Comprehensive lipid hydrolysis observation in anaerobic digestion

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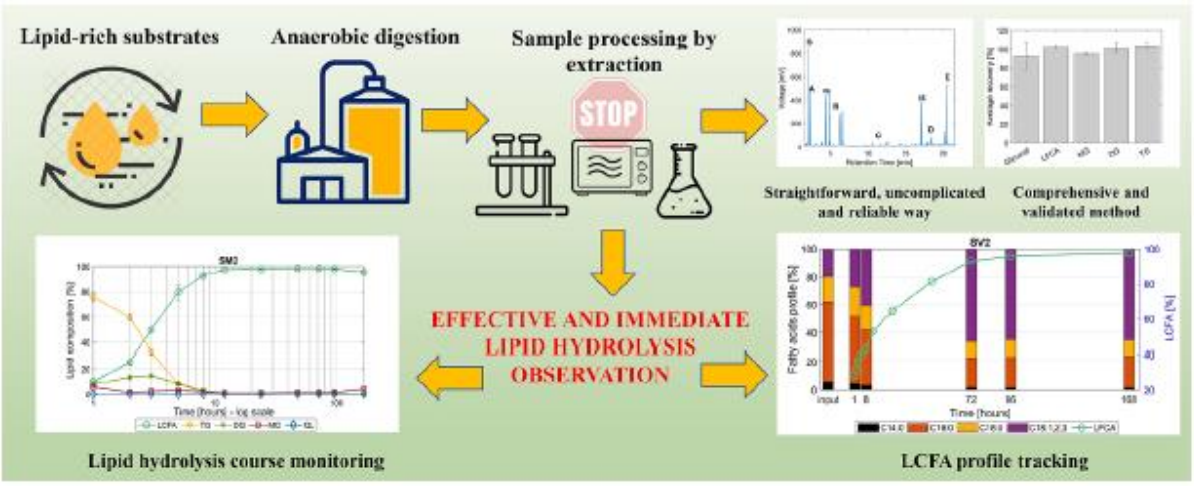
GRAPHICAL ABSTRACT



HIGHLIGHTS

- Lipid hydrolysis observation during anaerobic digestion of sludge.
- Microwave heating sample pretreatment to stop the reaction.
- Outstanding accuracy and repeatability of the complete analytical method.
- Monitoring the breakdown of glycerides in the course of the hydrolysis.
- Tracking free LCFA profile changes in the course of anaerobic digestion.

ABSTRACT

Lipid hydrolysis monitoring, including especially glycerides, is necessary for comprehending the anaerobic digestion process in lipid-rich substrates processing. This reaction has not been investigated in such detail so far, despite its potential to be crucial in assuring a stable process. This study suggested and thoroughly validated an uncomplicated method of monitoring lipid hydrolysis during anaerobic digestion, achieving recovery values >95 % with an average relative standard deviation <5 %.

Subsequently, the method was applied on the very first detailed observation of glyceride hydrolysis in the anaerobic sludge, tracking even changes in fatty acid profiles during anaerobic digestion. Results showed that lipid hydrolysis can take several days, thus likely affecting the whole anaerobic digestion of lipids. The method aims to provide answers to improve understanding of lipids' fate and their inhibition phenomena in anaerobic digestion.

Keywords: Lipid-rich substrate, long-chain fatty acids, glyceride, sludge, validation

1. Introduction

Anaerobic digestion (AD) is an environmentally friendly and cost-effective technology for the treatment of organic waste, where through the action of microorganisms, biogas as a by-product is produced and it can be subsequently used as a source of renewable energy (**Abomohra et al., 2022**). In the AD process, complex organic compounds are broken down by communities of microorganisms in the absence of oxygen (**Kadam and Panwar, 2017**). Anaerobic digestion can be considered a sustainable option for wastewater treatment in a variety of sectors, where a large amount of lipids is present.

It was extensively documented that lipid-rich substrates have a high biogas potential (**Palatsi et al., 2009; Rasit et al., 2015; Usman et al., 2020**). In the AD process, lipids, which are mostly glycerides, are hydrolysed by extracellular microbial lipases into long-chain fatty acids ($LCFA, C14 - C22$) and glycerol. $LCFA$ are further oxidised into acetate and hydrogen via p-oxidation and finally converted into methane (**Sani et al., 2022; Vargas-Muňoz et al., 2021**). However, the accumulation of $LCFA$ is widely recognised as a critical factor responsible for AD inhibition during the processing of lipid-rich feedstock (**Abomohra et al., 2022**). Consequently, numerous studies have been conducted to investigate the impact of LCFA concentration on AD, examining phenomena such as their degradation, inhibition mechanisms and changes in AD microbial communities (**Rasit et al., 2015; Usman et al., 2020; Zhu et al., 2019**). Interestingly, most of the studies neglected the impact of glycerides and hydrolysis itself, despite its significance to AD, which is further well illustrated by the reported impact of lipase pre-treatment on AD of lipid-rich wastes, where an improved process operation was usually observed (**Abomohra et al., 2022; Holohan et al., 2022; Rasit et al., 2015**). In addition, as indicated in recent literature through a comprehensive review (**Holohan et al., 2022**), it has been observed that when the fat concentration is significantly elevated, hydrolysis can emerge as the rate-limiting step within the anaerobic degradation process. This phenomenon is closely related to the nature of the surface area of the lipid droplets or particles. Moreover, lipid hydrolysis probably presents a major bottleneck in performing successful AD at low temperatures even in common municipal wastewater (**Petropoulos et al., 2018**).

