Methods for Analysis of Esterified and Free Long-Chain Fatty Acids in High-Lipid Food Processing Wastes

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Methods to screen the lipid composition of anaerobic food waste digester contents were developed. A colorimetric assay for long-chain fatty acid (LCFA) domains in mixed liquor measuring the partitioning of a lipophilic dye (Sudan III) between the lipid and an aqueous ethanol phase was validated using sheep tallow, deep water fish oil wax esters (orange roughy), olive oil, tripalmitin, tallow mixtures with phosphatidylcholine and cholesterol in a range of 0−10 g/L lipid, and mixed liquor from two different anaerobic digesters. Bacterial and yeast cells at up to 5 g/L did not interfere. The assay correlated well with the gravimetric lipid determination by the standard Bligh and Dyer method. Base-catalyzed esterification was employed as an alternative rapid method to trans-methylate esterified fatty acids in the presence of nonesterified lipid for gas chromatographic analysis. Derivatization and solvent extraction of methyl esters was followed by acidification of LCFA soaps and solvent recovery of the liberated fatty acids. Profiles were similar to those obtained using conventional silicic acid chromatography followed by methyl ester analysis.

Keywords: *Neutral lipid; LCFA; tallow waste; anaerobic digestion; process monitoring*

INTRODUCTION

Wastewater characterization by direct analysis of organic constituents has become important (Nielsen et al., 1992; Raunkjaer et al., 1994), and the main organic pools in wastewaters have been identified as protein, carbohydrate, lipid, and organic acids. Glyceride ester lipids (triglycerides, phospholipids, and glycolipids) have been recognized as major lipid components in domestic wastewater and industrial wastes (Raunkjaer et al., 1994; Cohen et al., 1994). Processing of certain deep water fish species such as orange roughy (*Hoplostethus atlanticus*) adds fish oil with unique wax ester lipids to seafood processing wastes (Buisson et al., 1982). Glyceride lipid, wax ester lipid, and long-chain fatty acid degradation by hydrolysis and fermentation to yield nonesterified ("free") long-chain fatty acids (LCFA) and volatile fatty acids (VFA) is a primary step in the conversion of lipid wastes to methane gas during anaerobic treatment (Cohen et al., 1994; Jarvis, 1995; Broughton et al., 1998; McInerney, 1988; Jarvis and Thiele, 1997a,b). However, high levels of free LCFA resulting from lipid fermentation are toxic for bacteria responsible for biogas production from the VFA, formate, and hydrogen plus carbon dioxide. Accumulation of free LCFA can lead to process failure (Hanaki et al., 1981; Broughton et al., 1998). Established standard methods cannot distinguish between esterified and free nonesterified LCFA (APHA et al., 1985). Also, the propensity of glyceride lipids, wax esters, and free LCFA to float demands rapid methods for routine monitoring of both esterified LCFA and free LCFA in digester contents to maintain process stability (Forster, 1992; Broughton et al., 1998; Jarvis and Thiele, 1997a).

To monitor and control conditions for optimal lipid digestion kinetics, we wished to develop methods to rapidly measure esterified LCFA and free LCFA in individual samples. Previously developed rapid HPLC methods allowed for detection of free LCFA in digester sludges but not for the simultaneous detection of fatty acid ester lipids (Jarvis and Thiele, 1997a) because only free carboxylic acid groups were derivatized by the method. Thus, a rapid complementary method to detect unhydrolyzed ester lipids would be very useful for confirmatory purposes and rapid process monitoring. Gravimetric and infrared methods according to *Standard Methods* (APHA et al., 1985; Raunkjaer et al., 1994) yield reproducible analyses for total oil and grease including free LCFA. However, they suffer from a low accuracy due to interference by coextracted nonlipid waste constituents and lipid masking effects by sludge material and are time-consuming, causing delayed operator responses during process control. Furthermore, they cannot distinguish between esterified lipids and lipid ester hydrolysates.

A total figure determining the relative abundance of LCFA esters in wastewaters and sludge samples may be sufficient to monitor hydrolysis of triglycerides and wax esters to LCFA on a routine basis. Although not recognized as a standard method for lipid measurement during wastewater analysis, lipid soluble dyes such as Sudan dyes have shown great potential for rapid semi-quantitative detection of "lipid droplets" in histochemical preparations (Conn, 1977), for tracing of wastewater grease particles in the ocean (Schulz et al., 1994), and for lipid analysis in food research (Heertje, 1993). Dye partitioning between a suitable solvent and the hydrophobic lipid particles in the waste materials and respective dye uptake may also be used to titrate the abundance of lipids in waste materials. Differential dye

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uptake by ester lipids and free LCFA would then allow selective quantification of esterified LCFA lipid domains.

