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Kinetics independent spectrometric analysis using non-linear calibration modelling and exploitation of concentration gradients generated by a flow–batch system for albumin and total protein determination in blood serum

Margarida C. Souza[c], Valdomiro L. Martins[c], Luciano F. Almeida[a], Osmundo D. Pessoa Neto[a], Edvaldo N. Gaião[b], Mario Cesar U. Araujo[a,*]

[a] Universidade Federal da Paraíba, CCEN, Departamento de Química, Caixa Postal: 5093, 58051-970, João Pessoa, Paraíba, Brazil
[b] Universidade Federal Rural de Pernambuco, Unidade Acadêmica de Serra Talhada, Brazil
[c] Universidade Federal do Amazonas, Instituto de Ciências Exatas e Tecnologia, Brazil

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ABSTRACT

An automatic method for kinetics independent spectrometric analysis is proposed in this study. It uses a non-linear calibration model that explores concentration gradients generated by a flow–batch analyser (FBA) for the samples, dye, and the single standard solution. The procedure for obtaining the gradients of the dye and standard solution is performed once at the beginning of analysis. The same procedure is applied thereafter for each sample. For illustration, the proposed automatic methodology was applied to determine total protein and albumin in blood serum by using the Biuret and Bromocresol Green (BCG) methods. The measurements were made by using a laboratory-made photometer based on a red and green bicolour LED (Light-Emitting Diode) and a phototransistor, coupled to a "Z" form flow cell. The sample throughput was about 50 h^{-1} for albumin and 60 h^{-1} for total protein, consuming about 7 μL of sample, 2.6 mL of BCG and 1.2 mL of biuret reagents for each determination. Applying the paired *t*-test for results from the proposed analyser and the reference method, no statistic differences at 95% confidence level were found. The absolute standard deviation was usually smaller than 0.2 g dL^{-1}. The proposed method is valuable for the determination of total protein and albumin; and can also be used in other determinations where kinetic effects may or may not exist.

1. Introduction

In order to emit diagnoses and prognostics accurately, health care professionals require reliable biochemical and clinical data and parameters. Several clinical biochemical parameters such as total protein, albumin, hemoglobin, cholesterol, urea, glucose, among others, are used in medical diagnosis. Albumin and total protein participate in the human metabolism and evaluation of their levels may indicate pathological conditions.

The determination of total protein and albumin is usually performed through the application of biuret and bromocresol green methods, respectively, and are widely employed in routine analysis by laboratories. However, manual methodologies present some inconveniences, such as contamination (due to constant handling of the samples and standard solutions), inaccuracies, low sample throughput; elevated consumption of reagents (increasing analytical costs), and labour. To overcome these problems, automatic flow analysers have been developed for determination of several clinical and biochemical parameters [1–7]. However, few studies describing flow systems for the determination of total protein and albumin [8–10] have been reported in the literature. An FIA system based on the biuret method applied to total protein determination was developed [7]. Although this system presents high throughput and can be applied to slow reactions with orders different than one or zero, it consumes elevated amounts of reagent, and uses six standard solutions starting from a concentrated reagent to obtain the analytical curve, this elevates the costs of analysis. Véras et al. [3] proposed an FIA system to quantify total protein in human blood serum exploiting concentration gradients. However, the proposed FIA system requires modifications and adjustments to its manifold for determining other biochemical parameters. These drawbacks can be overcome by using flow–batch analysers.

Flow–batch analysers (FBA) have gained great attention in recent years, which can be demonstrated by some key articles [11–21]. Flow–batch systems combine the useful and favourable characteristics of flow systems with well established classical batch mode approaches. A remarkable advantage of this hybridization is how easy it is to carry out almost all the classical batch mode

* Corresponding author.
E-mail address: laqa@quimica.ufpb.br (M.C.U. Araujo).

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methods with a new and advantageous automatic instrumental approach. This means maintaining the reliability of the classical batch mode methods while performing them with a modern, fully computer controlled and miniaturized mixing assembly accessory, which exchanges the use of large amounts of solutions for the micro-volumes, typically employed in flow systems.

