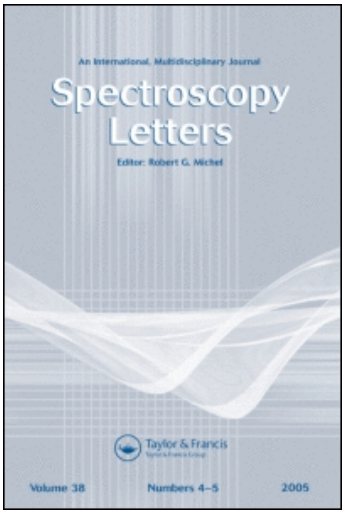

Spectroscopy Letters
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597299

Microquantification of Proteins by Spectrophotometry. Part I: From 190 nm to 1100 nm, Selection of Wavelengths

Pascal Baret[a]; Ariane Angeloff[a]; Claude Rouch[a]; Michel Pabion[a]; Frédéric Cadet[a]
[a] Laboratoire de Biochlmie, Faculté des Sciences, Université de la Réunion, Réunion, France-Dom

To cite this Article Baret, Pascal , Angeloff, Ariane , Rouch, Claude , Pabion, Michel and Cadet, Frédéric(1997) 'Microquantification of Proteins by Spectrophotometry. Part I: From 190 nm to 1100 nm, Selection of Wavelengths', Spectroscopy Letters, 30: 6, 1067 — 1088
To link to this Article: DOI: 10.1080/00387019708006708
URL: http://dx.doi.org/10.1080/00387019708006708

SPECTROSCOPY LETTERS, 30(6), 1067-1088 (1997)

MICROQUANTIFICATION OF PROTEINS BY SPECTROPHOTOMETRY. PART I : FROM 190 NM TO 1100 NM , SELECTION OF WAVELENGTHS

Key Words : far U.V., protein microquantification, enzyme.

Pascal BARET, Ariane ANGELOFF, Claude ROUCH, Michel PABION and Frédéric CADET*

Laboratoire de Biochimie, Faculté des Sciences, Université de la Réunion
15 avenue René Cassin. BP 7151, 97715 Saint-Denis Messag Cedex 9
Réunion, France-Dom.

ABSTRACT

Several methods for protein determination have been described. In almost all cases, a detection reagent is involved (Coomassie Blue reagent, bicinchoninic acid, Folin reagent). Proteins quantification determination by measurements in the U.V. region[1] have been

* to whom correspondence should be addressed

abandonned because of the weak sensitivity of the apparatus (concentrations of the order of 100 µg/ml) and because of the lack of precision at low wavelengths (far U.V.).

Owing to the progress in the performance of equipments, (i) we have been able to show that the reliability of current equipments allowed measurements in the more sensitivity range of proteins (190 to 220 nm) and beyond (220 to 1100 nm) and (ii) we have hence calculated the correlation factors between absorbance values and wavelengths for 17 proteins. It was found that 190 nm and 277 nm were the best wavelengths in far U.V. region for the protein quantification (correlation factor respectively of 0.69 and 0.62).

The concentrations of 16 proteins were predicted at 190 nm and 277 nm, with lactoglobulin (standard range between 0 and 35 µg/ml, and 0 to 3.5 µg/ml) and trypsinogen (standard range between 0 and 35 µg/ml) respectively as standards. The results were better (especially at 190 nm) than those achieved with the BCA method (indirect quantification of proteins with the Bicinchoninic Acid method), one of the mostly used method at the moment.

The main interest of such a quantification method is in its non damaging effects on proteins. Indeed, since HPLC is the mostly used purification methods, tiny quantities of proteins are recovered ; it is hence necessary to save them for physico-chemical characterisation (molecular weight, secondary and quaternary structures ...) and in case of enzymes, for kinetic studies.

INTRODUCTION

Among all the spectroscopic techniques used in biochemical analysis, U.V./Vis predominates for protein determination. Absorption spectra in U.V./Vis are due to electronic transitions, within molecules, between the ground state and the excited state (after light absorption). This absorption depends on wavelength itself, which in turn is function of the electronic structure of the molecule between the two states, and also of the chromophore environment.

The analysis is based on the study of molecule chromophores : proteins must be considered as a complex system of groups of chromophores. These can be divided into three types : the amide bond, the aromatic amino acids and others amino acid side chains.

The amide chromophore shows absorption peaks at many wavelengths, but the strongest ones are at 165 nm and 190 nm. Also aromatic amino acids are good chromophores : phenylalanine (phenyl residue : λ_{max} : 190 nm), tyrosine (phenol residue : λ_{max} : 192 nm and 220 nm), tryptophan (indole residue λ_{max} : 195-196 nm). Finally, there is another group of chromophores less sensitive than those of the first two groups : histidine (imidazole residue : λ_{max} : 190 nm and 212 nm) and sulfur-containing amino acids (as cysteine and methionine, which also absorb in the far U.V.)

