

# An accurate UV/visible method to quantify proteins and enzymes: Impact of aggregation, buffer concentration and the nature of the standard

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## ABSTRACT

A simple, fast and low cost UV/visible based method to quantify proteins or enzymes is presented. This method avoids some drawbacks found using conventional techniques. Representative proteins and enzymes such as bovine serum albumin (BSA), insulin (Ins.), *Rhizomucor meihei* lipase (RML), *Candida rugosa* lipase (CRL) and horseradish peroxidase (HRP) were assayed as model. Experiments revealed that the aggregation of the enzyme/protein molecules in aqueous solution was the main cause of inaccurate results obtained with the simple UV/visible method. It was determined that aggregation of proteins/enzymes in aqueous solutions follows a reversible mechanism that could be reverted by simple magnetic stirring treatment. The results achieved within this study warn about common error sources in protein quantification by UV/visible based methods and clearly shows the magnitude of the mistakes that could be achieved if aggregation and other factors are not considered.

**KEYWORDS:** enzyme, biocatalyst, adsorption, aggregation, UV/visible

## 1. INTRODUCTION

Enzyme immobilization has been widely studied during the last decades [1, 2, 3]. The quantification

of the immobilized enzyme results is crucial for 1) the evaluation of the efficiency of the immobilization procedure and 2) the quantification of the enzymatic activity of the immobilized biocatalyst in selected reactions in per mg of enzyme basis. Reported loading efficiencies for enzymes are determined by testing for residual amount of protein in the immobilization supernatant or by comparisons of enzymatic activity initially and after selected periods of contact with the immobilization support. The UV/Visible spectrophotometric methods used to quantify the protein in enzyme/protein immobilization protocols involve the reaction of lateral groups of the aminoacids of proteins: Bradford [4], Lowry [5, 6] methods and the BiCinchoninic Acid (BCA) assay [7, 8].

The use of ultraviolet absorbance measurement to quantify proteins or enzymes concentrations, without reaction with additional substrates, is the most simple and quick method. Unfortunately, inaccurate results are found when it is applied to supernatants of immobilization procedures [9, 10]. UV/visible-based methods assume that the protein/enzyme in solution is exactly the same before and after the immobilization procedure [11-13]. The decrease of absorbance of the protein/enzyme solution after certain time of contact with a support is correlated with the decrease of concentration. The immobilization step includes magnetic stirring in many reports or the use of shakers in others. When a calibration or standard solution of a protein/enzyme is prepared and measured in the UV/Visible spectrophotometer,

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the stirring time involved in the immobilization is not taken into account. Intermolecular interactions between proteins in aqueous solution lead to aggregates of variable sizes [14] and alter protein structure with loss of activity [15-18]. Detailed report of the experimental conditions about enzymatic or protein immobilization is required to allow reproducibility, such as recent published guidelines emphasized [19]. Recent publications highlight the errors introduced by UV/Visible methods in quantification of proteins during adsorption steps [20, 21].

The general aim of this contribution is to present a protocol for proteins (and enzymes) quantification during their adsorption and immobilization onto bare biopolymeric supports, employing a simple, fast and low cost UV/visible spectrophotometric method. The goal was to avoid or even minimize erroneous results derived from the aggregation of protein molecules, the presence of ionic moieties and the nature of the standard when proteins and/or enzymes are quantified by UV/Visible methods.

Proteins of different origin, average molecular weight and functions were studied such as bovine serum albumin (BSA), insulin (Ins.), *Rhizomucor meihei* lipase (RML), *Candida rugosa* lipase (CRL) and horseradish peroxidase (HRP). The magnitude of the committed mistakes when the above mentioned parameters are not considered is clearly stated. To this end original data on lipase immobilization and protein adsorption assays are also presented within this manuscript.

Finally, the information here discussed may be useful in the practice field of: preparation of biocatalysts by enzyme immobilization, the

formulation of pharmaceutical biomaterials and protein delivery systems (DDS) involving quantification of proteins/enzymes by UV/visible based techniques.

## 2. MATERIALS AND METHODS

Bidistilled water and phosphate buffer pH = 7 were employed as received. Chitosan (CS), commercialized as Chitoclear, was provided by Primex (Iceland). Analytical grade solvents provided by Dorwill (Argentina, SA) were used in all the described procedures. 100 ml of solution of phosphate buffer saline (PBS, pH = 7.4) was prepared from 137 mM NaCl, 2.7 mM KCl, 1.0 mM Na<sub>2</sub>HPO<sub>4</sub> and 1.76 mM KH<sub>2</sub>PO<sub>4</sub>. The insulin (porcine neutral insulin) was supplied by Betasint U-40 (Beta Laboratories, Argentina) as a 30% wt/v aqueous solution. Bovine serum albumin (BSA) was supplied by Laboratories Wiener (Argentina). Horseradish peroxidase and *Candida rugosa* Lipase (CRL) were donated by Amano Inc. (EEUU). *Rhizomucor meihei* lipase (RML) was kindly donated by Novozymes, as a concentrated solution (5000 U/ml). The Table 1 summarizes the proteins and enzymes employed within this work.

