Extracting Long-Chain Fatty Acids from a Fermentation Medium

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ABSTRACT: Several solvents were evaluated for extracting free long-chain FA (LCFA) from a fermentation medium. Chloroform, chloroform/methanol (1:1), hexane, and hexane/methyl *tert*-butyl ether (MTBE) (1:1) were evaluated as alternative extraction solvents. Parameters considered for optimizing LCFA recoveries included pH and ionic strength. Maximal LCFA recoveries were obtained by adding 2 mL of the hexane/MTBE (1:1) solvent mixture, 80 µL of 50% H_2SO_4, and 0.05 g NaCl to 1 mL of the aqueous sample and mixing for 15 min at 200 rpm. This method quantified saturated LCFA [capric acid ($C_{10:0}$) to stearic acid ($C_{18:0}$)] and unsaturated LCFA with 18 carbons [linoleic acid ($C_{18:2}$) and oleic acid ($C_{18:1}$)] with a 98 to 100% recovery. Caproic ($C_{6:0}$) and caprylic ($C_{8:0}$) acids were characterized by 27 and 76% recoveries, respectively.

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KEY WORDS: Extraction, fermentation, long-chain fatty acids.

Industrial effluents containing long-chain FA (LCFA) are treated aerobically or anaerobically before discharge into a receiving body of water. During treatment, LCFA are degraded into shorter-chain LCFA and acetic acid. LCFA can inhibit a variety of microbial populations and subsequently affect the treatment process stability (1–3). Analysis of the substrate LCFA and various by-products is important because the data are useful in determining reaction kinetics and predicting the performance of a waste treatment reactor.

Several methods have been developed to extract and analyze lipids and fats from various media. The formation of FAME is a routine method for LCFA analysis by GC (4). Another procedure considered for LCFA analysis includes extraction with a chloroform/methanol mixture followed by derivatization using 2-nitrophenylhydrazine hydrochloride with subsequent analysis by HPLC (5). A chloroform/methanol mixture also has been used to extract acidic phospholipids. Lysophospholipids and nonesterified FA were extracted from tissue samples, but low recoveries were observed (6,7). Inadequate results also have been reported from using acidified and alkaline solvents (8–10).

Some studies have evaluated using diethyl ether followed by an ethanol/diethyl ether mixture for extracting lipids from biological tissue (11). Sheppard *et al.* (12) also reported a comparative study of eight methods for extracting FA from a variety of food products. When comparing extraction with different solvents such as chloroform/methanol and diethyl ether, the latter proved more successful with regard to FA recovery. In comparison, chloroform/methanol and methylene chloride/methanol were the most effective for extracting neutral and polar lipids (13). Diethyl ether and a chloroform/methanol mixture were evaluated to determine the total lipid content consisting of FA, cholesterol, and other sterols from food products (14). Hubbard *et al.* (14) reported that sample pretreatment with hydrochloric acid provided excellent lipid recoveries. In many of these methods, the solvent mixture included a chlorinated compound and methanol.

The need for a rapid and simple procedure to extract FFA from a fermentation medium with the intention of eliminating chloroform and the LCFA methyl esters derivatization step was the driving force for this study. Because the LCFA degradation reaction produces even-carbon-number products, shorter-chain FA also were examined. The LCFA concentrations used for extraction represented the range of substrate used and products expected from other work involving FA fermentation (2,15). The objectives of this work were to evaluate several solvent mixtures for extracting free LCFA containing 6 to 18 carbons at several concentrations and to examine parameters such as pH, ionic strength, and solvent as a means of optimizing the extraction recoveries.

