
Determination of long chain fatty acids in anaerobic digesters using a rapid non-derivatisation GC-FID method

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ABSTRACT

A rapid non-derivatisation gas chromatographic (GC) method for quantification of palmitic, stearic and oleic acids was achieved using a flame ionisation detector and a highly polar capillary column at elevated temperature. These long chain fatty acids (LCFA) can accumulate in anaerobic digesters and a simple extraction method was also developed to permit a more rapid sample turn-around time, facilitating more frequent monitoring. The GC method was satisfactory in terms of peak separation, signal response, reproducibility and linearity range. The extraction method achieved recoveries of 103.8, 127.2 and 84.2% for palmitic, stearic and oleic acid respectively. The method was tested on digestate from mesophilic laboratory-scale digesters fed with source-segregated domestic food waste, and showed good repeatability between replicate samples. It was observed that the concentrations

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of stearic and palmitic acid in digesters routinely supplemented with trace elements were lower in proportion to the applied lipid loading than those without supplementation.

Keywords: Anaerobic digestion, food waste, GC-FID, long chain fatty acids, trace element

INTRODUCTION

In the anaerobic digestion process long chain fatty acids (LCFA) can be degraded via the ß-oxidation pathway to acetate and hydrogen, which are subsequently converted to methane (Weng and Jeris, 1976; Kim *et al.*, 2004). Despite this, LCFA have been reported in a number of studies to be inhibitory to methanogens, especially acetoclastic methanogens (Hanaki *et al.*, 1981; Angelidaki and Ahring, 1992; Lalman and Bagley, 2002). This has been attributed to their amphiphilic properties that allow them to be easily adsorbed onto a microbial surface, therefore impeding the passage of essential nutrients through the cell membrane (Henderson, 1973; Hwu *et al.* 1998; Alves et al, 2001; Pereira *et al.*, 2005).

There is some debate concerning the concentrations at which LCFA become inhibitory, and this may also depend on the digester operating mode and degree of acclimatisation. In batch experiments with granular sludge Koster and Cramer (1987) showed inhibition thresholds for methanogenesis at concentrations of 1.6, 2.4, 2.6, 2.6 and 6.75 mM for lauric, oleic, capric, myristic and caprylic acids respectively. Angelidaki and Ahring (1992) carried out thermophilic batch tests on cattle manure: addition of oleate and stearate at 0.7 and 1.8 mM respectively led to an increase in the lag period before biogas production, while at 1.8 and 3.5 mM methanogenesis was inhibited. No increase in tolerance was found using digestate that had been previously exposed to the LCFA and had successfully depleted it. This supported the findings of Koster and Cramer (1987), who also suggested that inhibition was concentration-dependent. Lalman and Bagley (2000, 2001), using unacclimated batch cultures at 21 °C, showed inhibition of acetoclastic methanogenesis by oleic and linoleic

acids at 0.11 mM but not by stearic acid at concentrations up to 0.35 mM; all three acids showed only slight inhibition of hydrogenotrophic methanogenesis.

Alves *et al.* (2001) tested for inhibition in a fixed bed digester at 35 ^{o}C continuously fed with 4.15 g l^{-1} sodium oleate at an organic loading rate of 8-9 kg COD m^{-3} day^{-1}, and showed it was efficiently converted to methane. Using granular sludge from fixed and expanded bed digesters Pereira *et al.* (2003, 2004) reported that LCFA had adverse effects on functionality, but also that the effect was reversible under appropriate conditions and LCFA could be efficiently converted. Palatsi *et al.* (2009, 2010) have more recently shown that the tolerance of anaerobic consortia towards LCFA could be improved by proper acclimation.

