Comparison of Catalysts for Direct Transesterification of Fatty Acids in Freeze–Dried Forage Samples

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ABSTRACT

Preparation of fatty acid methyl esters from forages comparing methanolic boron-trifluoride (BF_3) to methanolic hydrochloric acid (HCl) as a catalyst in single-step direct transesterification has not been reported. Our objective was to compare 1.09 M methanolic HCl to 1.03 M (7%) methanolic BF_3 as catalysts for direct transesterification of fatty acids in freeze-dried forage samples. Thin-layer chromatographic analysis revealed complete conversion of total lipid extracts to fatty acid methyl esters using both catalysts. Additionally, gas–liquid chromatography analysis confirmed similar ($P = 0.95$) total fatty acid concentrations for both catalysts. Regression analysis indicated that similar values for total concentration would be obtained ($P < 0.0001$; $r^2 = 0.96$; slope $= 0.98 \pm 0.03$) between the two catalysts. Concentrations of most individual fatty acids were similar ($P = 0.17–0.99$) for both catalysts. Summed weight percentages of identified fatty acids, as well as sum of unidentified peaks, were not affected ($P = 0.27$) by catalyst (91.8 and 8.7% vs. 90.8 and 9.2% for HCl and BF_3, respectively). We conclude that 1.09 M methanolic HCl is an appropriate substitute for 1.03 M methanolic BF_3 for preparation of fatty acid methyl esters from freeze-dried forage samples. This result is of interest because HCL is both less costly and less caustic than BF_3.

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Abbreviations: GLC, gas-liquid chromatography.

Procedures for preparing 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 is now discouraged due to high toxicity and carcinogenic properties (USEPA, 2002). Recognizing the hazards of benzene and toluene, Whitney et al. (1999) substituted these solvents with 7% (1.03 M) 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), it is highly 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 substantially less than BF_3.

Garcés and Mancha (1993) demonstrated that methanolic HCl is an efficient catalyst for preparing fatty acid methyl esters, with solubility of transmethylated end products a factor limiting its utility. However, we have overcome potential problems with

solubility by frequent vortex mixing of the sample while maintaining reaction temperature (Rule, 1997; Whitney et al., 1999). 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 methanolic BF_3 to methanolic HCl as a catalyst in **a** single-step direct transesterification of dried forages have not yet been reported. Thus, our objective was to compare 1.03 M methanolic BF_3 to 1.09 M methanolic HCl as catalyst for direct transesterification of fatty acids in freeze-dried forage samples.

MATERIALS AND METHODS
Sample Collection
A diverse range of fatty acid levels in forages was accomplished by collecting several different plant species in various stages of phenological development. Beginning in May and occurring every 3 wk until Oct. 2004, individual plant species were clipped 2.5 cm above the ground surface to attain 5.0 g of fresh tissue per species from a single enclosure (0.5 ha) located approximately 9 km northwest of Cheyenne, Wyoming. Samples included blue grama [*Bouteloua gracilis* (Kunth) Lag. ex Steud.], western wheatgrass [*Pascopyrum smithii* (Rydb.) A. Löve], needle-and-thread [*Hesperostipa comata* (Trin. & Rupr.) Barkworth ssp. *comata*], needleleaf sedge (*Carex eleocharis* L.H. Bailey), scarlet globemallow [*Sphaeralcea coccinea* (Nutt.) Rydb.], dalmation toadflax [*Linaria dalmatica* (L.) Mill.], and fringed sage (*Artemisia frigida* Willd.). 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 and were then stored in plastic containers with Teflon-lined caps at −20°C.

Total Lipid Extract for Qualitative Thin-Layer Chromatography
Freeze-dried samples (0.5 g) for each species were subjected to extraction with a mixture containing chloroform ($CHCl_3$), CH_3OH, and H_2O (1:2:0.8 v/v/v; Bligh and Dyer, 1959) in 29- by 123-mm screw-cap, borosilicate tubes with Teflon-lined caps. Extraction mixture volumes were individually adjusted (15 mL for blue grama, dalmation toadflax, needle-and-thread, needleleaf sedge, and western wheatgrass or 30 mL for fringed sage and scarlet globemallow) due to differences in the absorptive nature of the samples. Samples were continuously mixed by agitating tubes with 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 an aqueous solution containing 0.1 M 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 electronic mixer (Scientific Industries, Inc., Bohemia, NY). Tubes were then 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- by 125-mm screw-cap, borosilicate tube with a Teflon-lined cap. Residue in the

