HPLC analysis of VFAs, Furfural and HMF

Standard Operational Procedure

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Introduction

The following chromatographic analysis is developed in order to analyse volatile fatty acids and carbohydrate degradation products; furfural, hydroxymethylfurfural (HMF) and levulinic acid. The purpose of this method is to perform routine analysis of biogas and biomass pretreatment samples. Other purposes may also be applicable. The principle of separation of these analytes is that they are organic molecules with different hydrophobicity, and the separation principle is therefore based on reversed phase (RP) separation. It is important for efficient separation that pH in the mobile phase is below the pKa value of the analyte with the lowest pKa value as this will cause the analytes to be predominantly protonated. Due to a complex sample matrix and due to high water solubility for the shortest VFAs, the gradient profile is designed with relatively weak eluting conditions. Volatile fatty acids can be analyzed up to at least C7.

The sensitivity of the analysis is dependent on the injection volume and on the analyte. For VFAs at least 1 mM of each analyte is quantifiable at 1 µL injection. The sensitivity for HMF/furfural is approx. 1000 times better than for VFAs and therefore down to 0.001 mM is quantifiable in 1 µL injection. If lower concentrations of analytes are expected in the samples, larger injection volume may be applied (up to 10µL).

HPLC system

The analysis is performed on the Dionex Ultimate 3000 chromatographic system with UV detection. See relevant instrumental SOP for operation of this system.

Analytical column

The analytical column applied is Zorbax Eclipse Plus C18 from Agilent Technologies. For this SOP a 150x2.1 mm column is used (3.5 µm particles), equipped with guard column of the same brand, 12.5x2.1 mm, (5 µm particles). The column is based on usual reversed phase C18 technology (dimethyl-n-octadecyl double endcapped silica). The columns pH range is pH 2-9 and is fully compatible with usual RP-eluent systems like acetonitrile and methanol.

Column precautions

The normal operating back pressure of the column incl. guard column is in the range of 100-140 bars at 0.3 mL/min, depending on the concentration of organic solvent. Maximum operating pressure for the
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Column is 400 bars. Between runs, the column should be stored in 50/50 water/acetonitrile or methanol for shorter time storage, and 100% organic solvent for longer time storage.

Operating conditions

Column temperature: 40 °C

Flow conditions: 0.3 mL/min

Eluents: 100% Methanol (A)

2.5 mM H$_2$SO$_4$ (B)

Injection volume: 1 µL

Instrumental method: ZORBAX_BIOGAS_VFAs (Table 1). Note that there are two versions of the method, dependent on which pump you will use (method name ends by either RightPump or LeftPump).

Table 1: Gradient profile for Instrument method ZORBAX_BIOGAS_VFAs.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:00</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2:50</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2:51</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>25:00</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>25:01</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>35:00</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

The analytes of interest in this method have very different hydrophobic properties and due to the nature of the samples (sample matrix), the elution profile is starting with weak elution strength (isocratic 100% 2.5mM H$_2$SO$_4$) to achieve satisfactory retention/separation between the early eluting species (e.g. formic and acetic acid). After a short period of time, 2.5 min, the elution strength is increased slightly in order to elute the more hydrophobic analytes. Since the elution occurs at relatively mild conditions, re-equilibration to initial conditions is crucial and set to 10 minutes of the run time.
The analytes have different wavelength optima and therefore the instrumental method includes detection at two different wavelengths, namely 210 (for the organic acids) and 278 nm (HMF and furfural).

**Chromatographic run**

![Chromatographic profile](image)

*Figure 1: Chromatographic profile. 1 Formic acid, 2 Acetic acid, 3 Propionic acid, 4 Levulinic acid, 5 Impurity from levulinic acid std., 6 HMF, 7 Furfural, 8 Butyric acid, 9 Valeric acid. 1 µL injection, 210 nm*

**Sample preparation**

Samples must be acidified prior to analysis (pH <2.5). If the samples are not acidic by nature, H₂SO₄ is an appropriate acid to use. Apply as little volume as possible to avoid unnecessary sample dilution. NB! make sure signals are within the calibration range. Most often biogas samples do not need dilution, on the other hand you may need to increase injection volume for samples with low VFA-concentrations. Samples must be free from any particulate/insoluble matter and should preferably be colorless. If samples contain insoluble matter this MUST be removed either by centrifugation or by 0.22/0.45 µm filtration. Particularly for biogas samples an appropriate sample preparation is:

