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Facile analysis of short-chain fatty acids as 4-nitrophenyl esters in complex anaerobic fermentation samples by high performance liquid chromatography

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ABSTRACT

Short-chain fatty acids are crucial intermediates in the conversion of biomass to methane. Due to the complexity of raw biomass, volatile fatty acids (including *n*- and branched-chain compounds) as well as arylacetic and arylpropionic acids arise from digestion of carbohydrates, proteins and lipids. The development of a simple extraction procedure in combination with internal standardization and facile 4-nitrophenyl-labelling via oxalylchloride-generated acylchlorides enabled robust separation and quantification of the target compounds in crude biological samples like raw cattle manure and biogas fermenter contents. Detection limits of <100 μM and error rates of less than 4% for the quantification of individual compounds in a concentration range up to 50 mM for non-diluted samples suggest that the novel method might be of general advantage for the routine quantification of short-chain fatty acids in complex biological samples including complex fermentation media.

1. Introduction

Different microbial consortia and their syntrophic interrelation are involved in the production of biogas. Process stability and efficient biogas formation is only achieved when the environment provides optimum conditions for the participating microbes. Indicators for process balance are pH, redox potential and concentrations of crucial intermediates including short-chain fatty acids (SCFA). The latter have been recognized to be the most important early warning indicator of process disturbances in anaerobic fermentations, in particular the levels of isobutyric and isovaleric acids [1] and furthermore the ratio of butyric acid and its isoform [2].

In the past 35 years, several methods to identify and quantify individual short-chain fatty acids (SCFA) have been published, none of which fully meets the requirement of a simple, rapid, highly selective and sample matrix independent approach. The earliest method to separate and quantify individual SCFA from biological samples was by direct injection into the GC [3–6] or HPLC [7–10]. In some cases, a selection and cleaning step by solvent extraction preceded the separation via GC [11–15] or HPLC [16–18]. Other publications focused on concentrating the SCFA prior to gas chromatographic analysis with the aid of solid phase microextraction (SPME) [19–22] or static headspace procedures (HSGC) [23]. More recently, different scientific groups have focused on the development of online sensors [24–26]. In addition to the GC and HPLC approaches, a few studies have been carried out to establish chromatography independent approaches by development of capillary electrophoretical (CE) methods with indirect UV detection [27–29]. In general, however, the available methods are complex and costly [24] and, therefore, of limited use for application in frequent routine analysis necessary for supervision of biogas fermenters. Thus, most practical approaches published so far focused on the most abundant volatile fatty acids (VFA, *n*-C_2 to C_6 as well as isoC_4 to C_6) and do not consider other SCFAs derived from amino acid degradation, e.g. phenylacetic and phenylpropionic acid, hydroxyphenylacetic and hydroxyphenylpropionic acid, and indole-3-acetic and indole-3-propionic acid. Detection of these compounds, however, might be essential when the microbial communities are fed with complex substrates like facial slurries or manure.

Here, we present a protocol for the measurement of SCFA in biogas fermentation processes based on liquid manure by RP-HPLC. Previously described extraction protocols were optimized and combined with a new rapid and selective labelling method. The research was focused on a routinely manageable sample preparation step, which is applicable irrespective of the sample composition but allows selective and accurate quantification.

2. Experimental

2.1. Chemicals and materials

Acetic, propionic, *n*-butyric, *n*-valeric, *n*-caproic, *n*-pelargonic (internal standard), isobutyric, isovaleric, isocaproic, 2-methyl-butyric, 2-methyl-valeric, phenylacetic, phenylpropionic,

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hydroxyphenylacetic, hydroxyphenylpropionic, indole-3-acetic, indole-3-propionic acid (each >99%), 4-nitrophenol (PESTANAL® analytical grade) and oxalyl chloride (>99%) were purchased from Sigma–Aldrich (Schnelldorf, Germany). Concentrated analytical-grade HCl, NaCl (>99%) anhydrous diethyl ether (>99%), acetonitrile (>99.9%), triethylamine (>99%), pyridine (>99%), N,N-dimethylformamide (>99%) and methanol (>99%) were purchased from Carl Roth GmbH (Karlsruhe, Germany).

2.2. Instruments

The HPLC system consisted of a quarternary gradient pump S2100 (Sykam), connected to an autosampler S5200 (Sykam) for automated sample injection. The system was combined with a Sykam UV 2500 detector. Eluents were degassed with helium 5.0 prior use.

2.3. Standard and sample preparation

Samples (300 μL) were transferred into 1.5 mL polypropylene test tubes. Two spatula of NaCl (~0.2 g) and 100 μL of concentrated HCl were added and vigorously mixed with 800 μL of diethyl ether (containing 5 mM pelargonic acid as internal standard) for 1 min. The mixture was briefly centrifuged to remove residual water droplets from the ether phase.

