COMPARISON OF CATALYSTS FOR DIRECT TRANSESTERIFICATION OF FATTY ACIDS IN FREEZE-DRIED FORAGE SAMPLES

T. R. Weston[1], J. D. Derner[2], C. M. Murrieta[1], D. C. Rule[1], and B. W. Hess[1]
[1]Department of Animal Science, University of Wyoming, Laramie, WY 82071
[2]High Plains Grasslands Research Station, USDA-ARS, Cheyenne, WY, 82009

ABSTRACT: Our objective was to compare 1.09 M methanolic HCl to 14% BF_3 in methanol as catalysts for direct transesterification of fatty acids in freeze-dried forage samples. Samples included blue grama (*Bouteloua gracilis*), fringed sage (*Artemisia frigida*), western wheatgrass (*Pascopyrum smithii*), needle-and-thread (*Stipa comata*), dalmation toadflax (*Linaria dalmatica*), needleleaf sedge (*Carex eleocharis*), and scarlet globemallow (*Sphaelercea coccinea*). Thin layer chromatographic evaluation revealed complete conversion of total lipid extracts to fatty acid methyl esters using both catalysts. Additionally, GLC analysis confirmed similar ($P = 0.96$) total fatty acid concentrations for both catalysts. Concentrations of most identified fatty acids (13:0, 14:0, 16:0, 16:1, 17:0, 17:1, 18:0, 18:1, 18:2, 18:3, 19:0, 20:0, 20:1, 22:0, 22:1, 22:3, 24:0, 24:1, and 28:0) were similar ($P = 0.17$ to 0.99) for both catalysts. Concentrations of 14:0 tended to be greater ($P = 0.07$) for HCl but weight percentages of 14:0 did not differ ($P = 0.23$) between catalysts. Concentrations and weight percentages of 17:1 were less ($P < 0.0001$) for HCl compared with BF_3. Boron-trifluoride may cause partial isomerization of predominant fatty acids because the concentrations of unidentified fatty acids with GLC retention times of 8.0, 13.9, and 31.9 min were greater ($P = 0.005$ to 0.05) for BF_3; whereas, only the concentration of unidentified fatty acid eluding at 14.8 min was greater ($P = 0.02$) for HCl. Nevertheless, total concentration of unidentified fatty acids did not differ ($P = 0.71$) between catalysts. Additionally, total weight percentages of identified fatty acids and unidentified fatty acids were not affected ($P = 0.37$) by catalyst (91.2 and 8.8% vs. 90.6 and 9.4% for HCl and BF_3, respectively). It is also possible that BF_3 is more efficient at catalyzing methylation of less common or unusual fatty acids, but BF_3 costs $0.19 per sample more than HCl. We conclude that 1.09 M methanolic HCl is both a cost effective and appropriate substitute for 14% BF_3 in methanol for preparation of fatty acid methyl esters from freeze-dried forage samples.

Key Words: Fatty acids, Forages, Methyl esters

Introduction

Procedures for preparation of fatty acid methyl esters from forages using single-step transesterification have been well documented. Outen et al. (1976) demonstrated a one-step extraction and esterification method using benzene and 5% methanolic HCl. Sukhija and Palmquist (1988) later recommended that benzene or toluene be used for extraction and formation of methyl esters. The use of benzene and toluene are now discouraged due to high toxicity and carcinogenic properties (EPA, 2002). Recognizing the hazards of benzene and toluene, Whitney et al. (1999) substituted these solvents with 14% BF_3 in CH_3OH for direct transesterification of feedstuffs.

Although BF_3 has proven to be an effective catalyst for direct transesterification of fatty acids in forages (Whitney et al., 1999) and animal tissues (Rule, 1997), BF_3 is very volatile and can be toxic if inhaled (NIOSH, 2004). Boron-trifluoride also must be sealed with N_2 and stored in a cool, dark environment to maintain reactivity. In contrast, methanolic HCl is less volatile than BF_3, maintains shelf-life longevity without special preparation, and costs $101.50/L less than BF_3.

