**Presented at Poster Session PT03 – Inhibition**
**11th IWA World Congress on Anaerobic Digestion, 23-27 September 2007, Brisbane, Australia**

# A method to detect and quantify long chain fatty acids in liquid and solid samples and its relevance to understand anaerobic digestion of lipids

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**Abstract** A method for long chain fatty acids (LCFA) extraction, identification and further quantification by capillary gas chromatography was developed and its application to liquid and solid samples demonstrated. Linear calibration curves ($r^2$ between 0.997 and 1.000) were constructed for C12 to C18 LCFA, in the range from 25 to 1270 mg/L. All the acids were quantified in a single run using pentadecanoic acid (C15:0) as internal standard with response factors ranging from 0.79 to 1.09. Relative standard deviation values lower than 15% and mean LCFA recoveries above 90% were obtained. After validation, the usefulness of this method was demonstrated in a cow manure digester receiving pulses of an industrial effluent containing high lipid content. The knowledge obtained with the application of this method can contribute to a better understanding of LCFA adsorption and degradation processes that occur during the anaerobic digestion of lipids.
**Key Words**: Anaerobic digestion; lipids; liquid phase; long chain fatty acids; solid phase

## INTRODUCTION

In early literature it is suggested that LCFA, produced during hydrolysis of neutral lipids, exert a permanent toxic effect (Angelidaki and Ahring, 1992) and even a bactericidal effect on methanogens (Rinzema *et al*., 1994). Furthermore, LCFA inhibitory effect on the anaerobic microbial activity, at even low concentrations, has been often reported (Koster and Cramer, 1987; Hananki *et al.*, 1981; Hwu *et al.*, 1996; Lalman and Bagley, 2000, 2001, 2002; Shin *et al*., 2003). However, in later studies, Alves *et al*. (2001) observed that after being continuously fed with oleic acid (C18:1), anaerobic sludge that was encapsulated by a whitish matter, was able to efficiently convert to methane the accumulated substrate when incubated in batch assays at 37ºC, without any added carbon source, evidencing that the anaerobic consortium remained active in such conditions. It was further demonstrated that LCFA, provided they are associated with the sludge and not in the bulk medium, can be efficiently converted to methane and that the observed temporary decrease in the methanogenic activity after the contact with LCFA is a reversible phenomenon, being eliminated after the conversion to methane of the biomass-associated LCFA (Pereira et al., 2004, 2005). These new reported developments were the driving force to develop a method to identify and quantify the LCFA in the liquid phase as well as accumulated onto the biomass, during the AD process.

Some papers reported results of identification and quantification of LCFA in samples from anaerobic reactors fed with lipids/LCFA. Nevertheless, these analysis were performed only in the centrifuged and filtrate supernatant (Lalman and Bagley, 2000; Hwu *et al*., 1998; Fernández *et al*., 2005), and not in the solid matrix. Hence, a fast and effective method that is able to extract and quantify the LCFA adsorbed onto the solid matrix, i.e. biomass, and present in the liquid phase, i.e. supernatant, collected from anaerobic reactors, is required. In this paper, a method for LCFA extraction from liquid and solid samples and further quantification by capillary gas chromatography is described and validated. Furthermore, the application of the method to analyse samples of the solid and liquid phases from an anaerobic reactor fed with dairy cow manure during weakly pulses of an oily effluent from a can fish processing industry, is also presented as an example.

## METHODS

### Calibration

Calibration curves were produced from a series of standard solutions prepared with the following acids (puriss p.a. for GC analysis): lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), C18:1 and linoleic (C18:2), in a DCM solution. C15:0 was used as the LCFA internal standard (IS).