### 2.1. Methodology for construction of the calibration curves

Calibration curves for each protein/lipase were obtained using the method of the absorbance at 280 nm. The absorption at  $\lambda = 280$  nm is assigned to the presence of tyrosine, tryptophan and cysteine, whereas bands at lower  $\lambda$ , near 240 nm, are associated to the presence of tryptophan, tyrosine, phenylalanine, histidine, methionine and also to the peptidic bond.

**Table 1.** MW and identification of proteins and enzymes tested.

Protein/Enzyme	Abrev.	Average Molecular weight (KDa)
Insulin	Ins.	5,9
<i>Rhizomucor meihei</i> lipase	RML	39
Horseradish peroxidase	HRP	41
<i>Candida rugosa</i> lipase	CRL	60
Bovine serum albumin	BSA	66

Standard solutions were prepared from pure proteins or enzymes diluting to 3 ml with bidistilled water.

Magnetic stirring of the protein/enzyme solutions was performed during 180 min, the time commonly required for adsorption or immobilization procedures. The Abs. remained constant after 30 min. of stirring at roughly 500 rpm at room temperature.

Two different procedures were therefore implemented for the standard solution:

i) The absorbance value was measured directly, after total dissolution of standard protein, without stirring. This is the case of non-stirred calibration standards.

ii) The absorbance value was measured after magnetic stirring during 30 min at 1000 rpm.

The explored protein/enzymes concentration range was between 0.12 and 12 nanomol/mL of standard solution, considering the molecular weights reported in Table 1. The standard solution could be BSA or the same protein/enzyme to quantify.

## 2.2. Methodology for selected proteins adsorption on biopolymers

Chitosan (CS) was selected as support for the proteins's adsorption. The adsorptions assays were carried out using 12 mg of protein (Ins. and BSA) and 20 mg of support. The incubation was performed in 5ml of PBS, (pH = 7) or bidistilled water at 37°C under vigorous magnetic stirring during 180 min. Aliquots of 200 µL were withdrawn at different intervals of time from the supernatant and diluted to 3 ml with bidistilled water to measure the protein content in the supernatant solution by UV/Visible spectrometry.

The adsorption of HRP in CS was performed using 5 mg of enzyme and 100mg of support in 5 ml of bidistilled water. The solution was magnetically stirred during 180 min. at room temperature. The solution was centrifuged and the supernatant was analyzed by UV/visible spectrometry at 403 nm to determine HRP content.

For the RML immobilization, 0.5 mL of commercial solution from Novo (equivalent to

near 100 mg of precipitable protein (PP) using ammonium sulphate) was added to 5 mL of bidistilled water in a 10 mL vial. The variable of interest was total protein amount in the commercial lipase in this case.

200 mg of CS was incorporated to the lipase solution and vigorously stirred for 4h at room temperature. 200 µL of the RML/water solution was withdrawn before the addition of the solid. Immediately after the addition of the support and at the end of the immobilisation period, 200 µL of supernatant was further withdrawn. The samples were diluted up to 3 mL with bidistilled water for later UV/Visible analysis by Absorbance at 280 nm. Additional experiments were done to test for the impact of dilution in the UV/Visible spectra of buffer and buffer plus protein solution.

Malvern Zetasizer equipment was employed to verify the protein aggregation through the measurement of the hydrodynamic radio. Assays tending to characterize different aggregation states in protein standard solutions have been performed through Dynamic Light Scattering (DLS) tools included in Malvern Zetasizer equipment. The hydrodynamic radios of standard protein solutions with and without stirring have been assayed.

## 3. RESULTS AND DISCUSSION

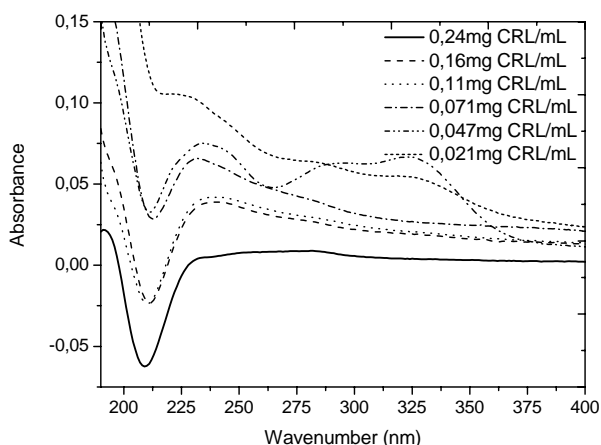
### 3.1. Protein quantification with non-stirred calibration standards

#### 3.1.1. Linear Abs-concentration response

A linear increment of the absorbance (Abs.) was detected on increasing concentrations of BSA, Ins. and RML.