EXPERIMENTAL PROCEDURES

Materials. Linoleic ($C_{18:2}$) (99%), oleic ($C_{18:1}$) (>99%), stearic ($C_{18:0}$) (99%), palmitic ($C_{16:0}$) (99%), myristic ($C_{14:0}$) (>99.5%), lauric ($C_{12:0}$) (>99.5%), capric ($C_{10:0}$) (>99%), caprylic ($C_{8:0}$) (>99.5%), and caproic ($C_{6:0}$) (99%) acids (Sigma Chemical Co., St. Louis, MO) were used for the extraction studies and to calibrate the gas chromatograph (HP 5890; Hewlett-Packard). Hexane, chloroform, diethyl ether, and methyl *tert*-butyl ether (MTBE) were HPLC grade (Caledon Laboratories, Georgetown, Ontario, Canada). GC carrier gases used were helium (99.999%) and nitrogen (99.999%) (BOC Gases, Toronto, Canada). Sodium chloride and concentrated sulfuric acid were reagent grade (VWR Canada, Toronto, Canada).

Solvent selection. The rationale for selecting a particular solvent or solvent mixture was based on several factors including the polarity of the LCFA under consideration, solvent toxicity, the LCFA K_{ow} values (octanol/water partition coefficients), solvent polarity index, and water solubility of the solvents (Tables 1, 2). Hexane, a nonpolar solvent with a low water solu-

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TABLE 1
Polarity Index and Water Solubility for Several Organic Solvents

Compound	Polarity index[a,b]	Water solubility (%) at 25°C
Hexane	0	0.001[c]
Chloroform	4.1	0.80[c]
MTBE	2.5	4.2[c]
Methanol	5.1	1116[d]

[a]The polarity index is a relative measure of the degree of interaction of the solvent with various polar test solutes.
[b]Data adapted from References 16 and 17.
[c]Data adapted from Reference 18.
[d]Data adapted from Reference 19. MTBE, methyl *tert*-butyl ether.

bility and polarity index, was predicted to extract the longer-chain FA more effectively than the shorter-chain acids such as $C_{6:0}$ and $C_{8:0}$. Chloroform, being much more polar than hexane, was expected to extract shorter-chain acids. Adding MTBE to hexane was predicted to provide the solvent mixture with a similar LCFA affinity as hexane, but with a greater capacity for extracting shorter-chain acids. Chloroform/methanol mixtures have been examined previously for lipid extraction (5). Based on these considerations, the four solvents evaluated were chloroform, chloroform/methanol (1:1), hexane, and hexane/MTBE (1:1).

(i) Method development for LCFA extraction. All samples for extraction were prepared in triplicate by adding specified amounts of an LCFA stock solution to 10 mL of an anaerobic culture in 20-mL serum bottles. The composition of the anaerobic medium was previously described (15). The culture contained 1,500 mg L^{-1} of anaerobic microbial biomass.

Samples (1 mL) were removed and transferred into 5 mL serum bottles containing 2 mL of an organic solvent. When pH adjustment was conducted, 80 μL of 50% H_2SO_4 was added. When salt was added, the amount was 0.05 g NaCl. After adding the solvent and adjusting the pH and ionic strength, the bottles were sealed with Teflon®-lined septa, secured with aluminum caps, and shaken using an orbital shaker at 200 rpm. The samples were then centrifuged for 5 min at 1,750 × *g* to separate the aqueous and organic layers.

LCFA analysis. Extracted samples (1 μL) were analyzed by GC using a Hewlett-Packard 5890 chromatograph equipped with an FID at 250°C, injector at 250°C, a 30 m × 0.53 mm diameter Nukol column (Supelco, Bellefonte, PA), and helium carrier gas flow at 5 mL min^{-1}. The oven temperature program was as follows: 0.5 min at 90°C, a 20°C min^{-1} ramp to 180°C, and a final hold at 180°C for 9 min. The effective detection limits ranged from 1 mg L^{-1} (in the bottle) for caproic to palmitic acids (C_6–C_{16}) and 2 mg L^{-1} for stearic, oleic, and linoleic acids (C_{18}).

