ELSEVIER

Journal of Chromatography A, 760 (1997) 271–277

JOURNAL OF
CHROMATOGRAPHY A

Short communication

High-performance liquid chromatographic analysis of free long chain fatty acids produced during lipolysis by anaerobic digestor sludge

Graeme N. Jarvis*, Jürgen H. Thiele

Waste Technology Research Centre, Department of Microbiology, University of Otago, P.O. Box 56, Dunedin, New Zealand

Received 26 June 1996; revised 18 September 1996; accepted 25 September 1996

Abstract

The development of sample extraction techniques in conjunction with application of a modified version of an established HPLC technique allowed the rapid estimation of concentrations of long chain fatty acids (LCFA) produced by the lipolytic activity of bacteria in anaerobic digestor sludges or experiments using the sludges as inocula. It was established that free long chain fatty acids will preferentially partition into certain phases in the sludge or experimental cultures. These data, and application of the developed techniques for monitoring of LCFA, may lead to the avoidance of toxicity and failure of full-scale anaerobic digestors treating high lipid content wastes in the future.

Keywords: Fatty acids

1. Introduction

Anaerobic digestors have been commonly used as a treatment for solids settled off from domestic sewage as well as microbial sludge from aerobic treatment plants. Indeed, anaerobic digestion has been an integral part of domestic sewage plants since the turn of the century [1,2]. Nowadays, digestors are finding more applied use, especially with respect to treatment of industrial waste waters [3]. It has been noted that in anaerobic digestors, most of the lipids associated with wastes and waste water are potentially biodegradable in both mesophilic and thermophilic anaerobic conditions [4]. However, the presence of lipids in both anaerobic and aerobic biological treatment systems is known to cause serious problems with respect to process stability [5–7]. This is due to the tendency for lipids to float in aqueous environments [8]. Several investigators have reported lipids and free long chain fatty acids (LCFA) to be inhibitory to the various bacterial trophic groups associated with the anaerobic digestor ecosystem [5,9–11]. Therefore, the anaerobic digestion of slaughterhouse wastes (and wastewater) is a challenging problem due to the high lipid content (up to 65% of the dry weight mass or volatile solids) associated with this type of waste [12,13].

In order to study the role of lipids in anaerobic digestion processes, we required a fast and effective chromatographic assay for determination of free LCFA, the major products from hydrolysis of neutral lipids by microbial activity in anaerobic digestors.

*Corresponding author. Present address: AgResearch Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand.

0021-9673/97/$17.00 Copyright © 1997 Elsevier Science B.V. All rights reserved
PII S0021-9673(96)00808-4

Here we report on development of methods for extraction of free LCFA from supernatant, biomass and lipid phases, and their application to in vitro experiments using anaerobic digestor contents and a neutral lipid substrate, in conjunction with a modified version of an existing HPLC method [14].

2. Experimental

2.1. Source of inocula

Sludge from a pilot scale mesophilic anaerobic digestor fed on meat waste at Waste Solutions Ltd. (Dunedin, New Zealand) was used as the inoculum source in the current study. The meat waste contained 60–70% volatile solids and consisted of the mince, offal and paunch from sheep carcasses. The digestor was operated for a three month period and maintained a stable pH of 7.8 prior to the sample being taken.

2.2. Reagents and chemicals

Capric (C10:0), lauric (C12:0), myrisitic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), palmitoleic (C16:1), margaric (C17:0), stearic (C18:0), oleic (C18:1), behenic (C22:0) and arachidic (C24:0) acids, and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (1-EDC-HCl) and 2-nitrophenylhydrazine (NPH) were purchased from Sigma (St. Louis, MO, USA). Analytical reagent grade acetonitrile was purchased from Ajax (Auckland, New Zealand). Analytical reagent grade methanol, ethanol and hydrochloric acid (HCl) were purchased from BDH Chemicals (Dorset, UK). Pyridine (analytical reagent grade) was purchased from Rhone Poulenc (Lyon, France). Unless otherwise stated, all other chemicals were purchased from Sigma (St. Louis, MO, USA), and were of analytical grade.

