

## 1 “Protein” Measurement in Biological Wastewater Treatment Systems: A Critical Evaluation

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9 **ABSTRACT:** Five commercially available assay kits were tested on  
10 the same protein sample with the addition of 17 different types of  
11 interfering substances typically found in the biological wastewater  
12 treatment, and a comparison of the use of these assays with 22  
13 different protein and peptide samples is also presented. It was  
14 shown that a wide variety of substances can interfere dramatically  
15 with these assays; the metachromatic response was also clearly  
16 influenced by different proteinaceous material. Measurement of the  
17 “protein” content in the effluent of an anaerobic membrane  
18 bioreactor was then carried out using these assay methods.

19 Quantitative results of the “protein” concentration in the different effluent samples, with or without spiked additions of  
20 Bovine Serum Albumin (BSA), showed considerable disagreement. We concluded that the “protein” measured in wastewater  
21 samples using standard colorimetric assays often shows false positive results and has little correlation to their real value. A new  
22 analytical method needs to be developed in order to gain greater insight into the biological transformations occurring in  
23 anaerobic digestion, and how soluble microbial products (SMPs) are produced.



### 1. INTRODUCTION

24 Understanding the composition of soluble microbial products  
25 (SMPs) and extracellular polymeric substance (EPS) present in  
26 wastewater treatment systems is becoming increasingly  
27 important because of their presence in effluents, and because  
28 these are the compounds that foul membranes in both aerobic  
29 and anaerobic membrane reactors. However, identification of  
30 SMPs and EPS is challenging because they are a mixture of a  
31 variety of unknown compounds that do not belong to a single  
32 well-defined group. The main components are believed to  
33 include “protein-like” compounds (<60%), carbohydrates (40–  
34 95%), lipids (<40%), DNA (<10%), aquatic humic substances,  
35 and small molecules.<sup>1–6</sup> In this paper we use the term  
36 “proteins” because despite the fact that many dated assays are  
37 still being used to evaluate protein concentrations, they are not  
38 designed for use in wastewater systems, and do not measure  
39 true protein concentration. In the last few decades, several  
40 studies have pointed out that “proteins” are the main  
41 compounds present in various wastewater systems,<sup>7,8</sup> and that  
42 they seem to be positively correlated with the aggregation  
43 process and flocculation ability.<sup>9–14</sup> Nevertheless, quantification  
44 of these “proteins” sometimes shows great variability, and such  
45 variations could be attributed to sludge origin and process  
46 treatments, with direct measurement of “protein” content in  
47 activated sludge varying between 224 and 462 mg protein/g  
48 VSS sludge.<sup>14,15</sup> One cause of variability in this quantification

could also be due to the choice of measurement (assay) 49  
method. 50

There are several colorimetric methods that are often used to  
51 analyze protein content; Kjeldahl,<sup>16</sup> Biuret,<sup>17</sup> Lowry,<sup>18</sup> Bicin-  
52 chonic acid (BCA)<sup>19</sup> and Bradford.<sup>20</sup> Besides being time- and  
53 sample-consuming, organic nitrogen compounds other than  
54 proteins will also be measured using Kjeldahl method; hence  
55 this procedure is not used often. The Biuret method is  
56 somewhat insensitive compared to the others, and therefore is  
57 not used for analyzing wastewater samples.<sup>7</sup> Owing to their  
58 simplicity and precision, the BCA, Lowry and Bradford  
59 methods have been used extensively in the direct analysis of  
60 “proteins” found in biological treated effluents in the past 20  
61 years, and are popular for first stage screening of “proteins” in  
62 wastewater systems.<sup>6,21–24</sup> Although these colorimetric or  
63 chromogenic assays putatively offer an insight into protein  
64 concentration, none effectively provides information on their  
65 qualitative identification. Moreover, colorimetric methods are  
66 likely to overestimate protein quantities since they only detect  
67 specific peptide bonds per se.<sup>6</sup> Thus, these methods could  
68 detect oligopeptides, polypeptides and/or other biological  
69 polymers in SMPs and EPS. In addition, not only are these 70

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71 protein assays markedly influenced by protein composition, but  
72 also a wide range of solutes can interfere with them.<sup>7,11,25,26</sup> As  
73 a result, the “proteins” measured by these chromogenic  
74 methods could be wrongly estimated; in addition, a detailed  
75 evaluation of these colorimetric protein quantification methods  
76 is often time-consuming and has thus received limited attention  
77 in the literature.<sup>27</sup> Hence the objective of this study was to  
78 evaluate the existing assay techniques, with and without these  
79 interfering solutes, to see whether they can accurately measure  
80 “proteins” in wastewater solutions. By comparing the perform-  
81 ance of these protein assays simultaneously and comprehen-  
82 sively with a large variety of proteins and peptides, and through  
83 a case study adding a ‘spike’ of BSA to effluent samples, the  
84 data generated will provide fresh insight into the analysis of  
85 protein-like material in wastewater, and help us understand in  
86 more depth the type and production of SMPs

## 2. MATERIALS AND METHODS

87 **Reagents and Chemicals.** All analytical grade chemicals  
88 were purchased from Sigma-Aldrich. Ultrapure water was  
89 obtained from a Milli-Q water process (Millipore Advantage  
90 A10).

