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Flow injection analysis: Rayleigh light scattering technique for total protein determination

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Abstract A novel flow injection analysis (FIA) method with Rayleigh light scattering (RLS) detection was developed for the determination of total protein concentrations. This method is based on the weak intensity of RLS of bromothymol blue (BB) (3',3''-dibromothymolsulfonephthalein) which can be enhanced by the addition of protein in weakly acidic solution. A common spectrofluorimeter was used as a detector. It was proved that the application of this method to quantify the total proteins in real samples by using bovine serum albumin was possible. The RLS signal was detected at $\lambda_{\text{ex}}=\lambda_{\text{em}}=572$ nm. The linear range was 7.0–70.0 $\mu\text{g mL}^{-1}$, the detection limit was 3.75 $\mu\text{g mL}^{-1}$, the reproducibility was 5.5% ($n=7$), and the sample throughput was 26 h^{-1} .

Keywords Rayleigh light scattering · FIA · Proteins

Introduction

Quantitative analysis of proteins is a basic requisite in biochemistry, since it is often used as a reference for the measurements of other components in biological samples. Many methods to determine proteins in different samples are available: the most often used are Coomassie brilliant blue (CBB) [1, 2], Lowry et al. [3], and bromophenol blue [4]. However, they have some limitations in terms of sensitivity, selectivity, stability, and simplicity. Therefore, a great number of assays have been developed, such as spectrophotometric [5, 6, 7, 8], spectrofluorimetric [9, 10], chemiluminescence [11], and electrochemical [12] methods. Recently sensitive methods for proteins and nucleic acids have been developed based on enhanced Rayleigh light scattering (RLS) [13, 14, 15].

According to Pasternack et al. [13], a particle, assumed to be spherical, absorbs and scatters light depending on its size, shape, and the refractive index of the surrounding medium. They have developed a technique to detect the intensity of light scattering by using a common spectrofluorimeter [16]. Several dyes present a weak Rayleigh light scattering intensity, which is enhanced through their binding with macromolecules such as proteins and DNA. By using RLS spectroscopy, organic dye probes used in spectrophotometric assays for proteins and nucleic acids can become much more sensitive. For instance, the dynamic range for bovine serum albumin (BSA) of the RLS method with bromophenol blue was 0.34–18.7 mg L^{-1} while the spectrophotometric procedure can just be used when protein concentrations are higher than 10 mg L^{-1} [17].

The aim of this work is concerned with the use bromothymol blue (BB) (3',3''-dibromothymolsulfonephthalein) as a binding reagent for protein determination because a high sensitivity to the RLS intensity was observed. By taking into account the advantages on automation of laboratory processes, an FIA system with RLS detection was developed and optimized in order to automate the determination of total proteins in urine and serum samples, with high sample throughput.

Experimental

Reagents

Analytical reagent grade chemicals and 18 $\text{m}\Omega$ water were used. Britton Robinson (BR) buffer solution pH 3.45 was prepared by mixing 100 mL of acidic BR solution (acetic acid 0.04 M, phosphoric acid 0.04 M, and boric acid 0.04 M) and 21 mL of sodium hydroxide 0.2 M.

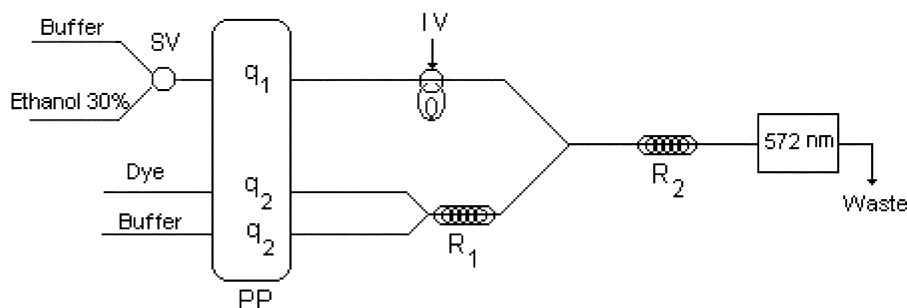
Standard bovine serum albumin (Wiener Lab.) was directly dissolved in water to prepare the stock solution. The working solutions were obtained by diluting the stock solution with water just before to use.

Bromothymol blue solution was prepared by dissolving 0.03 g in 20 mL of ethanol and diluting to 100 mL with water.

Wash solution was ethanol 30% (v/v).

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Fig. 1 FIA system for the determination of total proteins. *PP* peristaltic pump, q_1 1.35 mL min⁻¹, q_2 0.68 mL min⁻¹, *SV* selection valve, *IV* injection valve, $R_1=R_2=600$ mm



Instrumentation

An Aminco Bowman Serie 2 luminescence spectrophotometer equipped with a xenon lamp (150 W) and a 1-cm quartz cell (Hellma, QS 1.000) was used to obtain the RLS spectra. A Hellma 176752-QS flow cell with an inner volume of 25 μ L and 1.5-mm light path was used to measure the FIA signals. All the reaction coils of the FIA manifold were made of PTFE tubing (i.d. 0.5 mm). A Gilson Minipuls-3 peristaltic pump and a Rheodyne 5041 injection valve were used.