Despite the widely accepted importance of lipid hydrolysis, this reaction was rarely studied in detail, specifically in terms of tracking the concentration of glycerides, i.e. triglycerides (TG) and their hydrolysis intermediates, monoglycerides (MG) and diglycerides (DG), simultaneously with $LCFA$, and glycerol (GL) in the anaerobic digestate. Such lack of data is most probably caused by difficulties stemming from the complicated nature of lipid-rich anaerobic digestate. The system is present in the above-mentioned form of dispersion - lipid droplets can have a wide range of size and form even solidified particles or layers (**Rasit et al., 2015**). Consequently, reliable determination of lipid compounds poses a significant challenge. As a result, most studies focused solely on capturing $LCFA$ concentration (**Jiang et al., 2012; Usman et al., 2020; Vargas-Munoz et al., 2021**). These methods were

designed especially for the determination and monitoring of free $LCFA$ in AD; in some cases, $LCFA$ profiling was performed without distinguishing between bound (i.e. mostly glycerides) and free $LCFA$ (**Cirne et al., 2007; Usman et al., 2020**). In addition, a method designed for high-throughput lipid hydrolysis monitoring was suggested recently (**Van Gaelen et al., 2021**). However, this procedure with focus on $LCFA$ monitoring utilises only arbitrary units with limited reliability for $LCFA$ quantification. Therefore, none of these methods allow for simple direct monitoring of the lipid hydrolysis process during a single analysis.

As already noted, there is only a very limited number of studies which offer more profound insight into the phenomena of lipid hydrolysis in AD based on direct data. An old paper of (**Hanaki et al., 1981**) describes the rapid hydrolysis of milk fat during its AD, capturing total lipid content and total $LCFA$ in a solid fraction of the reaction mixture. Total fat was quantified via extraction using the classic method (**Bligh and Dyer, 1959**) with subsequent determination of $LCFA$ in the extract via gas chromatography (GC). The authors suggested that neutral fat was not inhibitory to AD. A similar method was employed 17 years later for the assessment of AD of sheep tallow with the main focus on free $LCFA$ assessment (**Broughton et al., 1998**). The only detailed studies that employed the laborious extraction-separation method of (**Kaluzny et al., 1985**) for determining neutral fat gravimetrically with subsequent determination of free $LCFA$ in the extract by GC, were by (**Masse et al., 2003; Masse and Masse, 2002**). The authors evaluated hydrolysis rate coefficients and concluded that neutral fat hydrolysis noticeably influenced lipid degradation (**Masse and Masse, 2002**). Most recently, (**Sakurai et al., 2023**) introduced a new multi-step method for analysing $LCFA$ in anaerobic digester sludge. Although this method determines the content of free and esterified $LCFA$s, it does not consider the content of the fatty fraction or the intermediate products of lipid hydrolysis. The method validation achieved $LCFA$ recoveries above 82 %, indicating rather unsatisfactory accuracy.

In summary, deep investigation of lipid hydrolysis during AD is impeded by the lack of reliable and simple analytical method. To date, no research has been published on the monitoring of the hydrolysis course of the lipid components in lipid-rich substrates, specifically in terms of tracking the concentration of glycerides (TG, DG and MG) simultaneously with $LCFA$ and glycerol in a single analysis from the anaerobic digestate. Moreover, current literature is surprisingly silent about the impact of glycerides on AD. Thus, the objective of this study was to propose and verify an uncomplicated extraction technique in combination with a GC analysis that facilitates dependable and effective quantification of the hydrolysis products of fat components present in lipid-rich substrates during their anaerobic fermentation and to utilise such a method for the very first detailed observation of lipid hydrolysis in AD.

2. Materials and methods

2.1. Analytical standards and reagents

The industrial AD sludges ($IADS$) were obtained from a local wastewater treatment plant, Zlín - Malenovice (Czech Republic, labelled SM1, SM2) and from the biogas station of Tanex s.r.o. (Czech Republic, labelled SV1, SV2) engaged in the production of glue. The basic characteristics of the sludges were determined (see supplementary material). The analytical standards used for calibration were set: glycerol (99.5 %), oleic acid (mixture of fatty acids purity 97 %, oleic acid min. 65 %) and solvent pyridine (99.8 %) were purchased at PENTA s.r.o. (Czech Republic); 1,4-butanediol (99 %, as an internal standard for quantification of glycerol), pentadecanoic acid (99.5 %, as an internal standard for $LCFA$ quantification), 1,2,3-tridecanoylglycerol (tricaprin, 99 %, as an internal standard for the quantification of mono-, di-, and tri-acylglycerols), 1-monopalmitoyl-rac-glycerol (monopalmitin, 99.5 %), 1-

monostearoyl-rac-glycerol (monostearin, 99.8 %), 1,2-dipalmitoyl-rac-glycerol (dipalmitin, 99.9 %), 1,2-distearoyl-rac-glycerol (distearin, 99.8 %); were purchased at Merck Life Science spol. s.r.o. (Czech Republic); palmitic acid (99 %), stearic acid (99.0 %) and solvents hexane, propan-2-ol, heptane, cyclohexane, methanol, chloroform and petroleum ether were obtained from IPL Uherský Brod (Czech Republic). Derivatisation agent bis(trimethylsilyl)trifluoroacetamide ($BSTFA$) was purchased at Restek (USA). Pure rapeseed oil (i.e. the Gustano brand, from a local grocery store) was used as the standard for triacylglycerol calibration and lipid hydrolysis observation.

2.2. Preparation of calibration standard solutions for chromatographic analysis

Calibration standard solutions were prepared in pyridine at five different concentration levels. Each solution contained all the compounds to be analysed. Monopalmitin and monostearin were used as standards for monoglyceride calibration, while distearin was used for diglyceride calibration. The stock $LCFA$ standard solution consisted of palmitic acid (30 %), stearic acid (30 %) and oleic acid (40 %). Pure rapeseed oil was used for separate triglyceride calibration. A mixture of internal standards (1,4-butanediol, pentadecanoic acid and tricaprin) and $BSTFA$ (a derivatisation agent) were added to each vial, followed by vigorous shaking and 15 min of derivatisation at room temperature. The volume was then diluted with n-heptane to reach the final volume of 5.5 mL. Further details regarding calibration procedure, linearity assessment, limits of detection (LOD) and quantification (LOQ) are available in (**Šánek et al., 2013**).