Analysis of the individual LCFA composition of isolated lipid classes is conventionally carried out by silicic acid based TLC or column chromatography of the extracted lipid (Sebedio et al., 1987) and gas chromatographic analysis of methyl ester derivatives employing boron trifluoride as the derivatizing agent (Bannon et al., 1982). The use of solid-phase extraction to speed separation of free and esterified LCFA (Perkins, 1991; Bateman and Jenkins, 1997) is an attractive alternative, although several preparative steps are still required. Base-catalyzed methanolysis is known to selectively transmethylate triglycerides (Frohlich, 1993) in the presence of free fatty acids (where reaction with the base to form soaps is stoichiometrically favored). Methylation of the soaps is then achieved via acid-catalyzed esterification so the total methyl esters may be recovered. The nature of this reaction sequence gives an opportunity to separate esterified fatty acids in the sludge samples from the free fatty acid soaps without the need for separate chromatography. The total mass of unhydrolyzed lipids may be estimated in this way by calculation.

Here we compare the use of the modified base-catalyzed methylation procedure for routine quantitative detection of individual esterified and free LCFA with the use of Sep-paks or silicic column chromatography. We also report the development and validation of a new Sudan III dye based colorimetric method for quantitative detection of LCFA esters in digester contents.

MATERIALS AND METHODS

Materials and Chemicals. Sheep tallow was obtained from a slaughterhouse and its purity ascertained by elemental and gas chromatographic analyses (Broughton et al., 1998; Jarvis and Thiele, 1997b). Partially saponified tallow was prepared as described elsewhere (Jarvis and Thiele, 1997a). Pure lipid materials, Sudan (III) and Tris (Trisma Base), were from Sigma Chemical Co.. Deep water fish wax esters (orange roughy oil) were obtained from the Department of Chemistry, University of Otago. All other chemicals were obtained from BDH (of at least analytical grade) unless otherwise stated.

Sample Preparation. Total Lipid Extraction. Mixed liquor samples (50 mL; 1.5% TS w/w) were taken via a sample port at selected times from a stirred 18 L anaerobic batch digester (Broughton et al., 1998) following the addition of emulsified (8% palmitate) tallow (0.5% w/v) and were processed immediately. Lipid material was extracted from the samples with the method of Bligh and Dyer (1959) as follows. Subsamples (~4 g wet weight) were dispensed into a separating funnel, and chloroform/methanol (1:2 v/v, 15 mL) was added. The contents were shaken for 1 min and allowed to stand for 1 h. Chloroform (5 mL) was added followed by distilled water (5 mL). The chloroform layer that separated was transferred into a preweighed flask and the chloroform evaporated in vacuo. For gas chromatographic quantification, pentadecanoic acid (2 mg) and methyl heptadecanoate (2 mg) were added in the chloroform fraction.

Procedures. *Column Chromatography.* Extracted total lipid subsamples (~15 mg) were dissolved in hexane/diethyl ether (75:25 v/v, 10 mL) and transferred onto a glass column (15 mm diameter) containing magnesium silicate (5 g, 60−100 mesh, Florisil) that had been equilibrated in hexane/diethyl ether (75:25 v/v). The column was washed sequentially with hexane/diethyl ether (75:25 v/v, 17 mL), diethyl ether/methanol (92:8 v/v, 23 mL), and diethyl ether/acetic acid (96:4 v/v, 15 mL) to selectively recover the nonpolar and polar lipid fractions (Perkins, 1991). Fatty acid methyl esters were prepared from the triglyceride fraction involving saponification followed by boron trifluoride/methanol esterification (van Wijngaarden, 1967). Solvents were removed under vacuum from the LCFA fraction, which was dissolved in diethyl ether (2 mL) for injection into the gas chromatograph.

Solid-Phase Extraction. The column chromatography procedure was followed by employing disposable Extract-Clean cartridges containing Florisil (500 mg, 2.8 mL, Alltech Associates, Deerfield, IL) but using $^1/_{10}$ of the sample amount applied in hexane/diethyl ether (75:25 v/v, 1 mL). The column was eluted with the same series of washes using 2 mL volumes. Fractions were similarly treated as above.

Transmethylation Using Sodium Methoxide. The method of Frohlich (1993) was adapted to selectively transmethylate the triglycerides and separate the LCFA underivatized. Lipid samples (~1−2 mg) were dissolved in methanol (1 mL), and sodium methoxide (10 μL, 25 wt % solution in methanol, Aldrich Chemical Co.) was added. The solution was mixed and refluxed (10 min). Distilled water (2 mL) and diethyl ether (2 mL) were added to the cooled solution, and the organic layer containing the methyl esters was removed and transferred to a sample vial (2 mL). The aqueous layer was backwashed with two further portions of diethyl ether and then acidified with a few drops of concentrated HCl to break down the fatty acid soaps. LCFA were recovered into diethyl ether (2 mL) and transferred to a sample vial.

Transmethylation Using Tetramethylammonium Hydroxide (TMAH). Lipid samples (~1−2 mg) were dissolved in diethyl ether (1 mL), and the method of Metcalfe and Wang (1981) was followed to transmethylate the triglycerides by the addition of TMAH (10 μL, 25 w/w% solution in methanol, Sigma Chemical Co.). The solution was mixed well by inversion and left to stand at room temperature (5 min). Distilled water (2 mL) was added, and the methyl esters were recovered in the organic layer and transferred to a sample vial (2 mL). The aqueous phase was treated as in the sodium methoxide procedure, and the LCFA were similarly collected.