91 **Modified Lowry Method.** The Lowry method (and its  
92 modifications) is based on a two-step procedure; initially,  $\text{Cu}^{2+}$   
93 ions are reduced to  $\text{Cu}^+$  by protein in alkaline medium,  
94 followed by the amplification stage, the reduction of the Folin-  
95 Ciocalteu reagent (phosphomolybdate and phosphotungstate)  
96 producing a characteristic blue.<sup>27</sup> Factors that play a role in the  
97 development of color are due, not only to the reduced copper-  
98 amide bond complex, but also to the interactions with specific  
99 amino acids such as tyrosine, tryptophan, and to a lesser extent  
100 cystine, cysteine, and histidine residues.<sup>28</sup>

101 The Thermo Scientific modified Lowry protein assay kit  
102 (23240) combines a stabilized formulation of the original  
103 Lowry reagent and the Folin-Ciocalteu Reagent. Bovine Serum  
104 Albumin (BSA) (1–1500 mg/L) from Thermo Scientific  
105 (23209) was used as the standard for calibration curve  
106 preparation. Two hundred micro liters of standard or sample  
107 was added to 1.0 mL of the Modified Lowry reagent at 15 s  
108 intervals. After 10 min of incubation at room temperature, 0.1  
109 mL of Folin-Ciocalteu Phenol reagent was added at 15 s  
110 intervals. The samples were incubated at room temperature for  
111 30 min and the absorbance of all the samples was measured at  
112 750 nm (Shimadzu UV-2600 UV/vis double-beam spectrom-  
113 eter).

114 **Bradford Method—Coomassie.** The ease and high  
115 sensitivity of the Bradford assay has driven its widespread use  
116 for the quantitation of protein in a wide variety of protein  
117 samples. The assay, first described by Bradford,<sup>20</sup> is based on  
118 the binding of the dye Coomassie Brilliant Blue G-250 at acidic  
119 pH to arginine, histidine, phenylalanine, tryptophan and  
120 tyrosine residues,<sup>30</sup> and hydrophobic interactions,<sup>31</sup> which  
121 result in a dye-protein complex with a metachromatic shift. The  
122 exact mechanism is still not fully understood, but the majority  
123 of the observed signal is due the interactions with lysine and  
124 arginine residues.<sup>30</sup>

125 The Thermo Scientific Coomassie protein assay kit (23200)  
126 is a quick Bradford method for the quantification of proteins  
127 that have a molecular weight greater than 3000 Da. Because the  
128 color response with Coomassie is nonlinear with increasing  
129 protein concentration, a standard curve (100–1500 mg/L) was  
130 prepared with each assay using a BSA standard (Thermo  
131 Scientific 23209). Thirty micro liters of standard or sample was

added to 1.5 mL of the Coomassie Reagent; after incubation for 132  
10 min at room temperature the absorbance was measured at 133  
595 nm. 134

**Bradford Method—Coomassie Plus.** The Coomassie 135  
Plus assay kit (Thermo Scientific 23236) improves the linearity 136  
of the color response and results in less protein-to-protein 137  
variation than other Bradford formulations. A standard curve 138  
with a dynamic range of 100–1500 mg/L was prepared using 139  
the BSA standard (Thermo Scientific 23209). Fifty micro liters 140  
of standard or sample was combined with 1.5 mL of the 141  
Coomassie Plus Reagent; for the most consistent results, 142  
samples were incubated for 10 min at room temperature before 143  
the absorbance measurement was carried out at 595 nm. 144

**Micro BCA Method.** This method replaces the Folin- 145  
Ciocalteu’s reagent as described in the Lowry method with BCA, 146  
and was developed by Smith et al.<sup>19</sup> This results in a protein 147  
assay with improved sensitivity and uses BCA to detect the  $\text{Cu}^+$  148  
ions generated by the reaction with protein at an alkaline pH.<sup>32</sup> 149  
The residues that reduce cupric ion include the cysteine, 150  
cystine, tryptophan, tyrosine, and the peptide bonds.<sup>19,33</sup> 151