Garcés and Mancha (1993) demonstrated that methanolic HCl is an efficient catalyst for preparing methyl esters, with solubility of transmethylated end products being a factor limiting its utility. Complete esterification of less soluble and less reactive lipids, however, can be accomplished if frequent vortex-mixing while heating is performed in a disciplined manner (Rule, 1997). Kucuk et al. (2001) modified the procedure of Whitney et al. (1999) by substituting BF_3 in CH_3OH with methanolic HCl for direct transesterification of lipids in feedstuffs; however, results comparing BF_3 in CH_3OH to methanolic HCl as catalysts in single-step direct transesterification have not yet been reported. Our objective was to compare BF_3 in CH_3OH to methanolic HCl as catalysts for direct transesterification of fatty acids in freeze-dried forage samples.

Materials and Methods

Sample Collection

Beginning in May and every 3 wk until October, whole plants were harvested in 2004 from a single enclosure (0.5 ha) located approximately 15 km northwest of Cheyenne, WY. Two transects (each 25 m) were randomly located within the enclosure and permanently established for the entire collection period. To harvest samples, 0.1 m^2 (0.2 × 0.5 m) quadrats were randomly located on each transect and individual plant species located within each quadrat having the same phenological stage of development were clipped 2.5 cm above the ground surface until 5.0 g/species (as-fed basis) was attained. Samples included blue grama (*Bouteloua gracilis*; BOGR, warm-season perennial grass), western wheatgrass (*Pascopyrum smithii*; PASM, cool-season perennial grass), needle-and-thread (*Stipa comata*; STCO, cool-season perennial grass), needleleaf sedge (*Carex eleocharis*; CAEL, cool-season perennial grasslike), scarlet globemallow (*Sphaelercea coccinea*; SPCO, perennial forb), dalmation toadflax (*Linaria dalmatica*;

LIDA, perennial forb), and fringed sage (*Artemisia frigida*; ARFR, sub-shrub). Clipped samples were immediately placed on dry ice, transported to the laboratory, and freeze-dried (Genesis Freeze Dryer, Virtis Co., Gardiner, NY). After freeze drying, samples were ground in a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA) to pass a 1-mm screen, pooled by species within each month, and stored in plastic containers with teflon-lined caps at -20°C.

Total lipid Extract for TLC

Freeze dried samples (0.5 g) for each species were subjected to extraction with (15 mL for BOGR, PASM, CAREX, and LIDA or 30 mL for ARFR and SPCO) a mixture containing $CHCl_3$, CH_3OH, and H_2O (1:2:0.8 vol/vol/vol; Bligh and Dyer, 1959) in 29×123 mm screw-cap, borosilicate tubes with teflon-lined caps. Volumes were individually adjusted (15 mL vs. 30 mL) due to the absorptive nature of ARFR and SPCO. Tubes were then placed on a Wrist Action Shaker (Burrell Corporation, Pittsburgh, PA) for 24 h. After extraction, 3.0 mL of $CHCl_3$, 1.5 mL of H_2O, and 1.5 mL of a solution containing aqueous 0.1 N HCl/2 M KCl were added to each tube and vortex-mixed for 15 s at a low speed, using a Fisher Vortex Genie 2 (Scientific Industries Inc., Bohemia, NY) electronic mixer with adjustable speed. Tubes were centrifuged (Beckman Model TJ-6 Centrifuge, Beckman Instruments Inc., Fullerton, CA) at $1300 \times g$ for 3 min to separate phases and the upper, aqueous phase was siphoned and discarded. The lower, $CHCl_3$ phase was transferred into a clean, hexane rinsed 16×125 mm screw-cap, borosilicate tube with a teflon-lined cap. Residue in the original tube was extracted twice with 2.0 mL of $CHCl_3$. Tubes were then placed in a Meyer N-Evap Analytical Evaporator (Associates Inc., South Berlin, MA) where $CHCl_3$ was evaporated under N_2 gas at 22° C. Extract residues within tubes were re-suspended in 2.0 mL of $CHCl_3$ and split by transferring 1.0 mL into a clean, hexane rinsed 16×125 mm tube. The $CHCl_3$ in each tube containing 1.0 mL of the split extract residue was then evaporated under N_2 gas at 22°C. Direct transesterification was performed by adding 2.0 mL of either 14% BF_3 in CH_3OH or 1.09 M methanolic HCl and 2.0 mL of CH_3OH to each tube. Tubes were placed on a hot block at 80° C for 1 h. Following 5 min of initial heating, tubes were individually vortex-mixed every 3 min for 1 h. Tubes were then allowed to cool for approximately 20 min. When tubes reached ambient temperature, 2.0 mL of H_2O and 2.0 mL of hexane (HPLC grade, Sigma-Aldrich, St. Louis, MO) were added to each tube followed by vortex-mixing for 15 s. After centrifugation at $1300 \times g$ for 3 min, the upper hexane phase was transferred to GLC auto-sampler vials containing a 1 mm bed of anhydrous sodium sulfate and sealed before loading. Thin layer chromatography was accomplished by loading 15 μL of each upper hexane phase sample onto a lane of a TLC plate (20×20 cm) coated with 250 μm Silica-gel G (Analtech, Newark, DE). The TLC plate was placed in a mobile phase mixture of petroleum ether, diethyl ether, and glacial acetic acid (85:15:1 vol/vol/vol) for 1 h. The plate was developed under I_2 vapors and visually assessed for band formation. Purified standards (Sigma-Aldrich, St. Louis, MO) of methyl-oleate, monoacylglycerols, diacylglycerols, and triacylglycerols were used for visual comparison of bands.