2.3. Reagent solutions

All reagents were prepared according to the previously published methods [14–16], except for the NPH-HCl (100 m*M*). Briefly, 20 ml of the NPH-HCl working solution was prepared by adding 0.306 g of NPH to a MilliQ water–100 m*M* HCl-ethanol solution (3:1, v/v). To this, 100 μl of concentrated HCl was added dropwise, and the final volume brought up to 20 ml by the addition of more MilliQ water–HCl-ethanol mix. The EDC-HCl working solution was prepared by adding together equal volumes of EDC-HCl stock solution (250 m*M*) in ethanol and pyridine (13%, v/v) in ethanol. All individual LCFA standards were prepared by dissolving the LCFA in absolute ethanol to obtain the required final concentration (10, 1, 0.1, or 0.01 mg ml^{-1}). Four different concentrations of mixed standard solutions composed of all fatty acids (C10:0–C24:0) were also prepared. The final concentration of each LCFA in each of the four mixed standards was 10, 1, 0.1 or 0.01 mg ml^{-1}, respectively.

2.4. Sample preparation

The procedures utilised for determination of free LCFA in samples from digestors treating slaughterhouse wastes are shown in Fig. 1. During in vitro experiments using digestor contents as inocula, the same procedures were employed to obtain data on total, biomass- and supernatant-associated free LCFA. In addition, the amount of free LCFA associated with an added neutral lipid (tripalmitin) used as bacterial carbon and energy sources was also determined. This was achieved by the removal of all supernatant and biomass from liquid cultures in Hungate tubes using a 27 gauge needle and syringe (BDH Chemicals). This technique avoided the loss of lipids which, being hydrophobic in aqueous solutions, tended to partition at the gas–liquid interface during the processing of the tubes, and were in fragments too large to be removed by the fine gauge syringe needle utilised. In all cases, margaric acid (C17:0) was used as the LCFA internal standard at a concentration of 10 g l^{-1}, and a total of 100 μl of subsample utilised in the derivatization step.

2.5. Derivatization procedure

The procedure was a modification of that previously employed to obtain 2-nitrophenylhydrazide derivatives of the fatty acids [14–16]. Briefly, to a 100-μl sub-sample (or 50 μl LCFA standard plus 50 μl ethanol), a 100-μl volume of 100 m*M* NPH-HCl

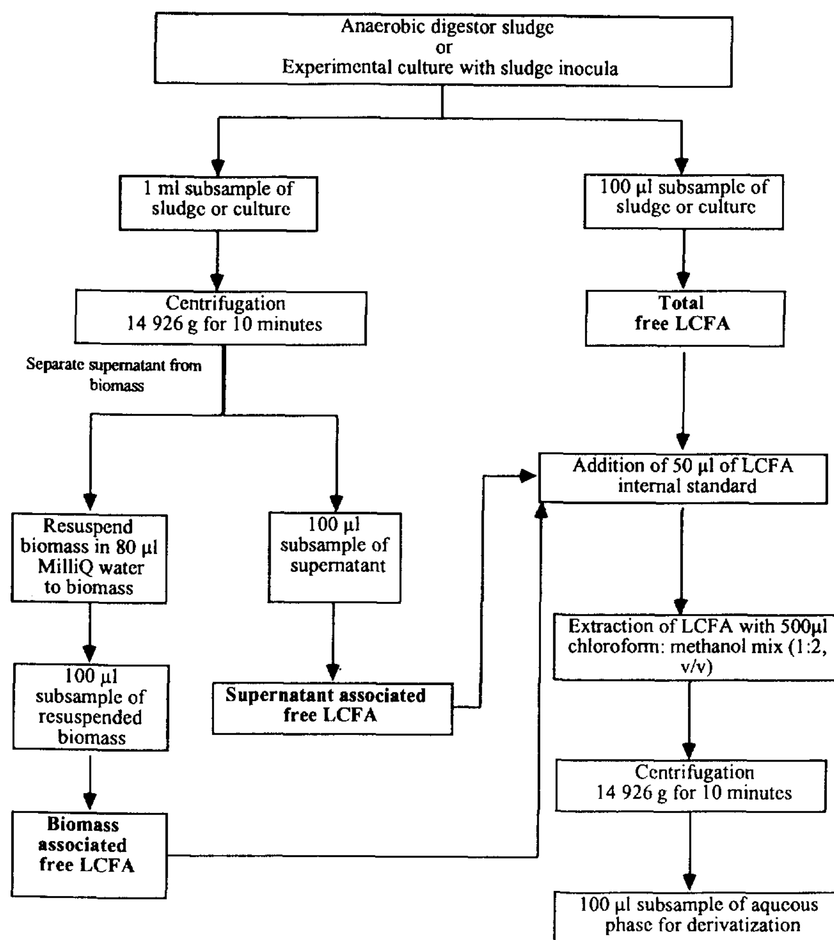

Fig. 1. Flow diagram of sample preparation prior to direct derivatization for anaerobic digestor sludge using a chloroform–methanol extraction technique.