A variation of the original BCA method is the Micro BCA 152  
protein assay (Thermo Scientific 23235), which is useful for 153  
dilute protein samples (0.5–20 mg/L). A BSA standard 154  
(Thermo Scientific 23209) was used for preparing the 155  
calibration curve. One milliliter of standard or sample was 156  
added to 1.0 mL of Micro BCA working reagent, and mixed 157  
thoroughly before incubation at 60 °C for 1 h. After cooling to 158  
room temperature, the samples were measured spectrophoto- 159  
metrically at 562 nm within 10 min. 160

**Pierce BCA Method.** The Pierce BCA protein assay 161  
(Thermo Scientific 23250), a variation of the original 162  
preparation, enables the quantification of total protein in 163  
samples while minimizing interference from reducing agents 164  
and enhancing sensitivity. A standard curve was generated in 165  
the range of 125 to 2000 mg/L using BSA (Thermo Scientific 166  
23209). Twenty-five micro liters of standard or sample was 167  
added to an equal volume of Reducing Agent-Compatibility 168  
Reagent solution, mixed thoroughly, and incubated at 37 °C for 169  
15 min. Subsequently, 1.0 mL of BCA working reagent was 170  
added to the samples, which was incubated at 37 °C for 30 min. 171  
After cooling to room temperature, the samples were measured 172  
spectrophotometrically at 562 nm within 10 min. 173

**Colorimetric Protein Analysis with Interferences.** 174  
Various researchers noted that while using these assays to 175  
determine “protein” concentrations in wastewater some solutes 176  
appeared to affect color development of the chromophore,<sup>7,34</sup> 177  
Seventeen compounds that were previously identified, or 178  
known to be present in wastewater samples,<sup>2,7,35–38</sup> represent- 179  
ing different classes of chemicals, were chosen as interfering 180  
substances. Each of them was deliberately added to known 181  
amounts (either 10 mg/L or 300 mg/L) of BSA, which was 182  
used as the standard protein, at concentrations of 1 mmol/L. 183  
The usual colorimetric protein analysis procedure was followed 184  
for all five commercial test kits, and a reference BSA standard 185  
containing no interferences was run concurrently for every 186  
batch. Calibration was also carried out using varying 187  
concentrations of stock standard protein solution (2 mg/mL, 188  
Thermo Scientific 23209). A statistical analysis for quad- 189  
uplicates was performed using the Student’s *t*-test in Excel. 190

**Metachromatic Response for Different Proteins and 191  
Peptides.** An attempt was made to determine any variations in 192  
the metachromatic response to different proteinaceous material 193  
with five protein assays. Eight different polyamino acid 194

Table 1. Linearity Range for Five Different Assays

assays	duration of reaction (min)	dynamic range (mg BSA/L)	linearity range (mg BSA/L)	regression coefficient	coefficients of multiple correlation ( $R^2$ )
modified Lowry	40	1–1500	1–125	$y = 0.0423x + 0.1197$	0.99946
micro BCA	60	0.5–20	0.5–20	$y = 0.00324x + 0.03193$	0.99980
Pierce BCA	10	125–2000	125–500	$y = 0.0007x + 0.08959$	0.99966
Bradford (Coomassie)	10	100–1500	125–750	$y = 0.00111x + 0.51937$	0.99932
Bradford (Coomassie Plus)	10	100–1500	125–500	$y = 0.00131x + 0.458$	0.99943

standards (poly-L-lysine, poly-L-proline, poly-L-arginine, poly-DL-aspartic acid, poly(glu, ala, tyr) 1:1:1, Poly(glu, ala, tyr) 6:3:1, Poly(arg-pro, thr) 1:1:1, Poly(arg-pro, thr) 6:3:1); a mixture of short chain peptide standards (gly tyr, val-tyr-val, tyr-gly gly-phe-met, tyr-gly gly-phe-leu, asp-arg-val-tyr-ile-his-pro-phe); a protein standard mixture (ribonuclease, cytochrome C, holo-transferrin, apomyoglobin) and various individual proteins (lysozyme, ovalbumin, apo-transferrin bovine, fetuin from fetal bovine serum,  $\alpha$ -acid glycoprotein) were purchased from Sigma-Aldrich. A known amount (either 10 mg/L or 300 mg/L) of the 16 standard sample was subjected to the assay procedures of all five commercial test kits. A BSA standard was also assayed concurrently as a reference for every batch, and calibration was also carried out simultaneously.

