2}$, 15.9% *trans*-10,*cis*-12-$C_{18:2}$, and 5.7% *trans*-9,*trans*-11-$C_{18:2}$ when methylated by the two-step procedure of Kramer (*3*) (Table 2). Exposure of the CLA fat source to methanolic HCl for 2 h in the one-step procedure caused considerable double-bond migration with conversion of both the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 dienes to the *trans*-9,*trans*-11 isomer. However, the percentages of the three conjugated dienes were not affected by prolonged incubation of the CLA fat source in sodium methoxide for 24 h at 100 °C (Table 2). Therefore, the modified two-step procedure was effective for the methylation of samples containing both fatty amides and conjugated dienes.

An animal feed sample was then spiked with 5% oleamide and methylated by using the modified two-step procedure to determine if conversion of amide to FAME was complete. Considerable amide remained in the spiked feed sample following its methylation in methoxide at the higher temperature and extended time (Table 3). Because the added oleamide was not pure and contained many fatty acids, its incomplete conversion to FAME with the modified two-step procedure reduced the recoveries of all fatty acids except $C_{18:2}$. The inability of the modified two-step procedure to fully convert oleamide to FAME in the feed sample, as it did for the oleamide standard (Table 1), may be related to nutrient components in the feed that might interfere with the basic (sodium methoxide) catalyst. The feed sample was fortified with a variety of protein and energy sources, minerals, and vitamins to meet the nutritional needs of lactating dairy cows.

The modified one-step procedure gave a fatty acid profile for the spiked feed sample that was devoid of amide and gave high recoveries for all fatty acids. The only exception was $C_{18:2}$, which was higher ($P < 0.05$) for the modified one-step procedure compared to its theoretical content.

Fatty amides have recently been used as feed supplements for ruminant species as a means to enhance the concentration of unsaturated fatty acids in meat and milk. Biohydrogenation of dietary unsaturated fatty acids by anaerobes in the rumen generally ensures that mostly saturated fatty acids reach the small intestine for absorption. However, ruminal microbes are unable to hydrogenate double bonds present in fatty amides so that their inclusion in animal diets provides an excellent vehicle for delivery of unsaturated fatty acids to the small intestine. For example, feeding oleamide to lactating dairy cattle increased oleic acid concentration in milk by 37% compared to feeding high-oleic canola oil (*5*). In studies with nonruminant species, such as the rat, fatty amides were not digested and absorbed intact, but instead were converted to fatty acids in the intestines, either through low pH in stomach contents or through the action of intestinal amidases (*6*).

Direct methylation procedures using acid or base catalysts are often used to determine total fatty acid concentration in feed and fecal samples collected from animal metabolism trials. However, as shown in this study, fatty amides appear separately from FAME when these direct methylation procedures are applied. This can lead to errors in interpreting the concentration and composition of total fatty acyl components present in these samples. It would be convenient if a direct methylation procedure were available that converted all fatty acyl components, including the simple amides of fatty acids, to FAME which then could be analyzed by GC for content and composition.

This study examined several modifications of existing direct methylation procedures to determine an approach for converting fatty amides to FAME. Increasing the time and temperature of incubation in sodium methoxide used in the two-step procedure was insufficient to quantitatively convert all fatty amides to FAME in a feed sample. However, the conversion of amides to FAME was complete when the incubation time in methanolic HCl was extended for the one-step procedure.

Feed and digesta samples obtained from animals fed fatty amides can be analyzed for total fatty acyl components, including those present as simple amides, according to the method of Sukhija and Palmquist (*2*) if the time of incubation in methanolic HCl is extended from 2 to 16 h. The increased exposure time to methanolic HCl will quantitatively convert fatty amides to FAME without changing the fatty acid profile. When soybean oil samples were methylated by using the one-step procedure for 2 or 16 h, there were no effects ($P > 0.05$) on the percentages of any saturated or unsaturated fatty acid (data not shown). A drawback of the 16

h one-step procedure is that it is not appropriate for analysis of conjugated dienes because they undergo extensive isomerization when this modification is used.

ABBREVIATIONS USED

CLA, conjugated linoleic acid; FAME, fatty acid methyl esters; GC, gas chromatography.

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