was added. The mixture was then vortex-mixed for 2 s prior to the addition of 200 µl of EDC-HCl working solution. Another vortexing step for 2 s followed, prior to the centrifugation of the samples for 1 min at 14 926 *g*. The samples were then incubated at 60°C for 10 min, then 16 µl of 15% (w/v) KOH was added, the samples vortex-mixed (2 s) and centrifuged again (1 min at 14 926 *g*), followed by another 60°C incubation for 5 min. The samples were placed in a cold water bath for 2 min. Then, 350 µl of sample supernatant was placed into sample cups (Roche Diagnostics, MD, USA) for HPLC analysis.

2.6. High-performance liquid chromatography

The analysis was undertaken using a system configuration of two LC1500 HPLC pumps, an LC1600 autosampler and a LC1200 variable-wavelength UV-Vis detector (GBC Scientific, Melbourne, Australia). The HPLC system was interfaced to a computer using a DP800 data interface (GBC Scientific). The detector was operated at a wavelength (λ) of 405 nm. Separation of LCFA was achieved using a 250×4.6 mm C_8 reversed-phase column packed with 5 µm Econosil (Alltech Asssociates, IL, USA). The column was operated at ambient room

temperature. The program operated by the computer led to a gradient system running from 30% (v/v) to 0% (v/v) methanol being used to separate the LCFA over the course of a chromatogram run time of 12 min. Two mobile phases were employed during the course of the present study. Mobile phase A consisted of two solvent reservoirs one being filled with 100% acetonitrile, and the other with 70% (v/v) acetonitrile in water. The second mobile phase utilized (mobile phase B), consisted of two solvent reservoirs: one being filled with a 1% (v/v) acetic acid in methanol, and the other with 1% (v/v) acetic acid in water. The flow-rate employed was 1.5 ml min^{-1}. The concentration of LCFA present in the samples and identity of the LCFA, were determined by comparison with known standard solutions and were calculated by the DP800 program based on the response factor for the internal LCFA standard and calibration curves constructed for each of the LCFA using peak-area values as the major analytical parameter. The lower detection limit for LCFA was 100 μM using the system under standard operating conditions.

3. Results and discussion

The original HPLC method was employed for analysis of free fatty acids associated with clinical samples (e.g. human serum), rather than environmental samples [16]. We have adapted the original method for use in studies of the hydrolysis of neutral lipids by consortia of anaerobic bacteria. One modification we have undertaken on the method has led to the reduction in time taken for derivatization of samples. With our modifications the derivatization procedure now takes 20 min to complete, compared to 35 min for the original method [14,16]. Overall the HPLC technique we have adapted from Miwa et al. [14,16] offers advantages over many other HPLC methods in that it utilises pre-column derivatization techniques (increasing sensitivity and selectivity of detection), and has a shorter chromatogram run time (less than 16 min compared to 40–70 min for other techniques) [14,16].

Examples of the results obtained from the application of this HPLC method (Figs. 2 and 3), indicate that it has a use in future studies using environmental