**Case Study.** In order to evaluate the applicability of these methods to wastewater samples, a test was performed using an added spike of BSA; this will reveal any possible interferences by adding known amounts of the standard protein to wastewater effluent. The effluent was collected at the outlet of a laboratory scale submerged anaerobic membrane bioreactor (SAMBR).<sup>39</sup> The SAMBR was operated at an hydraulic retention time (HRT) of 6, 4, and 2 h,  $35 \pm 1$  °C, and infinite sludge retention time (SRT). The reactor was continuously fed with a synthetic feed (500 mg COD/L) comprised of glucose, peptone, meat extract, and essential trace elements. Samples measured on the day of collection were not preserved, whereas other samples were refrigerated at 4 °C. The protein spiked into the effluent samples was BSA at either 10 mg/L or 300 mg/L. The precision of the assays was investigated by analyzing several replicates ( $n = 4$ ) with all five commercial test kits. A BSA standard was assayed simultaneously as a reference for every batch, and calibration was also carried out concurrently.

### 3. RESULTS AND DISCUSSION

**Working Range of Individual Assays.** Colorimetric protein assays are methods that use UV–vis spectroscopy to determine the concentration of protein, relative to a standard. An increase in the number of these assays has been observed over the last few decades, however, in the field of wastewater the BCA, Lowry and Bradford methods are still the most commonly used. These assays can be run at a high throughput using inexpensive reagents with equipment found in most laboratories. The reagents can either be economically prepared in bulk and stored for prolonged periods, or purchased from commercial sources such as Bio-Rad, Novagen, Roche, Sigma-Aldrich, and Thermo Scientific. It should be noted that different preparations of the same method may not give equal responses when using an identical protein.<sup>40</sup> The main advantage of using commercial sources is the improvement in

long-term repeatability and performance. However, each assay has its own advantages and disadvantages relative to sensitivity, ease of performance, linearity and accuracy. A comparison of the use of 5 commercial assays with the same protein sample is presented here; the linearity range of the five methods was tested and the comparative results are given in Table 1.

As can be seen in Table 1, it is often necessary to use more than one type of protein assay to cover a wide concentration range. The dynamic range of the assay was obtained based on the menu of a commercial product, and is not a rigorous measure of the accuracy range of the assay. Although the signal is adequately determined by the spectrophotometer, the accuracy and precision can vary beyond what is acceptable to report as a true measure of the concentration. A linearity study was performed in order to determine the linear reportable range. A single run testing of at least five concentrations was carried out in quadruplicate, and a linear regression equation was obtained. The result should not have an intercept significantly different from zero, and no value should deviate greatly from the others after the result is graphically and statistically analyzed. Hence the linearity range was obtained, where a linear response over a wide concentration range is produced, and the analyte concentration can be quantified with acceptable reliability and precision. Since it is a stricter measure and requires both sensitivity and accuracy, the linearity range is narrower than the dynamic range, and hence a more reliable measure of the accurate range of the concentration being quantified.

Superior linearity was observed for the Micro BCA method compared to the four other methods, and its dynamic range was similar to its linearity range indicating high sensitivity with the BSA protein sample. The protein-dye binding methods such as Bradford give sensitivities generally in the same linear range as the Pierce BCA method. Despite shorter preparation and reaction times than that of the Micro BCA, the other methods do not generate a linear response with BSA. Although shorter segments of their standard curve approximate linearity, a quadratic curve must be used to model the data over a wider range of concentrations for a more reliable and reportable result. A fourth or more polynomial equation is necessary in order to provide a better fit than that of a second-degree polynomial.

**Colorimetric Protein Analysis with Interferences.** Colorimetric protein analysis is the most widely accepted method for the determination of “protein” concentrations in wastewater samples, however, many solutes present in wastewater have been found to interfere with the determination of protein. Seventeen chemicals were selected to represent those most commonly found in wastewater samples, and were deliberately added at a concentration of 1 mmol/L to the BSA standard as interfering reagents. Table 2 provides a broad,

295 although not necessarily complete, list of compounds that were  
296 studied.