Gas Chromatography. Analyses were carried out using a Hewlett-Packard model 5890A gas chromatograph equipped with a flame ionization detector. Separation of both methyl esters and LCFA was performed on an FFAP capillary column (15 m, 0.54 mm i.d., Alltech Associates) in split mode (8:1). The temperatures were as follows: injector, 250 °C; detector, 250 °C; oven, 200 °C, programmed at 1 °C/min to 240 °C. The flow rates were as follows: helium (carrier), 6 mL/min; nitrogen (makeup), 25 mL/min; hydrogen, 40 mL/min; and air, 300 mL/min.

Dye Uptake Lipid Assay. Aqueous samples (1 mL) of stirred digester contents or aqueous suspensions of the pure lipid materials (see below) were mixed with Tris buffer (0.5 mL; 500 mM; pH 8.5) and Sudan III (3 mL; saturated solution at 20 °C diluted 1:2 in ethanol) and vortexed vigorously. The mixture was incubated for 10 min at 60 °C, vortexed, cooled on ice, and centrifuged (15 min, 12000*g*). The supernatant was decanted and the pellet washed with ethanol/water (70%; 4 °C). The washed pellet was redissolved in 3 mL of hexane heated to 60 °C (2 min) to extract the incorporated dye species and recentrifuged (5 min, 12000*g*). The supernatant was decanted and the absorbance at 500 nm determined in a Varian DMS 90 spectrophotometer. A calibration curve of 0−20 mg/mL lipid was prepared using the same protocol and respective aqueous emulsions of 0−20 g/L partially saponified sheep tallow (Jarvis et al., 1997a) as samples.

Testing of the Interference by Nonlipid Ester Materials on the Dye Uptake Lipid Assay. Concentrated stock solutions of the nonester lipids cholesterol and palmitic acid in 100% ethanol were added to emulsified tallow (Jarvis and Thiele, 1997a) to give final concentrations of 10 g/L tallow and 3 g/L of either cholesterol or palmitic acid. The response factors of respective dilutions of the mixtures were determined in the dye uptake lipid assay at 0, 2, 4, 6, 8, and 10 g/L tallow (= 0−13 g/L total lipid) and compared with the respective response factors of parallel assays using 0, 2, 4, 6, 8, and 10 g/L emulsified tallow alone. Interference by the nonester lipids

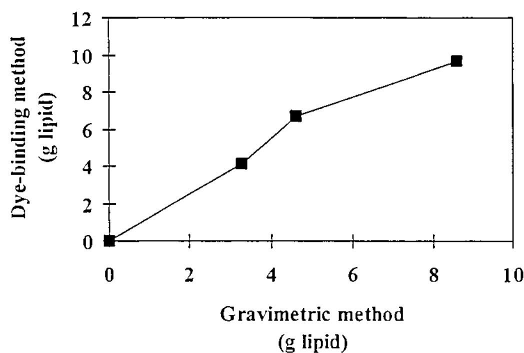

Figure 1. Recovery of added lipid levels in digester sludge determined according to the dye uptake colorimetric method and the standard gravimetric Bligh and Dyer method. Tallow was added at 0, 5, 7.5, and 10 g/L.

was measured comparing the response factors at each concentration with and without added nonester lipid.

Interference by microbial biomass (bacteria, yeast) and soluble carbohydrates (sucrose) on the dye binding assay was tested as follows: Ecoengineered anaerobic granular sludge consisting of bacterial cells (Thiele et al., 1990) was added to suspensions of emulsified tallow to give final concentrations of, respectively, 0, 1.5, 3, 4, 7, 9, 11, 13, and 15 g/L biomass (dry weight) and 8 g/L tallow. Commercial dry yeast was suspended in distilled water and added to emulsified tallow suspensions to give final concentrations of, respectively, 0, 1, 2, 3, 4, and 5 g/L biomass (dry weight) and 8 g/L tallow. Sucrose was dissolved in emulsified tallow suspensions to give final concentrations of, respectively, 0, 20, 40, 60, 80, and 100 g/L sucrose and 8 g/L tallow. The response factors of the respective mixtures were determined in the dye uptake lipid assay and compared with the respective response factors of parallel dye uptake assays using 8 g/L emulsified tallow alone. Interference was measured by comparing the response factors of each tallow suspension with and without added biomass or carbohydrate.