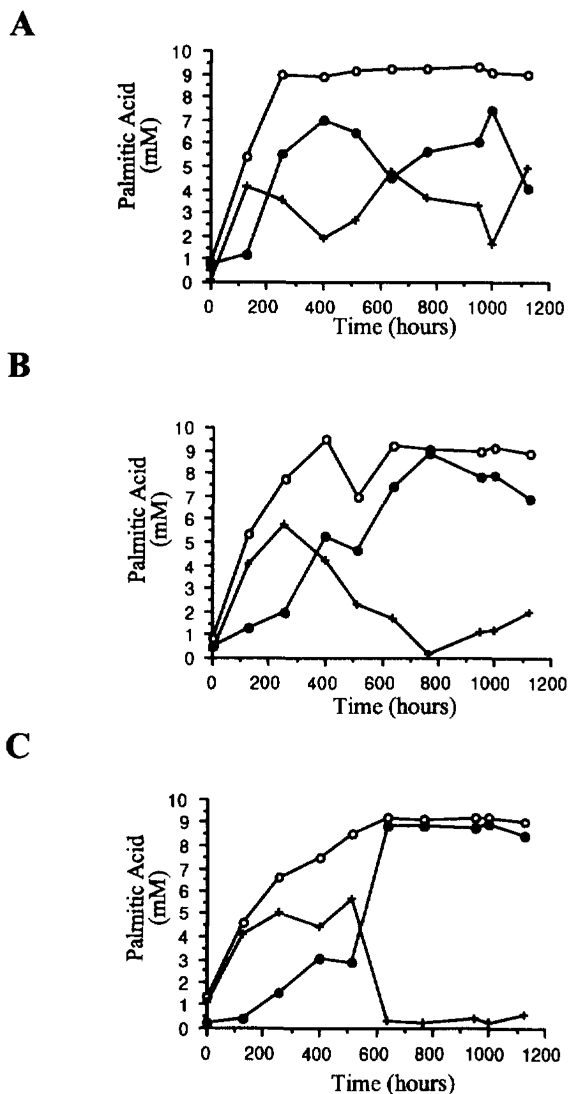

Fig. 2. Results illustrating the hydrolysis of tripalmitin into palmitic acid, and the LCFA partitioning into various phases in Hungate tubes containing tripalmitin-based medium inoculated with serial dilutions of anaerobic digestor. (A) Results for 10^{-3}, (B) 10^{-4} and (C) 10^{-5} dilutions of digestor sludge, respectively. The phases assayed for palmitic acid distribution at each time point were the lipid phase (+), biomass phase (●) and total sample (○). The experimental samples were incubated at 37°C with orbital agitation and a complete Hungate was sacrificed at the time points indicated for each sludge dilution to obtain the data on the free LCFA present in each of the three phases. Complete tripalmitin hydrolysis would yield 9.3 mM palmitic acid.