### Sample processing

The standards (DCM solution), liquid (aqueous solution) and solid (anaerobic biomass) samples were submitted to a similar procedure, ensuring that the organic phase and the aqueous phase always comprised equal amount (3.5 mL), in a total volume of 7 mL. For the standards and liquid samples, once homogenised, 2 mL were transferred into glass vials. Afterwards, 1.5 mL of the IS solution and 1.5 mL of HCl:1-Propanol (25 % v/v) were added. For the liquid samples, 2 mL of DCM was subsequently added, whereas, for the standard solutions, 2 mL of ultra-pure water was added instead. For the solid samples, a defined amount was transferred to the glass vials and dried for 12 hours at 85ºC. The content of the vial was weighed and the solutions of IS (1.5 mL), HCl:1-Propanol (1.5 mL), DCM (2 mL) and ultra-pure water (2 mL) were further added.

The mixture was vortex-mixed, to promote good contact between the two phases, and was digested at 100ºC for 3.5 hours. After digestion, the content of the vial was transferred with 2 mL of ultra-pure water to a different vial, rubber covered, and the contact between the two phases was further promoted. These new vials were kept in inverted position for 30 minutes, after which 1 mL of the organic phase was collected. 1µl of this sub sample was analysed by GC. This analysis was carried out in a GC system (CP-9001 Chrompack) equipped with a flame ionization detector (FID). LCFA were separated using an eq.CP-Sil 52 CB 30 m x 0.32 mm x 0.25 µm column (Teknokroma, Tr-wax), with He as the carrier gas at 1.0 mL/min. Temperatures of the injection port and detector were 220 and 250ºC, respectively. Initial oven temperature was 50ºC for 2 minutes, with a 10ºC/min ramp to 225ºC, and a final isothermal for 10 minutes.

### Validation procedure

The presented method was validated in terms of linearity, limit of detection and quantification, precision (repeatability and reproducibility), accuracy and selectivity. Linearity was evaluated by the correlation coefficient of the calibration curves obtained. Detection limit and quantification limit were estimated as the LCFA concentration for which the area of the chromatographic peak was equal to 3 and 10 times, respectively, the standard deviation of the most diluted standard. Reproducibility of the method was evaluated by the relative standard deviation (R.S.D., %) of the slope of 5 calibration curves constructed over a year period by 3 different annalists (3+1+1). Repeatability (measured as % R.S.D.) and accuracy (measured concentration/real concentration x 100) were assessed by means of LCFA recovery experiments performed in liquid and solid samples. Blank samples (supernatant and biomass from anaerobic reactors) with and without the addition of LCFA were also processed to test for interferences of the liquid and solid biological matrices, evaluating the selectivity of the method. An additional series of experiments was carried out to optimize the extraction of the LCFA from the solid phase to the organic phase. Table 1 and 2 summarizes the assays performed with liquid and solid samples, respectively. The solid samples consisted of anaerobic suspended (S) and granular (G) biomass collected from tree different reactors: (i) a lab scale reactor fed with sodium oleate, designated as biomass SL, which was expected to have a high amount of adsorbed/accumulated LCFA, because it was visibly encapsulated by a whitish matter; (ii) two wastewater treatment plants, designated as biomass S1 and S2; and (iii) an upflow anaerobic sludge blanket reactor treating effluent from a brewery company (Oporto, Portugal), designated as biomass G.

Table 1- Assays performed to validate the method for liquid (l) samples.

| Assay # | Oleic acid sodium salt (mg/L) | Sodium oleate powder (mg/L) | LCFA mixture (*) (mg/L) | Olive oil (mg/L) | C16:0 (mg/L) | C18:1 (mg/L) | Solvent |
|---|---|---|---|---|---|---|---|
| 1(l) | - | - | ≅500 | - | - | - | Water |
| 2(l) | - | - | ≅1000 | - | - | - | Water |
| 3(l) | 258 | - | - | - | | | Water |
| 4(l) | - | 590 | - | - | - | - | Water |
| 5A(l) | - | - | - | - | - | - | Supernatant |
| 5B(l) | - | - | ≅500 | - | - | - | Supernatant |
| 6(l) | - | - | - | 1173 | - | - | DCM |
| 7(l) | - | - | - | 1516 | 938 | 1184 | DCM |

(*) C12:0, C14:0, C16:0, C16:1, C18:0, C18:1 and C18:2.