Briefly, a dilution/mixing/reaction chamber, containing a magnetic stirring bar, is inserted in an FBA to allow all the chemicals to be thoroughly mixed, similar to classical batch analysis methods, before flowing to the detector for the monitoring of the analytical signal. As a result, efficient mixing and dilution of reagents, sample and any other solutions in a micro-litre range can easily be controlled through software. While most flow analysis systems usually require a specific apparatus assembly for each particular method, strongly limiting their widespread acceptance in routine analysis laboratories, a flow–batch system can be viewed as a universal purpose accessory tool easily attached to any conventional equipment for instrumental analysis. However, the main advantage of the flow–batch is that classical methods can be updated for better precision and speed. Perhaps the most outstanding characteristic of a flow–batch approach is the opportunity offered for developing analytical methods through software.

In this study, a methodology for kinetics independent spectrometric analysis using non-linear calibration modelling and exploitation of concentration gradients generated by a flow–batch system is advanced. To demonstrate the usefulness of this methodology, it was applied to the determination of total protein and albumin in human blood serum, using Biuret and Bromocresol Green (BCG), respectively. The methodology was used to determine total protein and albumin using kits easily found for sale in commerce. Such kits are sold with the reagents (biuret and BCG) and a single standard solution for calibration.

2. Experimental

2.1. Chemicals and samples

A commercial kit for determination of total protein in serum was purchased from *Labtest*, comprising the biuret reagent (1.86 mol L^{-1} NaOH plus 0.43 mol L^{-1} sodium and potassium tartarate—$KNaC_4H_4O_6$ plus 0.12 mol L^{-1} $CuSO_4$ plus 0.30 mol L^{-1} KI) and a single standard solution (4.0 g dL^{-1} bovine albumin stabilized with 10 g L^{-1} NaN_3).

A commercial kit for determination of albumin in blood serum comprised the same standard solution above and the BCG reagent (2.5 mmol L^{-1} bromocresol green reagent prepared in pH 4.0 buffer and in 0.82 mol L^{-1} lactic acid plus 1.5% (v/v) of Tween 80). This kit was purchased from Doles Reagents.

According to manufacturer suggestions these concentrated reagents were diluted 1:10 in distilled water for analysis using both the flow–batch system, and a reference method described below.

A $KMnO_4$ solution (10.0 mmol L^{-1}) prepared in distilled water and a malachite green solution (1.1 mmol L^{-1}) prepared in pH 3.5 buffer (12.8 mmol L^{-1} sodium acetate and 187.2 mmol L^{-1} in acetic acid) were used as dye in flow–batch determination of albumin and total protein, respectively.

The samples of blood serum were acquired in the Clinical Analyses Laboratory of the University Hospital at João Pessoa-Paraiba-Brazil. All solutions were prepared with freshly distilled water.

2.2. Reference methods

For comparative purposes, the reference methods for total protein and albumin determinations described by the commercial kit manufacturers (Labtest and Doles Reagents) were used to analyse all serum in study. The methods were carried out in three steps of absorbance measurements one each for blank, standard solution, and samples, which were measured employing a model 8453 Hewlett-Packard diode array UV–vis spectrophotometer at 630 and 560 nm wavelengths for protein and albumin determinations, respectively. The measurements were performed using the following procedure: initially, 2.5 mL of the diluted biuret or BCG reagents were mixed with 50 μL H_2O (for protein determination) or 10 μL H_2O (for albumin determination) and the resulting solution were used for blank absorbance measurements. Thereafter, reference solutions were prepared by mixing 2.5 mL of the diluted biuret or BCG reagents with 50 μL (for total protein determination) or 10 μL (for albumin determination) of the standard solution (C_R^o concentration). The absorbance of these reference solutions (A_R) was measured after 15 min (for protein determination) and 2 min (for albumin determination). The samples were similarly treated but the blood serum replaced the protein or albumin standard solution. After absorbance (A_S) measurement, the sample concentration C_S^o was calculated as $C_S^o = C_R^o A_S/A_R$.