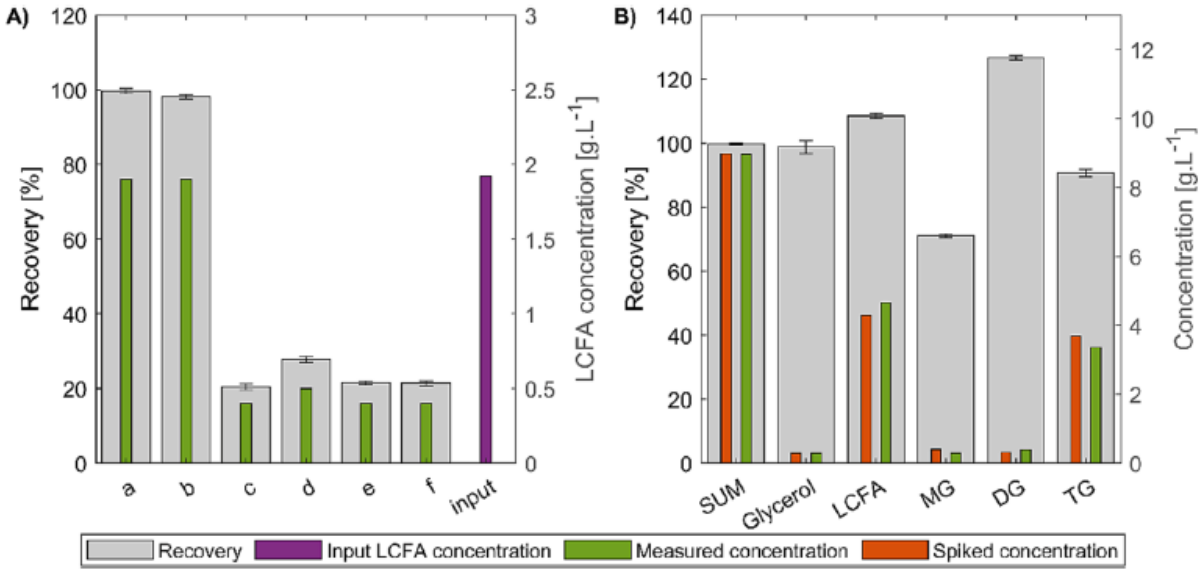


Fig. 1. Solvent system optimisation - obtained recovery assays (A), where a) mixture propan-2-ol: hexane (2:3, v/v), b) mixture methanol: chloroform (1:2, v/v), c) cyclohexane, d) petroleum ether, e) n-heptane and f) hexane; Initial recovery assays of the propan-2-ol: hexane mixture without sample pre-treatment (B).

2.3. Optimisation of the extraction efficiency of solvents

The extraction efficiency of selected solvents was tested on the IADS sample SV1, which had a high proportion of $LCFA$. The efficiency was evaluated after two extraction steps, as described: In the first step, 1 mL of $IADS$ was mixed with a solvent or a solvent mixture (n-heptane, hexane, cyclohexane, petroleum ether and mixture propan-2-ol: hexane (2:3, v/v) and methanol: chloroform (1:2, v/v)) in the ratio of 1:10 (v/v). The mixture was then subjected to intensive shaking for 2 h to ensure thorough extraction. After shaking, the mixture was centrifuged (6000 rpm, 15 min), allowing the separation of

the upper organic and lower water/solid fraction. In the second step, the lower water/solid fraction was subjected to another round of extraction under the same conditions, i.e. mixed with a fresh solvent or solvent mixture, shaken intensively for 2 h, and then centrifuged again. Following the second centrifugation, upper organic fraction was collected, and both organic fractions were further processed according to the specified methodology listed below. Finally, the processed samples were analysed using GC to determine the presence and concentration of target compounds. The extraction efficiency was subsequently evaluated as the percentage of $LCFA$ obtained after the extraction with the solvent to the input $LCFA$ content in $IADS$.

$$\text{Extraction efficiency}[\%] = \frac{c_{LCFA/solvent}}{c_{LCFA/input}}.100 \quad (1)$$

2.4. Sample pre-treatment

For the purposes of validating the extraction method and for the possibility of monitoring the degree of lipid hydrolysis in the real sludge sample, in order to receive the correct data, the sludge samples were pretreated before their extraction in terms of inhibition of enzyme activity. Two methods of inhibition were tested:

a) heating of the sludge sample on the laboratory hotplate to a temperature of 95 °C with a residence time of 10 min and subsequent slow cooling at ambient temperature,
b) microwave heating at oven wattage setting of 700 W (ETA 0208 90000). Sludge samples with a volume of 0.2 mL were heated for 15 s. In the case of pre-treatment of a large volume of sludge (50 mL) for samples spiking, gradual heating to the temperature of 95 °C was applied due to high foaming. The samples were then cooled to ambient temperature. Inhibition efficiency was compared with sludge samples without inhibition.

2.5. Preparation of samples for method validation

To verify the accuracy of the analysis with the most efficient extraction system, 5 g of the inhibited sludge using microwave heating were spiked with standards at three to four concentration levels. These levels corresponded to the linear range of each compound. To ensure the solubility and homogeneity of the spiked standards in the sludge, the mixture of standards was dissolved in the polar solvent pyridine. After adding the standard to the sludge sample, the resulting mixture was vigorously homogenised by shaking for one minute. Each sample was prepared in duplicate.

2.6. Extraction process of lipid components from sludge samples

To extract the lipid components, 0.2 mL of the sample was immediately pre-treated according to section 2.4b and mixed with a solvent mixture of propan-2-ol and hexane (2:3) in a ratio of 1:50 and shaken vigorously for 2 h. The extracted sample, along with the solvents, was then filtered through a 0.45 pm nylon syringe filter using a glass syringe. The filtered solution was left to evaporate under a stream of nitrogen, and the resulting sample was then prepared for GC analysis. Let it be noted that using a glass syringe is preferred, as contamination with oleamide originating from plastic syringes was observed and further confirmed.

2.7. Preparation of samples for chromatographic analysis

To the processed sample obtained by the extraction process described above, 0.8 mL of n-heptane, 0.1 mL of an internal standards solution for quantification of compounds and 0.1 mL of BSTFA were added. After at least one hour of derivatisation at room temperature, the sample was injected twice into the GC, analysed and evaluated. The chromatographic conditions were set according to (**Šánek et al., 2013**), where they are described in detail.