original tube was extracted twice with 2.0 mL of $CHCl_3$, with each $CHCl_3$ extract combined with the first extract. 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. Lipid extracts within tubes were resuspended in 2.0 mL of $CHCl_3$ and split by transferring 1.0 mL into a clean hexane-rinsed 16- by 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. Transesterification was performed by adding 4.0 mL of either 1.03 M methanolic BF_3 or 1.09 M methanolic HCl to each tube. Tubes were placed on a hot block at 80°C. 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 hexane phase was transferred to glass vials containing a 1-mm bed of anhydrous sodium sulfate and sealed to dry and store lipid extracts for later thin-layer chromatography analysis. Thin-layer chromatography was accomplished by individually loading 15 μL of each hexane phase with a microsyringe onto separate lanes of a thin layer chromatography plate (20 by 20 cm) coated with 250 μm Silica-gel G (Analtech, Newark, DE). The thin-layer chromatography plate was placed in a mobile phase mixture of petroleum ether, diethyl ether, and glacial acetic acid (85:15:1 v/v/v) 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 of Dried Forages
To begin the direct transesterification procedure, 1.0 mL of $CHCl_3$ containing 1.0 mg mL^{-1} of heneicosanoic acid was transferred into individual 29- by 123-mm screw-cap, borosilicate tubes and evaporated under N_2 gas at 22°C. Heneicosanoic acid, a 21-carbon saturated fatty acid, was used as an internal standard to quantify mass from peak areas for individual and total fatty acids. For calculation of total fatty acids, all peaks were assumed to be fatty acid methyl esters. Freeze-dried forage samples were individually weighed (0.5 g) into tubes containing the dried internal standard. Fatty acid methyl esters were prepared by adding 4.0 mL of 1.03 M methanolic BF_3 or 1.09 M methanolic HCl plus 4.0 mL CH_3OH directly to tubes containing samples. Due to their absorptive nature, fringed sage and scarlet globe mallow required more reagent for saturation. For these samples, 8.0 mL of either 1.03 M methanolic BF_3 or 1.09 M methanolic HCl and 8.0 mL of CH_3OH was used. Tubes were capped and placed in a water bath (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 extraction and 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 × g for 3 min; the upper hexane phase was transferred to gas–liquid chromatography (GLC) auto-sampler vials containing a 1-mm bed of anhydrous sodium sulfate and crimp 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 by 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^{-1}. 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^{-1}. Fatty acid methyl ester peaks were recorded and integrated using GC ChemStation software (Agilent Technologies, version A.09.03). Individual fatty acid methyl esters 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).

Statistical Analyses

Quantitative data were analyzed by ANOVA as a randomized block complete block design using the GLM procedure of SAS (SAS Institute, Cary, NY). The model included plant species and sampling date as blocks and catalyst (BF$_3$ and HCl) as the independent variable, with individual plant samples as experimental units. Least squared means are reported because blue grama was not present at the time of the first sample collection ($n = 82$). The REG procedure of SAS was used to determine r^2 of correlation coefficients and relationships between fatty acid levels for the two catalysts. Each value for BF$_3$ was the dependent variable, whereas each value for HCl served as the independent variable.

RESULTS AND DISCUSSION

For the qualitative thin-layer chromatography analysis, location of bands indicated that total lipid extracts from intact forage lipids had been completely converted to methyl esters using either 1.03 M methanolic BF$_3$ or 1.09 M methanolic HCl as catalyst for transesterification (Fig. 1). Although intensity of bands appear to differ between treatments, direct comparisons of band intensities were not possible because concentrations of total lipids loaded were not equal across lanes. Nevertheless, these results indicate that either methanolic BF$_3$ or methanolic HCl can be used as catalyst for transesterification of lipid extracts. The complete conversion of fatty acids in the intact plant lipids to fatty acid methyl esters was accomplished despite previous indications that 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).