#### 3.1.2. Non linear Abs-concentration response

A noticeable deviation of the linearity of Abs.-concentration plots was found for enzymes CRL and HRP. The deviations and changes in Abs. or spectra shapes are assigned to protein aggregation, based on the literature on the topic [22]. The concentration range employed in the calibration curves shown in Figure 1 was selected based on previous work of our group in lipases [23] or HRP. Even when the absorbance values are low, the results have been duplicated and shown a clear trend. The absorbance decrease at 235 nm increasing CRL concentration can be associated to



**Figure 1.** UV/visible spectra of solutions with different concentrations of CRL in bidistilled water.

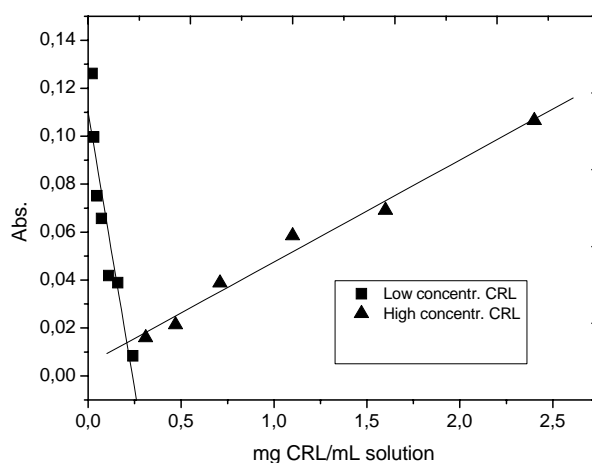
the aggregation of the protein molecules in the aqueous, non-buffered, solution [24].

Using low CRL concentrations (i.e. between 0.021 to 0.24 mg CRL/ml) the Abs. values are assigned to de-aggregated CRL (or minimally aggregated). The increase of the CRL concentration (without previous stirring of the solution) increases proportionally the aggregation, leading to a decrease in the Abs. values (see Figure 2).

In the high concentration range, increasing CRL concentration increased the amount of aggregates of similar sizes and the Abs. response is directly proportional to the CRL concentration. The method is quantifying CRL aggregates. It seems that after a threshold in the concentration of CRL the response in the UV/Visible is proportional to the concentration, even if protein aggregates are present. From Figure 2 this threshold for CRL at r.t in bidistilled water, without previous stirring, is 0.25 mg/ml [23-29]. Increasing the protein concentration increases the total volume occupancy by protein molecules (called “macromolecular crowding” [30]). Similar linear behavior has been reported in the case of  $\beta$ -lactoglobulin [31]. The dilution of the protein solutions reduces or avoids the aggregation [29]. This information correlates with our results for CRL below 0.25 mg/L.

### 3.2. Comparison of results with stirred or non-stirred calibration solutions in bi-distilled water

The calibration curves for the proteins obtained after the stirring process are compared with those

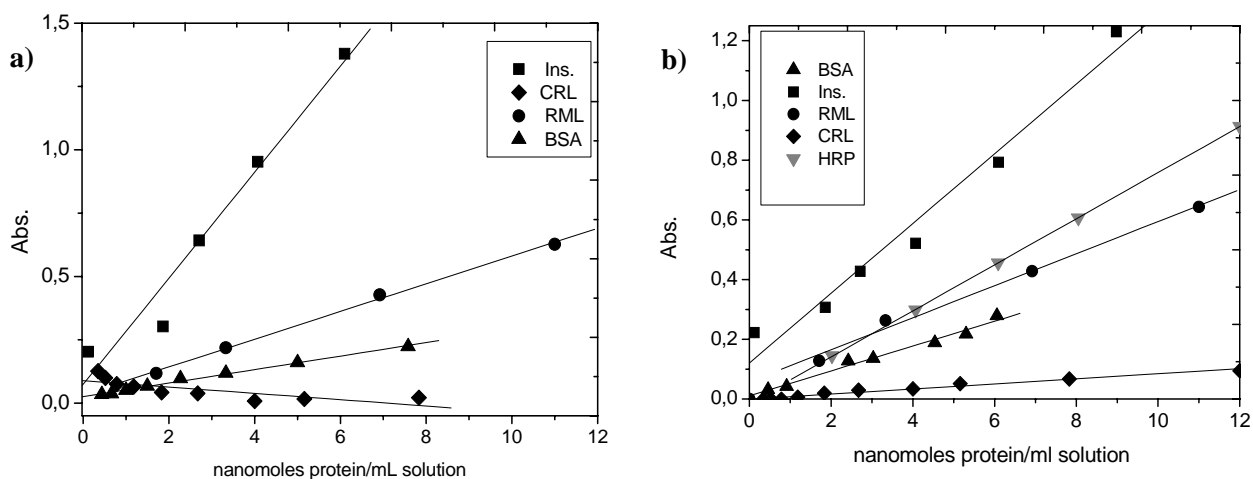


**Figure 2.** Calibration curves of CRL performed in two ranges of concentrations without stirring of the standard solutions.