Table 2- Assays performed to optimize and validate the method for solid (s) samples.

| Assay # | Biomass | TS (g) | Digestion time (h) | DCM (mL) | Added LCFA (C12:0, C14:0, C16:0, C16:1, C18:0, C18:1 and C18:2) (mg) |
|---|---|---|---|---|---|
| 1(s) | SL | ≅ 0.05 | 3.5 | 3.5 | - |
| 2(s) | SL | ≅ 0.05 | 5 | 3.5 | - |
| 3(s) | SL | ≅ 0.05 | 7 | 3.5 | - |
| 4(s) | SL | ≅ 0.05 | 3.5 | 4.0 | - |
| 5(s) | SL | ≅ 0.05 | 3.5 | 4.5 | - |
| 6(s) | SL | ≅ 0.1 | 3.5 | 3.5 | - |
| 7(s) | SL | ≅ 0.5 | 3.5 | 3.5 | - |
| 8(s) | S1 | ≅ 0.05 | 3.5 | 3.5 | - |
| 9(s) | S1 | ≅ 0.1 | 3.5 | 3.5 | - |
| 10(s) | S1 | ≅ 0.2 | 3.5 | 3.5 | - |
| 11A(s) | G | ≅ 0.05 | 3.5 | 3.5 | - |
| 11B(s) | G | ≅ 0.05 | 3.5 | 3.5 | ≅1 |
| 12A(s) | S2 | ≅ 0.05 | 3.5 | 3.5 | - |
| 12B(s) | S2 | ≅ 0.05 | 3.5 | 3.5 | ≅1 |

**Statistical analysis**

Single factor analysis of variances (ANOVA) was used to determine if significant differences existed between results obtained under different experimental procedures. Statistical significance was established at the $P < 0.05$ level.

**Application of the method to monitor anaerobic digestion of lipids**

A 26l mesophilic continuously stirred tank reactor (CSTR) was fed with dairy cow manure (1.4 gCOD/gTS) for 122 days, at an organic loading rate of 1.2 gCOD/L.day. The hydraulic retention time (HRT) was set at 26 days. From day 123 on, pulses of an oily effluent from a can fish processing industry (2.7 gCOD/gwaste and 99.8% of fat content) were added once a week, every 7 days. In those days, the organic loading rate applied to the reactor was of 5.0 gCOD/L.day. Chemical oxygen demand (COD) and total solids (TS) were determined according to Standard Methods (APHA et al., 1989). The fat content from the oily effluent was extracted with a mixture chloroform:methanol 1:2 (v:v) in a soxtec system, dried and weighed.

## RESULTS AND DISCUSSION

### Calibration and validation

The tested LCFA were detected by gas chromatography in a 25 minutes single run analysis, with a good separation between peaks. The generated calibration curves were linear over the concentration range studied (25.0-1270 mg/L) with coefficients of correlation ≥ 0.997 for all the analyzed LCFA. The R.S.D. of the slopes of the calibration curves generated for each LCFA, ranged between 4.1 to 13.3% (C18:0 and C12:0, respectively), verifying the day-to-day precision of the method.

*Liquid samples*

The results obtained from the recovery assays carried out with liquid samples (Table 1) achieved satisfactory yields in the tests performed to evaluate the extraction of LCFA from the aqueous phase. Mean recoveries in the range of 91-101% and 99-110% were obtained at LCFA concentration levels of about 500 (assay 1(l)) and 1000 mg/l (assay 2(l)), respectively, with good precision ($R.S.D._{(500)}$<9.5% and $R.S.D._{(1000)}$<3.0%). A high mean recovery of C18:1, i.e. 99%, was also attained from aqueous solutions prepared with oleic acid sodium salt (puriss p.a. ≥99%), with a R.S.D. value of 1.9% (assay 3(l)). Furthermore, analysis of a solution prepared with sodium oleate powder (assay 4(l)) revealed that oleic acid represented 80±1 % of the total LCFA detected, which corresponds to a mean recovery of 98% in relation to the minimum 82% of C18:1 expected, as specified by the manufacturer.