2.8. Lab-scale anaerobic digestion of rapeseed oil

A laboratory-scale experiment was conducted to study the anaerobic digestion of rapeseed oil and simulate an AD system with a high lipid content. Specifically, 0.38 g of rapeseed oil were added to the 130 g of $IADS$ SM1 sample. Mixing was applied, and the homogenisation of AD medium ran at room temperature in an inert nitrogen atmosphere for 5 min. To ensure uniform oil distribution (emulsion O/W) throughout the sludge sample, 0.34 g of xanthan gum emulsifier was subsequently added, as described by (**Putra et al., 2020**).

The anaerobic digestion process was carried out at ambient temperature, and samples were collected and analysed at the beginning of the anaerobic digestion process (at 20 and 50 min). Subsequently, a thorough lipid hydrolysis observations were conducted in a similar manner with sludges SM2 and SV2, which lasted 168 h. Samples were taken at selected intervals and processed as described in **sections 2.6 and 2.7**.

3. Results and discussion

3.1. Extraction process optimisation

Extraction of $LCFA$ and other lipid components from complex matrices is often a crucial step of their reliable quantification (**Saini et al., 2021**), as lipids may be present in a mixture with other compounds (non-lipid fraction) that can make analysis more tangled. The choice of solvent system is understood as the most critical factor in lipid extraction (**Saini et al., 2021**). Hence six different solvents and their mixtures were tested during the investigation of the effectiveness of various extraction agents. The test was conducted using $IADS$ SV1 sample obtained from a biogas station that operates in a glue manufacturing facility (Tanex s.r. o.). This sludge was known to have a high content of free $LCFA$ (1.9 g. L^{-1}), based on the GC analysis, making it an appropriate test material. The results of $LCFA$ determination after the first extraction step are depicted in **Fig. 1**A. It is evident that the tested single non-polar solvents, namely n-heptane, hexane, cyclohexane, and petroleum ether, exhibited comparable and very low extraction efficiency. Specifically, the efficiency of $LCFA$ single-step extraction reached less than 25 % on average with these solvents. This implies that the selected non-polar solvents did not effectively extract the $LCFA$ from the sludge, and even after the extraction, a significant amount of long chain fatty acids still remained in the solid residue.

Contrary to previous findings with single solvents, **Fig. 1**A reveals that solvent mixtures containing both polar and non-polar solvents had high $LCFA$ extraction efficiency. Specifically, when employing propan-2-ol: hexane mixture or methanol: chloroform mixture, comparably high efficiencies were achieved (99.6 % and 98.1 %, respectively). The first extraction step already yielded satisfactory efficiency, with subsequent confirmation of this fact in the second extraction step; no quantitatively significant proportion of long-chain fatty acids was extracted. Therefore, it is evident that the

combination of polar and non-polar solvents, such as the above-mentioned solvent mixtures, effectively facilitated the extraction of $LCFA$ from the sludge. Let it be noted that the efficacy of these tested solvent mixtures for lipid component extraction has been long known for isolating lipids from tissues (Bligh and Dyer, 1959; Hara and Radin, 1978), similarly complex sample matrices. In addition, the mixture of methanol: chloroform was used in the case of $LCFA$ determination in AD systems, e.g. (**Usman et al., 2020**).

The method utilising solvent mixtures was further optimised. This involved careful selection of the solvent system and determining the appropriate sample-to-solvent ratio, extraction time and mixing intensity. The goal was to achieve a reliable and simple single-step extraction technique suitable for subsequent analysis. It was observed that the evaporation step with methanol: chloroform took longer times compared to propan-2-ol: hexane. Considering the high toxicity of the methanol: chloroform mixture (**Lalman and Bagley, 2004**), the associated mixture of propan-2-ol and hexane was selected for further work. In addition, the sample-to-solvent ratio was adjusted to 1:50 in order to ensure complete lipid extraction on the one hand and to suppress possible overload of an analytical system by a potentially high concentration of non-lipid fraction of the sample on the other hand. Finally, the extraction time of 2 h of vigorous shaking proved to be sufficient. Overall, the preliminary tests demonstrated that the chosen extraction method (propan-2-ol: hexane 2:3 (v/v), 1:50 sample: solvent, 2 h of vigorous shaking) provided the best results in terms of extraction efficiency and accuracy while keeping an uncomplicated procedure. Therefore, these conditions were selected for subsequent thorough method validation.

Table 1 Recovery evaluation of monoglycerides in a sample extract after sample pretreatment.

Pre-treatment	Total MG concentration after spiking	Measured MG concentration after spiking	Recovery *
	[mg.L^{-1}]	[mg.L^{-1}]	[%± SD]
Raw sludge	60.7	36.8	60.6±5.3
Hotplate heating		54.7	90.1±2.1
Microwave heating		59.9	98.7±1.4

* *The results are presented as the mean of four samples, which were obtained through duplicate GC analysis.*

3.2. Sample pre-treatment optimisation

The initial results of analytes recovery depicted in **Fig. 1**B indicated an unexpected phenomenon. Triglycerides achieved a relatively lower recovery of 91 %; the recovery of MG was observed in the range of 60-70 %, on average. In contrast, recoveries of DG (130 %) and $LCFA$ (109 %) were higher. Nevertheless, the overall accuracy of the acquired data was corroborated by the fact that the total sum of all monitored analytes ("SUM"), presented as a recovery rate, reached 99.9 %. Consequently, this observation raised concerns about the ongoing hydrolysis during sludge sample spiking and especially lipid extraction, given the short time between spiking and start of the extraction during sample processing. The hydrolysis of TG present at a high concentration level explains the increase in DG and the corresponding increase of $LCFA$ concentration beyond the spiked amount, while

maintaining the overall concentration of added lipid compounds on constant value. To address the hydrolysis issue and accurately measure glyceride content in the sludge samples while preserving the lipid profile, further investigation and optimisation of the sample pre-treatment method was required. The presence of enzymes with high lipolytic activity had a significant impact on validating the extraction method. Hence, it became imperative to inactivate the enzymes in the sludge samples in order to prevent their continued action. Due to the thermal instability of most lipolytic enzymes, their catalytic activity at elevated temperatures tends to be lost (**Colla et al., 2015**), which seemed to be a suitable principle for the pretreatment of the sample.