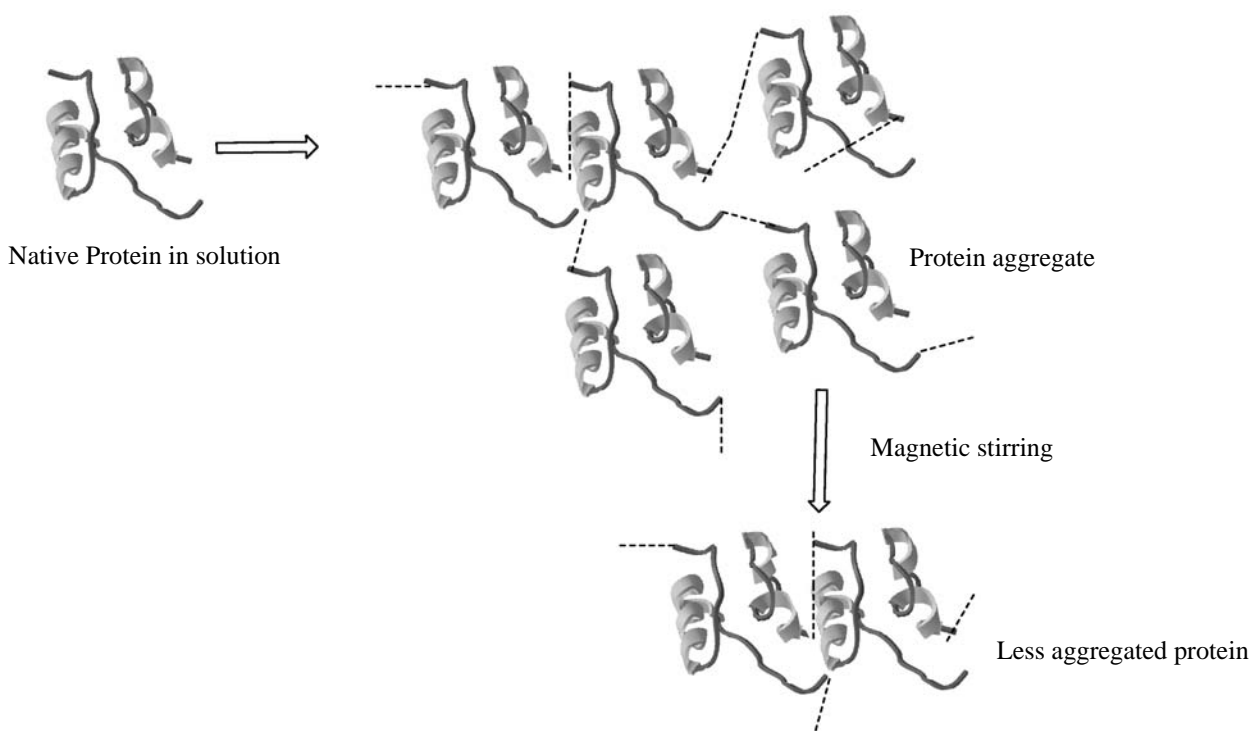
obtained from non-stirred solutions (always in bi-distilled water) in Figure 3. The stirring procedure leads to changes in the UV/Visible spectra, assigned to protein de-aggregation or protein aggregated in aggregates of reduced size (see Scheme 1). Magnetic stirring by several hours is commonly used in proteins or enzymes immobilization. Therefore the key requirement of exactly similar answer in UV/Visible if the same protein concentration is present at the beginning and at the end of an immobilization protocol (that includes stirring by hours) can not be met with non-stirred standard solutions, even if they are homogeneous.

Besides the problems related to protein quantification during lipase immobilization, the aggregation of proteins has been associated with neurodegenerative disorders [32-34]. For more specific and abundant information the authors recommend the reviews of Morris [22]; Roberts [35] and Mahler [36].

The proteins with the highest differences in molecular weight (insulin and BSA) were analyzed. Solutions in bi-distilled water were prepared to obtain BSA and Ins. concentration of 1 mg/ml using a final volume of 3 ml. Two different measurements were done for each protein. One of them was measured immediately after its preparation while the other was stirred during 30 min. The hydrodynamic radii ( $r$  in nm) measured in each solution are shown in Figure 4. It is important to



**Figure 3. a.** Calibration curves of different proteins and lipases performed without stirring of the standard solutions; **b.** Calibration curves of different proteins and lipases performed after stirring of the standard solutions.