For the quantitative GLC analyses comparing direct transesterification of dried forges using the two catalysts, mean concentrations of total fatty acids ($P = 0.95$) and most individual fatty acids ($P = 0.17$ to 0.99) were similar for both catalysts (Table 1). Concentrations of 14:0 and 22:0 tended to differ ($P \leq 0.08$), but weight percentages of these fatty acids did not differ ($P \geq 0.12$) between catalysts (Table 2). Concentrations and weight percentages of 17:1 were less ($P < 0.0001$) for HCl compared with BF$_3$. Except for 17:0 and 19:0, there was generally good agreement between catalysts for fatty acid concentrations ($P < 0.0001$; $r^2 = 0.70$–1.0) and weight percentages ($P < 0.0001$; $r^2 = 0.59$–1.0). Regression equations presented
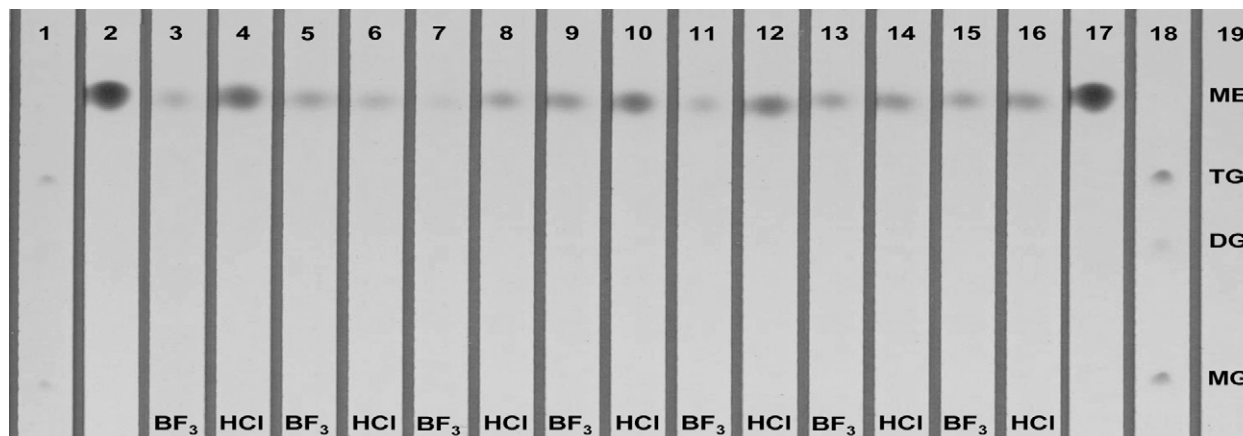

Figure 1. Thin-layer chromatogram of methyl esters (ME) prepared from total lipid extract using transesterification with 1.03 M methanolic boron-trifluoride or 1.09 M methanolic HCl. Lanes 1 and 18 represent monoacylglycerol (MG), diacylglycerol (DG), and triacylglycerol (TG) from purified standards. 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 hexane extract prepared from 0.5 g of each blue grama, fringed sage, western wheatgrass, needle-and-thread, dalmation toadflax, needleleaf sedge, and scarlet globemallow using total lipid extract followed by direct transesterification with 7% 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 blue grama, fringed sage, western wheatgrass, needle-and-thread, dalmation toadflax, needleleaf sedge, and scarlet globemallow 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. Extracts were not prepared to contain the same concentrations of total lipids; hence; fatty acid methyl ester concentrations would not have been equal across lanes.

in Table 1 and 2 indicate that values for fatty acid levels would be reasonably comparable if HCl were used instead of BF_3. Although results of regression analysis indicate that similar values for total concentration would be obtained ($P < 0.0001$; $r^2 = 0.96$) between the two catalysts, weight percentages of summed identified fatty acids would not be as comparable ($P < 0.0001$; $r^2 = 0.65$). Reporting of fatty acid concentrations would be preferred because these data are quantitative, whereas data expressed on a weight percentage basis merely indicate relative abundance of individual fatty acids. In addition to costing substantially more per sample than HCl, BF_3 in the form of its coordination complex with CH_3OH is a powerful Lewis acid and has serious drawbacks because of the formation of methoxy artifacts from unsaturated fatty acids when used in high concentrations with CH_3OH (Christie, 1993). Therefore, BF_3 may have caused partial isomerization of predominant fatty acids because the concentrations of unidentified peaks with GLC retention times of 8.0, 13.9, and 31.9 min were greater ($P \leq 0.03$) for BF_3, whereas the concentrations of unidentified peaks eluting at 10.6 and 14.8 min were greater ($P \leq 0.01$) for HCl (Table 3). Excluding the unidentified peaks with GLC retention times of 20.1 and 26.8, which had essentially identical concentrations and $r^2 = 1.0$ and 0.90, regression analysis revealed that concentrations of unidentified peaks eluting at 10.6 and 14.8

min would be more comparable than unidentified peaks eluting at other time points. Thus, uniformity of fatty acid methyl ester transesterification was likely better with HCl than BF_3. This notion also was supported by Christie (1993), who noted that all fatty acids are esterified at approximately the same rate by methanolic HCl and that differential losses of specific fatty acids are unlikely during the esterification step. Nevertheless, total concentration of unidentified peaks did not differ ($P = 0.71$) between catalysts. Additionally, total weight percentages of identified fatty acid methyl esters and unidentified peaks were not affected ($P = 0.37$) by catalyst.

