Matrix interference reduction for the analysis of carbohydrate in wastewater using H-point standard addition method
Ying Xu and Hongde Zhou

ABSTRACT

Soluble microbial products, consisting of protein, carbohydrate and humics, are generally considered as the main membrane foulants during the performance of membrane bioreactors. Nitrate and nitrite have been proved to affect the determination of carbohydrate when anthrone-sulfuric acid photometric method is used. In this study, three chemical analytical methods based on photometric assay, including the standard curve method, conventional standard addition method and H-point standard addition method, were assessed for the quantification of carbohydrate in order to reduce the interference. Three methods were carried out for both artificial and real wastewater sample analysis. The results indicated a significant amount of matrix interference, which could be eliminated through the use of H-point standard addition. This study proposed the H-point standard addition method as a more accurate and convenient option for carbohydrate determination.

Key words | carbohydrate analysis, matrix interference, standard addition method, standard curve method, wastewater

INTRODUCTION

Soluble microbial products (SMP), which are produced by mixed bacterial populations in bioreactors, are of crucial importance for biological wastewater treatment systems because of their significant impacts on both effluent quality and treatment efficiency (Liang et al. 2007). The group of SMP, mainly containing polysaccharides (PS) or carbohydrates, proteins, and humic substances, are commonly considered as the major cause of membrane fouling in membrane bioreactors (MBRs) (Drews 2010).

At present, the major method for SMP fractions determination is still the photometric analysis methods, which were modified by B. Frolund in 1996 (Frolund et al. 1996). The corrected Lowry method is usually employed to account for the influence of humic substances in the determination of protein, and the total carbohydrate content is often determined using the anthrone-sulfuric acid method or phenol-sulfuric acid method. Many cations (mainly calcium and magnesium) in wastewater cause the formation of precipitate or the reaction with Folin-phenol reagent, preventing proper color development (Peterson 1979). To eliminate interference from calcium and magnesium, Y. Shen et al. (Shen et al. 2013) used the modified Lowry method with two pretreatments based on cation exchange and dialysis, and proteins in standard solutions and in wastewater samples were successfully quantified with good reliability and reproducibility. For the determination of carbohydrate, the anthrone-sulfuric acid method has become the main method for carbohydrate determination rather than the phenol-sulfuric acid method because of the toxicity of phenol. However, matrix interference in activated sludge affects the accuracy of the conventional anthrone-sulfuric acid method. In chemical analysis, matrix refers to the components of a sample other than the analyte of interest. The matrix can have a considerable effect on the way the analysis is conducted and the quality of the results obtained; such effects are called matrix effects. Kimura et al. (Kimura et al. 2009) even suggests that the use of conventional methods for SMP analysis were not appropriate for investigation of membrane fouling phenomena in MBRs due to big errors (>50%). Drews et al. (Drews et al. 2007) found that the anthone-H$_2$SO$_4$ method was impaired by nitrate and nitrite in samples and proposed the corrected method, which was applied by many researchers. But the concentrations of nitrate and nitrite must be measured, which make the preparation of this method very complicated. Therefore,

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Ying Xu (corresponding author)
Key Laboratory of Marine Chemistry Theory and Technology, College of Chemistry and Chemical Engineering, Ocean University of China, Qingdao, Shandong 266100, China
E-mail: xuying.qd@163.com

Hongde Zhou
School of Engineering, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Key words | carbohydrate analysis, matrix interference, standard addition method, standard curve method, wastewater
there is an urgent need to improve the conventional photometric analysis methods with a focus on the elimination of matrix interference.

Standard addition method (SAM) is a calibration technique devised to overcome some problems of matrix effects in analytical measurement. C. Potvin tried to use SAM to eliminate the interference from the activated sludge matrix in wastewater samples (Potvin & Zhou 2011). Background interference and proportional effect are two types of matrix effects. When the matrix of the sample produces background interference in the determined analyte concentration, SAM cannot be used (Ellison & Thompson 2008). As a modification of the SAM, the H-point standard addition method (HPSAM) is proposed for simultaneous determination of two or three compounds that have strongly overlapping spectra. The method has been applied successfully for resolving binary mixtures of metal ions and pharmaceuticals with extensively or completely overlapping spectra. The method has been applied successfully for resolving binary mixtures of metal ions and pharmaceuticals with extensively or completely overlapping spectra. The method has been applied successfully for resolving binary mixtures of metal ions and pharmaceuticals with extensively or completely overlapping spectra. The method has been applied successfully for resolving binary mixtures of metal ions and pharmaceuticals with extensively or completely overlapping spectra.