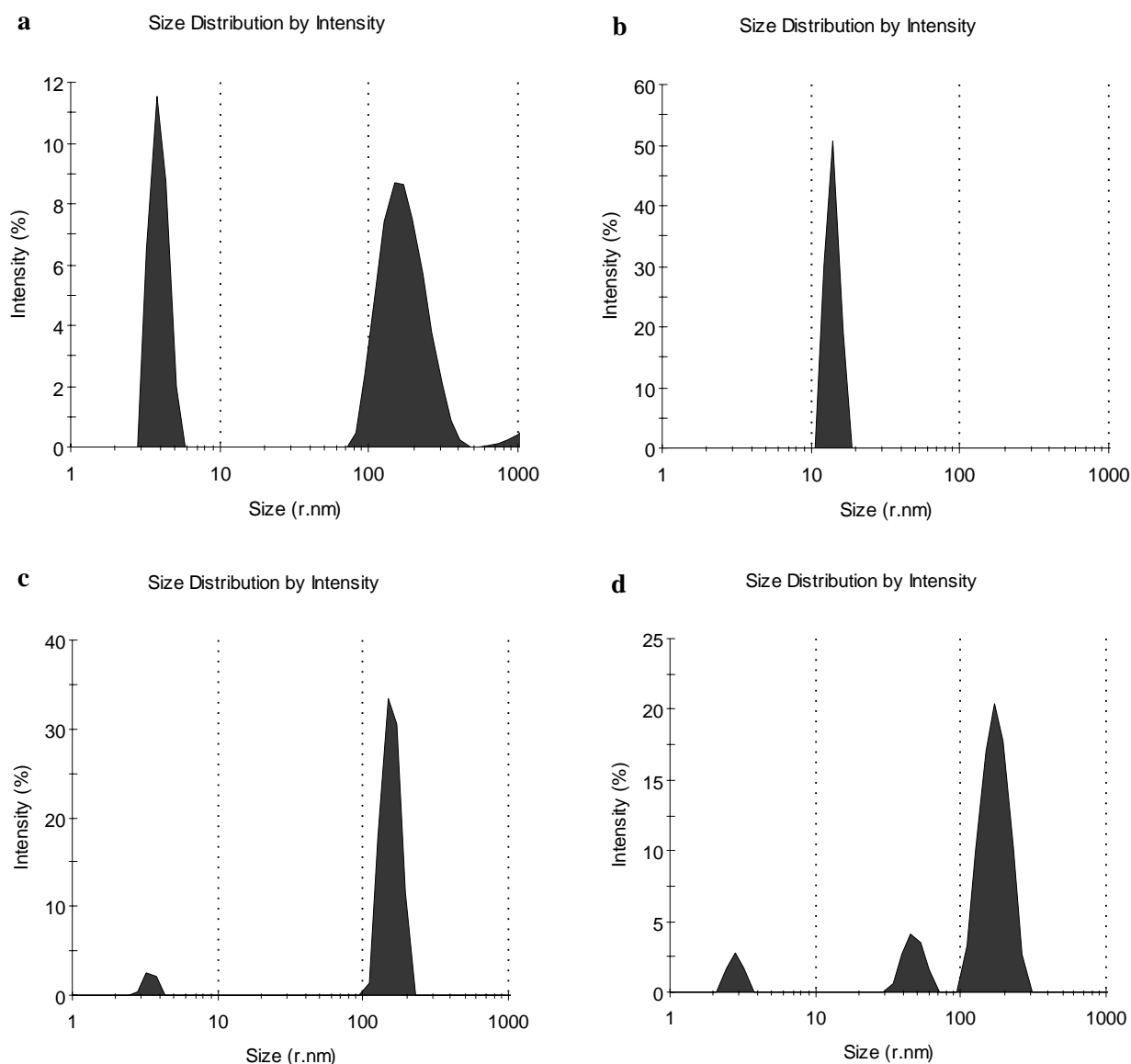
**Scheme 1.** Representation of the effect of the stirring on the protein aggregation.

note that the concentration of the assayed solutions was in the same order than the corresponding to the calibration curves.

It was found that the BSA radius varies from 57.34 nm using the non-stirred solution, to 10.97 nm when the same solution is stirred. It is

also worth noting, comparing Figure 4a and b, that the solution without stirring is polydisperse since aggregates of different sizes are detected.

An estimation of the average molecular weight (Mw) could be obtained from these r data. It reveals that in the case of non-stirred solution



**Figure 4.** Dynamic light scattering plots, radii ( $r$ ) vs. intensity (%) of: **a.** BSA solution (1 mg/ml bidistilled water) without stirring; **b.** BSA solution (1 mg/ml bidistilled water) with stirring; **c.** Ins. solution (1 mg/ml bidistilled water) without stirring; **d.** BSA solution (1 mg/ml bidistilled water) with stirring.

the Mw of BSA would be roughly 8655 kDa, indicating that enormous aggregation of the BSA molecules occurs in bi-distilled water. In the case of the stirred solution Mw value was 180 kDa. These assays reveal that the aggregation is highly reduced after the stirring treatment, in fact the Mw of stirred solution is 48 times lower than Mw of non stirred one for BSA. However, it is not eliminated. Remember that BSA Mw is 66 kDa.

In the case of Insulin the aggregation tendency is more marked. The  $r$  value of the non-stirred

solution is about 441.8 nm while the value descends to 244.5 nm when stirred solution is assayed, as it is shown in Figure 4c and d. This represents an increment of almost 225% in the hydrodynamic radio employing Ins. solution without stirring treatments. The average molecular weight calculated from  $r$  data suggest that the aggregation trend is higher than in the BSA case. The Mw calculated for non stirred insulin solution was almost  $1.028 \times 10^5$  kDa; while a Mw of  $2.56 \times 10^4$  kDa is reached using the stirred solution.

Remember that the Ins. has a Mw of only 5,9 kDa and it has a hexameric structure.

It is important to highlight that the method for estimating molecular weight from light scattering data is to measure the hydrodynamic size by DLS and then to estimate the molecular weight from a size vs. mass calibration curve - similar to the approach used in size exclusion chromatography. In this case it was used to better visualize the difference in the different aggregation states but it must be considered as an approximated Mw only, especially in the case of low molecular weight proteins such as Ins. [37].