**Table 2. Solutes That Can Interfere with Various Protein Assays<sup>a</sup>**

interfering solutes (1 mmol/L)	modified Lowry	micro BCA	Pierce BCA	Bradford (Coomassie)	Bradford (Coomassie Plus)
hexadecane	–	–	–	○	–
octadecene	○	–	–	●	○
<i>n</i> -hexadecanol	●	●	●	●	●
glucose	○	●	●	○	–
sucrose	–	–	–	○	–
sorbitol	–	–	–	–	–
urea	–	–	–	○	–
uric acid	●	●	○	○	–
acetic acid	–	–	–	○	○
butyric acid	–	–	–	–	–
hexanoic acid	–	–	–	–	●
palmitic acid	○	●	●	●	○
ascorbic acid	●	●	○	–	○
humic acid	●	●	●	○	●
squalane	–	–	●	●	●
dibutyl phthalate	–	–	–	–	–
2,6-Di- <i>tert</i> -butylphenol	●	●	○	○	○
all	●	●	●	●	●

<sup>a</sup>Key: ●, there is a statistically highly significant difference in measurement between standard w/o interfering solutes ( $p = 0.001$ ); ○, there is a statistically significant difference in measurement between standard w/o interfering solutes ( $p = 0.05$ ); –, there is no statistically significant difference in measurement between standard w/o interfering solutes.

297 Standard curves were first obtained with BSA concentrations  
298 varying from 0.5 to 500 mg/L; BSA was then assayed in parallel  
299 with or without the individual interfering substance. At the 95%  
300 confidence level ( $n = 4$ ,  $p = 0.05$ ), several compounds that  
301 interfered with the Bradford assay did not interfere with the  
302 others, causing an increase in the absorbance. Antioxidants such  
303 as ascorbic acid, uric acid, and substituted phenols can act as  
304 reducing agents and result in artificially high values for the  
305 protein concentration. At the 99.9% confidence level ( $n = 4$ ,  $p =$   
306 0.001) fatty alcohols such as hexadecanol appeared to result in  
307 an erroneous reading that almost certainly reflects on its  
308 interactions with the reagents. Similarly, it is noteworthy that  
309 the lipid-rich compounds such as palmitic acid interfere with  
310 protein assays, giving a false protein concentration. Box, and  
311 Randtke and Larson reported that humic substances interfere  
312 with the Lowry procedure,<sup>35,41</sup> and it seems like humic acids  
313 can be expected to interfere with all the colorimetric protein  
314 measurements due to its ability to complex metal ions,<sup>42</sup> and  
315 their heteropolycondensate structure that absorbs in the  
316 ultraviolet region.<sup>43</sup> Although the Coomassie and Coomassie  
317 Plus assays require little sample preparation time, they have  
318 little tolerance to most of the interfering substances, and result  
319 in significantly different “protein” concentrations. Interestingly,  
320 a plasticizer such as dibutyl phthalate, a common laboratory  
321 contaminant, shows no sign of interference.  
322 Although the mechanism of interference varied with the  
323 solute, they all resulted in strongly erroneous absorbance  
324 values. The easiest method for coping with interfering

compounds is to add them into the blank sample, and prepare  
a standard curve in their presence; however, this requires  
knowing the identity and amount of the interfering solute.  
Moreover, the presence of interfering solutes is difficult to  
adequately control for during colorimetric protein analysis.  
Considerable study of the interference by a compound over a  
range of appropriate concentrations for its effects on several  
different proteins is usually required. This is clearly infeasible  
and impractical due to the diverse range of chemical  
compounds, SMPs, and EPS found in biologically treated  
wastewater samples. The most common strategy for coping  
with interfering compounds is to remove them by selective  
isolation techniques,<sup>28</sup> and most low MW interfering  
substances can often be removed by dialysis. Another method  
is to precipitate the protein in acid and collect the precipitate by  
membrane filtration, although recovery of precipitated protein  
is not quantitative at low concentrations. Even though specific  
interfering substances can sometimes be removed prior to  
concentration determination, this adds additional steps to the  
overall procedure and can consequently result in dilution, or  
incomplete recovery of the original sample leading to errors.  
Hence, interfering substances are particularly troublesome  
when attempting to quantify protein content directly and  
reliably.

**Metachromatic Response with Different Proteins and Peptides.** Many of the traditional colorimetric protein assays depend on both protein quantity and composition, while another influential property is MW. In this work, we critically evaluated the variation in metachromatic response with five commercially available protein assays for a variety of synthetic polyamino acids, a mixture of short chain polypeptides, a mixture of protein standards and several protein samples using BSA as the reference protein. All the samples were analyzed at a fixed concentration within the quantitation range of each assay. Proteinaceous compounds with different MWs were also assayed to determine the sensitivity of the methods toward MW, and the results are summarized in Table 3.