In the **Table I**, a summary of different methods for protein determination is given. These methods are efficient but they have a major disadvantage as they include compounds that interact with

Table I : Different proteins quantification methods

METHOD	PRINCIPLE	Wavelength	Concentration Range	Reported Advantages	Reported Disadvantages
BIURET	dilute copper sulfate in strong alcali	540 nm	500 à 5000 µg/ml		
LOWRY[2]	copper sulfate and Folin reagent	750 nm	5 à 200 µg/ml	More sensitive than the biuret method	Susceptible to interference (notably from phenols)
LOWRY-LARSON[3]	copper sulfate	740 nm	3 à 200 µg/ml	Faster and slightly more sensitive than the standard Lowry method	
BRADFORD[4]	Coomassie Blue reagent in dilute acid solutions	595 nm	1 à 50 µg/ml	High sensitivity, speed and ease-of-use, few interfering substances	Sensitive to detergents, response different from a protein to another
BCA[5]	Bicinchoninic Acid	562 nm	0.5 à 400 µg/ml	Less susceptible to interference than the Lowry method and the reagent is more stable	Sensitive to ?,copper ?, thiols, ascorbic acid et ureic acid
Trinitrobenzene Sulfonate[6]	hydrolysis with HCl then addition of TNBS reagent	416 nm	0.5 à 100 µg/ml	Independent of the type of protein being assayed and it is more sensitive than the Lowry or Bradford methods	Hydrolysed ptrotein support
Direct U.V[7].	no reagent	280 nm	20 à 2000 µg/ml		
Methode de Woods[8]	direct	187 nm	0.1 à 25 µg/ml	Good sensitivity	
Waddell[9]	direct	215-225 nm	< 20 µg/ml		
Tombs[10]	direct	210 nm	2µg/ml		
Fluoroaldéhyde[a]	*o*-p htalaldéhyde	ex: 330-390 nm em: 436-475 nm	50 ng à 25 µg/ml	Compatible with most of detergents, ?, ?	Unstable fluorescent compound

[a] from Interchim notes. Abbreviations : DTT : DiThioThreitol, BCA : BiCinchoninic Acid, TNBS : TriNitroBenzene Sulfonate.

proteins (which are later on unusable). Since HPLC is mostly used as purification method, tiny quantities of proteins are recovered and it is necessary to keep them for physico-chemical characterisation (molecular weight, secondary and quaternary structures ...) and kinetic studies in case of enzymes.

This paper reports a direct method for determination of protein concentration, which prevents proteins from denaturation. After verification of the reliability of the spectrophotometer, the correlation factor between chemical values (protein concentrations) and every wavelength has been calculated. The most sensitive region were hence defined for quantification and measurements were carried out with proteins possessing different characteristics (molecular weight, hydrophobicity, secondary structure ...)

MATERIALS AND METHODS

The set of studied proteins was in **Table II**. Proteins were purchased from Sigma Chemicals.

Samples of proteins were prepared into aliquots (1.5 ml) in aqueous solution of MilliQ water (Waters Inc.). The preparation of the samples required specific equipements : pipetman Gilson (10 µl to 1000 µl). Measurements were achieved at room temperature (27 °C). There was no need for detection reagents because of direct quantification determination. Standards were prepared with stock solution of lactoglobulin at 1 mg/ml and a stock solution of trypsinogene at 1 mg/ml.

Table II: Set of studied proteins

Proteins	Reference SIGMA	M.W (Da)	Origin	Hydrophobicity[11]	Secondary structure[12]		
					% α screew	% β sheet	Disordered
α-chymotrypsin	C 4129	25000	Bovine	-	11	50	39
α-chymotrypsinogen A	C 4879	25700	-	1040	12	50	-
α-lactalbumin	L 5385	14200	Bovine	1150	45	5	50
β-galactosidase	G 6008	540000	E.Coli	1160	-	-	-
β-lactoglobulin	L 0130	35000	Bovine	1230	6	46	48
BSA	A 2153	68000	Bovine	1120	66	3	31
Casein κ	C 0406	29000	-	1200	7	37	56
Cytochrome C	C 2506	11700	Horse	1110	39	-	-
Hemoglobin	H 0256	64500	Mammals	-	86	0	14
Lysozym	L 6876	14400	Egg white	970	46	19	35
Ovalbumin	A 7641	45000	Chicken	1110	25	26	49
Papain	P 4762	21000	-	1150	28	24	48
Pepsin	P 6887	35000	-	1080	10	33	57
Phosphorylase B	P 6635	185000	Rabbit	-	-	-	-
Ribonuclease A	R 5000	13700	Bovine	870	23	46	31
Trypsin	T 7418	24000	Bovine	-	7	45	48
Trypsinogen	T 1143	24000	Bovine	940	9	56	35