Results from DLS reveal that: i-in both cases (stirred and non-stirred) both BSA and Ins. molecules are associated forming aggregates of different sizes; ii-the aggregation level is higher in the case of lower molecular weight protein; iii-that the level of aggregation is significantly reduced by the stirring treatment.

The reversibility of protein aggregation is a hypothesis supported by several authors [35, 38]. The strategy proposed here to revert (or minimize) the differences in the UV/Visible spectrum of aggregated vs. non-aggregated (or minimally aggregated) enzymes or proteins is a simple magnetic stirring of the protein/enzymes solution, freshly prepared in bi-distilled water by dissolution or dilution of the enzyme/protein, until constant absorbance is found at room temperature. This is applicable to calibration or standard solutions in enzymes or proteins immobilization protocols.

Almost no variation is found with or without stirring, using bi-distilled water as the media for dilution of the commercial RML (see Table 2). Even when the number of tested proteins is not high, the low molecular weight protein showed a high error in the quantification. For BSA and CRL, both of comparable Mn (near 60 kDa), the quantification showed errors by defect in the range of 30-36% comparing the use of stirred vs non-stirred standards. BSA aggregates easily because of the formation of incorrect inter-molecular salt bridges [39]. Every protein /enzyme is different and must be analyzed separately.

### 3.3. Effect on the protein identity on quantification

The Table 3 shows the  $\lambda_{\max}$  used to measure the protein/enzyme content by UV/visible. From these data it is evident that no differences are found in stirred and non-stirred standards solution (see entries 2 and 3 in Table 3). The use of  $\lambda_{\max}$  to measure Abs. is desirable but not necessary, since the shape of the curve is typical of a protein structure [40]. The differences observed in the spectra shapes for Ins. and BSA shown in Figure 5 indicate that different protein structures are present in stirred and non-stirred solutions. These protein structures are probably the monomeric (or less aggregated) state in stirred solutions vs. the more aggregated structure observed in non-stirred solutions [41].

The potential of further problems when reactions of lateral groups are involved is very high (Bradford, Lowry or BCA protocols). BSA is the

**Table 2.** Parameters derived from the linear fit of the calibration curves performed with and without stirring of the standard solutions.

Protein	Without stirring		With stirring		Error (%) <sup>(1)</sup>
	Slope	$r^2$	Slope	$r^2$	
Ins.	0.2017	0.9956	0.1168	0.9941	+72,7
RML	0.05475	0.9987	0.05363	0.9960	+2
HRP	( <sup>2</sup> )		0.07715	1	-
CRL	0.04257	0.9919	0.06077	0.9946	-30
BSA	0.02670	0.9967	0.04163	0.9914	-36

<sup>(1)</sup> The error in the slopes is calculated considering the true value as the obtained with stirring.

<sup>(2)</sup> Reproducible results, suitable to elaborate a calibration curve, were not obtained in this case. Values of absorbance were in the 0,01 range and lower for all the concentration range..

standard protein that comes with the protein assays when purchased as a kit. Absurd results could be obtained using the simple Abs. method to quantify immobilized lipase using BSA as standard when other proteins are intended to be quantified [41]. Commercially available BSA contains different amounts of monomeric (76,5%), dimeric (16,1%) and polymeric protein molecules (7,4%) [42, 43]. Stoscheck *et al.* claimed that to estimate the concentration of pure proteins can be very inaccurate depending on the principle of the assay, unless the same pure protein is used as a standard [41, 44]. Therefore, reactivity of lateral

groups of BSA with the reagents involved in the commercial kits is expected to be different than other proteins or enzymes.

### 3.4. Other error sources during protein quantification: Effect of the buffer and the effective ionic force

The Figure 6 compares the calibration curves of insulin performed in PBS and in bi-distilled water, without stirring of the standard solutions. From the plot in Figure 5 it is observed that the Abs. registered for the lowest Ins. concentration ( $2.7 \times 10^{-3}$  and  $7.02 \times 10^{-3}$   $\mu\text{mol/L}$  for the PBS and bi-distilled water curves, respectively) is very different. The spectrum obtained for Ins. using aqueous solution of PBS as solvent shows an Abs. value of 0.69 while with bi-distilled water the minimal Abs. value is 0.20 at 270 nm. This discrepancy could be associated to interferences of ionic products derived from the PBS ionization that may interact with the protein moieties. The absolute absorbance at zero concentration is 0,046 in bidistilled water whereas it is 0,536 in PBS.