2.3. Flow–batch analyser

The proposed analyser is shown in Fig. 1. The absorbance measurements were made by using a laboratory-made photometer based on a red and green bicolour LED (Light-Emitting Diode) and phototransistor, coupled to a "Z" form flow cell [22]. The red (λ_{max} = 630 nm) and green (λ_{max} = 560 nm) emissions of the bicolour LED were used for total protein and albumin determinations, respectively.

A model MCP78002-00 four channel Ismatec peristaltic pump and 1.85 mm i.d. Tygon® pumping tube were used. The transmission lines were constructed with 0.8 mm i.d. Teflon® tubing. A 2.0 mL laboratory-made gradient chamber (GC) [14] was constructed in Teflon®.

Three Cole Parmer three-way solenoid valves were used: valve V_1 was used to direct the sample, standard or dye solution into the GC; valve V_2 was utilized to direct the biuret reagent or water (for total protein determination), or BCG reagent or the pH 3.5 buffer (for albumin determination) into GC; and valve V_3 was used to select the stream flowing (water or GC mixture) through the photometer.

A PC microcomputer equipped with a laboratory-made parallel interface card was used to control the proposed analyser and to perform data acquisition and treatment. The software was developed in Labview® 5.1. An electronic actuator (EA) increased the power of the signal sent by the microcomputer in order to control the magnetic stirrer (MS) and the valves.

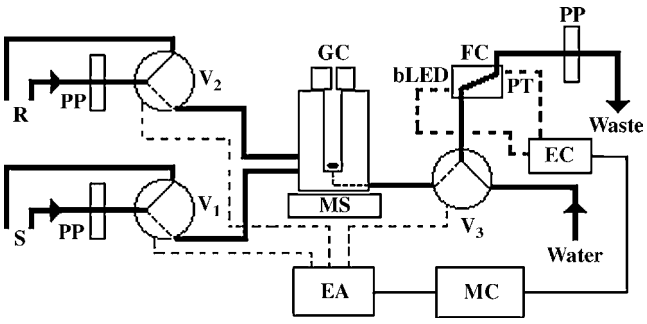

Fig. 1. Diagram of the flow–batch analyser: electronic actuator (EA); mixture chamber (GC); peristaltic pump (PP); magnetic stirrer (MS); solenoid valves (V_1, V_2, and V_3); laboratory-made photometer based on bicolour light-emitting diode (bLED), phototransistor (PT), "Z" form flow cell (FC) and electronic circuit (EC); microcomputer (MC); sample (S); standard solution (SS); water (W). The arrows indicate the direction of the fluids.

Table 1
q_{Si}, q_{Di}, q_{SSi} and t_{Si}, t_{Di}, t_{SSi} are the flow-rates and the times for the switching valve V_1 for insertion of the sample (S), dye (D) or standard solution (SS) inside the CG. q_{Ri}, q_{wi}, q_{BUi} and t_{Ri}, t_{wi}, t_{BUi} are flow-rates and the times for the switching valve V_2 for insertion of the reagent (biuret or BCG), water (W) or buffer (BU) inside the CG. v_{Ri} and v_{Si} are sample and reagent volumes inserted into the CG. q_{Rg} and t_{Rg} are the flow-rate and time for switching for valves V_2 and V_3 for the generation of the concentration gradients inside the CG and for the acquisition of $A_X(t)$ *versus* time curves (X = dye solution, standard solution or sample). t_{BL} and t_E, are times for switching valve V_3 for the blank (BL) signal acquisition and the CG emptying. t_H = time for homogenization of the solutions inside the CG. t_F is the time for switching valves V_1 and/or V_2 for channel filling.