To obtain the absorption spectra, a Hewlett Packard HP8452A spectrophotometer with a diode array detector was used.

Procedure

The FIA manifold is depicted in Fig. 1. A sample volume was injected into a buffer solution stream as a carrier. The dye (BB) solution stream merged with a buffer solution inside the reactor (R_1) to obtain a suitable pH for the forward reaction with proteins of the sample, in the reactor R_2 . The analytical signal was detected at $\lambda_{ex}=\lambda_{em}=572$ nm. A wash cycle between injected samples was necessary to obtain a good reproducibility and a stable baseline. For this purpose an ethanol solution 30% (v/v) stream was passed through the manifold, by switching the selection valve (SV) for 30 s.

Results and discussion

Spectral characteristics

The light scattering intensity was measured at 572 nm with 8-nm slit width for both excitation and emission radiation. The light scattering spectrum was obtained by scanning simultaneously with the same excitation and emission wavelengths. Figure 2 shows the RLS spectra of BB (0.03 g%) and BB (0.03 g%)+albumin (27 mg L⁻¹) obtained under the optimum experimental conditions. A low light scattering intensity of BB was observed between 450 and 700 nm. However, when the albumin was present, an important enhanced light scattering was observed at 572 nm. On the other hand, absorption spectra of BB (0.03 g%) and BB (0.03 g%)+albumin (27 mg L⁻¹) were obtained (Fig. 3) and it was observed that the RLS maximum was situated at the minimum absorption, so the reaction system can be treated as a transparent solution [18, 19].

Selection of FIA manifold

Different configurations for the FIA system were considered. The first configuration that was tested had a stream of buffer solution, which merged with a stream of BB so-

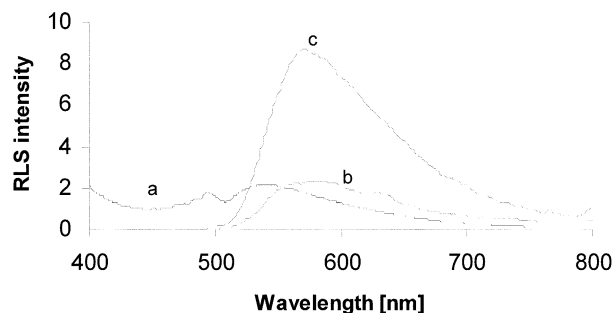


Fig. 2a–c Rayleigh light scattering spectra of a buffer solution, b BB, c BB+albumin

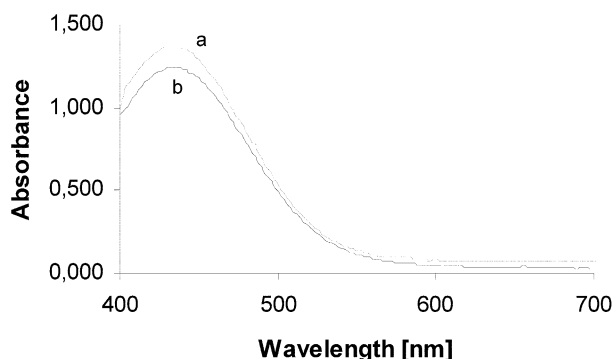


Fig. 3a, b Absorption spectra of a BB, b BB+albumin

lution. A sample volume of albumin was injected into this stream of BB mixed with buffer. The baseline obtained with this configuration was unstable, so it produced drawbacks on the reproducibility of signals. To overcome these drawbacks, a reactor was introduced into the manifold to allow better mixing between the two stream solutions, but the peak heights were not reproducible. Thus, a wash cycle was introduced between sample injections. The best reproducibility was attained with the FIA manifold shown in Fig. 1 and it was calculated as the RSD of five replicates of sample injections.

Optimization of chemical and FIA variables

The variables influencing the performance of the method were optimized by univariate method. Optimum conditions were selected to obtain maximum height of FIA sig-

nals, a stable baseline, and a good reproducibility (RSD of five injections of standards $\leq 1.5\%$).

Different dye compounds were tested as reagent (bromophenol blue, methyl red, phenol red, thymolsulfophthalein). Under the experimental conditions used in this method, BB reagent was selected because the baseline of FIA signals were smoothed and the reproducibility was better than those obtained with the other studied dyes.

The light scattering intensity of the BB+albumin was stable over the pH range 3.0–3.5, and any pH outside this range produces a decrease in the scattering intensity. Different buffer solutions were tested to obtain the suitable pH; the maximum signal was obtained with BR buffer solution (pH 3.45). BB reagent was prepared in ethanol/water, and this ratio was varied between 5 and 50% (v/v). With an optimum value of 20% (v/v), BB concentration was tested over the range 0.01–0.10 g% (w/v). A 0.03 g% of BB yielded the best results.