Validation of the Dye Uptake Lipid Assay with High and Low Lipid Content Digester Samples. For validation of the dye uptake method under realistic lipid digestion conditions with high total lipid contents (0−20 g/L), mixed liquor samples (100 mL) were taken at 0 h from four 18 L continuously stirred thermophilic anaerobic digesters (50 °C) seeded with anaerobic sludge from a slaughterhouse digester containing 5, 10, and 20 g/L emulsified sheep tallow as sole added source of carbon (Broughton et al., 1998). Duplicate subsamples (20 mL) from each mixed liquor sample were analyzed with the standard dye uptake lipid assay as described above. Nonspecific absorbance caused by endogenous mixed liquor lipids or coextracted colored constituents was determined in mixed liquor samples obtained from the respective control reactor (0 g/L tallow added). Duplicate mixed liquor samples were also analyzed by using the gravimetric lipid determination method as described elsewhere (Broughton et al., 1998), and the endogenous lipid pool of the sludge was determined from the respective control reactor mixed liquor (Figure 1). The analytical recovery efficiency of added triglyceride lipids in digester contents with low endogenous lipid pools was determined in parallel experiments in identical 18 L continuously stirred mesophilic digesters. These were operated for 6 months at 35 °C with daily doses of 1 g/L sodium acetate as sole source of carbon to deplete endogenous lipid pools. Samples (200 mL) thus acclimated to low lipid contents were spiked with emulsified sheep tallow (Jarvis and Thiele, 1997a), subsampled in duplicate (20 mL), and analyzed in parallel with the dye binding lipid assay and the gravimetric lipid determination method (see above). No correction for endogenous lipids of the mixed liquor was applied to the results (Table 1).

RESULTS AND DISCUSSION

A colorimetric lipid assay based on the uptake of lipophilic dyes from a solvent phase into biological lipid materials requires use of a dye with high selectivity for lipids, a polar solvent to force dye into the lipid phase, and conditions that allow for a rapid separation of residual dye bearing solvent and lipid materials. The solvent polarity and density for quantitative dye uptake and separation of stained lipid pellets and residual dissolved dye by centrifugation were thus optimized in a dilution series of partially emulsified tallow in various solvent/H_2O mixtures with Sudan III. A 2:1 ethanol/H_2O mixture produced deep red pellets from tallow, long-chain fatty acid wax esters (orange roughy fish oil), tripalmitin, olive oil, and various mixtures of tallow with cholesterol or phosphatidylcholine. At lower ethanol concentrations the lipid became brightly colored but could not be centrifuged to a pellet, and at higher ethanol concentrations easily centrifugable pellets were obtained but with very little dye uptake. Standard curves for tallow, fish oil wax esters, tripalmitin, and olive oil over the concentration range of 0−10 g/L lipid showed a linear relationship to absorption values at 500 nm (data not shown). Typical response coefficients of the dye uptake lipid assay for emulsified tallow were $0.09A_{500}$ g^{-1} L with a repeatability of ±5%. The relative response coefficients were 1.0, 0.96, 0.75, 0.46, and 0.0 for emulsified tallow, olive oil, tripalmitin, fish oil wax esters, and palmitic acid, respectively (data not shown).

Table 1. Comparison of Gravimetric (Bligh and Dyer) and Dye Uptake (Sudan Red) Methods for Quantifying Crude Lipid in Anaerobic Digester Sludge with Low Contents of Endogenous Lipids

material	lipid (g/L) gravimetric	lipid (g/L) dye uptake
mixed liquor (24 g/L solids)	0.7 ± 0.5	3.4 ± 0.2
mixed liquor + 10 g/L emulsified tallow added	5.8 ± 0.5	14.4 ± 0.4
10 g/L emulsified tallow	7.6 ± 0.5	10.9 ± 0.3
20 g/L partially (5%) saponified tallow (Sudan dye standard)	16.0 ± 0.5	20[a]
10 g/L palmitic acid in 50 mM Tris buffer, pH 8.5	7.0 ± 0.5	0

[a] By definition.

Table 2. Interference by Various Sludge Constituents on the Colorimetric Determination of Available Lipid in Sludge Samples Containing Tallow

condition	response factor (n = 6) mean ± SD	total solids, g/L
tallow only	0.036 ± 0.002	0−10
tallow plus		
phosphatidylcholine (3.33:1)	0.039 ± 0.006	0−6
cholesterol (3.33:1)	0.040 ± 0.002	0−6
yeast (2:1)	0.036 ± 0.001	0−13
palmitic acid (3.33: 1)	0.037 ± 0.002	0−6
bacterial sludge (>2:1)	0.036 ± 0.002	0−12
bacterial sludge (1:1)	0.031 ± 0.002	14−16
bacterial sludge (0.8:1)	0.027 ± 0.002	16−20

Table 3. Levels[a] of Free[b] and Esterified[c] LCFA (Millimoles per Liter) Recovered from Lipid Sludge

acid	silica column free	silica column bound	Extract-Clean free	Extract-Clean bound	sodium methoxide free	sodium methoxide bound	TMAH free	TMAH bound
14:0	0.806	0.170	0.797	0.049	0.793	0.031	0.788	0.042
16:0	9.15	0.440	8.84	0.179	9.55	0.104	9.55	0.122
18:0	4.43	0.330	4.10	0.109	4.53	0.092	4.19	0.081
18:1	1.60	0.280	1.50	0.100	1.40	0.077	1.60	0.067

[a] Average of four determinations. [b] As the free acid. [c] As the methyl ester.