Parameters	Parameters values	
	Albumin	Total proteins
$q_{Di} = q_{SSi} = q_{Si}$ (mL min^{-1})	0.284 ± 0.002	0.284 ± 0.002
$t_{Si} = t_{Di} = t_{SSi}$ (s)	1.5	1.5
$q_{Ri} = q_{Wi} = q_{BUi}$ (mL min^{-1})	7.005 ± 0.014	7.005 ± 0.014
$t_{Ri} = t_{Wi}$ (s)	–	3.0
$t_{Ri} = t_{BUi}$ (s)	15	–
v_{Ri} (mL)	1.751 ± 0.004	0.350 ± 0.002
V_{Si} (μL)	7.1 ± 0.1	7.1 ± 0.1
q_{Rg} (mL min^{-1})	1.281 ± 0.003	1.281 ± 0.003
t_{Rg} (s)	38	38
$t_{BL} = t_{Ea} = t_H$ (s)	3	3
t_{CF} (s)	5	5

2.4. Procedure

This procedure is carried out in six steps: channel filling (a), gradient acquisition of the dye, standard solution, and sample (b–d), and emptying of the GC (e). Before starting each step, the solution in each channel is pumped and recycled to its flask.

In the channel filling step, valves V_1 and V_2 are simultaneously switched ON during a time interval t_{CF} (Table 1) and the sample, standard or dye solution and the water, biuret or BCG reagent, respectively, are pumped towards GC. In fluid exchanges, this step should be performed always.

The emptying step (e) is always done at the end of each step (a) to (d) above, by switching ON valve V_3 for t_{Ea}. This step is always done using the maximum peristaltic pump flow-rate (28.8 mL min^{-1}).

Before starting the dye gradient acquisition step (Fig. 2a), the channel filling procedure is accomplished by pumping dye solution through the sample channel and water (for total protein determination) or the pH 3.5 buffer (for albumin determination) through the reagent channel. After, valve V_2 is switched ON during a time interval ($t_{BL} + t_{Wi}$) or ($t_{BL} + t_{BUi}$) and the water or buffer is pumped towards the GC. Following this, valve V_3 is switched ON during a time interval t_{BL}, the dye solution is aspirated towards the detector and the blank signal, BS_D, is measured and recorded. After this measurement, valve V_1 is switched ON during a time interval t_{Di} and the dye solution is pumped towards the GC. Subsequently, the homogenization during a time t_H is performed, valves V_2 and V_3 are activated simultaneously during time t_{Rg}, a concentration gradient of the dye solution is generated in the GC and an absorbance *versus* time curve ($A_D(t) \times t$) is recorded.

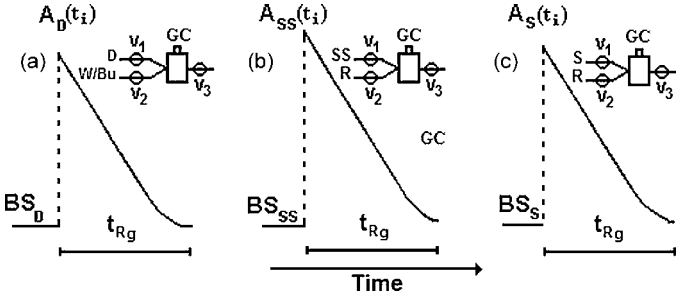

Fig. 2. Acquisition of the concentration gradient profiles. BS_D, BS_{SS} and BS_s, and $A_D(t) \times t$, $A_{SS}(t) \times t$ and $A_S(t) \times t$ are the baseline signals and absorbance *versus* time curves for (a) the dye, D, (b) standard solution, SS, and (c) samples, S, respectively. V_1, V_2, and V_3 are solenoid valves and the GC is the gradient chamber. For total protein determination, D, $KMnO_4$ solution; W, water; R, biuret reagent and SS, total protein standard solution. For albumin determination, D, malachyte green; Bu, pH 3.50 buffer; SS, albumin standard solution and R, bromocresol green. t_{Rg} is the time for switching for valves V_2 and V_3 for the generation of the concentration gradients inside the GC.