The objective of this study was to figure out which effect (proportional or background effect) played the most important role in the photometric methods for carbohydrate estimation. In order to eliminate or reduce the matrix effect, three methods, including conventional standard curve, SAM and HPSAM, were compared to determine the content of carbohydrate in supernatant of mixed liquor.

**MATERIALS AND METHODS**

**Sampling and analytical methods**

Mixed liquor samples were taken from three types of wastewater treatment facilities: a full scale conventional activated sludge (CAS) plant, an extended aeration activated sludge plant (EAS), and a pilot scale MBR in Guelph, Ontario (Canada). Table 1 summarizes the main plant process parameters. During shipping, the samples were kept at 4 °C. Chemical oxygen demand (COD) was conducted by HACH method 8000, with high range vial (20–1,500 mg/L), using a DRB200 spectrophotometer (HACH Company, USA). Mixed liquor suspended solids (MLSS) were determined based on Standard Methods (Eaton et al. 2005). The extraction of SMP was performed within 24 h after sampling according to Frølund et al. 1996. Mixed liquor samples were centrifuged at 3,700 rpm, 30 min, 4 °C, followed by filtration through a 0.45 μm glass filter and collection of the supernatant as SMP samples. The total carbohydrate content was determined using the anthrone-sulfuric acid method with glucose as the standard (Morris 1948). Samples were measured at 625 nm using a HP UV/Vis spectrophotometer (HP, USA).

### Table 1 | The summary of typical parameters of three types of plants

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CAS</th>
<th>EAS</th>
<th>MBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRT (d)</td>
<td>12</td>
<td>12</td>
<td>15–20</td>
</tr>
<tr>
<td>HRT (hr)</td>
<td>5–7</td>
<td>10–12</td>
<td>5–6</td>
</tr>
<tr>
<td>Influent COD (mg/L)</td>
<td>250–280</td>
<td>200–400</td>
<td>400–600</td>
</tr>
<tr>
<td>MLSS (g/L)</td>
<td>2–3.5</td>
<td>3–4</td>
<td>9–11</td>
</tr>
<tr>
<td>pH</td>
<td>7.7–7.9</td>
<td>7.6–7.8</td>
<td>7.3–7.6</td>
</tr>
</tbody>
</table>

SRT, sludge retention time; HRT, hydraulic retention time.

**Standard curve method**

Standard curve method (SCM) was carried out based on Gaudy 1962, and as modified by Raunkjaer et al. (Raunkjaer et al. 1994) and Drews et al. (Drews et al. 2007). According to Drews et al. (Drews et al. 2007), nitrite and nitrate can influence the measurement of carbohydrates. The concentration of carbohydrate was calculated by:

\[
C_{PS} = C_{PS, meas} - 0.099C_{NO_3-N} - 1.9C_{NO_2-N}
\]

where \(C_{PS}\) is real concentrations of PS or carbohydrate, \(C_{PS, meas}\) is the measured value by the anthrone-sulfuric acid method, \(C_{NO_3-N}\) is the concentration of nitrate, and \(C_{NO_2-N}\) is the concentration of nitrite.

Nitrate-N was measured by HACH method 10020 for the range of 0.2–30.0 mg/L. Nitrite-N was measured by HACH method 8507, for the range of 0.002–0.300 mg/L.