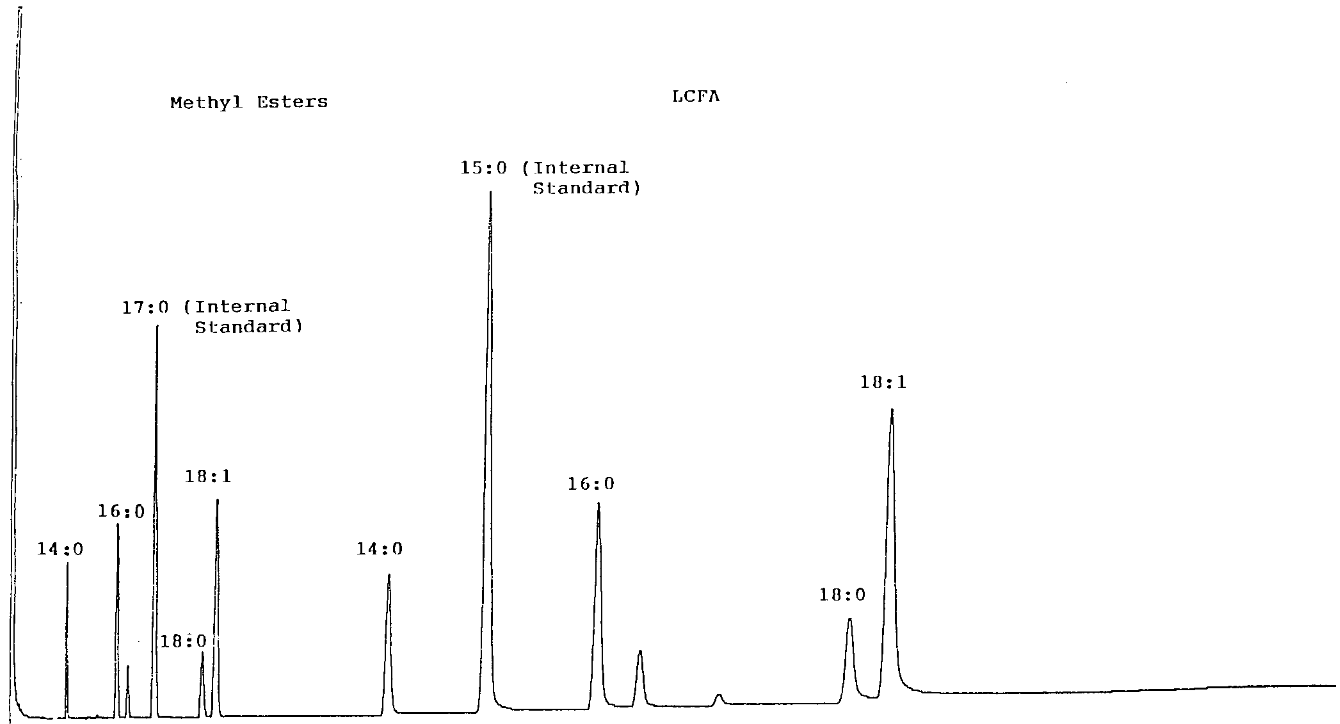

Figure 2. Separation of LCFA methyl esters and free acids.