Spectra were achieved with a Hewlett Packard spectrophotometer (HP 8453 U.V./Vis) . The optical performances are : 190 nm and 1100 nm wavelength range, 1 nm slit width, < 0.05 % stray light, baseline flatness < 0.001 A. Thereafter, absorbance values were treated as "*dx*" files under compatible *PC*, each file corresponding to a protein.

Blank was made on MilliQ water.

QS Hellma cuvettes was used for UV-visible spectra. This cuvettes have more than 80% transmission for wavelengths from 190 to 2500 nm.

RESULTS AND DISCUSSION

Reliability of the spectrophotometer

One of the arguments commonly used against measurements in the far U.V. is instrumental limitations and reliability of absorbance values between 190 nm and 200 nm. Since then, equipments have been continually improved and we proposed to verify if this point of view is valid as ever. **Figure 1** gives the standard deviation calculated for five successive measurements of BSA (Bovine Serum Albumin) solution (2.5 µg/ml) of over the wavelength range covered by the spectrophotometer.

The results show that the spectrophotometer is quite reliable on the range studied. The standard deviation calculated between 190 nm and 200 nm is different from zero, but its value is however extremely low

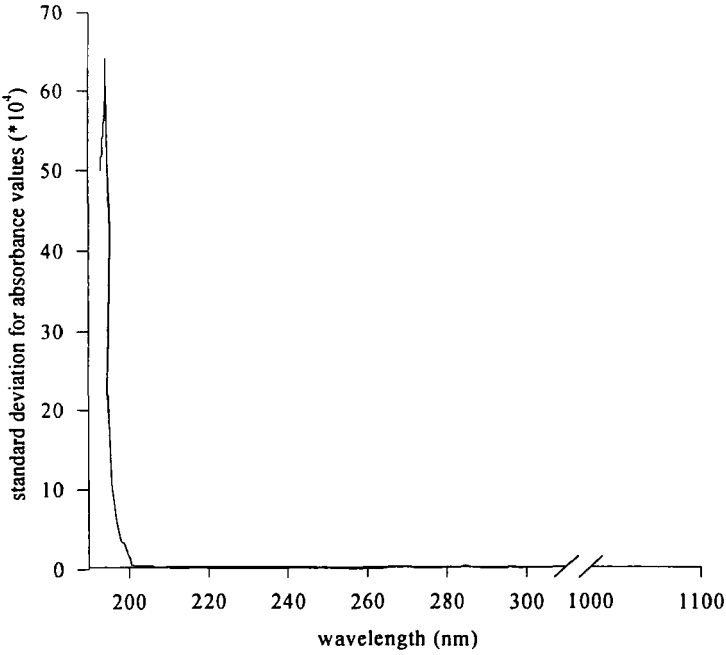

Figure 1 : reliability of the spectrophotometer

($6.3.\ 10^{-3}$) : the mistake made on the absorbance value readed is quite insignificant : 0.28 % of the absorbance (2.2 +/- 6.3. 10^-3 absorbance units).

Choice of suitable wavelength :

The proposal of a new method for determination of proteins depends on the choice of a suitable wavelength. The first operation consisted in choosing the best wavelength. In first instance, we have collected the spectra of samples of the various proteins at the concentration of 25 µg/ml (**Figure 2, a-d**). Expanded curves shown that : (i) all the