Supernatant samples, collected from an anaerobic reactor, with and without the addition of LCFA were also analysed to test for interferences of the liquid biological matrix. No LCFA were detected in the pure supernatant samples (assay 5A(l)). After processing this samples supplemented with the standard LCFA at a concentration level of about 500 mg/l (assay 5B(l)), mean recoveries above 96% and R.S.D. lower than 9.0% were obtained. Additionally, comparison of the chromatograms obtained after processing the matrix solution, i.e. supernatant (assay 5A(l)), and the matrix solution to which the analytes had been added (assay 5B(l)) revealed no interferences of the biological liquid matrix in the LCFA analysis.

Olive oil solutions were also analysed to ensure that the method procedure did not promote hydrolysis of neutral lipids. The obtained results confirmed the desired condition, as no free LCFA were detected after processing the pure olive oil solutions (assay 6(l)). Moreover, high oleic (and palmitic acid recoveries (105 and 112%, respectively) were achieved after processing olive oil solutions supplemented with the two acids (assay 7(l)).

*Solid samples*

To optimise LCFA extraction from the solid matrix (Table 2), different digestion times, volumes of organic phase and amounts of dry sample were studied, using biomass expected to be highly loaded with biomass-associated LCFA, designated as SL (assays 1(s) to 7(s)). No statistically significant differences at a 0.05 level ($P$=0.075) were observed when a dry amount of ≅ 0.05 g of this biomass was submitted to different digestion times of 3.5 (assay 1(s)), 5 (assay 2(s)) and 7 hours (assay 3(s)). Likewise, when tested for different organic phase volumes of 3.5 (assay 1(s)), 4.0 (assay 4(s)) and 4.5 mL (assay 5(s)), the detected LCFA content showed statistic similar results ($P$=0.429). Furthermore, in all experiments the amount of C16:0 detected represented 86±1% of the total LCFA extracted. However, when the amount of dry biomass was increased to ≅ 0.1 g (assay 6(s)) and ≅ 0.5 g (assay 7(s)) a decrease on the LCFA content detected to 30% and 85%, respectively, was found. Nevertheless, in both cases, the percentage of C16:0 detected in the total LCFA extracted was identical, i.e. 85±1%. From the obtained results, the solid phase LCFA extraction procedure was set-up with 3.5 hours of digestion and 3.5 mL of organic phase, as previously established for the liquid phase extraction procedure. The amount of dry biomass used in this analysis should be such that allows complete LCFA extraction from the solid phase to the organic phase, therefore

depending on the amount of LCFA present in the sample. For highly LCFA loaded biomass, i.e. up to a maximum of approximately 500 mg LCFA/gTS, as observed for biomass SL, a dry amount as low as 0.05 g should be used in order to avoid LCFA extraction saturation. Due to the heterogeneity associated to the random LCFA accumulation into the biomass, high standard deviations can be obtained when analysing biomass samples.

To validate the lower sensitivity of the method, biomass expected to have a low LCFA content, designated as S1, was also tested using different amounts of dry sample (assays 8(s) to 10 (s)). The results obtained showed no statistically significant differences ($P$= 0.609) in the LCFA content detected when analysing dry amounts of 0.05, 0.1 or 0.2 g, further demonstrating that the described method is feasible even when the biomass has a total LCFA content as low as 3 mg/gTS. In this case, dry sample amounts higher than 0.05 g should be used in order to increase the obtained precision (% R.S.D).

Anaerobic biomass samples with and without the addition of LCFA were also analysed to test for interferences of the solid biological matrix. No LCFA were detected in the granular biomass (assay 11A(s)), whereas in the suspended biomass (assay 12A(s)) a total LCFA content of 18 mg/gTS was found. The individual LCFA contents present in this biomass were discounted in the experiments performed with the biomass samples fortified at individual LCFA amounts of about 1mg. In these experiments, LCFA mean recoveries above 93%, with R.S.D.<11%, were attained for both biomass types. As previously found for the biological liquid matrix, no interference of both biological solid matrices in the LCFA analysis was observed.