The traditional gas chromatography method for LCFA determination requires free fatty acids to be derivatised to a methyl ester (FAME). This approach was introduced by Morrison and Smith (1964) and similar methods are still used (Masse *et al.* 2002; Palatsi *et al.*, 2009). A two-step procedure is required: firstly methylation free fatty acids under high temperature with a suitable catalyst; then extraction of the derivatised fatty acids using a solvent. Methylation enhances the volatility and reduces activity of the free fatty acid. Morrison and Smith (1964), Angelidaki (1990), Chou *et al.* (1996) and Masse *et al.* (2002) used a catalyst prepared by dissolving Boron Fluoride, a very toxic gas, into methanol. Other workers (Eras *et al.*, 2004; Palatsi *et al.*, 2009) have used the less toxic Clorotrimethylsilane (CTMS)-methanol, but CTMS reacts violently with water requiring lyophilisation of all samples before extraction, with a significant increase in sample preparation time. Two less dangerous reagents, HCl:1-propanol and methanolic HCl, are reported in Neves *et al.* (2009) and Sönnichsen and Müller (1999) respectively, and good methylation has been achieved.

Irrespective of the catalyst selected, the methylation step requires a long reaction time (from 1-16 hours) at high temperature (90-100 °C). For routine monitoring of LCFA where a high sample throughput and a short turnaround time are essential, these methods are therefore not very suitable. There is also a concern that with small sample sizes, a complicated procedure is likely to be less accurate (Sönnichsen and Müller, 1999).

The purpose of the current work was to develop a quick and reliable gas chromatographic technique to analyse LCFA without a derivatisation step. The method was then tested for analysis of samples from laboratory-scale mesophilic digesters treating source segregated food waste with and without trace element (TE) addition.

MATERIALS AND METHODS

LCFA method development

Standards and reagents. Analytical grade palmitic (C16:0) and oleic (C18:1) acids were obtained from Fisher Chemical, UK. GC grade Stearic acid (C18:0) of ≥98.5% purity was obtained from Sigma-Aldrich, UK. Hexane (high performance liquid chromatography (HPLC) grade), Methyl tertiary butyl ether (MTBE) (HPLC grade), sodium chloride (analytic grade) and sulphuric acid (analytic grade) were purchased from Fisher Chemical, UK. Each standard was prepared by dissolving the LCFA into a 1/1 hexane-MTBE mixture. These were prepared at 50, 100 and 250 mg l^{-1} and either kept in a sealed gas-tight bottle or prepared freshly before each analysis.

LCFA extraction. The procedure was modified from that of Neves *et al.* (2006) and Lalman and Bagley (2000). A known weight of around 1.5 g of digestate was added to a 50 ml centrifuge tube, followed by 0.05 g NaCl, 0.2 ml of 50% H_2SO_4, and 5 ml of 1/1 Hexane-

MTBE mixture. The centrifuge tube was closed and the contents mixed vigorously with a vortex mixer (FB15024, Fisher Scientific). The tube was then placed in an ultrasonic bath (Crest Ultrasonic CP1100, UK) for 20 minutes. The contents of the tube were allowed to separate and 2 ml of the upper layer was carefully transferred into a 2 ml tube and centrifuged for 5 minutes at 20,800 rcf (Eppendorf 5417C); the clear organic layer was used in gas chromatographic analysis.

GC method. The method was developed on a gas chromatograph (Shimazdu GC2010, Shimazdu, UK) fitted with a flame ionisation detector (FID) using a highly polar capillary BP-21 (FFAP) column 0.25 mm × 30 m, 0.25 µm thickness (SGE Forte GC, UK). The optimum instrument parameters were found to be: FID 280°C with H_2 and air flows of 40 and 400 ml min^{-1} respectively; makeup flow: 30 ml min^{-1} (helium); column flow: 2.0 ml min^{-1} (helium); oven temperature: initial 160 °C, ramp rate 10 °C min^{-1}, final 225 °C, final hold 20 minutes; injection volume 1 µl.

Validation procedure. Precision of the method was evaluated based on reproducibility and repeatability (Miller and Miller, 1993; Caulcutt and Boddy, 1983), indicated by relative standard deviation (RSD, %). To check reproducibility over time, three mixed standard solutions containing palmitic, oleic, and stearic acids at individual acid concentrations of 50, 100 and 250 mg l^{-1} were injected 6 times over a one-month period. To confirm repeatability single samples taken from two food waste digesters operating at different organic loading rates were subdivided into 6 sub-samples, each of which was extracted and each extract run in triplicate on the GC. To validate the extraction efficiency, three digestate samples were prepared and each spiked with 0.1 mg palmitic, stearic and oleic acid; these were recovered

and analysed using the above methods with percentage recovery based on the difference between spiked and unspiked samples.