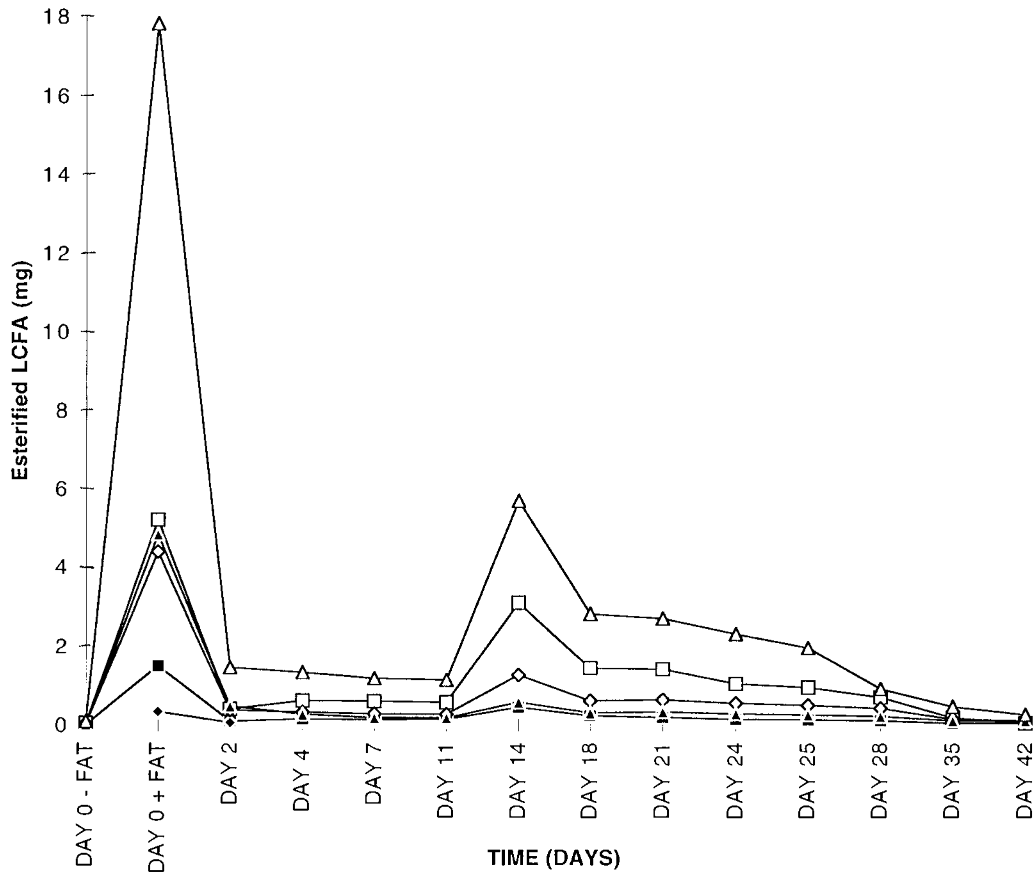

Figure 3. Levels of bound LCFA (methyl esters) during tallow fermentation in an anaerobic batch reactor: (■) C14; (□) C16; (◆) C16:1; (◇) C18; (▲) C18:1; (△) total.

Pure palmitic acid was not stained in the assay (Table 1). Dye uptake by mixtures of emulsified tallow with cholesterol (3.3:1, w/w), emulsified tallow with egg yolk lecithin (phosphatidylcholine; 3.3:1, w/w), and emulsified tallow with palmitic acid (3.3:1, w/w) were compared side by side with pure emulsified tallow over a concentration range of 0−10 g/L total tallow in the sample (Table 2). The relative response coefficients compared to pure tallow (per gram of tallow) were 1.1, 1.08, and 1.0, respectively. This showed that cholesterol, which

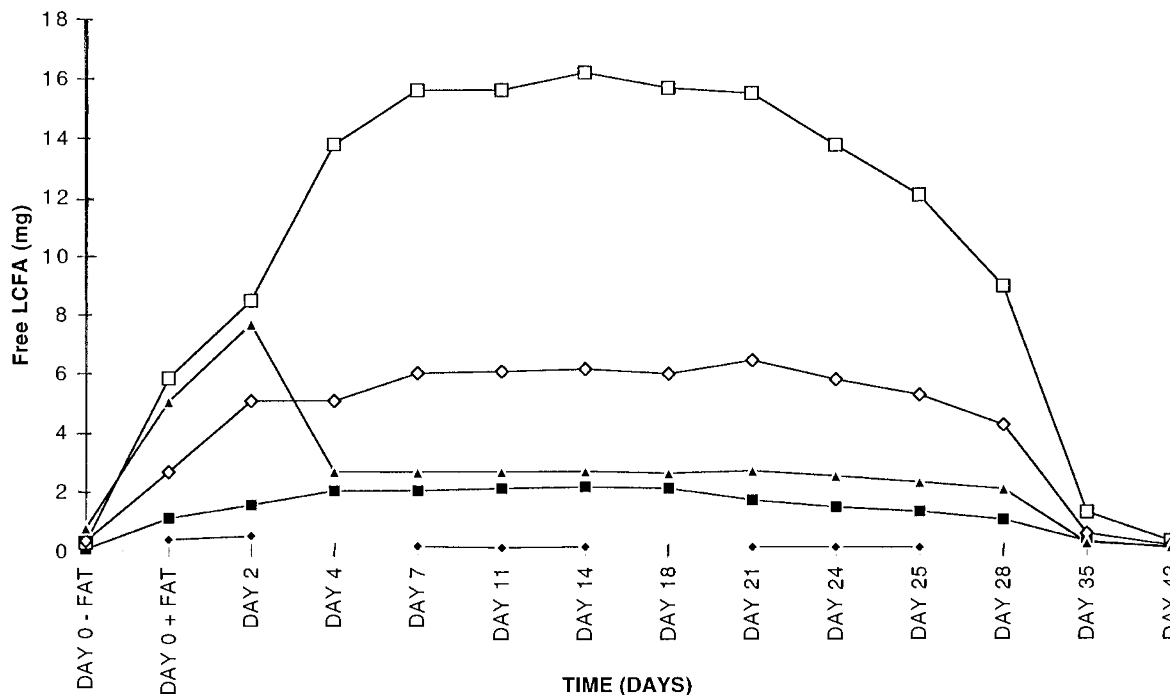

Figure 4. Levels of free LCFA during tallow fermentation in an anaerobic batch reactor: (■) C14; (□) C16; (◆) C16:1; (◇) C18; (▲) C18:1.

is known to intercalate between the fatty acid tails of lipids and egg yolk lecithin, had no effect on tallow dye uptake despite increasing the total lipid content 4-fold. Similarly, nonesterified free LCFA (palmitic acid) did not react in the dye uptake assay even when presented in a mixture with glyceride lipid materials (tallow). This corroborated the findings presented in Table 1. Among the tested glyceride ester lipids, tallow (melting point = 50−55 °C) and olive oil (melting point = 15 °C) showed higher response coefficients than tripalmitin (0.75; melting point = 66 °C), supporting the hypothesis that ester lipids with melting points below the assay temperature (60 °C) showed higher dye uptake.