A similar procedure is carried out in order to obtain BS_{SS} and BS_s signals, and $A_{SS}(t) \times t$ (Fig. 2b) and $A_S(t) \times t$ (Fig. 2c) absorbance *versus* time curves for the standard solution and samples. However, water is replaced by biuret reagent and buffer by BCG reagent, when the standard solution or samples are used instead of the dye solution. Moreover, $t_{SSi} = t_{Si} = t_{Di}$; ($t_{BL} + t_{Wi}$) or ($t_{BL} + t_{BUi}$) are replaced by ($t_{BL} + t_{Ri}$), with $t_{Ri} = t_{Wi}$ (for total protein determination); and $t_{Ri} = t_{BUi}$ (for albumin determination).

The dye solution gradient acquisition step must be done always without reaction and a linear relationship between dye concentration and absorbance measurements (obedience to Beer's law) must always be provided (see non-linear calibration modelling section below).

Owing to the hydrophobic characteristic of PTFE used in the GC, valves, and transmission lines of the flow–batch manifold, cleaning steps were unnecessary for this study. Indeed, this aspect was investigated by delivering malachite green solution into the GC for 15 s and then emptying the GC through the photometer. Afterwards, the procedure was repeated with water and no blank signal change (carryover) was detected in the photometer. The same was observed when $KMnO_4$ solution was used instead of the malachite green. However, if in other applications some organic substance present in the sample matrix were to adhere to the surface of the PTFE materials, carryover might be observed and then a cleaning step would be needed. Cleaning steps can be performed in triplicate by switching valve V_1 ON and pumping a cleaning solution towards the GC for 4.0 s. After that, valve V_3 switched ON and the GC content drained towards the discard flask for 3.0 s using the maximum peristaltic pump flow-rate.

3. Non-linear calibration modelling

The instantaneous concentrations of the sample, dye and standard solution are obtained by associating time in the gradient concentration profile to an instantaneous dilution factor $f_X(t_i)$ [2]. This factor defines the relationship between the original concentration of the standard solution (X = SS), and/or the sample (X = S), and/or the dye solution (X = D) in its container (C_A^o) and their instantaneous concentrations in the detection point ($C_X(t_i)$).

$$f_X(t_i) = \frac{C_X(t_i)}{C_X^o} \quad (1)$$

If the convectional processes are dominant in the GC and the diffusion processes are negligible, $f_X(t_i)$ may be considered independent of the chemical nature of the X solution and of the sample matrix. In this case, $f_D(t_i) = f_{SS}(t_i) = f_S(t_i)$ and the expressions below are valid;

$$\frac{C_{SS}(t_i)}{C_{SS}^o} = \frac{C_D(t_i)}{C_D^o} \quad (2)$$

and

$$\frac{C_A(t_i)}{C_A^o} = \frac{C_D(t_i)}{C_D^o} \quad (3)$$

Otherwise, for the same analyte, sites with the same absorbance imply the same concentration in the $A_S \times$ time and $A_{SS} \times$ time tracings (Fig. 2), although the detections occur in different times.

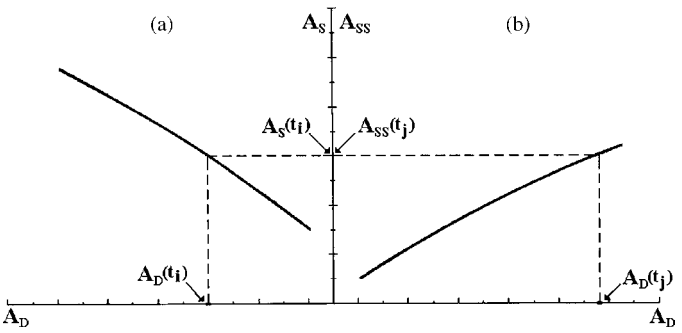

Fig. 3. Graphs relating analytical signals from sample *versus* dye solution (a) and standard *versus* dye solution (b) for the determination of total protein or albumin in blood serum. A_S, A_{SS} and A_D represent absorbance of the sample, standard and dye solutions; each point represents the absorbance related to a given t instant.