From Table 3, it is clear that there is a noticeable difference between all the samples, even when their concentration was identical to that of the reference protein, BSA, and each of the assays tested exhibited some degree of varying response toward different proteins. Some of the factors that could possibly contribute to this difference are the amino acid sequence, isoelectronic point (pI), its three-dimensional structure, and the presence of certain side chains or prosthetic groups. These results highlight the fact that the metachromatic response is predominantly dependent on amino acid content. In particular, the presence of a few specific residues enhances color development in the colorimetric protein assays, and hence the dissimilar metachromatic response. For example, color formation is highly dependent on the arginine amino residues in the binding of Coomassie Blue to the protein. Furthermore, residues like tryptophan and tyrosine in all of the di-, tri-, or polypeptides are capable of reducing cupric ions to cuprous ions in the BCA reaction, and consequently gave a stronger response than other residues. Moreover, it was demonstrated that peptides with a molecular mass of less than 3000 Da did not form a complex in the Bradford assay, and this could result in very serious errors of omission; low MW SMPs and EPS such as short chain peptides would fall into this category.

It is also remarkable that a large variability was measured between the BSA standard and the five proteins (lysozyme, ovalbumin, apo-transferrin bovine, fetuin from fetal bovine

Table 3. Effect of Composition on Metachromatic Response

protein or polypeptide sample	molecular weight (Da)	reading (mg BSA/L) $\pm$ SD					
		modified Lowry	micro BCA	Pierce BCA	Bradford (Coomassie)	Bradford (Coomassie Plus)	
reference protein	bovine serum albumin	$\sim$ 66 500	$10 \pm 1.4$	$10 \pm 0.9$	$300 \pm 11$	$299 \pm 8.3$	$300 \pm 1.3$
polymeric amino acids	poly-L-lysine	1000–5000	$6.4 \pm 1.4$	$9.1 \pm 0.8$	$303 \pm 25$	$22.5 \pm 6.6$	$19.3 \pm 5.7$
	poly-L-proline	1000–10 000	$0.1 \pm 1.2$	$0.2 \pm 0.2$	$1.00 \pm 2.1$	$21.1 \pm 5.4$	$17.9 \pm 7.9$
	poly-L-arginine	5000–15 000	$3.3 \pm 1.3$	$7.5 \pm 0.2$	$129 \pm 20$	$995 \pm 8.6$	$912 \pm 10$
	poly-DL-aspartic acid	2000–11 000	$0.7 \pm 1.0$	$0.2 \pm 0.2$	$0.40 \pm 2.1$	$20.9 \pm 6.1$	$17.3 \pm 7.3$
	poly(glu,ala,tyr) 1:1:1 <sup>a</sup>	20 000–50000	$10 \pm 2.1$	$14 \pm 0.5$	$675 \pm 41$	$109 \pm 10$	$183 \pm 11$
	poly(glu,ala,tyr) 6:3:1	20 000–50000	$3.6 \pm 1.0$	$10 \pm 0.5$	$340 \pm 48$	$0.6 \pm 0.8$	$46.8 \pm 5.1$
	poly-(arg-pro,thr) 1:1:1	5000–20 000	$7.7 \pm 2.1$	$13 \pm 0.1$	$175 \pm 26$	$619 \pm 26$	$594 \pm 15$
short chain peptide standard mixture	poly-(arg-pro,thr) 6:3:1	10 000–30000	$2.3 \pm 1.4$	$6.4 \pm 0.2$	$120 \pm 19$	$981 \pm 20$	$922 \pm 21$
	Gly-tyr	238.2	$46 \pm 1.0$	$19 \pm 0.4$	$412 \pm 14$	$13.1 \pm 2.9$	$12.5 \pm 7.9$
	Val-tyr-val	379.5					
	Tyr-gly gly-phe-met	573.7					
	Tyr-gly gly-phe-leu	555.6					
protein standard mixture	Asp-arg-val-tyr-ile-his-pro-phe	1046.2					
	ribonuclease A	$\sim$ 13 700	$27 \pm 0.6$	$18 \pm 0.6$	$432 \pm 10$	$362.1 \pm 14$	$360 \pm 17$
	cytochrome C	$\sim$ 12 000					
	holo-transferrin	76 000–81000					
proteins	apomyoglobin	$\sim$ 16 900					
	lysozyme	14 388	$18.3 \pm 0.3$	$15 \pm 0.3$	$567 \pm 15$	$186 \pm 8.6$	$410 \pm 6.3$
	ovalbumin	45 000	$11.6 \pm 0.4$	$12 \pm 0.4$	$410 \pm 5.8$	$148 \pm 5.5$	$307 \pm 5.7$
	$\alpha$ -acid glycoprotein	41 000–43 000	$9.0 \pm 1.2$	$6.9 \pm 0.1$	$255 \pm 12$	$132 \pm 6.7$	$172 \pm 1.9$
	Apo-transferrin bovine	76 000–81 000	$9.5 \pm 1.1$	$11 \pm 0.2$	$415 \pm 17$	$346 \pm 6.5$	$389 \pm 2.4$
	Fetuin from fetal bovine serum	48 400	$6.7 \pm 0.8$	$7.0 \pm 0.2$	$255 \pm 6.8$	$191 \pm 2.9$	$210 \pm 2.0$

<sup>a</sup>Molar ratio of random copolymers of amino acid.