The dye binding assay hence showed low selectivity for polar lipids and non-LCFA ester lipids. Effective dye uptake by mixtures of polar and neutral glyceride lipids reflected mainly the neutral lipid present, and respective response coefficients were virtually indistinguishable from the response with pure neutral lipids. Possible interferences caused by high concentrations of soluble carbohydrates (sucrose; 0−10%) and bacterial and yeast cells (up to 5 g/L added to an 8 g/L tallow solution) were insignificant (<5%; Table 2). This suggested that the dye uptake lipid assay could be a useful tool for rapid screening of neutral glyceride lipid levels in complex waste materials and biological sludges. The method will not detect free fatty acids.

Controlled experiments in operating anaerobic digesters were thus used to test the accuracy of the dye uptake lipid assay under practical conditions in comparison to the gravimetric Bligh and Dyer lipid extraction method. Nonspecific dye uptake by the mixed liquor in the absence of added lipid waste caused a colorimetric response equivalent to 3.4 g/L or 14% (w/w) of the sludge solids content (Table 1). This result was higher than obtained with the gravimetric method (Table 1) and indicated some interference from mixed liquor constituents at high solids concentrations (24 g/L).

When the digester contents were spiked with 10.0 g/L emulsified tallow, the dye binding lipid assay gave 11.0 ± 0.3 g/L lipid after the respective background interference (3.4 g/L) was subtracted (Table 1). When tested in direct comparison with the dye binding assay, the gravimetric method underestimated spiked lipid concentrations in sludge with low levels of endogenous lipids (Table 1). The low lipid recovery by extraction may have been due to specific masking effects of the sludge matrix in lipid digestion systems with low lipid contents (Broughton et al., 1998). When compared in sludge environments with high endogenous lipid levels of 5−15 g/L (Figure 1), both methods correlated well (Figure 1). Use of the dye uptake assay for monitoring of residual lipid levels in continuous anaerobic fermentations of 10 g/L tallow emulsions (Fraser, 1994) gave also absolute carbon recoveries of 84%. These data support the conclusion that the rapid dye binding assay is suitable for continuous rapid monitoring of neutral glyceride ester lipids in industrial effluent (for example, meat, dairy, butter, margarine, confectionery, olive oil, and palm oil processing industries).

Results obtained for the levels of methyl esters (representing esterified fatty acids present as triglycerides in the original sample) and LCFA determined by the four alternative chemical methods are shown in Table 3. Values obtained for the LCFA are consistent for all four methods. The difference in yields for the bound fatty acids is due to differential separation of intact triglycerides and the C17:0 methyl ester (chosen as internal standard) into the two less polar fractions for the column separation method. This led to overestimation of levels that would have been countered by selecting a triglyceride standard and is not a concern with the direct methylation procedures.

Base-catalyzed transesterifications therefore present an attractive alternative to the solid phase extraction procedures. Separation of LCFA and methyl esters in situ is convenient, and results show that ester hydrolysis is not occurring, nor is methylation of the LCFA similarly a problem. Gas chromatographic conditions may be chosen such that the two sets of analyses could

be performed simultaneously by co-injection, with the LCFA eluting well after the methyl esters (see Figure 2). Alternatively, both free and bound LCFA could be extracted by acidifying the reaction products prior to extracting the methyl esters.

Although the results appear similar, the sodium methoxide method may be preferable to the TMAH. This is due to the possibility of TMA soaps being transformed into methyl esters in the gas chromatographic injector (Metcalfe and Wang, 1981) should free acids be carried over in the first diethyl ether fraction or insufficient acid be added to break the soaps prior to extraction of the LCFA. The added precaution of drying the Bligh and Dyer chloroform extracts over anhydrous sulfate was taken to ensure the sodium methoxide was not compromised and that saponification of methyl esters cannot occur (McCreary et al., 1978).

Use of the sodium methoxide methylation procedure to monitor the production of LCFA from batch digesters is presented in Figures 3 and 4. Changes in the relative amounts of individual LCFA can be assessed as well as time course compositional differences between free and bound fatty acids. In this manner we were able to support the possibility of conversion of oleic acid to stearic and palmitic acids to relieve oleic acid toxicity prior to the gas production phase of digester activity (Broughton et al., 1998).

The two methods thus allow complementary screening of high-lipid waste sludges for LCFA and neutral lipid content. They can be performed on the same sample for the purpose of process monitoring or for more detailed information on lipid composition and quantification.

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LITERATURE CITED

APHA, AWWA, and WPCF. *Standard Methods for Examination of Water and Wastewater*, 16th ed.; American Public Health Association, American Water Works Association, and Water Pollution Control Federation: New York, 1985.

Bannon, C. D.; Breen, G. J.; Craske, J. D.; Hai, N. T.; Harper, N. L.; O'Rourke, K. L. Analysis of fatty acid methyl esters with high accuracy and reliability. III. Literature review of and investigations into the development of rapid procedures for the methoxide-catalysed methanolysis of fats and oils. *J. Chromatogr.* **1982**, *247*, 71−89.