Consequently, if $A_S(t_i) = A_{SS}(t_j)$ (see Fig. 3), then

$$C_S(t_i) = C_{SS}(t_j) \quad (4)$$

If the Eqs. (2) and (3) are inserted in the Eq. (4), the following equation is obtained:

$$C_S^o = \frac{C_D(t_j)}{C_D(t_i)C_{SS}^o} \quad (5)$$

With a linear relationship between absorbance and concentration (obedience to Beer's law) for dye solution, then Eq. (5) can be rewritten as:

$$C_S^o = \frac{A_D(t_j)}{A_D(t_i)C_{SS}^o} \quad (6)$$

In the sample gradient, n points $A_S(t_i)$ can be used from its application in Eq. (6), obtaining thus, n C_S^0 values. The final calculation of the analyte concentration is estimated as the mean value of n values of C_S^0:

$$\overline{C_S^o} = \frac{\sum_{i=1}^{n} C_S^o}{n} \quad (7)$$

4. Results and discussion

4.1. Flow–batch parameters

The insertion flow-rates of sample and reagent into the GC were evaluated considering the reagent/sample volumetric ratio (v_R/v_S) of the reference methods. For albumin determination, $v_R/v_S = 5000\,\mu L/20\,\mu L = 250$ and for total protein determination, $v_R/v_S = 5000\,\mu L/100\,\mu L = 50$. Also taken into account was, the precision of the volumes delivered to the GC, as well as the maximum internal volume of the GC (about 2.0 mL). A 7.0 μL sample volume for both determinations was elected, due to the elevated value of v_R/v_S for albumin determination. For total protein determination, larger sample volumes can be taken, however, a 7.0 μL was also chosen in order to reduce the sample and reagent consumption.

Because the errors associated with valve activation depend on its minimum response time, which is about 20 ms (according to the valve manufacturer's data), and the errors inherent to computational processing, a 1.5 s switching ON time for the valve V_1 (t_{si}) was chosen in order to assure a precision for the inserted sample volume (v_{si}) into the GC. To add 7.0 μL using $t_{si} = 1.5$ s, a sample channel flow-rate (q_{Si}) roughly 0.28 mL min^{-1} is necessary. To estimate this flow-rate, valve V_1 was switched ON for 10 s, water (density = 0.9965 ± 0.0001 g cm^{-3} about 27 °C) was pumped into a flask and its weight was measured. The q_{Si} was then estimated at 0.284 ± 0.002 mL min^{-1} ($n = 10$). Thus, the v_{si} can be estimated at 7.1 ± 0.1 μL by using this estimated flow-rate and $t_{si} = 1.5$ s. Employing this estimated volume v_{si} for albumin determination, the inserted reagent volume v_{Ri} inside the GC must be around 1.75 mL in order to maintain v_R/v_S around 250, as established by the reference method. Having a higher analytical frequency and good precision in compromise, the BCG reagent channel flow-rate (q_{Ri}) must be about 7.0 mL min^{-1}. By using the same procedure that was utilized for the sample channel, the q_{Ri} was then estimated at 7.005 ± 0.014 mL min^{-1} ($n = 10$) and the v_{Ri} at 1.751 ± 0.004 mL by employing this same flow-rate and $t_{Ri} = 15.0$ s.

For total protein determination, a v_{Si} about 7.0 μL was also used as described above. The volume v_{Ri} of biuret reagent added inside the GC must be approximately 350.0 μL in order to maintain $v_R/v_S = 50$, as established by the reference method. The v_{Ri} was estimated at 1.751 ± 0.004 mL by using the same estimated q_{Ri} for the albumin determination and $t_{Ri} = 3.0$ s. In Table 1 are summarized other flow–batch parameters for both determinations, which were chosen aiming at larger sample throughput and good precision in the measurements.