During the preliminary treatment of the sample prior to extraction, two methods for thermal inactivation of the $IADS$ sample were tested (refer to section 2.4 a, b). Table 1 displays the results of assessing the recovery of MG, following the sludge inactivation since MG were the most vulnerable substances (see supplementary material).

Based on the recovery results obtained for monoglycerides and considering the quick and straightforward nature of the pre-treatment of the sample using the microwave heating method, this approach was chosen and subsequently employed for all further sample pretreatments. For the record, the hotplate heating was lengthy, and its effectiveness was not particularly compelling (see **Table 1**).

The impact of the described observation goes beyond the sole analytical method. It indicates that lipolytic enzymes remain active even when present in highly concentrated solvents. In this case, hydrolysis is most probably facilitated by the substantial elimination of mass transfer barriers through applying a solvent system and relatively intensive mixing. In contrast, the mass transfer between practically insoluble nonpolar lipid particles and the polar water phase/microorganism aggregates can be the rate-limiting step of the entire process in the native state of the AD sludge (**Holohan et al., 2022; Ware and Power, 2016**). Additionally, the elimination of hydrolysis during sample processing is key to obtaining accurate data, even in the case of free $LCFA$ analysis. Nevertheless, such verification has rarely been performed in the literature published so far.

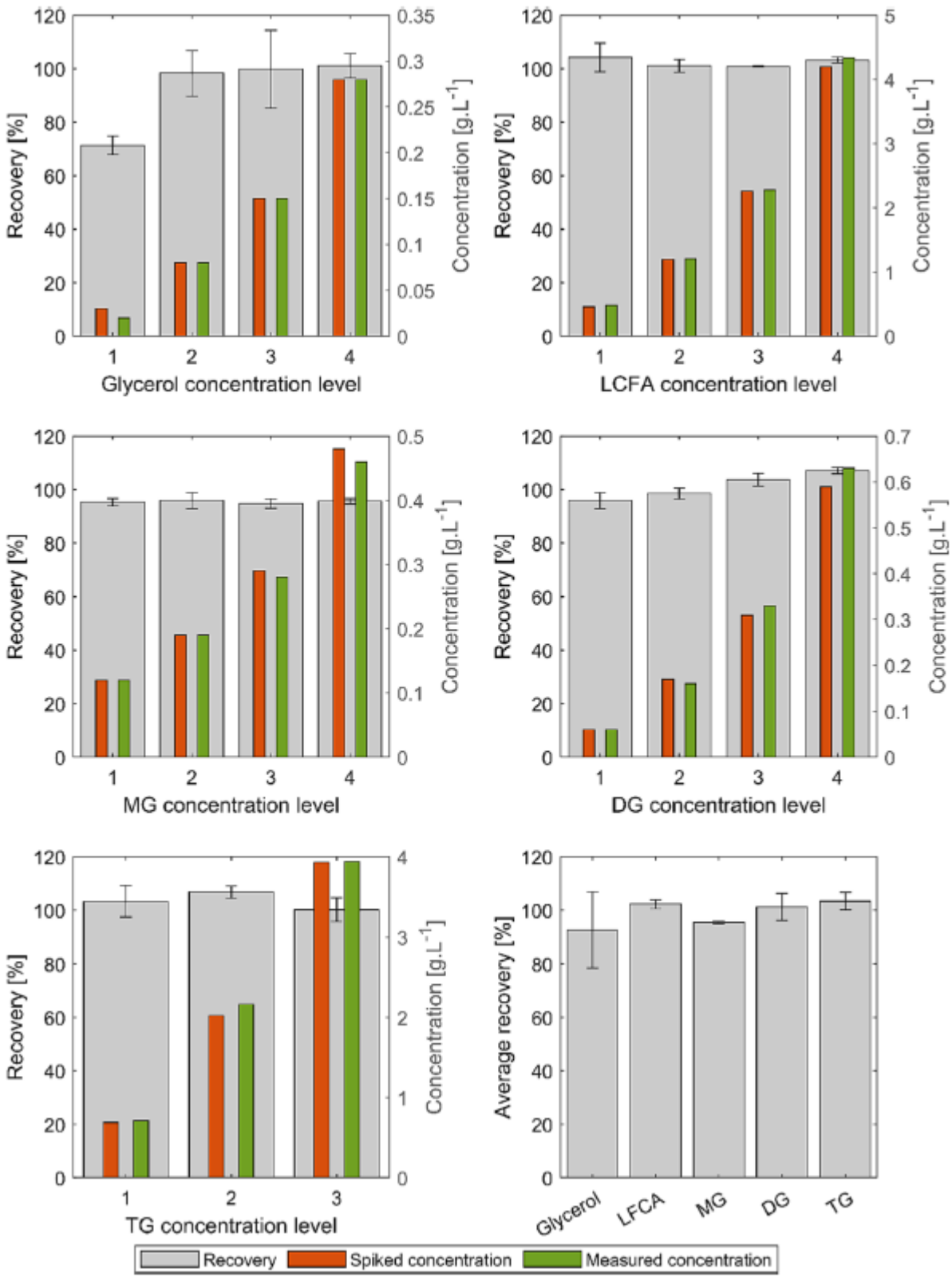


Fig. 2. Recovery assays of the complete analytical method.

3.3. Analytical method validation

Previous discussion highlights that validation of the method is essential for obtaining trustworthy picture of the course of lipid hydrolysis in AD. First of all, chromatographic conditions were verified to

ensure good separation of all analytes of interest (GL, TG, MG, DG and $LCFA$), their calibration was subsequently performed, and a linear range of concentrations, LOD and LOQ were evaluated (see **supplementary material**). Here, the primary difficulty faced by the proposed approach was to guarantee a satisfactory level of accuracy within the desired large concentration ranges designed to cover the complete course of lipid hydrolysis, i.e. from 100 % content of triglycerides to 100 % content of $LCFA$. Overall, the obtained calibration curves were linear within the required concentration ranges with coefficients of correlation ≥0.996 for all the analytes.