To wash the system, different percentages (v/v) of ethanol were tested and 30% was the optimum.

The FIA variables (flow rates, reactor lengths, sample volume, and time for the wash cycle) were optimized. The studied range and optimum values are shown in Table 1.

Analytical application

A linear calibration graph from 7.0 to 70.0 $\mu\text{g mL}^{-1}$ of bovine serum albumin was obtained. The analytical curve was $Y=0.3098x+0.6913$ (Y =scattering intensity and x =concentration ($\mu\text{g mL}^{-1}$)) with $R^2=0.9997$. The reproducibility

Table 1 Optimization of FIA variables

Variable	Studied range	Optimum value
Carrier flow rate (mL min^{-1})	0.70–1.50	1.35
BB flow rate (mL min^{-1})	0.52–1.20	0.68
Buffer flow rate (mL min^{-1})	0.52–1.20	0.68
Reactor length (R_1) (mm)	200–800	600
Reactor length (R_2) (mm)	50–800	600
Sample volume (μL)	50–300	250
Time for the wash cycle (s)	30–80	30

Table 2 Slopes of the calibration curves obtained with proposed method and standard addition method^a

Proposed method	Standard addition method	
	Urine	Serum
0.272 \pm 0.004 (0.999)	0.305 \pm 0.010 (0.996)	0.255 \pm 0.007 (0.999)
0.289 \pm 0.007 (0.997)	0.312 \pm 0.010 (0.995)	0.310 \pm 0.006 (0.998)
0.270 \pm 0.005 (0.999)	0.242 \pm 0.005 (0.998)	0.298 \pm 0.010 (0.999)
0.309 \pm 0.003 (0.999)	0.271 \pm 0.004 (0.999)	0.309 \pm 0.008 (0.996)
0.298 \pm 0.009 (0.996)	0.344 \pm 0.004 (0.999)	0.311 \pm 0.004 (0.999)
0.306 \pm 0.007 (0.998)	0.313 \pm 0.010 (0.996)	0.284 \pm 0.009 (0.995)

^aRegression coefficient (R^2) is given in parenthesis

Table 3 Determination of total proteins in real samples

Samples	Proposed method (g L^{-1}) \pm SD ($n=5$)	Standard method ^a (g L^{-1})	Error (%)
Urine 1	2.13 \pm 0.23	2.10	1.4
Urine 2	0.89 \pm 0.06	0.91	-2.2
Urine 3	1.30 \pm 0.12	1.28	1.6
Urine 4	3.05 \pm 0.25	3.00	1.7
Serum 1	48.9 \pm 0.8	49.0	-0.2
Serum 2	6.92 \pm 0.42	7.00	-1.1
Serum 3	12.2 \pm 0.5	11.9	2.5
Serum 4	5.78 \pm 0.28	5.70	1.4

^aTurbidimetric method for urine samples and Biuret method for serum samples

of the proposed method was calculated by running 6 calibration curves on different days and with different conditions (standard solution, reagent solution, etc.). The RSD was 5.5%.

Detection limit estimated ($S/N=3$) was 3.7₅ $\mu\text{g mL}^{-1}$ and sample throughput was 26 h⁻¹.

Owing to the complexity of the serum and urine matrix, matrix-matched standard solutions could not be prepared. Thus, the standard addition method can be advantageous because it was performed with the sample [20]. In these cases, the interference of the matrix can introduce systematic errors on the analytical determination. These systematic errors can be indicated by a comparison between the slope of the standard addition calibration line and the slope of calibration line obtained with bovine serum albumin. If the matrix does not interfere, both lines must have the same slope. Table 2 shows the slopes of calibration lines obtained with the proposed method and with the standard addition method applied to urine and serum samples. The comparison of the slopes was done by applying an ANOVA test. From the values ($F_{\text{calculated}}=0.11$ and $F_{\text{tabulated}}=3.7$) obtained, no significant differences were observed for a 95% confidence level. Therefore, the application of this method to quantify the total proteins in the samples by using bovine serum albumin calibration line is possible.

To validate the quality of the obtained results, several real samples were analyzed by using the proposed procedure without sample treatment. The same samples were analyzed in a biochemical laboratory by the turbidimetric method (urine) [21] and Biuret automated method (serum) [22]. The results were compared and a good agreement between them was observed. Table 3 shows the corresponding errors and they were within 2% of standard methods.

Conclusion

The Rayleigh light scattering technique is useful and sensitive for the determination of proteins in different samples. This can be done on a common spectrofluorimeter. Based on the fact that the weak RLS intensity of BB dye

can be enhanced greatly by the addition of proteins, a novel automated method for protein determination was developed by using FIA methodology. Thus, the proposed method is a simple, fast, and inexpensive analytical technique to determine total proteins in urine and serum samples compared with other general methods.

Moreover, there is no sample matrix interference, which is important to determine the total protein in real samples.

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