In addition to partial isomerization, the unidentified peaks 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). Such 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

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

Fatty acid retention time	Fatty acid	Catalyst		ANOVA		Regression	
		BF_3	HCl	SEM	P value	r^2	(slope ± SE + intercept ± SE)
min		— mg g^{-1} DM‡ —					
6.6	13:0	0.139	0.135	0.004	0.55	0.84	1.01 ± 0.07 + 0.002 ± 0.01
10.0	14:0	0.290	0.331	0.009	<0.01	0.80	0.84 ± 0.07 + 0.01 ± 0.02
15.8	16:0	3.204	3.288	0.081	0.46	0.91	0.90 ± 0.05 + 0.24 ± 0.15
16.6	16:1	0.234	0.251	0.011	0.25	0.75	0.80 ± 0.07 + 0.03 ± 0.02
17.9	17:0	0.085	0.073	0.006	0.17	0.10	0.71 ± 0.31 + 0.03 ± 0.02
18.6	17:1	0.121	0.077	0.003	<0.01	0.73	1.09 ± 0.10 + 0.04 ± 0.009
19.7	18:0	0.500	0.499	0.171	0.96	0.86	1.00 ± 0.06 + 0.004 ± 0.04
20.3	18:1	0.243	0.236	0.124	0.97	1.00	1.05 ± 0.002 − 0.007 ± 0.003
21.1	18:2	4.704	4.725	0.793	0.99	1.00	1.00 ± 0.008 − 0.02 ± 0.06
21.8	19:0	0.066	0.073	0.005	0.36	<0.01	0.10 ± 0.10 + 0.06 ± 0.008
22.3	18:3	8.518	8.687	0.239	0.62	0.97	0.99 ± 0.03 − 0.08 ± 0.26
22.8	20:0	0.244	0.233	0.010	0.42	0.94	1.01 ± 0.04 + 0.009 ± 0.01
23.2	20:1	0.059	0.063	0.006	0.58	0.70	0.76 ± 0.08 + 0.01 ± 0.007
25.5	22:0	0.310	0.282	0.011	0.08	0.93	1.13 ± 0.05 − 0.007 ± 0.02
25.8	22:1	0.734	0.757	0.071	0.82	0.99	0.94 ± 0.02 + 0.02 ± 0.02
28.0	22:3	0.297	0.276	0.012	0.21	0.76	0.93 ± 0.08 + 0.04 ± 0.03
28.2	24:0	0.274	0.264	0.018	0.68	0.84	0.91 ± 0.06 + 0.03 ± 0.02
28.7	24:1	0.082	0.079	0.021	0.90	0.99	1.04 ± 0.02 + 0.0007 ± 0.004
36.3	28:0	0.262	0.233	0.022	0.38	0.90	1.18 ± 0.06 − 0.02 ± 0.02
Total		22.402	22.493	1.121	0.95	0.96	0.98 ± 0.03 + 0.39 ± 0.63

[†]A diverse range of fatty acid levels in forages was accomplished by collecting several different plant species in various stages of phenological development (n = 82). Values for BF_3 were dependent variables.

[‡]DM, dry matter.

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 unidentified peaks in the forage lipids of the current study.

CONCLUSIONS

Fatty acid methyl esters can be prepared by one-step direct transesterification of forage lipids using methanolic hydrochloric acid as effectively as with methanolic boron-trifluoride as catalyst. The significance of this