Next, the accuracy and repeatability of the complete analytical method (i.e. sample pre-treatment, its extraction and subsequent GC analysis) were verified using a real industrial sludge sample obtained from a local wastewater treatment plant in Zlín - Malenovice - SM1. The comprehensive findings of recovery assays are presented in **Fig. 2**. The figure demonstrates that the complete quantification range for each compound was thoroughly investigated, and highly satisfactory recoveries were obtained.

Table 2 Observation of lipid hydrolysis in a laboratory AD lipid-rich system by means of the validated method; comparison of analysis without and with sample pretreatment (raw and inactivated sludge SM1, respectively).

Compound	Raw SM1 sludge	Inactivated SM1 sludge
	[% w/w ± SD]	[% w/w ± SD]
Total time 20 min of fermentation		
LCFA	13.4±1.7	10.7±0.5
Diglycerides	10.8±0.3	9.5±0.3
Triglycerides	72.4±2.1	76.2±0.6
Monoglycerides	3.3±0.2	3.4±0.2
Glycerol	0.1±0.01	0.2±0.01
The sum of all compounds [g.L^{-1}]	3.7±0.1	3.7±0.1
Total time 50 min of fermentation		
LCFA	34.4±0.1	31.7±0.6
Diglycerides	16.2±0.2	16.3±0.5
Triglycerides	43.5±1.5	47.6±1.1
Monoglycerides	5.6±1.6	4.2±0.1
Glycerol	0.3±0.01	0.1±0.01
The sum of all compounds [g.L^{-1}]	3.5±0.1	3.6±0.1

**The results are presented as the mean of two samples, which were obtained through duplicate GC analysis.*

The only unfavourable result is the low recovery for glycerol (approx. 70 %) in the LOQ area; however, this fact can be attributed to the rapid occurrence of acidogenesis, where glycerol is converted to acetate (**Cirne et al., 2007**). This phenomenon plays a substantial role in explaining the observed decrease in recovery rates for glycerol at these low concentrations. Let it be noted that this fact has been verified several times, and in the case of not-deactivated sludge, the GL recovery rarely exceeded 50 %. However, when higher glycerol concentrations in the sludge sample were tested, this phenomenon was not observed, and very good recoveries were achieved. For the other analytes under observation, namely MG, DG, $LCFA$, and TG, the proposed single-step extraction procedure resulted in average recovery values ranging from 95 % to 104 % across the entire concentration range. Moreover, the recovery relative standard deviations (RSD) were generally low. The highest average

RSD was observed for glycerol, reaching nearly 8 %. On the other hand, compounds such as MG, DG, and $LCFA$ exhibited RSD values below 3 %. In the case of TG, the average deviation was approximately 4 %.

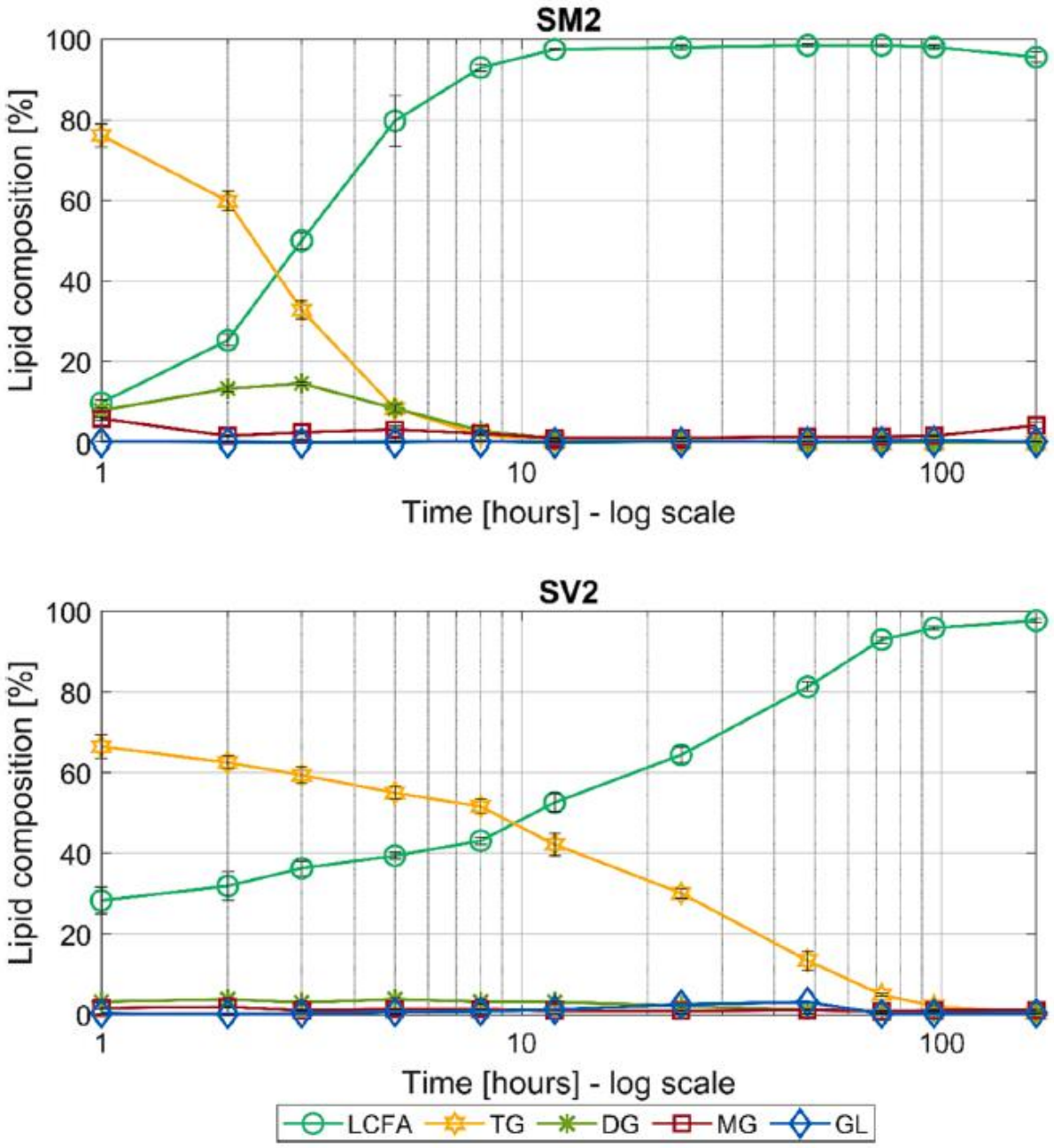


Fig. 3. The course of lipid hydrolysis in the fresh AD sludges.