Anaerobic digesters and feedstock

The digesters used in this work were part of a larger study to assess the effect of trace element (TE) additions on the stability and performance of food waste digestion (Banks *et al.* 2012). The digesters were fed on food waste collected from Biocycle digestion plant in Shropshire, UK and processed by passing it through a macerating grinder (S52/010, IMC Ltd, UK). Feedstock characteristics are shown in Table 1. One of the digesters used had no TE addition and was operated at an organic loading rate (OLR) of 1.8 g VS l^{-1} day^{-1}. The second digester was operated at 5.5 g VS l^{-1} day^{-1} and supplemented with Se, Mo, Co.

RESULTS AND DISCUSSION

GC method calibration and validation

The GC analysis showed good reproducibility for peak amplitude and retention time for the three fatty acids used as standards. A typical chromatogram is shown in Figure 1 and the RSD of peak responses for the six runs conducted over a one-month period are given in Table 2. The RSD values obtained were low compared to the 20% which might be considered acceptable (Shah *et al.*, 1992). Under the flow conditions used the variations in retention time windows were ±0.016, 0.017, and 0.018 minutes for palmitic, stearic and oleic acid respectively with mean values of 13.4, 19.7 and 21.0 minutes.

The calibration curves plotted for the three standards were linear over the concentration range studied, with correlation coefficients $R^2 \geq 0.99$ for all the analysed LCFA. The slopes of the regression equations obtained are shown in Table 2.

Extraction procedure and repeatability with single samples

In the LCFA extraction procedure a 1/1 Hexane and MTBE mixture was chosen because this has a lower flash point than hexane and was found to give a better peak response than other potential solvents. Methanol and ethanol were also tested as alternative solvents, but neither gave a satisfactory peak response.

Table 3 shows the results for the three LCFA quantified in replicated digestate samples with triplicate injections. The unsupplemented control had lower LCFA concentrations than those in the TE supplemented sample, with slightly lower %RSD values. LFCA concentrations mainly reflected the lipid loading rate, which was three times higher for the TE supplemented digester than for the control.

Recovery efficiency

The average recovery from the LCFA spiked into digestate samples was 103.8%, 127.2% and 84.2%, for palmitic, stearic and oleic acid respectively (Table 4).

The method reported showed that a highly polar capillary column used at high temperature can give good peak separation and signal response without the need for methylation of the sample. The sample preparation time was significantly reduced (45 minutes on average), allowing a much higher sample throughput.

In the digesters studied the values of LCFA recorded may not necessarily reflect the actual accumulation of these compounds in the digestate. LCFA have been observed to accumulate as discrete inclusions forming around inert material such as fruit pips. Analysis of these inclusions by x-ray diffraction (XRD) showed the deposits to consist mainly of salts of LCFA

(unpublished data). The measured LFCA values therefore reflect the proportion miscible in the digestate which had not been hydrolysed in the degradation process. Considering, however, that at the time of sampling the digesters had been receiving food waste for a period of almost 2 years at a lipid concentration of around 150 g kg^{-1} VS, it seems probable that the degree of degradation is quite high. This view is supported by the studies of Angelidaki and Ahring (1992) and (Masse *et al.* (2002) who suggested that in an anaerobic environment the lipid load to the digester is readily hydrolysed to free LCFA and glycerol. Subsequently the free LCFA are oxidised by acidogenic bacteria through ß-oxidation (Masse *et al.*, 2002) which leads to the final formation of simple volatile fatty acids and hydrogen. However, β-oxidation is thermodynamically unfavourable under standard conditions due to its positive Gibbs free energy (equation 1), therefore requiring constant removal of the reaction products (Fox and Pohland, 1994).