Triplicate calibration standards for LCFA analysis of 1, 2, 3, 4, 5, 10, 20, 30, 40, 50, and 100 mg L^{-1} were prepared in a 1:1 mixture of hexane/MTBE using a 1500 mg L^{-1} LCFA stock solution. The stock solution was prepared with caproic, caprylic, capric, lauric, myristic, palmitic, stearic, oleic, and linoleic acids in diethyl ether (Sigma Chemical Co.).

At the beginning and end of each set of samples for GC analysis, a solvent blank followed by a series of standards was placed in the queue to verify the calibration. To check for sample carryover, a calibration standard of differing concentrations followed by a solvent blank was placed between every six to seven samples.

TABLE 2
K_{ow} Values for Several LCFA[a]

Compound	K_{ow}[b]	Compound	K_{ow}[b]
Linoleic	7.51	Lauric	5.0
Oleic	7.73	Capric	4.02
Stearic	7.94	Caprylic	3.03
Palmitic	6.96	Caproic	2.05
Myristic	5.98		

[a]Predicted using the KOWWIN™ software available from Syracuse Research Corporation (Syracuse, NY).
[b]K_{ow} = (concentration in octanol)/(concentration in water). LCFA, long-chain FA.

RESULTS AND DISCUSSION

The extraction results for chloroform, chloroform/methanol (1:1), and hexane are depicted in Figures 1–3. No pH adjustment or salt addition was conducted, and the samples were shaken for 15 min. For chloroform alone, the percent recoveries for FA with ≥10 carbons were >90% for cultures receiving 56.3 and 112.5 mg L^{-1} LCFA. However, lower recoveries, 70–94%, were obtained for cultures receiving 5.63 mg L^{-1}. Recoveries for $C_{8:0}$ were much lower, ranging down to 30% for cultures receiving 5.63 mg L^{-1}, whereas $C_{6:0}$ recoveries were less than 26% for all concentrations examined.

The chloroform/methanol mixture provided similar recoveries to chloroform for the 56.5 and 112.5 mg L^{-1} concentrations of the longer-chain LCFA (≥10 carbons). At the lowest concentration, 5.63 mg L^{-1}, the chloroform/methanol mixture provided significantly better recovery (95% confidence) for $C_{6:0}$ and $C_{8:0}$ (Table 3), greater than 90% recovery for both acids. The recoveries of $C_{10:0}$–$C_{14:0}$ were significantly lower for the chloroform/methanol mixture, however—as low as 80% recovery for $C_{12:0}$. Using hexane provided recoveries (93 to 100%) similar to those with chloroform and the chloroform/methanol solvent mixture for samples receiving 112.5 mg L^{-1} FA with ≥10 carbons. The recoveries for $C_{6:0}$ and $C_{8:0}$ were 15 and 76%, respectively. At the lowest concentration, 5.63 mg L^{-1}, hexane provided significantly poorer recovery (95% confidence) than the chloroform/methanol mixture for all but $C_{16:0}$.

Although effective for this application, especially when mixed with methanol, chloroform is a known carcinogen even at low concentrations (21). Under the 1986 U.S. Environmental Protection Agency (EPA) Guidelines for Carcinogen Risk Assessment, chloroform is classified as Group B2, a probable human carcinogen, based on sufficient evidence of carcinogenicity in animals (21–23). Additionally, chloroform is toxic to the microorganisms that conduct the fermentations (24,25). In comparison, hexane is not classified as a carcinogen and MTBE is classified as Group 3, a chemical for which the evidence of carcinogenicity is inadequate or limited in experimen-