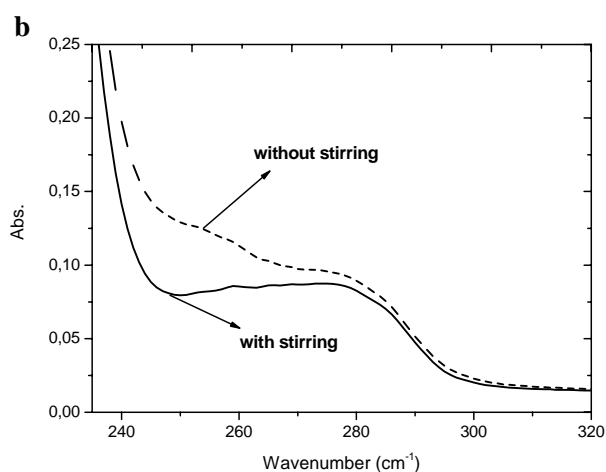
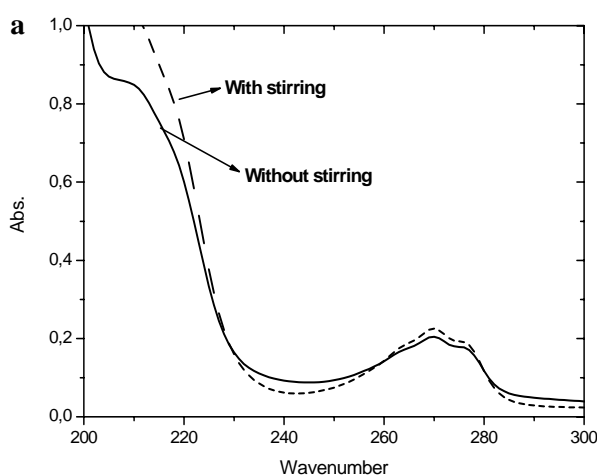
Hofmeister [45] reported that inorganic salts and ions showed different abilities of precipitating proteins. The UV/Visible spectra of buffers and diluted buffers show that there is an important “negative band” that increases with dilution and decreases with the increase of the buffer concentration (pH 7 buffer). The increase of protein or buffer concentration decreases the importance

**Table 3.**  $\lambda_{\text{max}}$ . corresponding to the maximum Absorbance value used to elaborate the calibration curves using the standards solutions with and without stirring.

Protein/ Enzyme	$\lambda_{\text{max}}$ . (nm) without stirring	$\lambda_{\text{max}}$ . (nm) with stirring
Ins.	267	270
BSA	276	276
CRL	235 <sup>(1)</sup> 278 <sup>(2)</sup>	235 <sup>(1)</sup> 276 <sup>(2)</sup>
RML	254	254
HRP	402	402

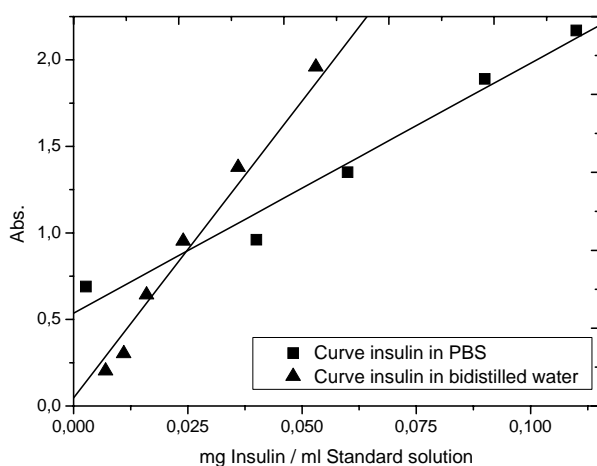
<sup>(1)</sup>Low concentration

<sup>(2)</sup>More concentrated



**Figure 5. a.** UV/visible spectrum of standard solutions of insulin performed with and without stirring; **b.** UV/visible spectrum of standard solutions of BSA performed with and without stirring.





**Figure 6.** Calibration curves of Ins. in bi-distilled water and in PBS media without stirring of the standard solutions.

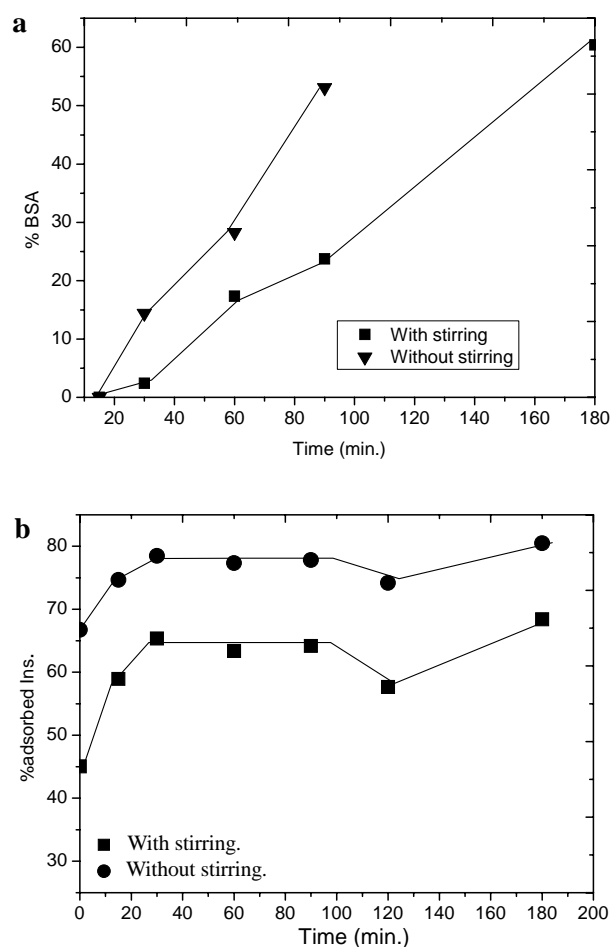
of this band (Results not shown). The adsorption of ions from the commercial solid powder on the CRL is a plausible explanation of the apparent “negative band” from 180 to 230 nm in the CRL spectra of a concentrated CRL solution shown in Figure 1.