$$\text{n-carboxylic acid} \longrightarrow \text{(n-2) carboxylic acid} + CH_3COOH + 2\ H_2\ \Delta G_0 = +\ 48\ \text{kJ mol}^{-1} \quad (1)$$

Methanogenesis provides the syntrophic complement to the process by using acetate, formate and hydrogen. The concentration of LCFA found in the TE supplemented digester was higher than that in the non-supplemented control which may reflect the difference in lipid loading between the two digesters. Proportional to the load, however, the concentrations of palmitic and stearic acids in the non-supplemented digesters were higher as was the total VFA concentration, further supporting the view that TE supplementation was required to prevent an accumulation of intermediate products (Ferry, 1999; Ragsdale and Pierce, 2008). The concentrations of palmitic, stearic and oleic acid of 1.0, 1.9 and 0.7 mM found in the TE supplemented digestate are below the values suggested as inhibitory in other studies (Koster and Cramer, 1987; Angelidaki and Ahring, 1992; Lalman and Bagley, 2002).

CONCLUSIONS

A reliable gas chromatographic method was developed and validated for quantification of palmitic, stearic and oleic acid without the requirement for further sample methylation. During repetitive runs, the relative standard deviations (RSD) of the results were satisfactory. Good LCFA recoveries were shown using a spike addition of LCFA to digester sludge. The simplicity of the sample preparation procedure reduces analysis time which would make the routine analysis of LCFA in digestate samples more realistic as a monitoring tool. Digestate samples from food waste digesters at different lipid loads and with and without trace element addition showed LCFA concentrations below values considered inhibitory in other studies, but concentrations of palmitic and stearic acid were lower in the TE supplemented digester in proportion to the lip[id loading applied than in the unsupplemented control.

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Table 1. Characteristics of food waste substrate

pH (1:5)	4.71 ±0.01
Total solids, TS (% wet weight (WW))	23.74 ±0.08
Volatile solids, VS (% WW)	21.71 ±0.09
VS (% TS)	91.44 ±0.39
Total organic carbon (TOC) (% TS)	47.6 ±0.5
Total Kjeldahl nitrogen (TKN) (% TS)	3.42 ±0.04
Lipids (g kg-1 VS)	151 ±1
Crude proteins (g kg-1 VS)	135 ±3

Table 2. Peak area shift in sequential injections of standards and calibration curve parameters