Direct Transesterification

For the direct transesterification procedure, 1.0 mL of $CHCl_3$ containing 1.0 mg/mL of heneicosanoic acid (21:0; internal standard) was added to pre-weighed 29×123 mm screw-cap, borosilicate tubes with teflon-lined caps and evaporated under N_2 gas at 22° C. Freeze-dried forage samples were individually weighed (0.5 g) into tubes containing the internal standard. Fatty acid methyl esters were prepared by adding either 4.0 mL of either 14% BF_3 in CH_3OH or 1.09 M methanolic HCl and 4.0 mL CH_3OH directly to tubes containing samples. Due to their absorptive nature ARFR and SPCO required more reagent for saturation. For these samples, 8.0 mL of either 14% BF_3 in CH_3OH or 1.09 M methanolic HCl and 8.0 mL CH_3OH was used. Tubes were capped and placed in water bath incubator (Isotemp 220, Fisher Scientific, Pittsburgh, PA) at 80° C. After 5 min of initial heating, tubes were individually vortex-mixed every 3 min for 1 h and then allowed to cool. Sukhija and Palmquist (1988) suggested that transesterification may remain incomplete if forage samples are not frequently vortex-mixed at slow speeds. Emphasis was placed on vortex-mixing individual tubes every 3 min to ensure complete transesterification (Rule, 1997). After tubes cooled to ambient temperature, 4.0 mL of H_2O and 4.0 mL of hexane were added followed by vortex-mixing for 15 s. Tubes were centrifuged at $1300 \times g$ for 3 min; the upper hexane phase was transferred to GLC auto-sampler vials containing a 1 mm bed of anhydrous sodium sulfate and sealed.

Fatty acid methyl esters were separated by GLC using an Agilent 6890 Gas Chromatograph (Agilent Technologies, Wilmington, DE) equipped with a flame ionization detector and a 30 m $\times$ 0.32 mm (i.d.) fused siloxane capillary column (BPX-70, 0.25 μm film thickness, SGE, Inc. Austin, TX). Oven temperature was maintained at 110° C for 5 min, and then increased to 200° C at 5° C/min. Injector and detector temperatures were 200° C and 225° C, respectively. Hydrogen was the carrier gas at a split ratio of 25:1 and a constant flow rate of 1.0 mL/min. Fatty acid peaks were recorded and integrated using GC ChemStation software (Agilent Technologies, version A.09.03). Individual fatty acids were identified by comparing retention times with known fatty acid methyl ester standards (Nu-Chek Prep. Inc., Elysian, MN and Matreya Inc., Pleasant Gap, PA).

Statistics Analyses

Quantitative data were analyzed as a one-way ANOVA using GLM procedure of SAS (SAS Institute, Cary, NY). The model included treatment (BF_3 in CH_3OH and methanolic HCl in CH_3OH) as the independent variable.