**Standard addition method**

The SAM aims to measure the concentration of a particular analyte in a sample while accounting for matrix effects caused by other constituents of the sample (Ellison & Thompson 2008). The samples were added to a series of
volumetric flasks with various volumes of a standard solution of analyte. The aliquots were diluted with Milli-Q water to ensure an identical dilution ratio. The dilution ratio used in this study was 2/3 because it seemed to give a recovery that fluctuated around 100% in most cases (Potvin & Zhou 2011). The standard addition curve was typically produced by plotting concentration values against absorbance values for each dilution of standard solution. The x-intercept of the curve indicated the diluted concentration of analytes in the unknown sample.

**H-point standard addition method**

Some recovery loss or the change of signal in analytical chemistry is caused by concomitant substances which are independent of analyte concentration. This effect is referred to as the ‘translational effect’ or ‘background interference’. A translational matrix effect is the result of substances besides the analyte influencing the value of the baseline of the analytical measurement. On the other hand, some recovery loss or the change of signal is proportional to the concentration of analyte, which is called the term ‘rotational effect’ or ‘proportional bias’ (Thompson & Ellison 2005) (Figure 1). A rotational matrix effect influences the degree of response from the same amount of analyte in solution between two or more different matrices (Ellison & Thompson 2008).

The HPSAM is proposed in order to permit both rotational effect and translational effect present in the samples (Reig & Falcó 1988). As shown in Figure 2, HPSAM is based on the measurement of standard addition lines at two analytical signals, which cross at one common point H (C_H, A_H) (Hund et al. 1999). C_H is the concentration of the unknown analyte and A_H is the signal due to the interference, respectively.

The method implies that the spectra of the analyte X and the interferent Y should be those shown in Figure 3 in the wavelength interval (λ_1, λ_2). At these selected wavelengths the analyte signals must be linear with the concentrations and the interference signal must remain equal, in the case where the analyte concentrations are changed, the analytical signal obtained from the mixture containing the analyte and the interference should be equal to the sum of the individual signals of the two components.

Two standard addition curves obtained at two wavelength λ_1 and λ_2 are described as follows:

(2) \[ A_1 = b_1 + a_1 C_s \]

(3) \[ A_2 = b_2 + a_2 C_s \]

where b_1 and b_2, the absorbance in the samples at wavelength λ_1 and λ_2; a_1 and a_2, the slope of the standard addition curves at wavelength λ_1 and λ_2; C_s, the standard solution of analyte added.
The two lines intersect at H point \((-C_H, A_H)\):

\[
b_1 + a_1(-C_H) = b_2 + a_2(-C_H) \tag{4}
\]

So \(C_H\) can be given by:

\[
C_H = \frac{-a_2 - a_1}{b_2 - b_1} = C_x \tag{5}
\]

where \(C_x\), the concentration of analyte in the sample.

Hence, the absorbance of interference \(Y\) can also be obtained from Equation (6):

\[
A_H = b_1 + a_1(-C_H) \tag{6}
\]

The concentration of \(Y\) can be calculated by a standard curve of the pure interference at wavelength either \(\lambda_1\) or \(\lambda_2\).

### Accuracy control

To compare the effectiveness of three analytical methods, each method was carried out on artificial water samples and on real mixed liquor samples (in which the concentrations of carbohydrate were unknown). The artificial samples were prepared in the laboratory using standard solutions, including glucose (\(\alpha\)-glucose, biological reagent grade, Sigma, USA), bovine serum albumin (BSA, biotech grade, Fisher Biotech, USA) and humic acid salts (HA, Aldrich Chemical Company Inc., USA) as the components of SMP as well as nitrite and nitrate (shown as Table 2), and attempted to mimic the analyte concentrations found in typical wastewater samples. Triplicates of each test were performed to ensure accuracy in the measurement.

### RESULTS AND DISCUSSION

#### Interference of nitrate and nitrite on carbohydrate determination

Figure 4 illustrates the full scan wavelength spectra of glucose standard solution (20 mg/L) and the mixed standard solutions of nitrate (20 mg/L) and nitrite (1 mg/L). As can be seen, there was no peak spectra overlapping between glucose and nitrate/nitrite observed in the full scan wavelength spectra. But the background matrix, nitrate and nitrite, significantly affected the baseline of the glucose spectra, which means that nitrate and nitrite make translational matrix effects on carbohydrate analysis. In this case, both standard curve and SAMs might be used to reduce the interference for carbohydrate determination. According to the principle of HPSAM, a wavelength interval (670 nm, 690 nm) was chosen to calculate the concentration of glucose, shown as Figure 5.