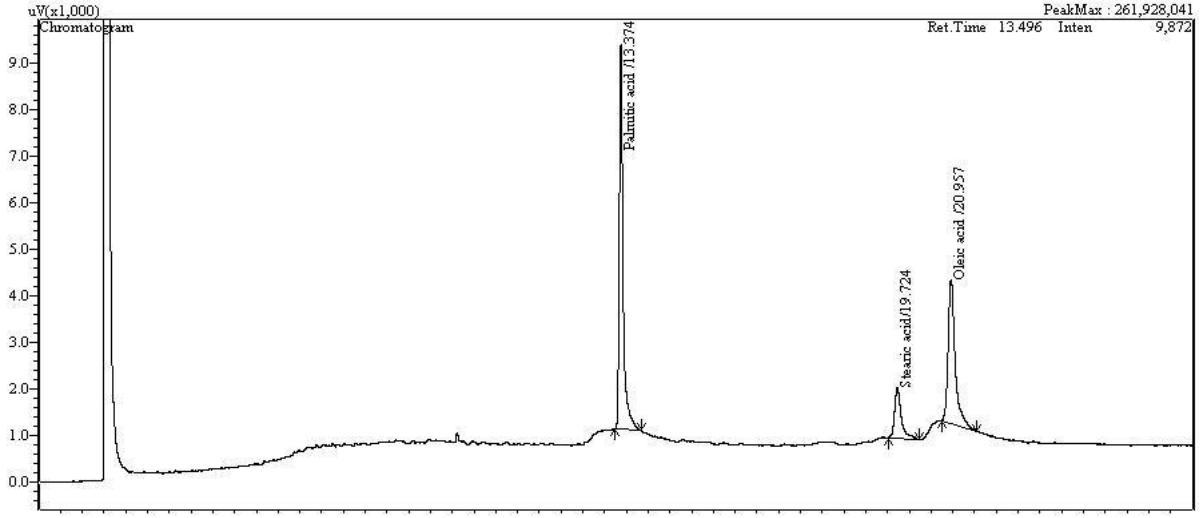
	mg l^{-1}	**Run1**	**Run2**	**Run 3**	**Run 4**	**Run 5**	**Run 6**	**Average**	**SD**	**%RSD**
	50	52268	49481	49972	51130	46892	51154	50150	1873	3.74
	100	90426	108081	99801	110980	97601	109874	102794	8173	7.95
Palmitic	250	249226	253405	268318	245168	257543	278050	258618	12403	4.8
	Slope	1002	1008	1099	953	1056	1131	1042	67	6.40
	R^2	0.9965	0.9984	0.9995	0.9960	0.9999	0.9999	1.0000		
	50	13389	11486	15043	10210	15398	12561	13015	2017	15.5
	100	20233	23367	25481	21871	25298	25872	23687	2277	9.61
Stearic	250	58161	53420	59879	57348	53475	56045	56388	2595	4.6
	Slope	231	208	225	236	190	214	217	17	7.85
	R^2	0.9900	0.9987	0.9997	1.0000	0.9999	0.9963	1.0000		
	50	37540	34936	31971	35190	34578	37540	35293	2087	5.91
	100	68213	64066	65469	57543	67564	65423	64713	3828	5.92
Oleic	250	141673	136942	167305	123628	154432	142110	144348	15000	10.39
	Slope	514	504	677	442	595	520	542	82	15.17
	R^2	0.9977	0.9985	1.0000	1.0000	0.9993	0.9997	0.9996		

Table 3. LCFA concentrations (mg l^{-1}) in subsamples from control and TE-supplemented food waste digesters (three injections)