Results and Discussion

For the TLC analyses, total lipid extracts from intact forage lipids had complete conversion to methyl esters using 1.09 M methanolic HCl or 14% BF_3 in CH_3OH as catalysts for transesterification (Figure 1). These results

indicated that either BF_3 or methanolic HCl can be used as a catalyst for direct transesterification of lipid extracts.

For the GLC analyses, concentrations of total fatty acids ($P = 0.96$) and most identified fatty acids ($P = 0.17$ to 0.99) were similar for both catalysts (Table 1). Concentrations of 14:0 tended to be greater ($P = 0.07$) for HCl but weight percentages of 14:0 did not differ ($P = 0.23$) between catalysts (Table 2). Concentrations and weight percentages of 17:1 were less ($P < 0.0001$) for HCl compared with BF_3. It is possible that BF_3 is more efficient at catalyzing methylation of less common fatty acids, but BF_3 costs \$0.19 per sample more than HCl. Additionally, BF_3 in the form of its coordination complex with CH_3OH is a powerful acidic catalyst and has serious drawbacks due to the formation of methoxy artifacts from unsaturated fatty acids when used in high concentrations with CH_3OH (Christie, 1993). Therefore, BF_3 may cause partial isomerization of predominant fatty acids because the concentrations of unidentified fatty acids with GLC retention times of 8.0, 13.9, and 31.9 min were greater ($P = 0.005$ to 0.05) for BF_3, whereas only the concentration of unidentified fatty acid eluding at 14.8 min was greater ($P = 0.02$) for HCl. Christie (1993) also noted that all fatty acids are esterified at approximately the same rate by methanolic HCl and differential losses of specific fatty acids are unlikely during the esterification step. Certain classes of simple lipids are not soluble in methanolic HCl (Christie, 1993); however, extensive vortex-mixing while heating results in complete methylation of these simple lipids when methanolic HCl is used (Rule, 1997). Nevertheless, total concentration of unidentified fatty acids did not differ ($P = 0.71$) between catalysts. Additionally, total weight percentages of identified fatty acids and unidentified fatty acids were not affected ($P = 0.37$) by catalyst.

In addition to partial isomerization, the unidentified fatty acids listed in Table 3 could be "unusual" fatty acids (Moire et al., 2004) synthesized with hydroxyl, epoxy, acetylenic, or carboxylic functional groups, as well as those with conjugated unsaturated bonds (Jaworski and Cahoon, 2003). The unusual fatty acids can also be incorporated into triacylglycerols for storage (Moire et al., 2004). For freshly harvested forages, detection of unusual fatty acids with shorter chain lengths would most likely be low due to β-oxidation of unusual fatty acids to maintain integrity of the lipid membrane (Millar et al., 1998). Derivatives of peroxisomal β-oxidation can also have unusual conformity. For example, Moire et al. (2004) reported that peroxisomal β-oxidation of 18:3 resulted in intermediates 14:3, 12:2, 10:2, 8:1 and 6:0. Those authors also noted that peroxisomal β-oxidation of 18:1 resulted in 14:1, 12:0, 10:0, 8:0, and 6:0. Further investigation would be required to identify these unusual fatty acids. As a reference, the retention times of unidentified fatty acids have been reported in relation to predominant fatty acids (Table 3).

Implications

Fatty acid methyl esters can be prepared by using hydrochloric acid in a direct transesterification procedure. This catalyst is both cost effective and an appropriate alternative to boron-trifluoride for methylation of most common fatty acids.

Literature Cited

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911-917.

Christie, W. W. 1993. Preparation of ester derivatives of fatty acids for chromatographic analysis. Pages 69-111 in Advances in Lipid Methodology-Two. ed. W. W. Christie, Oily Press, Dundee.

EPA. 2002. Integrated risk information system on benzene. National Center for Environmental Assessment, Office of Research and Development, Washington, DC. Available: http://www.epa.gov/iris/subst/0276.htm.

Garcés, R., and M. Mancha. 1993. One-step lipid extraction and fatty acid methyl esters preparation from fresh plant tissues. Anal. Biochem. 211:139-143.