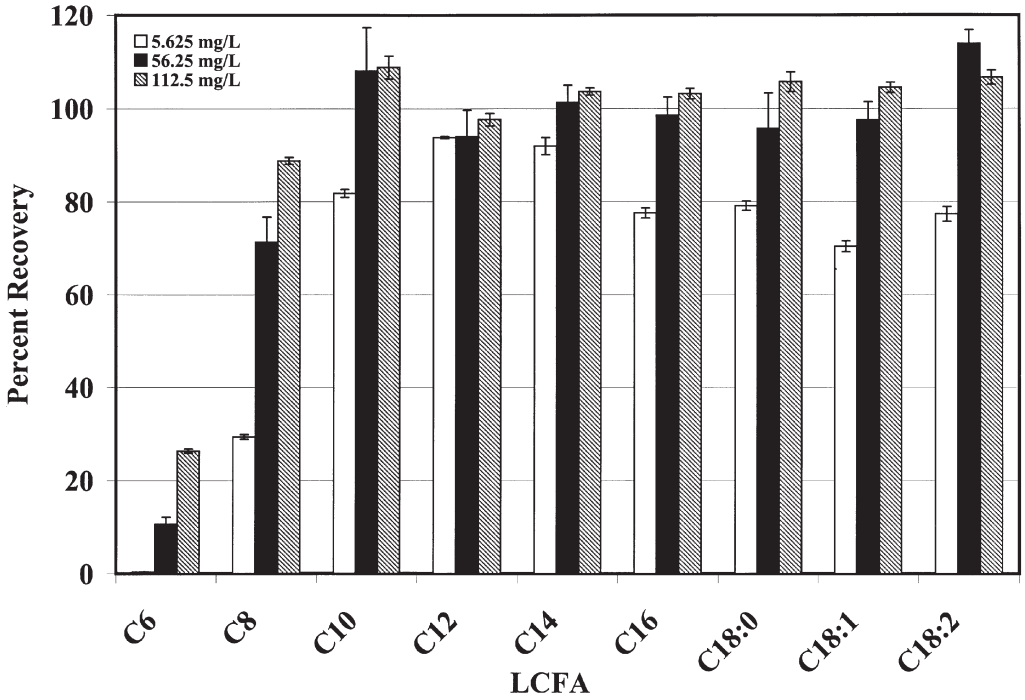

FIG. 1. Percentage of long-chain FA (LCFA) extracted into chloroform. Averages for triplicate samples; error bars represent the 95% confidence interval for the samples.

tal animals (26,27). Neither compound is known to affect the fermentative microorganisms. Although not entirely risk-free—hexane has a low flash point (28) and is classified as a hazardous air pollutant (29–32)—hexane and hexane/MTBE may be safer for laboratory analysis. Therefore, pH adjustment and salt addition were examined to improve the extraction recovery for hexane and the hexane/MTBE solvent mixture. The lowest concentration of LCFA proved particularly challenging for the hexane extraction (Fig. 3) and was examined further.