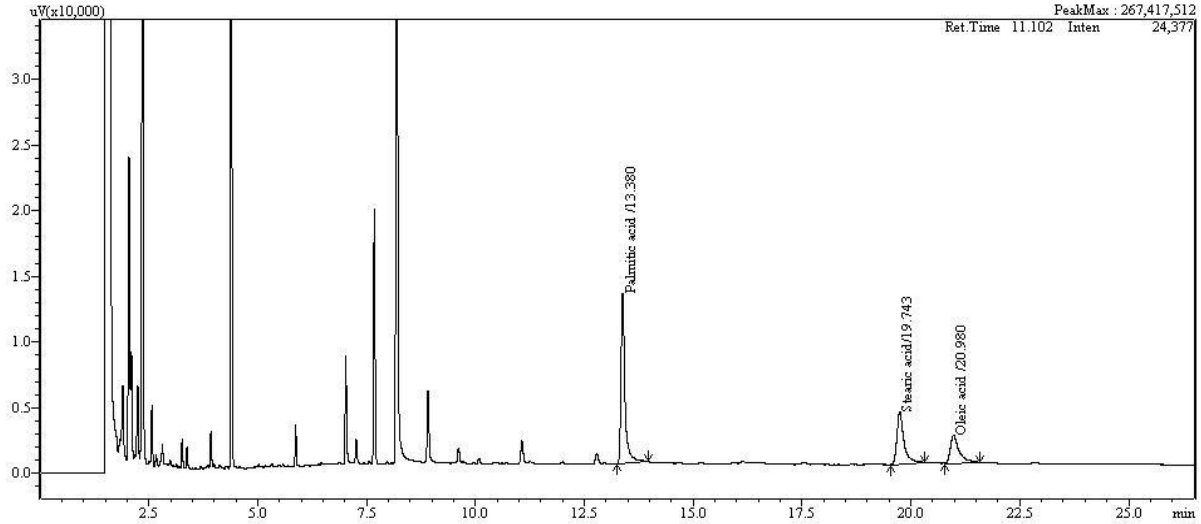
		Unsupplemented control F1 (OLR=1.8 g VS l^{-1} day^{-1})				TE supplemented 1 F5 (OLR=5.5g VS l^{-1} day^{-1})				TE supplemented 2 R3 (OLR=3g VS l^{-1} day^{-1})			
Subsample	LCFA	1#	2#	3#	Ave.	1#	2#	3#	Ave.	1#	2#	3#	Ave.
1	Palmitic	133.3	130.6	129.1	131.0	205.2	201.3	197.5	201.3	49.6	49.0	48.5	49.0
	Stearic	292.6	290.9	289.4	291.0	402.5	394.7	388.3	395.2	114.5	111.6	112.1	112.8
	Oleic	75.5	71.6	69.1	72.1	134.1	129.9	126.6	130.2	11.0	11.0	10.7	10.9
2	Palmitic	110.6	110.4	111.1	110.7	253.7	240.5	257.4	250.5	45.4	49.2	45.5	47.3
	Stearic	271.2	271.2	272.6	271.6	487.9	466.8	501.5	485.4	113.0	115.2	115.0	114.1
	Oleic	50.6	48.4	48.5	49.2	181.2	161.0	179.4	173.9	19.6	20.1	18.5	19.9
3	Palmitic	117.4	116.9	118.7	117.7	346.8	272.6	302.7	307.4	48.8	48.8	49.2	48.8
	Stearic	281.8	281.7	285.3	282.9	667.8	527.8	590.2	595.3	119.5	118.6	118.3	119.0
	Oleic	51.4	54.2	54.4	53.3	248.1	182.7	206.8	212.5	9.6	7.9	7.7	8.7
4	Palmitic	96.0	95.5	95.6	95.7	249.2	241.6	281.7	257.5	46.1	46.7	46.2	46.4
	Stearic	235.5	233.9	232.8	234.1	489.2	476.3	555.6	507.0	116.2	117.6	117.7	116.9
	Oleic	35.9	34.0	34.4	34.8	162.4	157.3	190.2	170.0	7.2	5.9	5.6	6.5
5	Palmitic	138.5	136.8	136.8	137.4	219.1	247.2	243.6	236.6	52.1	53.0	52.2	52.5
	Stearic	323.3	318.9	316.3	319.5	430.0	491.9	484.1	468.6	107.7	109.8	109.1	108.7
	Oleic	55.4	54.5	53.3	54.4	151.9	178.3	174.9	168.4	13.7	13.8	15.0	13.7
6	Palmitic	118.9	107.8	104.4	110.4	335.4	377.4	325.2	346.0	49.6	47.3	56.7	48.4
	Stearic	281.1	255.7	247.3	261.4	772.8	867.8	752.4	797.7	120.2	113.0	112.1	116.6
	Oleic	68.9	61.5	59.8	63.4	281.0	316.5	272.3	289.9	8.2	8.1	7.6	8.2
Average		**average**	**stdev**	**% RSD**	**%RSD without the outlier**	**average**	**stdev**	**% RSD**		**average**	**stdev**	**% RSD**	**%RSD without the outlier**
	Palmitic	**117.1**	15.2	12.9	10.1	**266.6**	51.9	19.5		**48.7**	2.1	4.3	4.5
	Stearic	**276.8**	28.8	10.4	7.8	**541.5**	141.1	26.1		**114.7**	3.7	3.2	3.6
	Oleic	**54.5**	12.7	23.3	15.7	**190.8**	55.1	28.9		**11.3**	4.9	43.0	28.9

Table 4. Extraction recovery of the spiked samples

	Spike recovery		
	Palmitic	Stearic	Oleic
Replicate 1	108.9%	121.8%	64.7%
Replicate 2	110.2%	133.9%	75.8%
Replicate 3	92.4%	125.8%	112.2%
Average	103.83%	127.17%	84.23%



a) Representative chromatogram using a 50 mg l^{-1} standard LCFA mix



b) Chromatogram of identified LCFA from a digestate sample

Figure 1. Typical chromatograms for extracted LCFA