The accuracy results obtained from the extraction method utilising the solvent mixture of propan-2-ol and hexane undeniably demonstrate a remarkable efficiency in the retrieval of the lipid fraction from AD sludge. To the best of the authors' knowledge, no alternative validated approach enabling such quantitative observation of individual glyceride hydrolysis in AD sludge was presented so far.

3.4. Lipid hydrolysis observation

Set of lab-scale anaerobic fermentation experiments with a high concentration of lipids (3-4 g.L^{-1}) was conducted to verify the suggested and validated method for monitoring hydrolysis of lipid-rich sludge and to gain most probably the very first complete data of this reaction. A sludge with high lipolytic activity (SM1) was chosen for the purpose of complete method verification and triglyceride concentration was increased by rapeseed oil addition to the sludge. **Table 2** summarizes the results of lipid hydrolysis within 1 h of reaction and documents the impact of sample pre-treatment on the obtained data. The composition of lipid fraction is presented in mass percent.

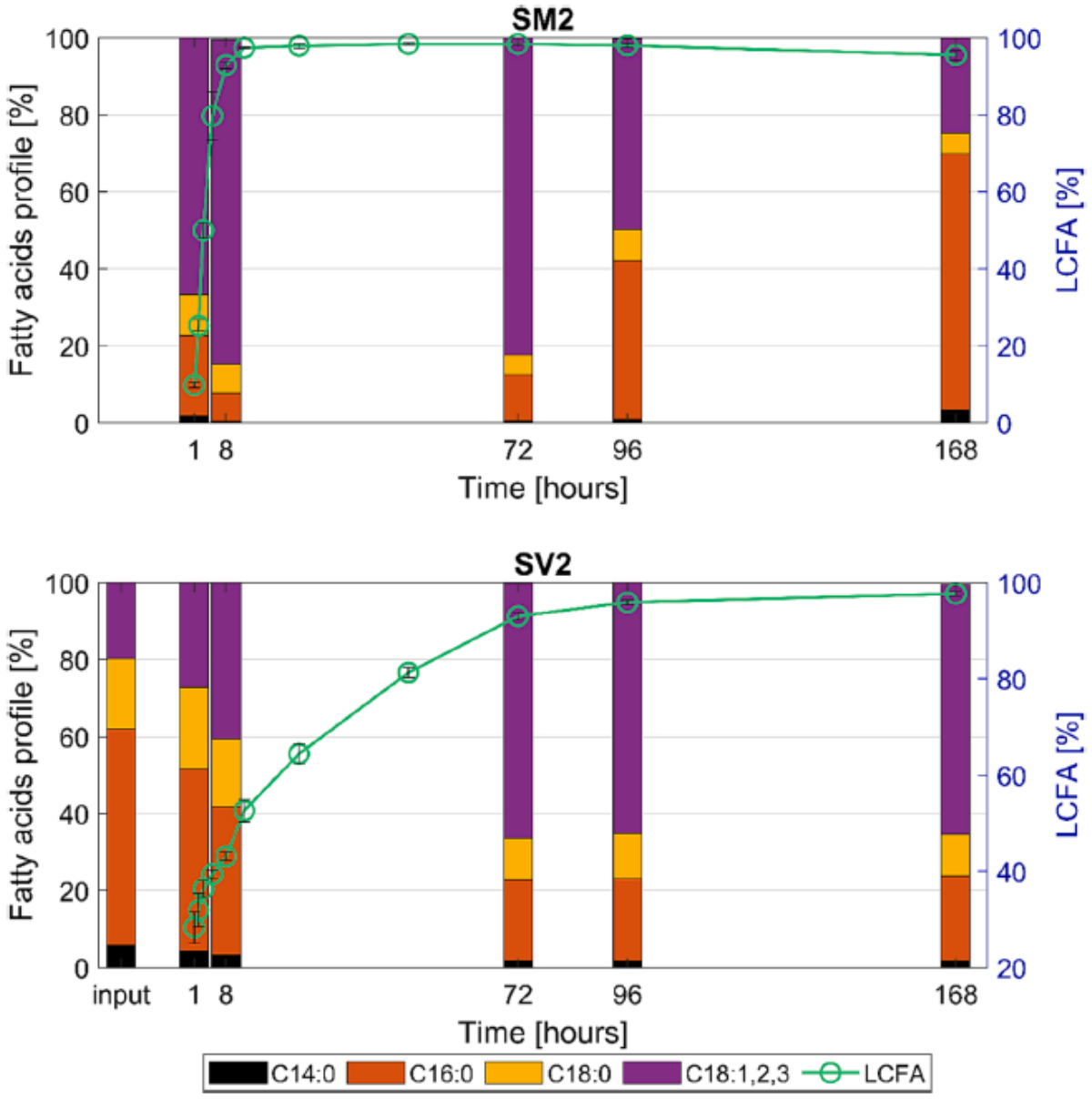


Fig. 4. Evolution of free $LCFA$ profile during AD; $LCFA$ content in lipid fraction during AD is highlighted in green.

As can be seen, the hydrolysis of the lipid fraction occurred very rapidly as more than 50 % of the added triglycerides underwent hydrolysis within the first 50 min of AD. This was confirmed by an increased content of triglyceride hydrolysis reaction products - MG, DG, and especially $LCFA$. The results have also substantiated that the deactivation of sludge during sample preparation does not noticeably

impact its properties, e.g. no degradation of the lipid components due to elevated temperatures during sample heating was observed. This was verified by quantification of the total sum of all lipid compounds present in the sludge sample. Specifically, no significant differences in total sum of all lipids were recorded (see **Table 2**).