**Application of the method to monitor anaerobic digestion of lipids**

The proposed method was applied to monitor LCFA degradation/accumulation in an anaerobic CSTR reactor treating dairy cow manure, when submitted to pulses of an oily effluent from a can fish processing industry. Analysis of the fed wastes revealed that LCFA represented 3% and 77% of the COD in the cow manure and in the oily effluent, respectively (Table 3). Previous to the pulses, analysis of the digested manure showed a LCFA reduction of 80%, when compared to the fresh manure. This reduction was identical for all LCFA detected in the analysed samples.

Table 3- LCFA content in the reactor feed.

| LCFA | Cow manure (mg COD/gTS) | Oily effluent (mg COD/ gwaste) |
|---|---|---|
| C14:0 | 3 | 19 |
| C16:0 | 14 | 260 |
| C16:1 | 0 | 27 |
| C18:0 | 25 | 75 |
| C18:1 | 0 | 891 |
| C18:2 | 0 | 790 |

Successive pulses of the oily effluent were applied to the reactor on days 123, 130, 137, 144, 151 and 158. In each applied pulse, the main LCFA fed to the reactor were C18:1 (43.2±0.4%), followed by C18:2 (38.3±0.5%) and C16:0 (12.5±0.1%) (Table 3). During the pulses trial, no LCFA were detected in the liquid phase collected from the reactor, suggesting a fast accumulation of these compounds into the solid phase. This finding is in accordance with Hanaki et al. (1981) that reported that these compounds could fast adsorb to the biomass, within 24 hours. In this study, LCFA accumulation onto the biomass was confirmed by the results obtained from LCFA analysis in the solid matrix collected from the bottom of the reactor. The LCFA detected in this phase were C14:0, C16:0, C18:0, C18:1 and C18:2. It was further shown that C16:0 and C18:1 were the main LCFA adsorbed/accumulated onto the sludge, jointly accounting for about 60 to 100% of the total LCFA detected in the solid phase (Figure 1). Along the trial period, an overall decreased in the accumulation of C18:1 in the solid phase was observed, which became more evident in the last two

pulses. These data suggests that the conversion of C18:1, the main LCFA fed to the reactor, by the adapted biomass became faster and more effective along the successive pulses. Conversely, the accumulation of C16:0 in the solid phase suggests that degradation of this LCFA, under these conditions, is less effective. Nevertheless, the specific content of C16:0 accumulating in the last two pulses was relatively low, ranging from 7-18 mgCOD/gST. The present method for LCFA detection and quantification constitutes a valuable tool to identify key intermediates in the still obscure anaerobic accumulation/degradation of LCFA as was shown in the given example.

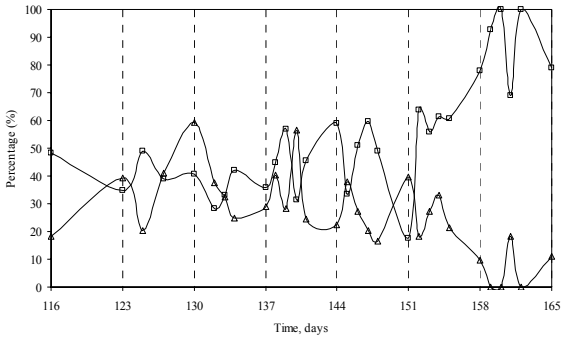


Fig.1. Percentage of C16:0 (□) and C18:1(△) in the total LCFA detected in the solid phase present in the bottom of the reactor.

## CONCLUSIONS

The analytical method reported is based on the extraction and gas cromatographic analysis of LCFA present in solid and liquid samples. R.S.D. values lower than 15% and mean LCFA recoveries above 90% were obtained. After validation, the usefulness of this method was demonstrated in a cow manure digester receiving pulses of an industrial effluent containing high lipid content. The application of this method will contribute to a better understanding of LCFA adsorption and degradation processes that occur during the anaerobic digestion of lipids.

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