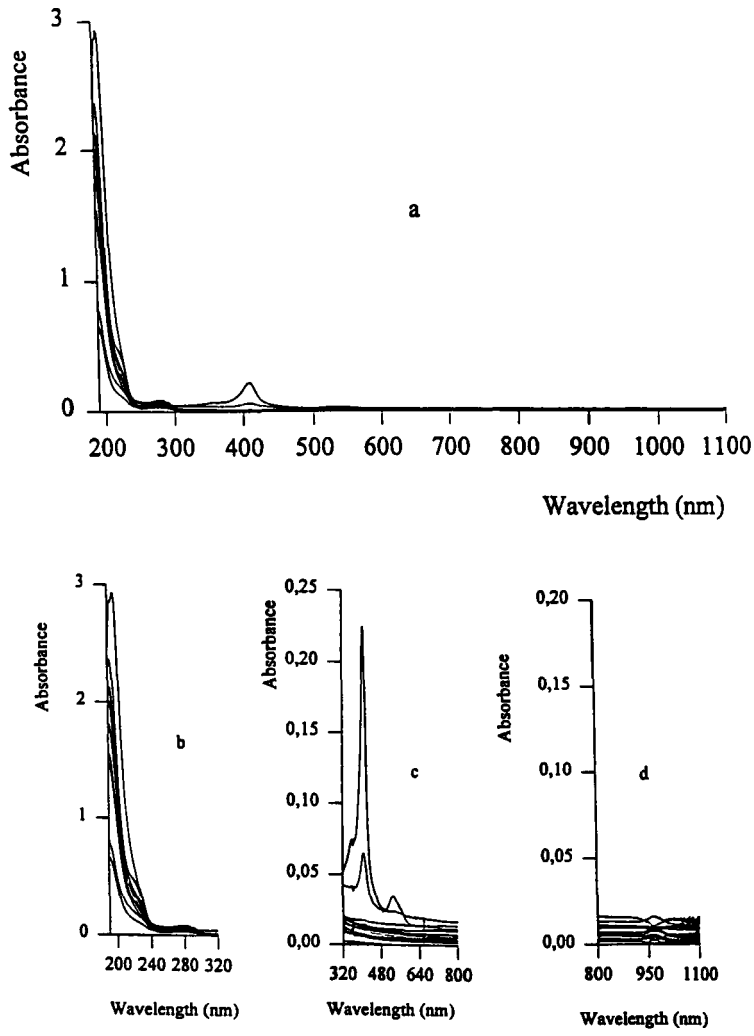

Figure 2 : Spectra from 190 to 1100 nm of 17 proteins (25µg/ml)

(a) Total spectra.

Details from : (b) 190 to 320 nm.

(c) 320 to 800 nm.

(d) 800 to 1100 nm.

standards showed strong absorbance in the region 190-320 nm, (ii) only two of the standards showed strong absorbance in the region 320-800 nm, (iii) the absorbance exhibited in the 800-1100 nm range is very slight and is observed for only few proteins. Proteins have been choosen in such a way to have a large range of molecular weight (11 700 Da to 185 000 Da), large scale of hydrophobicity (970 to 1230 units) and varied secondary structures.

When analysing the resulting spectra, we can notice that they are quite differents from one another. However, we can see that the most intense response for all protein solutions to a U.V. light exposure is located in the region of the far U.V.

Figure 3 gives the standard deviation of the absorbance values for the whole proteins between 190 nm and 1100 nm. It illustrates that the highest sensitive zone is located between 190 nm and 220 nm. As far as sensivity is concerned, 280 nm appears to be quite uninteresting.

With the aim of determining the most appropriate wavelength(s) for our study, we have calculated the correlation factor between the chemical values (solutions at 25 μg protein/ml) and each wavelength (**figure 4**). The correlation factor goes from 0.027 to 0.69 respectively for 1098 nm and 190 nm. This illustrates that the response of proteins in terms of intensity and absorbance values is more correlated at 190 nm than at 1098 nm. Others wavelengths with a correlation factor near 0.6 can be noticed : 195 nm (correlation factor : 0.61), 219 nm (correlation factor : 0.59), 277 nm (correlation factor : 0.62).

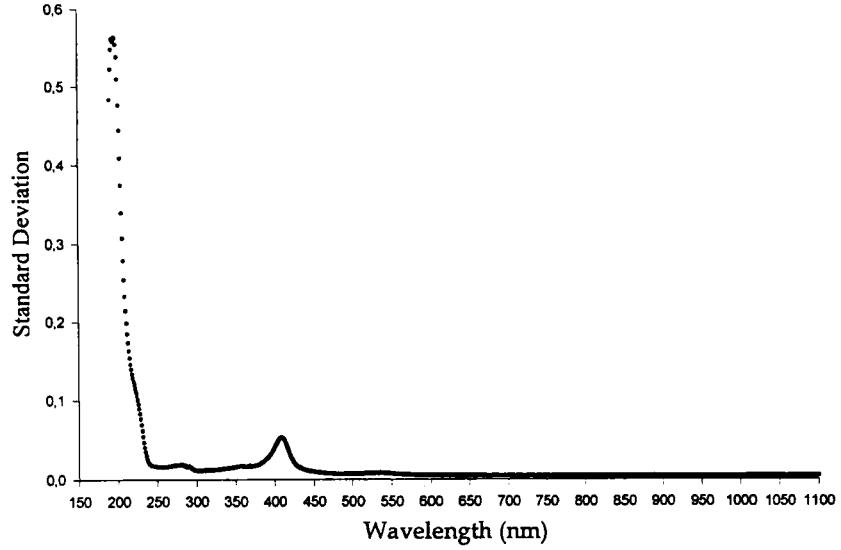

Figure 3 : Standard deviation for absorbance values of the 17 proteins as a function of wavelength