Table 4. Average reading in the effluent from each sample under various HRTs

HRT (h)	COD (mg/L)	reading (mg BSA/L) $\pm$ SD				
		modified Lowry	micro BCA	Pierce BCA	Bradford (Coomassie)	Bradford (Coomassie Plus)
6	19.12	$5.6 \pm 0.8$	$6.8 \pm 0.1$	$6.5 \pm 1.8$	$1.9 \pm 2.0$	$15.2 \pm 1.8$
4	31.16	$8.3 \pm 0.5$	$10.3 \pm 0.3$	$16.5 \pm 2.1$	$3.0 \pm 1.2$	$16.6 \pm 0.8$
2	40.65	$9.5 \pm 0.5$	$23.7 \pm 0.8$	$31.5 \pm 1.4$	$3.5 \pm 0.7$	$18.7 \pm 2.4$
6 (SA <sup>a</sup> )		$24.6 \pm 1.4$	$19.2 \pm 0.1$	$369 \pm 22$	$379 \pm 15$	$373 \pm 5.4$
4 (SA <sup>a</sup> )		$32.9 \pm 1.1$	$20.4 \pm 0.2$	$373 \pm 24$	$384 \pm 9.9$	$375 \pm 4.4$
2 (SA <sup>a</sup> )		$33.1 \pm 2.8$	$25.9 \pm 0.5$	$388 \pm 20$	$386 \pm 11$	$380 \pm 5.2$

<sup>a</sup>Standard Addition of BSA at 10 mg/L for Modified Lowry and Micro BCA, and 300 mg/L for the others.

serum,  $\alpha$ -acid glycoprotein). However, the standard derivation (SD) suggests that this variation in concentration between the different proteins was not due to repeatability, but rather to the individual colorimetric assay responses being dependent on the amino acid content of the protein. Moreover, the protein concentration measured was highly sensitive to the degree of glycosylation of the protein analyzed, and the specific sugars present in the protein.<sup>44</sup> Comparison of an equal amount of lysozyme (0% carbohydrate content), fetuin (22.9% carbohydrate content) and acid glycoprotein (41.4% carbohydrate content) suggests that the nonglycosylated form of the protein generally gave higher responses. This difference was observed for all of the assays analyzed, which suggests this was not an erroneous result.

Taken together, since the assay responses are in fact dependent on the amino acid content of the protein, extreme caution should be exercised in using BSA to estimate the

concentration of unknown “proteins” in a wastewater sample due to the large variability observed. It has been documented that the colorimetric assays require an appropriate protein standard to obtain a good estimate of the concentration present, and the ideal protein standard to use would be the same protein being assayed.<sup>28</sup> In practice, we often do not know what protein or polypeptide we are looking for, and there is not always a matched protein standard available, especially in wastewater analysis.

**Case Study.** A study was carried out in order to evaluate the applicability of these methods to actual wastewater samples; samples at 3 different HRTs were characterized by measuring their COD and “protein” content. The effluent samples were taken under the conditions shown in Table 4 and analyzed with various colorimetric protein assay methods.

HRT is an important operational parameter that impacts on treatment performance, and affects SMP production and hence

422 membrane fouling in a SAMBR. From Table 4 it is clear that, as  
423 expected, the effluent COD increased with decreasing HRT  
424 and an increase in “protein” concentration with all assays was  
425 also observed. While a low operating HRT is desirable for  
426 anaerobic reactors in order to reduce their overall footprint, it  
427 enhances biomass growth and the accumulation of SMPs  
428 leading to membrane fouling.<sup>45</sup> Using a modified Lowry  
429 method, Chae et al. also observed a similar trend with the  
430 “protein” concentration increasing with decreasing HRT.<sup>46</sup>  
431 However, these five methods gave widely varying readings; at 6  
432 h HRT the “protein” concentration varied by a factor of 8  
433 (1.9–15.2), the highest measured with the Bradford  
434 (Coomassie Plus) assay. At 2 h HRT the highest was obtained  
435 using the Pierce BCA kit, and the ratio was even greater at 9. It  
436 is true that all assays showed an increase in “protein” with  
437 decreasing HRT, but each ratio varied markedly, with a small  
438 increase of 23% from 6 to 2 h with Bradford (Coomassie Plus),  
439 while the largest increase was 485% with the Pierce BCA assay.  
440 Hence, while all these assays predicted a general trend, there  
441 was little or no agreement between them in terms of absolute  
442 concentrations, or relative increases.