Jaworski, J., and E. B. Cahoon. 2003. Industrial oils from transgenic plants. Curr. Opin. Plant Biol. 6:178-184.

Kucuk, O., B. W. Hess, P. A. Ludden, and D. C. Rule. 2001. Effect of forage:concentration ratio on ruminal digestion and duodenal flow of fatty acids in ewes. J. Anim. Sci. 79:2233-2240.

Millar, A. J., M. Wrischer, and L. Kunst. 1998. Accumulation of very-long-chain fatty acids in membrane glycerolipids is associated with dramatic alterations in plant morphology. The Plant Cell. 11:1889-1902.

Moire, L., E. Rezzonico, S. Goepfert, and Y. Poirier. 2004. Impact of unusual fatty acid synthesis on futile cycling through β-oxidation and on gene expression in transgenic plants. Plant Physiol. 134:432-442.

NIOSH. 2004. The registry of toxic effects of chemical substances: borane-trifluoro. Available: http://www.cdc.gov/niosh/rtecs/ed22b6b8.html.

Outen, G. E., D. E. Beever, J. S. Fenlon. 1976. Direct methylation of long-chain fatty acids in feeds, digesta and faeces without prior extraction. J. Sci. Food Agric. 27:419-425.

Rule, D. C. 1997. Direct transesterification of total fatty acids of adipose tissue, and of freeze-dried muscle and liver with boron-trifluoride in CH_3OH. Meat Sci. 46:23-32.

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

Whitney, M. B., B. W. Hess, J.E. Kaltenbach, H. J. Harlow, and D. C. Rule. 1999. Direct transesterification of lipids from feedstuffs and ruminal bacteria. Can. J. Anim. Sci. 79:247-249.

Table 1. Fatty acid concentrations of freeze-dried forage samples using boron-trifluoride in methanol or methanolic hydrochloric acid as catalysts for direct transesterification

Fatty acid[a] retention time	Fatty acid	Catalyst BF$_3$	HCl	SE[1]	P-value
--- min ---		------- mg/g of DM -------			
6.6	13:0	0.137	0.133	0.008	0.76
10.0	14:0	0.289	0.330	0.016	0.07
15.8	16:0	3.174	3.259	0.111	0.59
16.6	16:1	0.230	0.247	0.017	0.46
17.9	17:0	0.085	0.073	0.006	0.17
18.6	17:1	0.120	0.076	0.007	< 0.0001
19.7	18:0	0.505	0.504	0.024	0.97
20.3	18:1	0.251	0.244	0.129	0.97
21.1	18:2	4.760	4.781	0.835	0.99
21.8	19:0	0.066	0.073	0.005	0.39
22.3	18:3	8.368	8.537	0.478	0.80
22.8	20:0	0.245	0.234	0.012	0.53
23.2	20:1	0.059	0.063	0.007	0.63
25.5	22:0	0.311	0.282	0.014	0.17
25.8	22:1	0.739	0.762	0.074	0.83
28.0	22:3	0.297	0.276	0.014	0.29
28.2	24:0	0.273	0.263	0.019	0.69
28.7	24:1	0.087	0.083	0.027	0.92
36.3	28:0	0.261	0.233	0.025	0.43
Unidentified		2.054	1.950	0.200	0.71
Total		22.311	22.403	1.228	0.96

[1]n = 82.

Table 2. Fatty acid weight percentages of freeze-dried forage samples using boron-trifluoride in methanol or methanolic hydrochloric acid as catalysts for direct transesterification

Fatty Acid	Catalyst BF$_3$	HCl	SE[1]	P-value
	------ g/100g of total fatty Acids ------			
13:0	0.64	0.63	0.03	0.67
14:0	1.53	1.76	0.13	0.23
16:0	14.97	15.31	0.25	0.33
16:1	1.04	1.13	0.05	0.28
17:0	0.40	0.35	0.02	0.16
17:1	0.58	0.36	0.02	< 0.0001
18:0	2.25	2.23	0.08	0.82
18:1	0.81	0.80	0.28	0.97
18:2	18.52	18.48	1.25	0.98
19:0	0.32	0.36	0.03	0.37
18:3	39.18	39.88	1.25	0.69
20:0	1.14	1.08	0.05	0.49
20:1	0.27	0.30	0.04	0.64
22:0	1.50	1.36	0.08	0.23
22:1	2.93	2.92	0.26	0.98
22:3	1.46	1.35	0.09	0.40
24:0	1.31	1.24	0.11	0.68
24:1	0.47	0.46	0.15	0.95
28:0	1.32	1.19	0.14	0.51
Total identified	90.64	91.19	0.42	0.37
Unidentified	9.36	8.81	0.42	0.37

[1]n = 82.