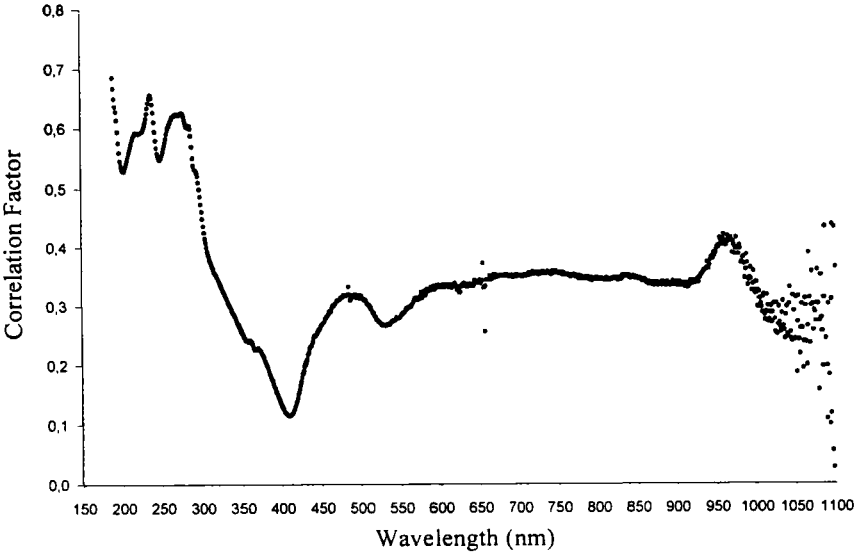

Figure 4 : Correlation factor between absorbance values and protein concentrations as a function of wavelength

Table IIIa : determination of the standard at 190 nm and 195 nm

Protein (25 µg/ml)	A 190 nm	Protein (25 µg/ml)	A 195 nm
ovalbumin	2.43063	ovalbumin	2.87840
papain	2.21516	papain	2.23891
trypsin	2.0496	trypsin	1.99985
lactalbumin	2.03266	lactalbumin	1.99200
casein	2.01196	casein	1.92649
lysosym	1.98436	lysosym	1.87080
cytochrome c	1.95892	cytochrome c	1.86473
trypsinogen	1.94240	trypsinogen	1.82669
pepsine	1.91817	pepsine	1.79798
chymotrypsinogen	1.88268	chymotrypsinogen	1.74977
galactosidase	1.77012	galactosidase	1.65740
lactoglobulin	**1.76690**	**lactoglobulin**	**1.61631**
ribonuclease	1.73619	ribonuclease	1.38138
BSA	1.53720	BSA	1.30597
chymotrypsin	1.47001	chymotrypsin	1.00272
hemoglobin	0.78049	hemoglobin	0.68854
phosphorylase	0.66010	phosphorylase	0.58647
mean	**1.77339**	**mean**	**1.66967**
standard deviation	0.45706	**standard deviation**	0.55841

Hypochromism is a phenomenon long familiar in the nucleic acid field. A similar situation is known to prevail in α-helical polypeptides and proteins. Gases have a system of absorption bands, the Schumann-Runge bands, which extend from just below 200 nm downward. Below about 195 nm, it is well known that the oxygen bands become intense. Nevertheless, despite these interfering phenomenons (hypochromicity due to secondary structure and oxygen absorption), 190 nm remains the most correlated wavelength to the family of 17 proteins.

Table IIIb : Choice of the standard at 219 nm and 277 nm

Protein (25 µg/ml)	A 219 nm	Protein (25 µg/ml)	A 277 nm
ovalbumin	0.64596	ovalbumin	0.04224
papain	0.47412	papain	0.07662
trypsin	0.38878	trypsin	0.06160
lactalbumin	0.47713	lactalbumin	0.07241
casein	0.39362	casein	0.05053
lysosym	0.49113	lysosym	0.08803
cytochrome c	0.40595	cytochrome c	0.07394
trypsinogen	**0.35727**	**trypsinogen**	**0.05406**
pepsine	0.34149	pepsine	0.04584
chymotrypsinogen	0.39832	chymotrypsinogen	0.06720
galactosidase	0.36660	galactosidase	0.05987
lactoglobulin	0.30037	lactoglobulin	0.03557
ribonuclease	0.16119	ribonuclease	0.03230
BSA	0.26102	BSA	0.03498
chymotrypsin	0.30371	chymotrypsin	0.05049
hemoglobin	0.20419	hemoglobin	0.06503
phosphorylase	0.13437	phosphorylase	0.03471
mean	**0.35913**	**mean**	**0.05561**
standard deviation	0.12818	**standard deviation**	0.01682

Water (and water vapor) is also known to absorb around these wavelengths. Nevertheless, water absorbs below 190 nm, the absorbance becoming important and very large at 183 nm and below. The interfering effect of water is weak at the selected wavelength : 190 nm[13].