Part of the ether phase (200 μL) was transferred to 1.5 mL screw capped glass vials and mixed with a solution of 250 mM oxalyl chloride in N,N-dimethyl formamide/acetonitrile 1:100 (v/v) (200 μL). Then, derivatization reagent (50 mM 4-nitrophenol in 500 mM pyridine/acetonitrile, 800 μL) was added and mixed. The samples were directly inserted into the autosampler rack for measurement.

2.4. Measurement of standards and samples

20 μL from each sample was injected via autosampler. Binary gradients with 20 mM triethylamine/acetate buffer; pH 4.8; 35% (v/v) acetonitrile (solvent A) and the same buffer with 80% (v/v) acetonitrile (solvent B) were used for separation on a Synergi Polar-RP (80 Å, 4 μm, 250 mm × 4.6 mm) column at 45 °C. All measurements were performed with a flow rate of 1 mL/min and detection at 295 nm. The method started with a 2 min isocratic interval of 100% solvent A, followed by a linear increase of 0.9% solvent B per min. Then, three isocratic intervals with 20% (7 min), 32% (4 min) and 100% solvent B (6 min) followed. Pre-equilibration time with solvent A prior sample application was 4 min.

2.5. HPLC calibration

For calibration, a stock solution containing 200 mM of each acetic, propionic, *n*-butyric, *n*-valeric, *n*-caproic, isobutyric, isovaleric, isocaproic, hydroxyphenylacetic, hydroxyphenylpropionic, phenylacetic, phenylpropionic and indole-3-acetic acid was prepared. From this stock solution, calibration standards ranging from 0 to 50 mM were prepared. In each case, 300 μL of standard solution was used for extraction and preparation of the 4-nitrophenyl esters according to Sections 2.3 and 2.4.

3. Results and discussion

The detections of SCFAs in complex biological samples like facial slurries or manure called for a major selection step prior to analysis. Alkalinizing the sample with $NaHCO_3$ and freeze drying of the respective sodium salts resulted in a significant loss of sensitivity, poor reproducibility and slowed down sample preparation and analysis significantly. Solvent extraction with diethyl ether circumvented these problems when previously described protocols [11–18] were adapted to the needs: as previously reported, the non quantitative phase transfer of acetic and propionic acids and especially the volatility of diethyl ether lead to reproducibility problems in terms of calibration and quantification [14]. The addition of saturating concentrations of sodium chloride to the samples prior extraction significantly increased phase transfer of acetic and propionic acids. A good reproducibility was reached by adding an internal standard (pelargonic acid) directly to the ether extraction phase, which allowed correction for volume losses of the ether phase and circumvented problems related to the recovery of acetic and propionic acids. The internal standardization did not only correct for the evaporation of ether during sample preparation and analyses of multiple samples, but also rendered the quantification independent of the loaded sample volume. The concentration values determined by the injection of 10 μL, 20 μL or 40 μL of a single sample were identical within a range of <2% (Supplemental material).

The nitrophenyl esters of carboxylic acids are readily detected at 295 nm. Their preparation according to the method presented herein takes only a few minutes as compared to previously described derivatization methods which usually take more than 1 h [16,30,31]. The SCFA-nitrophenyl esters were synthesized in a two-step procedure: First, the free acids were converted very cleanly to the respective acyl chlorides by oxalyl chloride in acetonitrile containing 1% (v/v) of N,N-dimethylformamide as a protic solvent. The acid chlorides thus formed were subsequently converted to the nitrophenyl esters by excess of 4-nitrophenol dissolved in acetonitrile containing pyridine as a base. Samples thus obtained were clear, stable (for at least several weeks) and, therefore, well suited for automated HPLC analysis.

The results of retention time determination for individual nitrophenyl esters and the generated calibration data are summarized in Table 1. Acceptable separation was achieved for most of the analytes in question (Fig. 1b) and only a small number of conflicts remained: The HPLC method described was not able to separate isovaleric and 2-methylbutyric acid. These two SCFAs are oxidation products of the proteinogenic amino acids leucin and isoleucin, respectively. Both compounds are isomers and, therefore, equal in their biogas forming potential. Also not separated were isocaproic, 2-methylvaleric and indole-3-propionic acids. Isocaproic acid and indole-3-propionic acid are reduction products of the amino acids leucin and tryptophan, while 2-methyl-valeric acid has not been described as a fermention product of anaerobe microbes. While leucin is an abundant constituent of proteins, tryptophan is rather rare. Thus, the biogas forming potential of the latter is quantitatively almost negligible. As a consequence of these findings, 2-methylbutyric, 2-methylvaleric and indole-3-propionic acids were omitted from the calibration standards.