As expected, the hexane extraction without augmentation

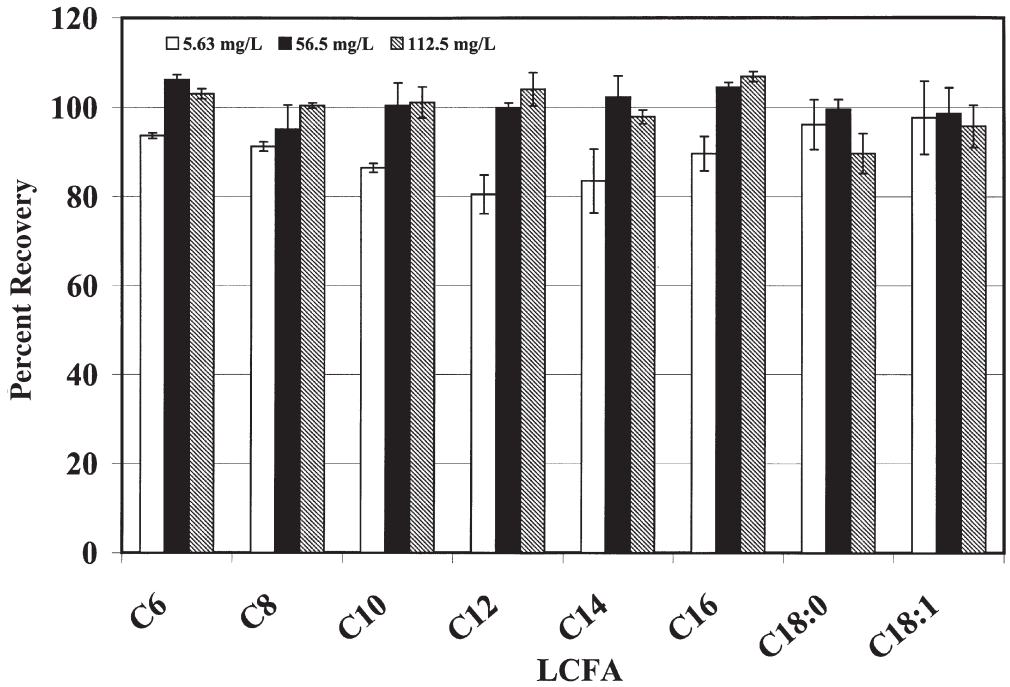

FIG. 2. Percentage of LCFA extracted into chloroform/methanol. Averages for triplicate samples; error bars represent the 95% confidence interval for the samples. For abbreviation see Figure 1.

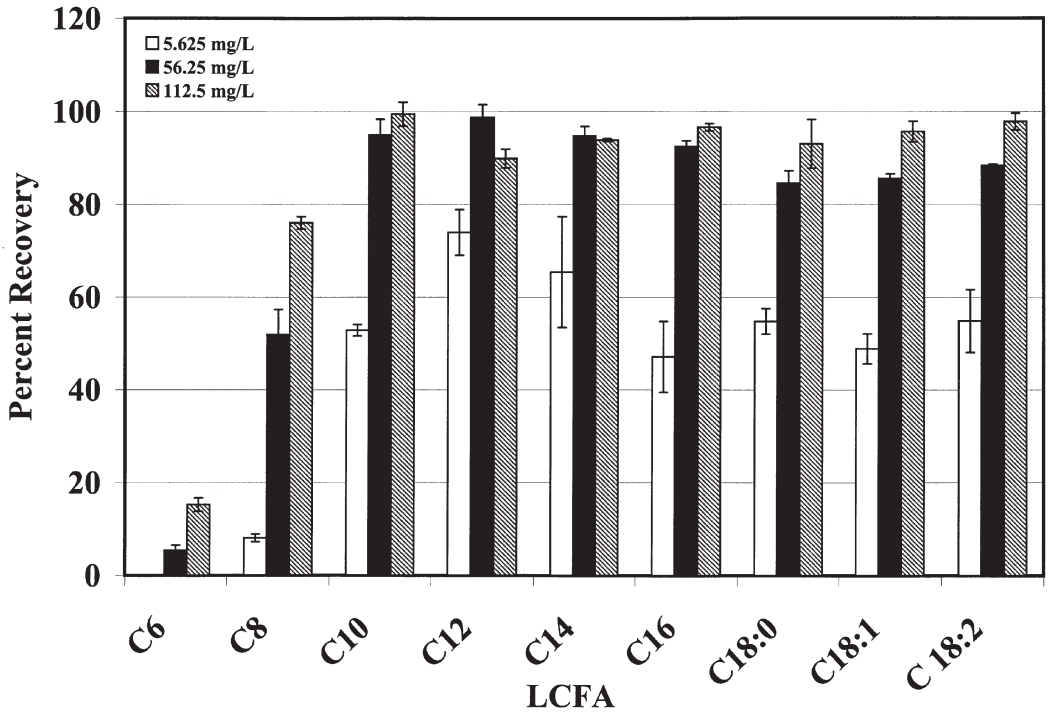

FIG. 3. Percentage of LCFA extracted into hexane. Averages for triplicate samples; error bars represent the 95% confidence interval for the samples. For abbreviation see Figure 1.

provided the lowest recoveries, from 66 to 77%, for all LCFA with ≥12 carbons (Fig. 4). Decreasing the pH so as to convert the LCFA to their acid form and decrease their aqueous solubility and adding NaCl to further decrease their solubility significantly improved extraction with hexane, providing recoveries of 82 to 93% for LCFA with ≥12 carbons (Fig. 4). For $C_{10:0}$, pH and NaCl addition provided an even greater improvement in recovery, up to 64% vs. 31% without pH and NaCl addition, but for $C_{6:0}$ and $C_{8:0}$, there was no improvement (Table 3).