Choice of the standard

The aim was to choose a standard for the wavelengths mentioned before. In **Table III a and b** absorbance values of protein solutions at 25 µg/ml are reported.

The comparison between absorbance values for each protein to the mean value allowed us to choose lactoglobulin for the low wavelengths, and trypsinogen for the highest ones. Standard curves have been established for each one :

- 0 to 35 µg/ml protein for each standard (**figure 5 and 7**)
- standard curve for lactoglobulin between 0 and 3.5 µg/ml (**figure 6**).

Indeed, because of low sensitivity at 277 nm, it was not possible to carry out protein determination at low concentrations (from 0 to 3.5 µg/ml) at this wavelength.

From the prediction equations related to each standard curve, we have predicted the concentration of a verification set of proteins (this set is made of the 16 remaining proteins and the standard as a reference) at protein concentration of 25µg/ml. The predictions, reported in the **Table IV**, fluctuated :

- from 8.5 µg/ml to 34.91 µg/ml, the mean value is 25.09 +/- 6.82 µg/ml for the predictions at 190 nm
- from 12.52 µg/ml to 44.51 µg/ml, the mean value is 25.9 +/- 9.65 µg/ml at 277 nm ;

The standard deviation calculated for the predictions, expressed as a percentage of the mean value, is respectively 27.17 % and 37.27 %.

As for the BCA method, one of the mostly used method for protein determination at present, this rate is 31.23 % (**Table V**). On the other hand, the comparison between the predicted concentrations at 190 nm and 277 nm, pointed out that the first predictions are better in terms of

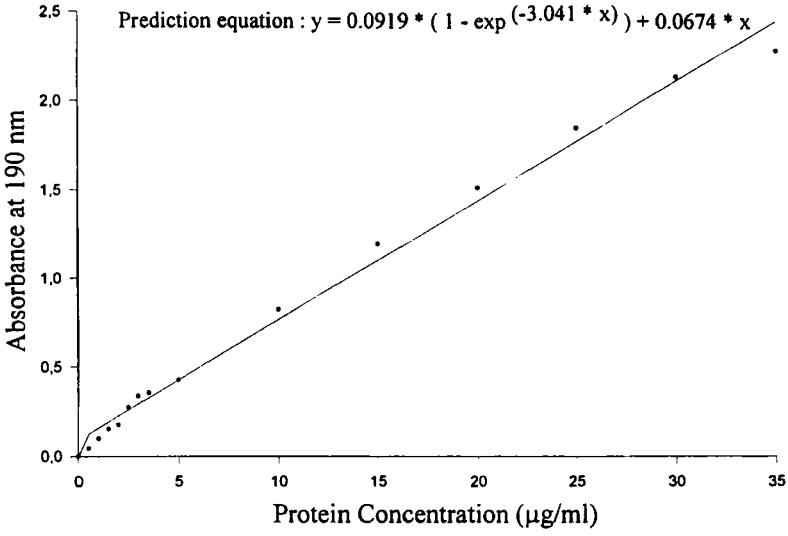

Figure 5 : Standard curve for lactoglobulin at 190 nm
(range of protein concentration : 0 to 35 µg/ml)

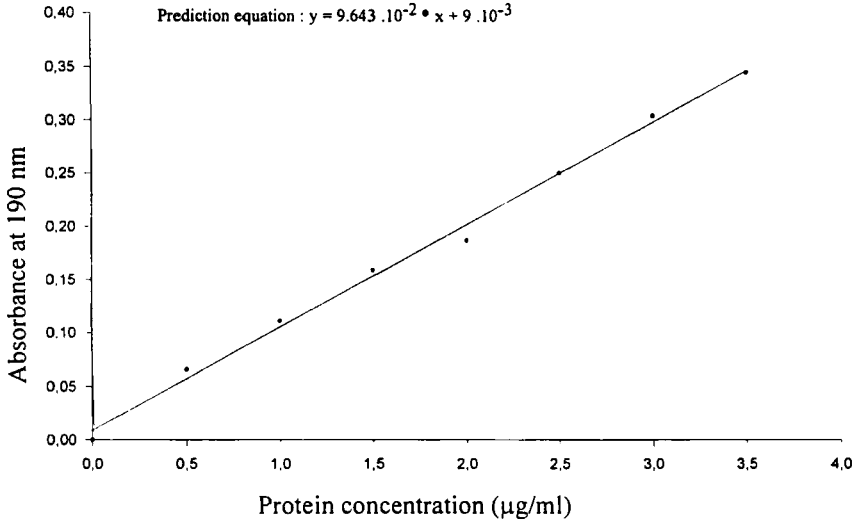

Figure 6 : Standard curve for lactoglobulin at 190 nm
(concentration range : 0 to 3.5 µg/ml)