As shown in Fig. 1a, plots of detector response area ratios of individual nitrophenyl esters and internal standard versus the concentration ratio of these compounds were nicely linear in a molar ratio between 0, 1 and 10. Thus, the slope of these plots was used to calculate the SCFA concentrations in unknown samples. As expected, extraction effectiveness of individual compounds within the homologous series decreased with increasing polarity of the derivative while the number of carbons exhibited no predictable effects (e.g. the extraction efficiency of isobutyric acid was lower than that of acetic acid) (see slopes in Table 1). Detection limits were determined at a signal-noise ratio of 10:1 measuring different standard dilutions, which lead to concentrations ranging from 45 to 115 μM for the VFA and 23 to 52 μM for the aromatic SCFA (see Table 1).

Using the calibration data, the SCFA concentrations in raw cattle manure (Fig. 1c) and a laboratory biogas fermenter (Fig. 1d) were determined in order to elucidate the precision of the novel method.

Table 1
Quantitative parameters for HPLC analysis of selected SCFA.

4-Nitrophenyl derivative	t_R (min)	Det. limit (mM)	Calibration				Liquid manure sample
			Slope *b*	Intercept *a*	S_a	R^2 adj.	Reproducibility[a]
Acetate	13.5	0.077	0.244	−0.027	0.014	0.9990	81.1 ± 1.99 mM (2.5%)
Propionate	19.1	0.067	0.279	−0.022	0.009	0.9997	23.25 ± 0.53 mM (2.3%)
n-butyrate	25.2	0.052	0.365	−0.014	0.011	0.9997	8.46 ± 0.22 mM (2.5%)
n-valerate	31.7	0.047	0.393	−0.024	0.019	0.9993	1.35 ± 0.04 mM (3.0%)
n-caproate	38.1	0.045	0.412	−0.004	0.008	0.9999	0.399 ± 0.016 mM (4.0%)
Isobutyrate	24.7	0.115	0.163	−0.009	0.007	0.9994	2.46 ± 0.08 mM (3.4%)
Isovalerate	30.5	0.093	0.199	0.013	0.009	0.9994	–
Isocaproate	37.1	0.054	0.350	−0.016	0.014	0.9995	–
Hydroxyphenylacetate	19.5	0.044	0.211	0.024	0.008	0.9996	N.D.
Hydroxyphenylpropionate	22.8	0.028	0.330	0.007	0.005	0.9999	0.045 ± 0.002 mM (4.2%)
Phenylacetate	33.9	0.052	0.179	0.025	0.011	0.9988	2.47 ± 0.1 mM (4.0%)
Phenylpropionate	38.4	0.039	0.243	0.027	0.007	0.9998	2.06 ± 0.08 mM (3.8%)
Indole-3-acetate	32.3	0.023	0.391	−0.003	0.002	0.9995	N.D.

Estimates of the calibration validity are given by the standard deviation of the intercept (S_a) and the adjusted correlation coefficient (R^2 adj.). The estimated linear calibration function is given for each analyte by the linear coefficients *a* (intercept estimate) and *b* (slope estimate).

[a] Reproducibility was determined as the percentage deviation of the mean concentration obtained from 10 different extractions of liquid manure.

Therefore, crude samples were screened to remove large particular matter and subsequently split in ten individual sub-samples for derivatization and HPLC analysis. As summarized in Table 1 for the manure sample, the independent determinations yielded similar concentrations for individual nitrophenyl esters with errors below 4%. The precision of the novel method is readily evident by the observations made with manure samples which were analyzed and spiked with 10 mM of each acetic and propionic acids in order to