However, it has been observed that the extraction process of the samples without deactivation is still accompanied by slight hydrolysis of the triglycerides most probably due to the persistent lipolytic activity of the sludge also during sample extraction itself, as was already noted above. This is substantiated by the observed changes, particularly in the triglyceride proportions and in the proportion of hydrolysis products, specifically $LCFA$ and diglycerides. In this specific case, the difference in triglyceride concentration was approximately 4 % w/w. In other words, if the sample is not properly pre-treated (i.e. inactivated) at the time of its withdrawal, the composition of its lipid fraction at the time of the analysis can be substantially different from the original, depending on the lipolytic activity of the specific sludge, time passed between sampling and analysis and overall analytical procedure. As a result, there is a real danger of acquiring completely wrong data without thorough validation and verification of the experimental methodology.

The complete course of lipid hydrolysis was captured with two fresh AD sludges originated from different facilities by means of a validated method. **Fig. 3** documents that in the case of sludge SM2, nearly all glycerides were converted to LCFA within 8 h after adding the rapeseed oil to the sludge. The figure has logarithmic time scale in order to better illustrate very fast hydrolysis in SM2. The reaction intermediate DG achieved maximum concentration in 3 h highlighting the consecutive nature of triglyceride hydrolysis reaction. On the other hand, concentration of the reaction second intermediate, MG, was low and most of the time slightly fluctuating below 3 % without reaching clear maxima. This observation suggests that the hydrolysis of MG is considerably faster than the hydrolysis of TG and even DG, which can probably be attributed to stronger surfactant properties of MG in the studied system. Overall, TG hydrolysis by SM2 sludge was very fast. Unfortunately, a comparison with data in literature is difficult since the reactions were usually studied in more extensive time frame - usually days, not hours. Nevertheless, limited old data on the degradation of milk fat (**Hanaki et al., 1981**) indicated a similar rate of lipid hydrolysis.

In contrast, hydrolysis of the added rapeseed oil by sludge SV2 was considerably slower, as revealed in **Fig. 3**. Here, the initial amount of free $LCFA$ present in the sludge must be taken into account as these $LCFA$ are not the product of the rapeseed oil hydrolysis. As a result, the content of TG at the beginning of the reaction was around 80 %; thus, approximately 60 % of the added TG were hydrolysed after 24 h, and TG were still present in the reaction mixture after 3 days of AD. In the case of this sludge, no DG maximal concentration was observed; DG behaved similarly as MG in the SM2, likely because of slower TG hydrolysis in SV2. Glycerol content remained low in both SM2 and SV2, indicating that glycerol degradation by AD is much faster than $LCFA$, as already pointed out. Finally, let it be noted that lipid hydrolysis in both SM2 and SV2 was significantly influenced by thorough emulsification of the added rape-seed oil, which was performed to facilitate a reasonable method verification. Hence, a hydrolysis of TG without such emulsification would probably take noticeably longer.

The presented data of hydrolysis by SM2 and SV2 gained at the same experimental conditions reveals that there can be a significant difference in rate of glyceride hydrolysis between different AD microbial consortia, and confirms that especially TG hydrolysis can be noticeably slow. Consequently, the impact of glyceride presence and lipid hydrolysis on AD system shall be assessed individually, for each specific AD system under study. The possibility of slow neutral fat hydrolysis was indicated by other scarce studies capturing lipid hydrolysis to a limited extent. Specifically, (**Masse and Masse, 2002**) documented that around 30 % of neutral fat (i.e. unhydrolyzed) was present in the sludge after more

than 40 h of AD, similarly as in the case of SV2. More recently, (**Sakurai et al., 2023**) observed that major fraction of $LCFA$ remained unhydrolysed (esterified) on the 4th day of AD in their system, and significant content of esterified $LCFA$ was still present even after ten days of AD. Hence, lipid hydrolysis can notably influence whole AD of lipids in such a case -e.g. (**Moukazis et al., 2018**) reported that 80 % of maximum methane production was achieved within the first 7 days of AD in the case of slaughterhouse waste with high lipid content (above 50 %). Furthermore, (**Ware and Power, 2016**) hypothesised that the limited rate of lipid hydrolysis prevented the inhibition of AD through consequent elimination of $LCFA$ accumulation, according to the analysis of methane production kinetics. The presented method for lipid hydrolysis observation, therefore aims to help to find answers to these questions to better understand the very important phenomena of lipid inhibition in AD.

Apart from lipid hydrolysis, the proposed method can capture free $LCFA$ profiles to a large extent. **Fig. 4** shows changes in fatty acid profiles during rapeseed oil hydrolysis by SM2 and SV2. In case of SV2, free $LCFA$ profile of the input sludge is presented due to its considerable $LCFA$ content. As can be seen, notable changes in $LCFA$ profile occurred during hydrolysis by SM2. Despite rapeseed oil containing predominantly unsaturated $LCFA$, the content of saturated palmitic acid (C16:0) grows over time, reaching more than 60 % after 7 days. This finding is consistent with literature - many authors noted this behaviour, which is typical for lipid degradation by AD (**Holohan et al., 2022; Sakurai et al., 2023; Usman et al., 2020**). This is usually attributed to the conversion of unsaturated $LCFA$ to saturated ones with their subsequent β-oxidation. Surprisingly, a similar anticipated change in $LCFA$ profile was not detected in SV2 during the time frame of the experiment, highlighting the different properties of both utilised inocula.

4. Conclusion

Detailed triglyceride hydrolysis performed in AD was captured. It was shown that there can be significant differences in the rate of glyceride hydrolysis between individual AD biomass. The TG hydrolysis can take several days and likely influences the whole AD process in this case. Rare observations of TG, DG and MG hydrolyses into $LCFA$ and GL were recorded thanks to the suggested and thoroughly validated method. Ceasing of the AD was crucial for gaining reliable data and, therefore, a trustworthy picture of lipid hydrolysis. The method aims to provide answers to a better understanding of lipid inhibition phenomena in AD.

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