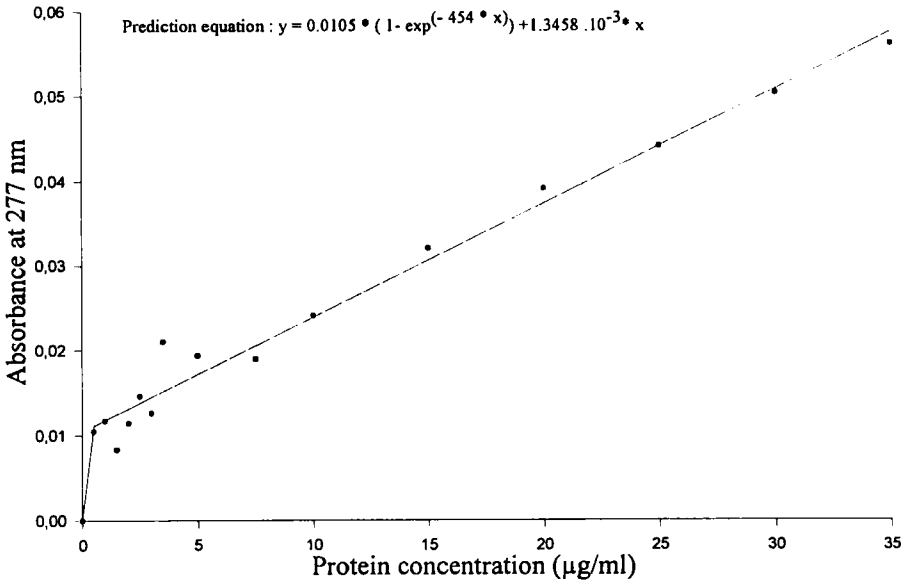

Figure 7 : standard curve for trypsinogen at 277 nm (concentration range : 0 to 35 µg/ml)

accuracy (mean value close to 25 µg/ml) and more precise (as the standard deviation is lower at 190 nm than at 277 nm). The predictions of the verification set have also been done for proteins at 1 µg/ml in order to validate our method as microquantification one (**Table VI**).

For the determination of low protein concentrations, the method reported here appears to be better than the BCA method **(Table V)**, as shown before.

We can also notice that the results achieved from Scopes equation[14-15] (used for direct protein determination by double reading at 205 nm

TABLE IV : Predicted concentrations of the verification set at 190 nm and 277 nm

Protein (25 µg/ml)	A 190 nm predicted	A 277 nm predicted
BSA	21.58	14.06
casein	28.47	22.99
chymotrypsin	20.57	22.95
chymotrypsinogen	26.71	32.53
cytochrome c	27.87	36.44
galactosidase	25.05	28.36
hemoglobin	10.26	31.30
lactalbumin	28.97	35.55
lactoglobulin	**25.00**	14.37
lysozym	28.27	44.51
ovalbumin	34.91	18.24
papain	31.69	37.98
pepsin	27.26	20.29
phosphorylase	8.50	13.91
ribonuclease	24.55	12.52
trypsin	29.23	29.33
trypsinogen	27.62	**25.00**
mean	25.09	25.90
standard deviation	6.82	9.65
%	27.17	37.27

Table V : values from BCA method[5]

Protein (1200 µg/ml)	
BCA method	**A 562 nm**
Avidin	1.12
BSA	1.38
Chymotrypsin	1.62
Gelatin	0.56
Immunoglobulin G	1.46
Insulin	1.68
Ribonuclease A	1.86
Mean	1.38
Standard deviation	0.43
%	31.23

TABLE VI : predicted concentrations of the verification set at 190 nm

Protein (1 µg/ml)	A 190 nm predicted
BSA	1.143
casein	1.170
chymotrypsin	1.213
chymotrypsinogen	1.142
cytochrome c	1.042
galactosidase	1.187
hemoglobin	0.845
lactalbumin	1.269
lactoglobulin	1.000
lysozym	1.150
ovalbumin	1.919
papain	1.431
pepsin	1.160
phosphorylase	0.903
ribonuclease	1.129
trypsin	1.041
trypsinogen	1.033
mean	1.16
standard deviation	0.24
%	20.41

and 280 nm) are less valid than those obtained with BCA method and our direct determination at 190 nm (**Table VII**).