443 In addition, the values shown in Table 4 for protein content  
444 after the addition of a BSA “spike” are generally higher than  
445 expected (a spike of 10 mg/L was added to the Modified Lowry  
446 and Micro BCA, and 300 mg/L to the Pierce BCA and  
447 Bradford methods). This clearly shows that all the samples  
448 must contain solutes that interfere with the protein  
449 determination for all the five assays to varying degrees.  
450 Moreover, the use of BSA as a reference standard could also  
451 possibly introduce errors since it is clearly not an appropriate  
452 protein standard for estimating proteinaceous material present  
453 in the wastewater sample.

454 **Proteins versus Proteinaceous Material.** Proteins are  
455 large biopolymers that are composed of  $\alpha$ -amino acids which  
456 can polymerize through condensation and form dipeptides,  
457 tripeptides, oligopeptides, or polypeptides. Proteins consist of  
458 one or more polypeptide chains ranging in length from ~40 to  
459 34 000 amino acids residues<sup>47</sup> and since the average mass of an  
460 amino acid residue is ~110 Da, proteins can have molecular  
461 masses that range from ~10 to over 3700 kDa. Moreover,  
462 proteins are constantly being degraded by a variety of catabolic  
463 pathways during biological treatment processes, adding a  
464 dynamic component to the system, and therefore, based on  
465 the results obtained in this study, and information discussed  
466 earlier, it is understandable that if BSA was chosen as the  
467 reference standard, the result should be analogized and  
468 reported as mg BSA/L despite the fact that they are not the  
469 actual protein mass concentrations in the samples. Hence it is  
470 clear that enumerating changes in “proteins” measured with the  
471 assays tested above, with varying operational parameters is very  
472 likely to be misleading in published papers, and the data lacks  
473 credibility. Increasingly, many authors are starting to use the  
474 term “protein-like materials”,<sup>6</sup> although a more suitable term  
475 could be “proteinaceous” material, whose definition includes  
476 any materials relating to, resembling, or being proteins that are  
477 synthesized or decomposed by bacteria or eukaryotic  
478 organisms, and describes all forms of polypeptides/pro-  
479 teins.<sup>48,49</sup>

480 Based on the results of this study, we have shown that a wide  
481 variety of solutes can interfere with colorimetric protein assays.  
482 In addition, the metachromatic response of these assays is  
483 clearly influenced by sample composition, and hence the  
484 proteinaceous material measured by these methods could be

wrongly estimated. Given the complexities, vast dynamic range  
of proteinaceous material abundances, and analytical limitations  
associated with traditional colorimetric assays, these five  
colorimetric methods for protein determination are therefore  
not recommended for the measurement of “proteins” in  
wastewater samples.

**Future Prospects.** In recent years, fluorescence excitation–  
emission matrix (EEM) spectroscopy has been applied to  
investigate “protein” concentrations in activated sludge in a  
sequencing batch reactor.<sup>50</sup> However, limited independent  
testing of this methodology prevents a full critical analysis.  
Increasingly, rapid advances in mass spectrometry-based  
“omics” techniques enable protein cataloging, analyses of  
protein localization, and uncovering the pathways behind  
environmental cellular processes.<sup>51</sup> In particular, state-of-the-art  
proteomics technologies provide detailed information about the  
protein profile<sup>51</sup> whereas metaproteomics offers the ability to  
characterize the global protein complement of environmental  
microbiota at a given point in time.<sup>52</sup> Despite the capability of  
these techniques, only a handful of quantification assays can be  
considered established, and often these require with equipment  
far too expensive for routine application. Pioneering studies in  
environmental proteomics have successfully revealed links  
between protein diversity and ecological functions in simple  
microbial communities in the laboratory.<sup>51,53</sup> Nonetheless, such  
applications are limited to microbial ecology, and none have  
been used to analyze wastewater samples. While still in its  
infancy, metaproteomics studies of activated sludge and  
wastewater treatment plants have qualitatively revealed a  
number of interesting cytoplasmic proteins, but very few  
studies have used a quantitative approach.<sup>51,53</sup> It is important to  
push the boundaries of technological innovation in wastewater  
treatment, and hence a new method needs to be developed  
quickly to replace these old and inaccurate techniques to  
accurately identify and quantify proteinaceous material found in  
wastewater so that more detailed investigations can be carried  
out on SMP production and membrane fouling.

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### Notes

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