4.2. Extrapolations in concentration gradients

One hundred points for the concentration gradients ($A_D(t_i) \times t$, $A_{SS}(t_i) \times t$ or $A_S(t_i) \times t$) were always measured and recorded for time t_{Rg}. However, the number of points used in the Eq. (7) for the calculation of the concentration of the analyte was smaller than this value, normally between 40 and 90, in order to avoid extrapolation problems in data treatment. These problems occur when the maximum absorbance value (A_{Smax}) of the sample profile is larger than the A_{SSmax} of the standard solution profile (Fig. 4a) or when the minimum absorbance value (A_{Smin}) of the sample profile is smaller than the A_{SSmin} of the standard solution profile (Fig. 4b). The extreme cases are illustrated in the Fig. 3c ($A_{Smin} > A_{SSmin}$) and Fig. 3d ($A_{Smax} < A_{SSmin}$) and they require dilution of the sample (in the first case) or of the standard solution (in the second case). This dilution may be performed automatically in flow–batch systems by suitable modifications of the time parameters.

4.3. Kinetics behaviour

Determinations of albumin and total protein are based on reactions which present different kinetic behaviour (Fig. 5). The non-linear calibration model built in this study was able to determine both analytes even when presenting different kinetic behaviours. The calibration is performed by obtaining analytical signals using exactly the same procedure for the dye solution, standard solution and sample. Both analytical signals of sample and standard solution are plotted against the dye solution curve (Fig. 3), thus, the gradient generated into the GC is calibrated. As the kinetics of the sample and standard solutions are the same, their curve profiles are also the same, thus calibrating the kinetic effects. The proposed methodology may be applied for any reaction, not taking into consideration kinetics behaviour.

4.4. Determination of albumin and total protein

Albumin was determined in seven blood serum samples ($n = 5$) using the flow–batch analyser and the reference method ($n = 3$) and total protein was quantified in eight samples in the same way. Concentration values and the standard deviations obtained for both determinations are presented in Table 2.

The mean values obtained with the FBA agreed well with those of the reference method. At a confidence level of 95%, no statistical difference was observed between them when the paired t-test was applied. The overall absolute standard deviation obtained in the determinations for both methods is 0.1 g dL^{-1}.