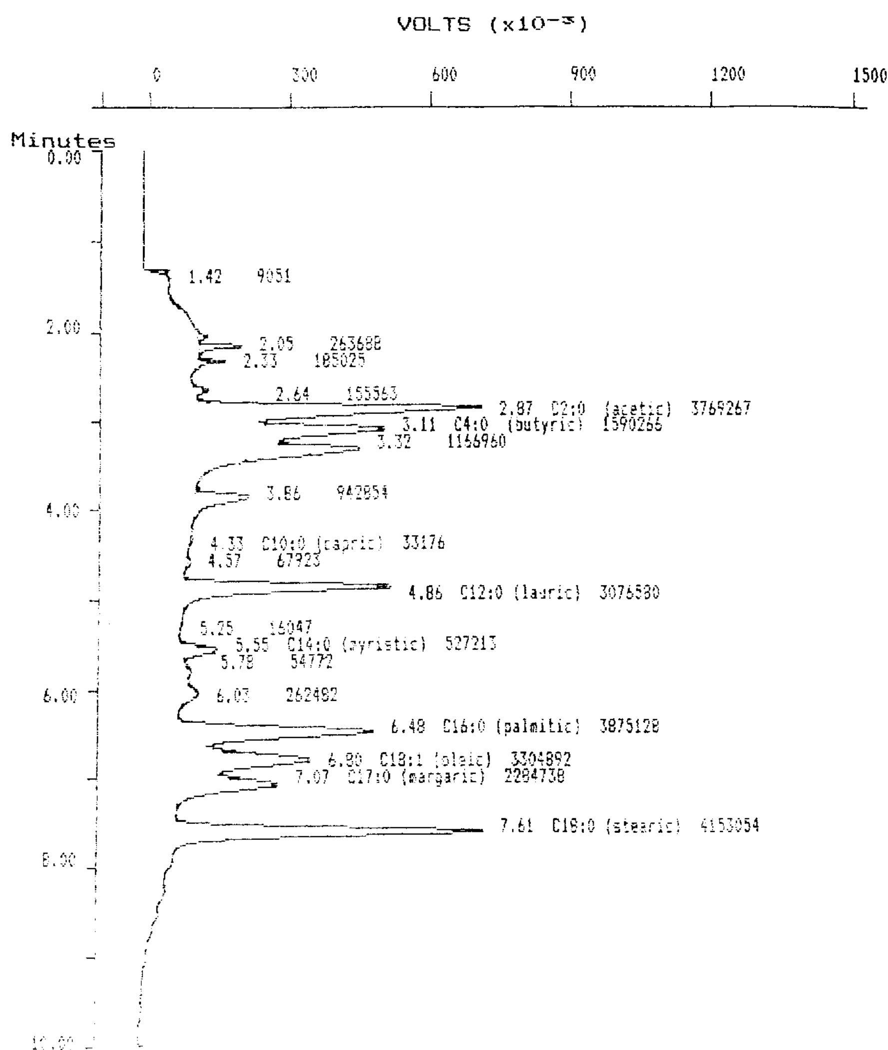

Fig. 3. Chromatogram of the free fatty acid profile of tallow-based medium inoculated with a 10^{-2} dilution of anaerobic digestor sludge. In the sample chromatogram, the LCFA peaks were identified, the retention times recorded and areas below the curve (ABC) calculated by the DP800 program (GBC Scientific, Melbourne, Australia). Unknown peaks also had retention times and ABC calculated, and margaric acid (10 mg ml^{-1}) was used as the internal standard.

samples. The data obtained (Fig. 2.) indicated that with respect to microbial hydrolysis of neutral fats (using tripalmitin as a model neutral fat), there exists a "partitioning " of free LCFA in the biomass and lipid phases of anaerobic digestor sludges [17]. Little or no free LCFA were found to be associated with the supernatant phase. These preliminary in vitro results have important ramifications not only on the analytical procedures used, but also on conclusions that one draws from such studies. For example, measurement of LCFA levels using only the supernatants from in situ or in vitro anaerobic digestor experiments will lead to misleading data being obtained on the LCFA levels present. This observation supports data from a previous study where it was noted that LCFA accumulated rapidly within a day in the digestor sludge, but neither accumulated rapidly nor to the same level in the supernatant fraction [5]. The sample chromatogram (Fig. 3) illustrates the baseline noise and separations of

different LCFA in an experimental sample using tallow as the neutral lipid carbon source for bacterially-mediated lipolysis by anaerobic digestor contents. The chromatogram showed that whilst some baseline noise occurred (due to unidentified compounds in the digestor contents), integration of the LCFA was achieved by the DP800 program.

The accuracy and sensitivity of the HPLC method (Table 1) were also checked by comparison of the results for an LCFA profile obtained from saponified tallow samples by HPLC [17], compared to both GC analysis of the same saponified tallow (M. Broughton, personal communication, 1995), and previously published results of tallow LCFA profiles using GC analyses [18,19]. Regardless of the chromatographic method employed (Table 1), the predominant LCFA present in the tallow were shown to be palmitic, oleic and stearic acids. Comparable LCFA profiles (Table 1) were obtained by both chromatographic procedures regardless of source, (either the present study or previously published results) of the data (Table 1). The GC analyses of tallow could account for 92.3–96.6% of the LCFA based upon weight % values. In comparison, the HPLC analyses were able to account for 99.6±2.3% of the LCFA (Table 1). The use of either of the two mobile phases employed in the current study was not observed to affect the results when experimental samples were analysed using both mobile phases [17].

These data indicate that the HPLC method gives comparable results to those obtained by GC analyses. Therefore, the HPLC method would seem to be as reliable and accurate as the more commonly employed GC methods. The rapid analysis time, simple extraction protocols and ability to analyse different fractions of sludge samples from anaerobic digestors, will allow more data to be obtained on this ecosystem in order to gain further insight into the process of lipolysis and lipid or LCFA inhibition. Therefore it is hoped that in the future, by careful monitoring of LCFA levels in anaerobic digestor contents using the analytical protocols and techniques established here, that the avoidance of complete process failure may be achieved when treating high lipid content wastes or wastewaters.