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		ANOVA		Regression	
	BF_3	HCl	SEM	P value	r^2	(slope ± SE + intercept ± SE)
	— g 100 g^{-1} total fatty acids —					
13:0	0.65	0.63	0.02	0.53	0.80	1.00 ± 0.08 + 0.02 ± 0.05
14:0	1.53	1.76	0.10	0.12	0.93	0.83 ± 0.04 + 0.08 ± 0.08
16:0	15.01	15.35	0.22	0.27	0.96	0.94 ± 0.03 + 0.54 ± 0.48
16:1	1.05	1.14	0.04	0.13	0.59	0.59 ± 0.08 + 0.38 ± 0.09
17:0	0.40	0.35	0.02	0.16	0.15	0.70 ± 0.25 + 0.16 ± 0.09
17:1	0.58	0.36	0.01	<0.01	0.71	1.09 ± 0.11 + 0.19 ± 0.04
18:0	2.22	2.20	0.06	0.76	0.88	1.04 ± 0.06 − 0.06 ± 0.15
18:1	0.79	0.78	0.27	0.97	1.00	1.05 ± 0.005 − 0.02 ± 0.01
18:2	18.37	18.33	1.11	0.98	1.00	1.01 ± 0.008 − 0.22 ± 0.17
19:0	0.32	0.36	0.03	0.35	0.02	0.10 ± 0.08 + 0.28 ± 0.03
18:3	39.51	40.22	0.78	0.52	0.97	0.98 ± 0.02 + 0.16 ± 1.03
20:0	1.13	1.07	0.05	0.42	0.91	0.96 ± 0.05 + 0.09 ± 0.06
20:1	0.27	0.30	0.04	0.61	0.75	0.69 ± 0.06 + 0.07 ± 0.03
22:0	1.50	1.36	0.07	0.18	0.96	1.14 ± 0.04 − 0.06 ± 0.06
22:1	2.89	2.88	0.26	0.98	0.98	0.98 ± 0.02 + 0.07 ± 0.12
22:3	1.45	1.34	0.08	0.36	0.80	0.98 ± 0.08 + 0.13 ± 0.12
24:0	1.31	1.25	0.10	0.64	0.88	0.95 ± 0.05 + 0.13 ± 0.08
24:1	0.45	0.43	0.12	0.93	0.98	1.05 ± 0.02 − 0.01 ± 0.02
28:0	1.33	1.20	0.14	0.45	0.91	1.17 ± 0.06 − 0.07 ± 0.09
Total identified	90.77	91.32	0.34	0.27	0.65	0.80 ± 0.10 + 18.07 ± 8.42

[†]A diverse range of fatty acid levels in forages was accomplished by collecting several different plant species in various stages of phenological development (n = 82). Values for BF_3 were dependent variables.

Table 3. Concentrations and relative retention times of unidentified gas-liquid chromatography peaks in freeze-dried forage samples using methanolic boron-trifluoride or methanolic hydrochloric acid as catalyst for direct transesterification.[†]

Fatty acid retention time	Catalyst		ANOVA		Regression	
	BF_3	HCl	SEM	P value	r^2	(slope ± SE + intercept ± SE)
Min	— mg g^{-1} DM[‡] —					
8.0	0.050	0.031	0.004	<0.01	0.37	0.95 ± 0.19 + 0.02 ± 0.009
8.9	0.044	0.044	0.005	0.96	0.29	0.61 ± 0.15 + 0.02 ± 0.009
10.6	0.106	0.128	0.005	<0.01	0.79	0.86 ± 0.07 − 0.004 ± 0.01
13.9	0.045	0.023	0.005	<0.01	0.37	0.66 ± 0.13 + 0.03 ± 0.006
14.8	0.118	0.153	0.081	<0.01	0.59	0.64 ± 0.08 + 0.02 ± 0.01
20.1	0.799	0.799	0.124	1.0	0.99	1.00 ± 0.02 − 0.002 ± 0.02
26.8	0.056	0.055	0.003	0.90	0.67	0.89 ± 0.10 + 0.007 ± 0.006
29.5	0.050	0.041	0.005	0.22	0.09	0.50 ± 0.23 + 0.03 ± 0.01
31.3	0.202	0.155	0.006	0.07	0.02	0.006 ± 0.02 + 0.04 ± 0.007
31.9	0.034	0.013	0.007	0.03	0.17	0.77 ± 0.26 + 0.13 ± 0.02
33.9	0.044	0.034	0.009	0.39	0.04	−0.09 ± 0.05 + 0.02 ± 0.005
Other[§]	0.486	0.454	0.039	0.57	0.04	−0.07 ± 0.04 + 0.07 ± 0.02

[†]A diverse range of fatty acid levels in forages was accomplished by collecting several different plant species in various stages of phenological development (n = 82). Values for BF_3 were dependent variables.

[‡]DM, dry matter.

[§]Other = sum of remaining unidentified peaks.

finding is that methanolic hydrochloric acid is less hazardous to use, has a longer shelf life, and is less costly than methanolic boron-trifluoride.

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