$$C_{(mg/ml)} = DO_{205nm} / [27 + 120*(DO_{280nm}/DO_{205nm})]$$

CONCLUSION

The results show that it is possible to develop far U.V assay for protein determination. Accuracy, precision and sensitivity are

TABLE VII : predicted concentrations with Scopes relation

Protein (25 µg/ml)	Predictions
BSA	29.83
casein	30.49
chymotrypsin	34.72
chymotrypsinogen	34.52
cytochrome c	74.67
galactosidase	35.21
hemoglobin	36.82
lactalbumin	6.99
lactoglobulin	25.00
lysozym	29.68
ovalbumin	24.12
papain	37.44
pepsin	12.02
phosphorylase	35.49
ribonuclease	35.77
trypsin	29.32
trypsinogen	39.91
mean	32.47
standard deviation	14.00
%	43.13

essential for such a direct method of protein quantification. We establish that microquantification is possible with good accuracy and precision at the most sensitive wavelength : 190 nm. As interfering substances are concerned, we noticed that interference is maximum at our working wavelength : 190 nm[16]. In the case of protein solutions with interfering substances, complementary methods as BCA method or Bradford dying can be used. Indeed, these methods present few or no interferences at their working wavelength (respectively 562 nm and 595 nm) nevertheless they are destructive. We propose in the

following paper a non destructive procedure, so that microprotein quantification (and particularly enzyme) can be carried out when the sample to analyse contains interfering substances.

Acknowledgements

This work was supported by a grant from the Ministère de la Recherche et de la Technologie and the Conseil Régional de la Réunion.

REFERENCES

1 - A. C. Gornall, C. J. Bardwill, M. M. David, (**1949**), *J. Biol. Chem.*, **177**, 751.

2 - O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, (**1951**), "Protein measurement with the Folin phenol reagent", *J. Biol. Chem.*, **193**, 265-275.

3 - E. Larson, B. Howlett, A. Jagendorf, (**1986**), "Artificial reductant enhancement of the lowry method", *Anal. Biochem.*, **155**, 243-248.

4 - M. M. Bradford, (**1976**), "A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding", *Anal. Biochemistry*, **72**, 248-254.

5 - P. K. Smith, R. I. Hermanson, A. K Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, D. C. Klenk, (**1985**), "Measurement of protein using bicinchoninic acid", *Analytical Biochemistry,* **150**, 76-85.

6 - A. K. Hazra, S. P. Chock, R. W. Albers, (**1984**), "Protein determination with trinitrobenzene sulfonate : a method relatively independent of amino acid composition", *Analytical Biochemistry*, **137**, 437-443.

7 - Warburg, Christian, (**1942**), *Biochem. Z.*, **310**, 384.

8 - A. H. Woods; P. R. O'Bar, (**1970**), "Absorption of proteins and peptides in the far ultraviolet", *Science*, **167**, 179-181.

9 - W. J. Waddell; M. D. Chapel Hill; N. C., (**1956**), "A simple ultraviolet spectrophotometric method for the determination of protein", *J. Lab. Clin. Med.*, **48**, 311-314.

10 - M. P. Tombs, F. Souter, N. F. Maclagan, (**1959**), "The spectrophotometric determination of protein at 210 mμ", *Biochem. J.*, **73**, 167-171.

11 - C. C. Bigelow, (**1967**), "On the average hydrophobicity of proteins and the relation between it and protein structure", *J. Theoret. Biol.*, **16**, 187-211.

12 - M. Safar, (**1995**), "Comparaison des plages spectrales de l'infrarouge proche et moyen pour l'étude des produits agro-alimentaires", thèse de Doctorat, spécialité : physico-chimie des bioproduits, Université de Nantes.

13 - W.B. Gratzer (**1967**), "Ultraviolet absorption spectra of polypeptides" In Poly-alpha-amino acids : proteins models for conformation studies *in* Biological macromolecules vol 1, 1967, p 177-238, ed G. Fasman, published by Decker.

14 - R. K. Scopes, (**1974**), "Measurement of protein by spectrophotometry at 205 nm", *Analytical Biochemistry*, **59**, 277-282.

15 - A. P. Demchenko, (**1981**), "Ultraviolet Spectroscopy of Proteins", Ed. Springer-Verlag, 306 pages.

16 - P. Baret, C. Robert and F. Cadet (**1997**), "Microquantification of proteins by spectrophotometry. Part II : Application procedure for complex mixture containing interfering substances", *Spectroscopy Letters*, submitted.

Date Received: February 18, 1997
Date Accepted: April 9, 1997