Adding MTBE to hexane increased the polar characteristics of the extracting solvent (Table 1) and significantly improved the recovery vs. hexane alone (Fig. 4, Table 3) for all but $C_{16:0}$. This improvement was equal to the improvement gained by pH adjustment and NaCl addition for LCFA with ≥12 carbons, but the hexane/MTBE mixture provided significantly improved recovery of FA with fewer than 12 carbons (Table 3). The hexane/MTBE mixture augmented with pH adjustment and NaCl addition provided the best recoveries of the four hexane extraction alternatives examined (Fig. 4). In particular, the recoveries of all the LCFA examined except $C_{14:0}$ and $C_{18:1}$ were significantly improved (95% confidence). Furthermore, essentially 100% recovery of all LCFA containing 10 carbons or more was observed.

The hexane/MTBE mixture with pH adjustment and NaCl addition for ionic strength adjustment provided consistently high extraction recoveries (approximately 100%) for FA with

TABLE 3
Extraction Recoveries for 5.63 mg L^{-1} LCFA in Different Solvent Systems

Solvent system	Caproic ($C_{6:0}$)	Caprylic ($C_{8:0}$)	Capric ($C_{10:0}$)	Lauric ($C_{12:0}$)	Myristic ($C_{14:0}$)	Palmitic ($C_{16:0}$)	Stearic ($C_{18:0}$)	Oleic ($C_{18:1}$)	Linoleic ($C_{18:2}$)
Chloroform	0.3 ± 0.0[a]	29.4 ± 0.5[a]	81.8 ± 0.9[a]	93.8 ± 0.2[a]	91.9 ± 1.8[a]	77.6 ± 1.1[a]	79.1 ± 1.0[a]	70.4 ± 1.2[a]	77.4 ± 1.6[a]
Chloroform/methanol	93.7 ± 0.6[b]	91.2 ± 1.0[b]	86.5 ± 1.0[a]	80.5 ± 4.3[b]	83.5 ± 7.2[a]	89.6 ± 3.9[a]	96.2 ± 5.6[b]	97.7 ± 8.2[b]	NA
Hexane	ND	20.5 ± 0.5[c]	31.4 ± 0.7[b]	66.3 ± 5.0[c]	76.7 ± 7.1[b]	74.7 ± 7.7[a]	71.6 ± 6.4[a]	75.4 ± 10.3[a]	69.9 ± 10.1[a]
Hexane + NaCl + pH adjustment	ND	24.2 ± 2.2[c]	63.9 ± 8.4[c]	93.2 ± 4.3[a]	92.4 ± 4.8[a]	89.5 ± 5.5[a]	90.6 ± 4.6[b]	89.5 ± 6.0[b]	81.7 ± 7.6[a]
Hexane/MTBE	17.3 ± 0.2[c]	32.5 ± 1.6[a]	81.1 ± 0.9[a]	89.2 ± 5.4[a]	85.9 ± 7.1[a]	87.2 ± 7.4[a]	87.5 ± 5.4[b]	90.9 ± 6.9[b]	89.6 ± 5.2[a]
Hexane/MTBE + NaCl + pH adjustment	26.6 ± 2.2[c]	75.7 ± 4.4[d]	106.0 ± 6.4[d]	105.1 ± 5.2[d]	101.4 ± 5.2[a]	100.0 ± 5.4[b]	104.0 ± 8.7[c]	101.8 ± 6.0[b]	98.4 ± 9.9[b]

[a]All values are averages for triplicate samples. Data set pairs labeled using dissimilar letters (a, b, c, d) within the same columns are statistically different as evaluated at the 95% confidence interval using Tukey's procedure (20). The mixing time was 15 min for all samples. NA, data unavailable; ND, not detected; for other abbreviations, see Tables 1 and 2.