Table 3. Concentrations and relative retention times of unidentified fatty acids in freeze-dried forage samples using boron-trifluoride in methanol or methanolic hydrochloric acid as catalysts for direct transesterification

Fatty acid retention time	Catalyst BF$_3$	HCl	SE[1]	P-value	Relative retention time 16:0	18:2	18:3
-- min --	---- mg/g of DM ----				--------------- min ---------------		
8.0	0.049	0.030	0.007	0.05	-7.8	-13.1	-14.3
8.9	0.042	0.043	0.008	0.98	-6.9	-12.2	-13.4
10.6	0.105	0.126	0.008	0.07	-5.2	-10.5	-11.7
13.9	0.043	0.022	0.006	0.005	-1.9	-7.2	-8.4
14.8	0.117	0.152	0.011	0.02	-1.0	-6.3	-7.5
20.1	0.818	0.819	0.182	1.00	+4.3	-1.0	-2.2
26.8	0.055	0.056	0.003	0.91	+11.0	+5.7	+4.5
29.5	0.051	0.042	0.005	0.24	+13.7	+8.4	+7.2
31.3	0.205	0.158	0.019	0.08	+15.5	+10.2	+9.0
31.9	0.035	0.014	0.007	0.03	+16.1	+10.8	+9.6
33.9	0.044	0.034	0.009	0.39	+18.1	+12.8	+11.6
Other[2]	0.490	0.458	0.040	0.57	NA	NA	NA
Total	2.054	1.950	0.200	0.71	NA	NA	NA

[1]n = 82.
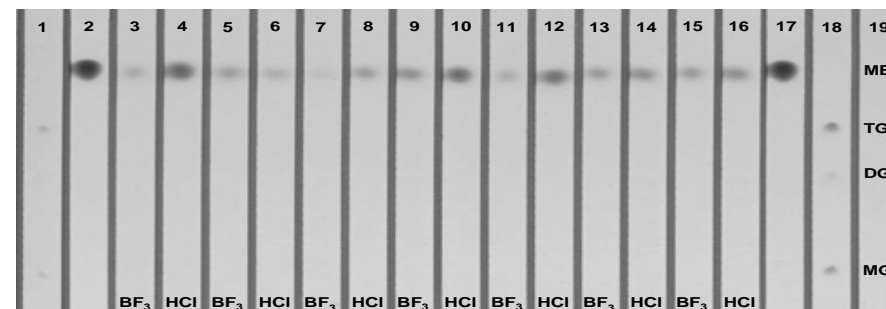[2]Other = sum of non-major peaks.

Figure 1. Thin layer chromatogram of methyl esters (ME) prepared from total lipid extract using direct transesterification with 14% boron-trifluoride or 1.09 M methanolic HCl. Lanes 1 and 18 represent monoacylglycerol (MG), diacylglycerol (DG), and triacylglycerol (TG) from purified standards. Lanes 2 and 17 represent a purified ME standard (methyl oleate). Lanes 3, 5, 7, 9, 11, 13, and 15 represent 15 µL of fatty acid ME prepared from 0.5 g of each sample (BOGR, ARFR, PASM, STCO, LIDA, CAEL, and SPCO, respectively) using total lipid extract followed by direct transesterification with 14% BF$_3$ in CH$_3$OH. Lanes 4, 6, 8, 10, 12, 14, and 16 represent 15 µL of fatty acid ME prepared from 0.5 g of each sample (BOGR, ARFR, PASM, STCO, LIDA, CAEL, and SPCO, respectively) using total lipid extract followed by direct transesterification with 1.09 M methanolic HCl. Lane 19 depicts the location of ME, TG, DG, and MG on each lane.