### 3.5. Impact of stirring treatments and the identity of the standard on quantification of proteins in adsorption or immobilization procedures

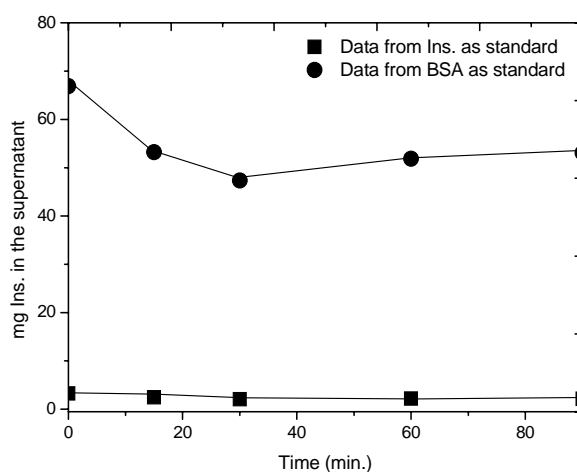
The results of the adsorption of BSA and insulin onto chitosan are presented in the Figure 7. The use of non-stirred calibration solutions leads to erroneous estimation of the efficiency of protein adsorption or efficiency of enzyme immobilization using the simplest method for protein quantification.

The same procedure should be implemented in the treatment of the standard solutions to construct the calibration curve to assure the presence of the same species in solution, *of the same protein than the one to quantify*. Special care should be taken looking at the stirring time and stirring design, temperature and media ionic force [46, 47].

In Figure 8 the magnitude of the error in protein adsorption is illustrated when using BSA as standard -in this case- for the determination of the amount of insulin adsorbed in chitosan support. The initial amount of insulin obtained using the BSA calibration curve gave levels of insulin in solution considerably higher than the *real* initial



**Figure 7. a.** Adsorption profile of BSA in CS; **b.** Adsorption profile of Ins. in CS.



**Figure 8.** Adsorption profile of Ins. in CS, using Ins. and BSA as standard.

**Table 4.** Immobilization data, expressed as mg enzyme/ml sol., calculated from stirred and non-stirred calibration curves and using different protein as standard.

mg enzyme/ml sol. <sup>(1)</sup>			Error (%) <sup>(3)</sup>	mg enzyme/ml sol. <sup>(2)</sup>		Error (%) <sup>(4)</sup>
(5)	Enzyme as standard	BSA as standard		With stirring	Without stirring	
HRP	0.55	1.72	+215	0.55	~536	+980
RML	0.12	0.25	+107	0.12	0.145	+20

<sup>(1)</sup>Calculated from curves using the same enzyme or BSA as standards, with stirring of calibration solutions.

<sup>(2)</sup>Calculated from curves corresponding to each enzyme, with and without stirring.

<sup>(3)</sup>Calculated considering the true value as the Enzyme as standard

<sup>(4)</sup>Calculated considering the true value the one obtained with stirring of the standard solution

<sup>(5)</sup>Initial amount of lipases: 20.5 mg protein in RML/ml solution; 1 mg HRP/ml solution.

amount of protein incubated for the adsorption test (12 mg).

The results of HRP and RML immobilization in chitosan are presented in Table 4. When using BSA as standard and with stirring of the standard solutions, the percentage of error for HRP and RML is 215 and 107% respectively. Tremendous mistakes can be made because in many cases absurd results are not evident. A huge inaccuracy in the determination of the amount of immobilized HRP was found using non-stirred standard solutions (+980%), whereas in the case of the RML the associated error was about +20% (see data on Table 4).

### CONCLUDING REMARKS

This contribution warns about three particular sources of mistakes in quantification of proteins using a simple UV/Visible method: i-the use of a protein as standard different than the one to quantify; ii-the lack of stirring of the standard solution before the obtention of the calibration curve. This step should be done with the same design than the sample (i.e. shaking or magnetic stirring).iii-the lack of control of the ionic force in the sample and the blank solutions used in the spectrophotometric method (in terms of kind and effective concentration of ions, when buffers are used). These mistakes can be avoided (or minimized) with i-previous stirring of the standard solution until constant spectra shape and absorbance are found; ii- the selection of an adequate stirring time to reach the constant absorbance; iii-the use of the same protein as

standard than the one to quantify; iv- the adequate control of the blank and sample effective ionic force and ionic concentration v- using bidistilled water as an immobilization media instead of buffered media. If buffer is used at the immobilization solution and the sample includes buffer, the blank should include it also, at the same dilution than the sample. However, this procedure only minimizes the potential mistake, but it can not avoid it completely. Due to the unexpected and unpredictable change of the buffer ions concentration related to ions adsorption on the proteins in the solution, the best solvent and blank are bi-distilled water. Otherwise, a careful study of the impact of the buffer concentration in the enzyme/protein solution UV/Visible spectra should be included.

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