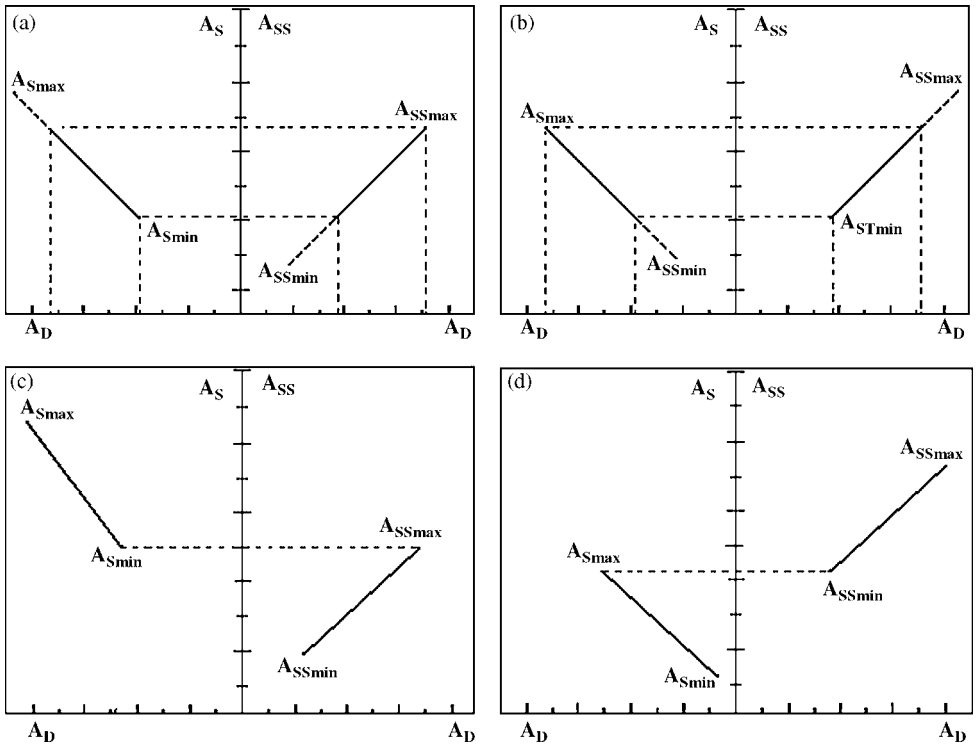

Fig. 4. Extrapolation problems in the gradients of the sample ($A_S(t) \times t$) and of the standard solution ($A_{SS}(t) \times t$). In the cases (a) and (b), the points above A_{Smax} and below A_{STmin} respectively, are discarded. In (c) and (d) it is necessary to dilute the sample or standard solution in order to avoid extrapolation problems.

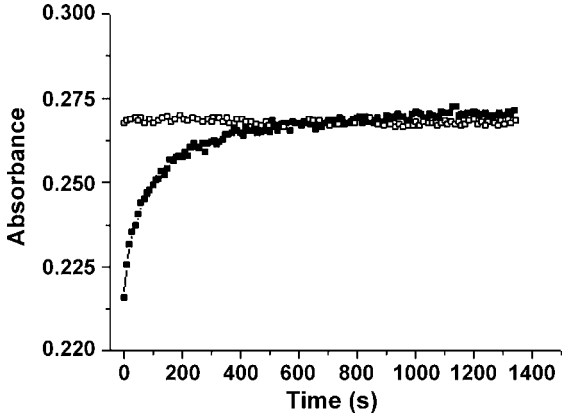

Fig. 5. Kinetic behaviour for total protein (■) and albumin (□) determination.

Table 2
Albumin and total protein concentrations (g dL^{-1}).

	Albumin		Total protein	
Sample	Flow–batch	Reference method	Flow–batch	Reference method
1	4.4 ± 0.1	4.3 ± 0.1	6.3 ± 0.2	6.3 ± 0.1
2	3.7 ± 0.1	3.6 ± 0.2	6.8 ± 0.1	6.8 ± 0.2
3	3.6 ± 0.0	3.9 ± 0.0	7.2 ± 0.2	7.1 ± 0.1
4	4.3 ± 0.1	4.4 ± 0.1	6.1 ± 0.1	6.2 ± 0.1
5	3.8 ± 0.1	4.1 ± 0.1	6.6 ± 0.1	6.6 ± 0.1
6	3.8 ± 0.1	3.9 ± 0.1	7.0 ± 0.0	7.1 ± 0.1
7	4.2 ± 0.1	4.2 ± 0.0	6.6 ± 0.1	6.6 ± 0.1
8	4.4 ± 0.1	4.3 ± 0.1	6.3 ± 0.1	6.3 ± 0.1

With the FBA, the sample throughput was 50 h^{-1} for albumin and 60 h^{-1} for total protein approximately, consuming about 7.0 μL of sample in both determinations and 2.6 mL of BCG reagent and 1.2 mL of biuret reagent.

5. Conclusions

In this study, a flow–batch analyser that exploits concentration gradients for determination of total protein and albumin in blood serum involving slow kinetics reactions was developed. This analyser was applied successfully to these parameters by using only one standard solution available in commercial kits, with low consumption of samples and reagents.

This system also presents the advantage that no manifold alterations are necessary, allowing to perform determinations of other parameters, by changing the operational parameters (time control of the valves, for instance) through the control software, giving great flexibility to the analytical method chosen.

The problems inherent in slow kinetics reactions were avoided with the appropriate use of automated analytical procedure and of non-linear calibration model proposed for the analyser. For both applications, the results were always in good agreement with those of the reference methods.

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