Acknowledgments

This study was funded by grants from Waste Solutions Ltd., Dunedin (New Zealand) and the Otago Research Committee. The authors wish to thank Dr. Tico Cohen and Mr. Mike Broughton of

Table 1
The fatty acid profile of tallow used in the current study analysed by HPLC and GC (GC analysis 3) compared to previously published data (GC analyses 1 and 2), illustrating the reliability and accuracy of the HPLC method

Fatty acid	HPLC analysis[a] (weight %)	GC analysis 1[b] (weight %)	GC analysis 2[b] (weight %)	GC analysis 3[b] (area %)
10:0–14:0	7.6±0.5	3.4	4.6	4.4
14:1	0.0	0.9	0.0	0.0
15:0	0.0	0.6	0.0	0.9
16:0	20.9±0.1	20.0	24.9	21.9
16:1	0.0	2.7	4.2	1.3
17:0	0.0	1.8	0.0	1.7
17:1	0.0	1.3	0.0	0.0
18:0	29.1±0.8	30.0	18.9	22.3
18:1	42.0±0.9	33.0	36.0	35.4
18:2	0.0	1.0	3.1	0.0
18:3	0.0	0.8	0.6	0.0
20:0–24:0	0.3±0.0	1.1	0.3	0.0
Total	99.6±2.3	96.6	92.3	87.9

[a] The HPLC data represents the average of triplicate analyses (±standard deviation) of the tallow.

[b] The GC data were obtained from the following sources: GC analysis 1: Davey et al. [19]; GC analysis 2: Anderson [18]; GC analysis 3: M. Broughton, personal communication, 1995.

Waste Solutions Ltd., for supplying the anaerobic digestor sludge and GC data used in the study.

References

[1] D.E. Hughes, What is Anaerobic Digestion? An Overview, Applied Science Publishers, Essex, 1980.
[2] H. Sahm, Advances in Biochemical Engineering/Biotechnology, 1 (1984) 83–115.
[3] L. Huber and G. Metzner, Examples of Industrial Waste Water Treatment, VCH Publishers, Weinheim, 1986.
[4] F. Roy, G. Albagnac and E. Samain, Appl. Environ. Microbiol., 49 (1985) 702–705.
[5] K. Hanaki, T. Matsuo and M. Nagase, Biotech. Bioeng., 23 (1981) 1591–1610.
[6] I.W. Koster, Biol. Wastes, 22 (1987) 295–301.
[7] T. Komatsu, K. Hanaki and T. Matsuo, Wat. Sci. Tech., 23 (1991) 1189–1200.
[8] A. Rinzema, Anaerobic Treatment of Wastewater with High Concentrations of Lipids or Sulfate, Wageningen Agricultural University, Wageningen, 1988.
[9] D.I. Demeyer and H.K. Henderickx, Biochim. Biophys. Acta, 137 (1967) 484–497.
[10] I. Angelidaki and B.K. Ahring, Applied Microbiol. Biotech., 37 (1992) 808–812.
[11] A. Rinzema, M. Boone, K. van Knippenberg and G. Lettinga, Water Environ. Res., 66 (1994) 40–49.
[12] R.I. Mackie, B.A. White and M.P. Bryant, Crit. Rev. Microbiol., 17 (1991) 449–478.
[13] M.J. McInerney, in A.J.B. Zehnder (Editor), Biology of Anaerobic Microorganisms, Wiley, New York, 1988, pp. 373–415.
[14] H. Miwa, C. Hiyama and M. Yamamoto, J. Chromatogr., 321 (1985) 165–174.
[15] H. Miwa, M. Yamamoto and T. Momose, Chem. Pharm. Bull, 28 (1980) 599–605.
[16] H. Miwa, M. Yamamoto, T. Nishida, K. Nunoi and M. Kikuchi, J. Chromatogr., 416 (1987) 237–245.
[17] G.N. Jarvis, The Microbiology of Tallow Hydrolysis during Anaerobic Digestion, University of Otago, Dunedin, 1995.
[18] B.A. Anderson, in A.M. Pearson and T.R. Dulson (Editors), Edible Meat By-Products. Advances in Meat Research, Elsevier Applied Science, London, 1988, pp. 32–39.
[19] C.L. Davey, P.R. Johnstone and J.E. Swan, New Zealand Tallow – Its Potential, Duromark Publishing, Auckland, 1983.