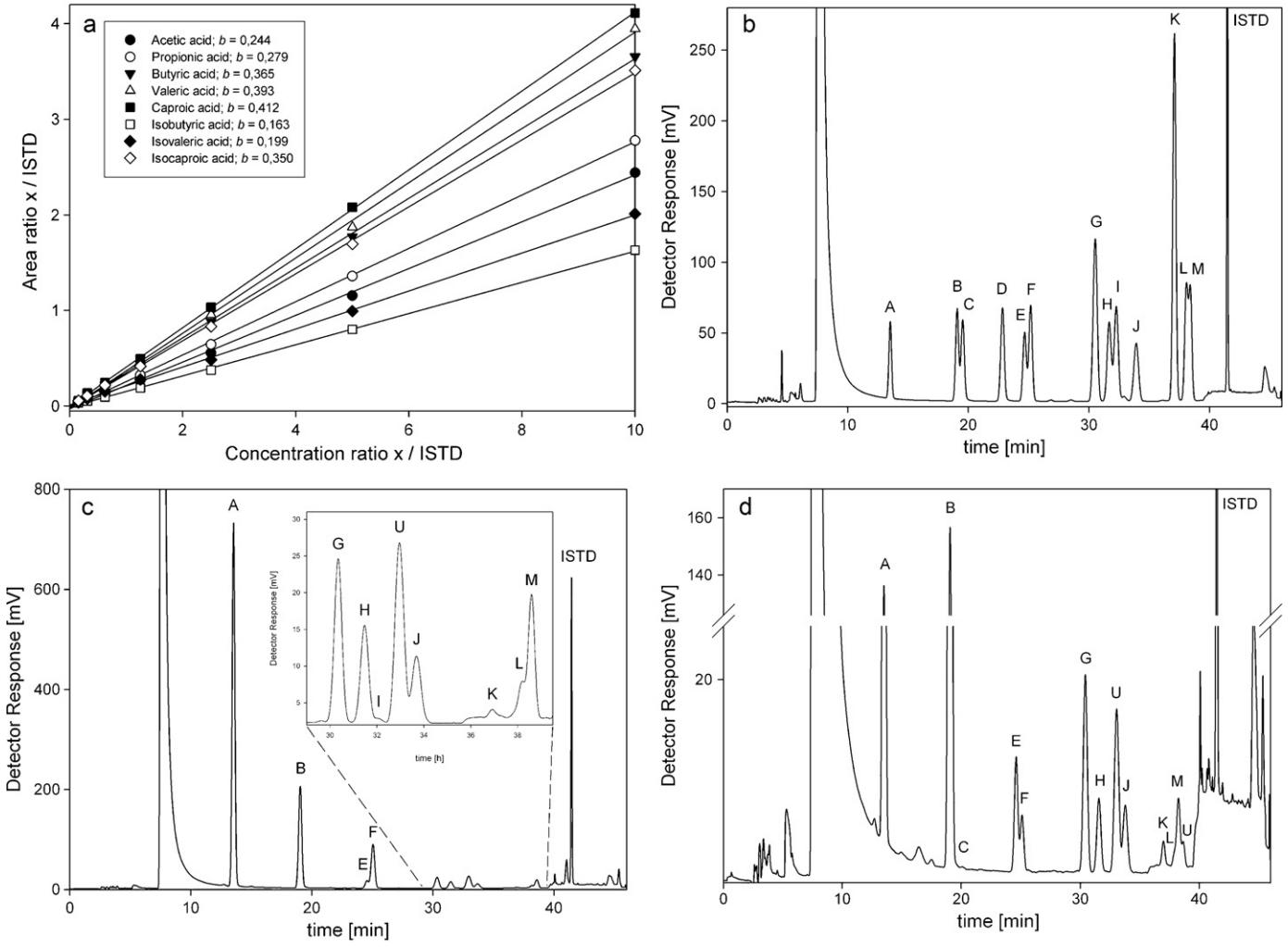

Fig. 1. (a) Linear calibration curves exemplarily for the VFA and chromatograms of extracts from (b) a standard mixture with 5 mM of each SCFA, (c) liquid manure and (d) a biogas fermenter sample based on liquid manure. A = acetic acid, B = propionic acid, C = hydroxyphenylacetic acid, D = hydroxyphenylpropionic acid, E = isobutyric acid, F = *n*-butyric acid, G = isovaleric and 2-methyl-butyric acid, H = valeric acid, I = indole-3-acetic acid, J = phenylacetic acid, K = isocaproic, 2-methyl-valeric and indole-3-propionic acid, L = caproic acid, M = phenylpropionic acid, U = unknown, ISTD = internal standard (pelargonic acid).

confirm that the concentration changes induced by spiking were reflected in the experimental results. Indeed, these samples yielded the calculated values within an error range ≤2.2% (Supplementary material).

4. Conclusions

The protocol for quantitative determination of SCFA via HPLC presented in this communication was optimized for samples from biogas fermenters and facial slurries, but might be applicable for a larger selection of crude biological samples including raw fermentation media, stool or other "dirty" samples. The method circumvents technical demanding and time consuming sample workup procedures. Upon manual performance, the full extraction and derivatization procedure takes less than 30 min for up to 24 samples and represents, therefore, an excellent alternative to elaborate and costly online measurement techniques recently employed in this field of analytical chemistry. Moreover, the method has a substantial potential for automated "in place" derivatization, which can be performed with many commercially available autosamplers equipped with pre-column derivatization liquid handling capability.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.06.093.

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