Bateman, H. G.; Jenkins, T. C. Method for extraction and separation by solid-phase extraction of neutral lipid, free fatty acids, and polar lipid from mixed microbial cultures. *J. Agric. Food Chem.* **1997**, *45*, 132−134.

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

Broughton, M. J.; Thiele, J. H.; Birch, E. J.; Cohen, A. Anaerobic batch digestion of sheep tallow. *Water Res.* **1998**, *32*, 1423−1428.

Buisson, D. H.; Body, D. R.; Dougherty, G. J.; Eyres, L.; Vlieg, P. Oil from deep water fish species as a substitute for Sperm Whale and Jojoba oils. *J. Am. Oil Chem. Soc.* **1982**, *59*, 390−395.

Cohen, A.; Broughton, M. J.; Jarvis, G. N.; Thiele, J. H. Anaerobic treatment of high lipid wastes. *Proceedings of the New Zezland National IAQW Conference: Developments in Water Science & Technology*; 1994; 9 pages.

Conn, H. J. Sudan III. In *H. J. Conn's Biological Stains: A handbook on the nature and uses of the dyes employed in the biological laboratory*, 9th ed.; Lillie, R. D., Ed.; Williams & Wilkins: Baltimore, MD, 1997; pp 95−105.

Forster, C. F. Oils, fats and greases in wastewater treatment. *J. Chem. Technol. Biotechnol.* **1992**, *55*, 402−404.

Fraser, N. S. Process parameters of microbial tallow hydrolysis during anaerobic digestion. M.Sc. Thesis, University of Otago, Dunedin, New Zealand, 1994.

Frohlich, O. Fats and compounds in fat containing foods. In *Capillary Gas Chromatography in Food Control and Research*; Wittkowski, R., Matissek, R., Eds.; Technomic: Lancaster, PA, 1993.

Hanaki, K.; Matsuo, T.; Nagase, M. Mechanism of inhibition caused by long chain fatty acids in anaerobic digestion processes. *Biotechnol. Bioeng.* **1981**, *23*, 1591−1610.

Heertje, I. Microstructural studies in fat research. *Food Struct.* **1993**, *2*, 77−94.

Jarvis, G. N. The microbiology of anaerobic tallow hydrolysis. Ph.D. Thesis, University of Otago, Dunedin, New Zealand, 1995.

Jarvis, G. N.; Thiele, J. H. High performance liquid chromatographic analysis of free long chain fatty acids produced during lipolysis by anaerobic digester sludge. *J. Chromatogr. A* **1997a**, *760*, 271−277.

Jarvis, G. N.; Thiele, J. H. Qualitative Rhodamine B assay which uses tallow as a substrate for lipolytic obligately anaerobic bacteria. *J. Microbiol. Methods* **1997b**, *29*, 41−47.

McCreary, D. K.; Kossa, W. C.; Ramachandran, S.; Kurtz, R. R. A novel and rapid method for the preparation of methyl esters for gas chromatography: application to the determination of the fatty acids of edible fats and oils. *J. Chromatogr. Sci.* **1978**, *16*, 329−331.

McInerney, M. J. Anaerobic hydrolysis and fermentation of fats and protein. In *Biology of Anaerobic Microorganisms*; A. J. B. Zehnder, A. J. B., Ed.; Wiley: New York, 1988; Chapter 8, pp 373−417.

Metcalfe, L. D.; Wang, C. N. Rapid preparation of fatty acid methyl esters using organic base-catalyzed esterification. *J. Chromatogr. Sci.* **1981**, *19*, 530−535.

Nielsen, P. H.; Raunkjaer, K.; Norsker, N. H.; Jensen, N. Aa.; Hvited, J. T. Transformation of wastewaters in sewer systems—a review. *Water Sci. Technol.* **1992**, *25*, 17−31.

Perkins, E. G. *Analyses of Fats, Oils and Lipoproteins*; American Oil Chemists' Society: Champaign, IL, 1991; pp 122−137.

Raunkjaer, K.; Hvited-Jacobsen, T.; Nielsen, P. H. Measurement of pools of protein, carbohydrate and lipid in domestic wastewater. *Water Res.* **1994**, *28*, 251−262.

Schulz, T. J.; Marczan P. J.; Fane A. G. Behaviour of sewage effluent oil and grease in the ocean. *Water Environ. Res.* **1994**, *66*, 800−804.

Sebedio, J. L.; Astorg, P. O.; Septier, C.; Grandgirard, A. Quantitative analyses of polar components in frying oils by the Iatroscan thin-layer chromatography-flame ionisation detection technique. *J. Chromatogr.* **1987**, *405*, 371−378.

Thiele, J. H.; Wu, W.; Jain, M. K.; Zeikus, J. G. Ecoengineering high rate anaerobic digestion systems: Analysis of syntrophic biomethanation catalysts. *Biotechnol. Bioeng.* **1994**, *35*, 990−999.

van Wijngaarden, D. Modified rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Anal. Chem.* **1967**, *39*, 848−849.

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