1. Centrifuge the samples as hard as possible.
2. Take an aliquot of the supernatant into an eppendorf tube and centrifuge a second time at max. speed (15,000 rpm for table centrifuges).
3. Take an aliquot of the supernatant and add a little amount of strong sulfuric acid (72%) (2 µL to approx. 200 µL sample) and leave the sample on the table for a few minutes. The added volume
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does not need to be exactly precise as long as the volume of acid is much smaller (< 1/100 times) than volume of sample. Carbon dioxide formation will occur and result in a pressure buildup (fizz) and pressure may need to be released from the eppendorf tubes.

4. The acidification will also precipitate appreciable amounts of colorants etc. and therefore the samples should be centrifuged hard or filtered one last time before dispensed into HPLC vials (note that air easily is entrapped in the bottom of the HPLC vials, air is removed by flicking tubes repeatedly).

Calibration

The calibration range is different for VFAs and HMF/furfural. VFAs should be analysed in a range from 1 mM to 100 mM. The calibration curve is linear to at least 500 mM at 1 µL injection, but poor peak shape is observed for butyric and valeric acid at concentrations above 250 mM. For low concentration samples, larger injection volume may be applied. Up to 10 µL injection is applicable, but this could affect the peak shape negatively. Figure 2 shows an example of a calibration curve for VFAs. Limit of detection (LOD) and limit of quantification (LOQ) in absolute amounts for VFAs are listed in table 2.

Table 2: LOD/LOQ values for VFAs measured at 210 nm

<table>
<thead>
<tr>
<th>VFA</th>
<th>LOD (nmol)</th>
<th>LOQ (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formic acid</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>0.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>1.2</td>
<td>3.9</td>
</tr>
</tbody>
</table>
Figure 2: Calibration curve for VFAs at 1 µL injection and detection at 210 nm

The sensitivity for HMF and furfural are significantly better than for VFAs and at 278 nm a suitable calibration range for these two analytes is from 0.01 mM to 5 mM. At high concentrations base line separation may not be achieved, and calibration based on peak height may be more applicable than calibration on peak area. Both methods provide good fit, since the peak shapes for HMF and furfural are fairly good. Figure 3 and 4 show examples of calibration for HMF and furfural both for area and peak height calibration. HMF and furfural have similar limit of detection (LOD) and limit of quantification (LOQ) and these are 0.3 nmol and 1 nmol, respectively.

Always provide a new set of standards for the analytes of interest in every sequence and perform a recalibration in the processing method. The calibration curves provided here are meant as inspiration. An existing processing method may be found on the instrument and is named “VFA-Furdehydes-biogas”.
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Figure 3: Calibration curve for HMF at 1 µL injection and detection at 278 nm

Area: $y = 43,742x$
$R^2 = 0.9999$

Height: $y = 371.78x$
$R^2 = 0.9996$

Figure 4: Calibration curve for furfural at 1 µL injection and detection at 278 nm

Area: $y = 43,706x$
$R^2 = 0.9999$

Height: $y = 352.24x$
$R^2 = 0.9998$

Trouble shooting

Identify if any immediate precautions should be taken and then call instrument super users (currently Jane W. A. or Bjørge W., IKBM-PEP).
**Column regeneration**

Biogas samples are complex matrices that will contain many different components other than those of interest for the analysis. These can be observed either as background noise (unstable baseline) or they will simply stay on the column because the eluting conditions are too weak to remove them all during a normal analytical run. It is therefore necessary to end EVERY sequence with a regeneration/cleaning program so that residual compounds sticking to the column will be removed. If this is not done, the quality of the analysis will decrease very fast because column capacity will be exceeded. The cleaning program contains a longer elution profile with high elution strength and should be included as the last program in a sequence running a blank sample. The name of the cleaning program is: ZORBAX_BIOGAS_CLEAN_RightPump/LeftPump

**Column storage conditions**

Column storage times longer than 5 days, should be done in 100% ACN/MeOH. For shorter storage times lower organic solvent concentration is applicable, but please replace any buffers with water. This is important for the column, but also for the instruments lifetime!

**Editorial log**

First edition. 2012.01.27