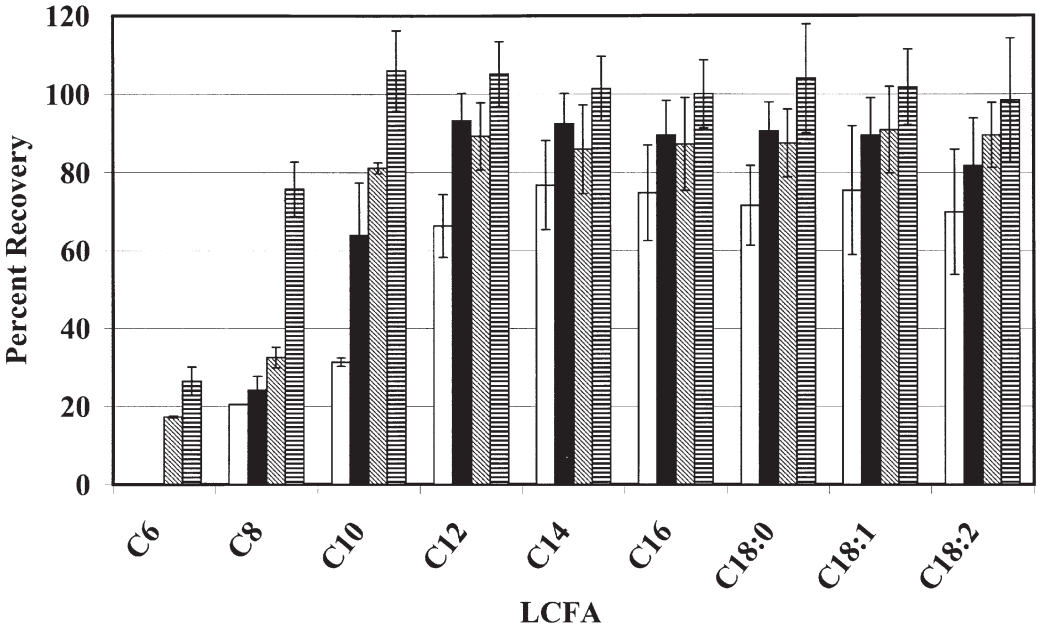

FIG. 4. Percentage of recovery for 5.63 mg L^{-1} C_6 to C_{18} in hexane and hexane/MTBE (15 min mixing at 200 rpm). Averages for triplicate samples; error bars represent the 95% confidence interval for the samples. Open bar: hexane; solid bar, hexane with NaCl and pH adjustment; diagonally lined bar, hexane/MTBE; horizontally lined bar, MTBE with NaCl and pH adjustment. MTBE, methyl *tert*-butyl ether; for other abbreviation see Figure 1.

≥10 carbons (Table 3). In particular, this method provided significantly improved recoveries (95% confidence) vs. both chloroform and chloroform/methanol for $C_{10:0}$, $C_{12:0}$, $C_{16:0}$, and $C_{18:0}$ and equal recoveries to one or the other of the chloroform alternatives for $C_{14:0}$ and $C_{18:1}$. For $C_{6:0}$ and $C_{8:0}$, the chloroform/methanol mixture was clearly preferable, although the hexane/MTBE mixture with pH adjustment and NaCl addition provided the next-highest extraction recovery for $C_{8:0}$.

The best extraction protocol for a given medium will depend not only on extraction recovery but also on other factors. In cases where chloroform-based extractants are less desirable, the hexane/MTBE method with pH adjustment and NaCl addition developed here may be more appropriate. The decreased extraction recoveries for $C_{6:0}$ and $C_{8:0}$ are compensated by the significantly improved recoveries of the longer-chain LCFA.

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