#### Carbohydrate analysis in artificial wastewater samples

The concentrations of glucose in artificial wastewater samples were respectively obtained by SCM, SAM and HPSAM, and were compared with the known concentration values (20, 30, 60 mg/L respectively). Figure 6 illustrates the calculated

<table>
<thead>
<tr>
<th>Table 2</th>
<th>The composition of artificial SMP solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition (mg/L)</td>
<td>S1</td>
</tr>
<tr>
<td>(\alpha)-Glucose</td>
<td>20</td>
</tr>
<tr>
<td>Nitrate</td>
<td>20</td>
</tr>
<tr>
<td>Nitrite</td>
<td>1</td>
</tr>
<tr>
<td>Protein</td>
<td>10</td>
</tr>
<tr>
<td>Humic acid</td>
<td>20</td>
</tr>
</tbody>
</table>

Figure 4 | Full scan wavelength spectra of the mixed solution with glucose (20 mg/L), nitrate (20 mg/L) and nitrite (1 mg/L).

Figure 5 | Plot of HPSAM for determination of carbohydrate in the selected wavelength interval (670 nm, 690 nm).
values of glucose in three batches of blind mixed samples using SCM, SAM and HPSAM methods. As shown in Figure 6, the concentrations of glucose measured by SAM were almost twice the true values, which suggested that SAM should be avoided in the determination of carbohydrate with nitrate and nitrite present due to the low accuracy compared with other methods. Similar results were obtained by SCM and HPSAM, and both the methods gave higher accuracy at higher glucose concentration (e.g. 60 mg/L), with error <5%. At lower concentrations (S1 and S2), HPSAM was better than SCM, although the error was still over 40%.

These results reinforced the conclusions made by Drews et al. (Drews et al. 2007) that nitrates and nitrites have a translational matrix effect on carbohydrate analysis. Meanwhile, the change of results was not proportional to the concentration of glucose, which means ‘rotational effect’ or ‘proportional bias’ were not observed for carbohydrate analysis under the influence of nitrate and nitrite.

Overall, according to the measurement of carbohydrate in known concentration mixed samples, both modified SCM and HPSAM were options for the determination of carbohydrate, while SAM should be avoided due to the huge error. The concentrations of carbohydrate can be obtained by HPSAM instead of SCM to reduce the amounts of preparation, due to no nitrate and nitrite concentrations being required.

**Carbohydrate analysis in real wastewater samples**

The concentrations of carbohydrate in real wastewater samples were obtained by SCM and HPSAM, and the phenol-sulfuric acid method was also carried out as a referee. As shown in Figure 7, the concentrations of carbohydrate obtained by standard curve were in the range of 50–60 mg/L, two to three times higher than the results of HPSAM (18–31 mg/L). But the latter was much closer to the results of the phenol-sulfuric acid method for carbohydrate determination. In a word, the HPSAM approach provides an alternative method with improvement in both the accuracy and precision of carbohydrate determination. Correcting for matrix interference, measuring carbohydrate requires less operation involved due to the omission of nitrate and nitrite measurement.

**CONCLUSIONS**

The SCM corrected by Drew’s formula was performed alongside the standard addition methods (SAM and HPSAM) for carbohydrate analysis on many different samples including artificial and real wastewater samples. The results of carbohydrate analysis in artificial wastewater samples implied both SCM and HPSAM gave higher accuracy at all glucose concentrations than SAM. SAM should be avoided for carbohydrate analysis under the influence of nitrate and nitrite. Large differences in the concentration of carbohydrate in actual wastewater as determined by SCM compared to HPSAM indicated a huge matrix interference, which can be eliminated through the use of HPSAM. The carbohydrate concentrations obtained by HPSAM in real wastewater samples were closer to the concentration range obtained by the phenol-sulfuric acid method. Therefore, this study proposed HPSAM as a more accurate and convenient option for carbohydrate determination.

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