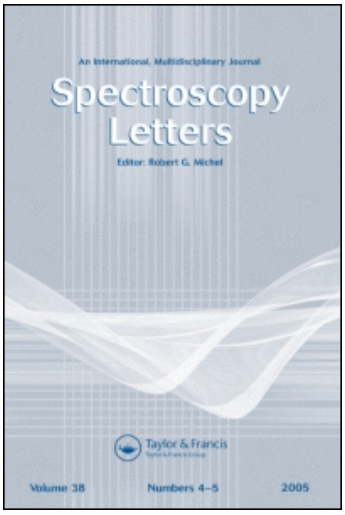

Spectroscopy Letters
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597299

Microquantification of Proteins by Spectrophotometry. Part II : Application Procedure for Complex Mixture Containing Interfering Substances

Pascal Baret[a]; Christine Robert[a]; Frédéric Cadet[a]
[a] Université de la Réunion, Laboratoire de Biochimie, Cédex, Réunion, FRANCE-DOM

To cite this Article Baret, Pascal , Robert, Christine and Cadet, Frédéric(1998) 'Microquantification of Proteins by Spectrophotometry. Part II : Application Procedure for Complex Mixture Containing Interfering Substances', Spectroscopy Letters, 31: 3, 487 — 501
To link to this Article: DOI: 10.1080/00387019808002744
URL: http://dx.doi.org/10.1080/00387019808002744

SPECTROSCOPY LETTERS, 31(3), 487-501 (1998)

MICROQUANTIFICATION OF PROTEINS BY SPECTROPHOTOMETRY. PART II : APPLICATION PROCEDURE FOR COMPLEX MIXTURE CONTAINING INTERFERING SUBSTANCES

KEY-WORDS : microquantification, UV-visible spectroscopy, proteins

Pascal BARET, Christine ROBERT and Frédéric CADET*

Laboratoire de Biochimie, Université de la Réunion, 15 avenue René Cassin B.P. 5175, 97715 Saint Denis Messag. Cédex 09, Réunion FRANCE-DOM

Fax : (Intl) 262 93 81 66 email : cadet@univ-reunion.fr

ABSTRACT

In a previous paper we have shown that it was possible to quantify protein solutions at very weak concentrations directly by UV-visible spectroscopy. Nevertheless the protein quantification could not be

* to whom correspondence should be addressed

possible if there is any trace of interferents left in the solution. So it is necessary to eliminate all the interferents to make the measure at 190 and/or 277 nm possible. We have developped a method based on the use of Microcon membranes and centrifugation. Interferents could be eliminated from protein solutions after four centrifugations at 13 000 g during 5 min. This procedure allowed the recovering of proteins, with 80 to 99 % yield, and thus making microquantification possible.

This method is particularly interesting for enzymatic solutions after a purification procedure by HPLC where very tiny quantities of protein are recovered. This protocol has been tested on three enzymes ; enzymatic activity recovered after four centrifugations was quite high for PEPcase and malic enzyme (71 and 64 % respectively).

INTRODUCTION

Several methods of protein quantification have already been proposed. Among them, the Bradford (1976), trinitribenzene sulfate (Hazra *et al.*, 1984) or bicinchoninic acid, BCA, (Smith *et al.*, 1985) methods are the most recents. For almost all of these methods the use of chemical products is necessary leading to protein denaturation.

In a precedent paper (Baret *et al.*, 1997) we have shown that it was possible to quantify proteins directly by UV-visible spectrophotometry at 190 and 277 nm. These wavelengths presented the best correlation factor between absorbance and protein concentration for the 17 proteins tested. No chemical product is

necessary for this method. However it has been tested for the proteins prepared in MilliQ water. When a protein protein purification is undertaken, buffer solutions containing salts or detergents are usually used. These constituants present high absorbance values at 190 and 277 nm.

In this present work we have proposed a protocol to eliminate substances which could interfere with the quantification of proteins. This protocol allowed the preservation of molecule integrity (structure, enzymatic activity, ...).

MATERIALS AND METHODS

Products : MDH was from Boehringer Mannheim

Malic enzyme, PEPcase, lactoglobulin, cytochrome c, chymotrypsinogen, trypsinogen, glycin, CHAPSO, Tween 20 and Triton X-100 was from Sigma

Microconcentrators Microcon was from Amicon

Centrifugation was performed on Sigma 3K20, rotor n° 12154.

MilliQ water was obtained with the Ultra Pure water system Alpha Q from Millipore Inc.

Enzymatic activity assay : PEPcase activity was followed at 340 nm with a Philips PU 8730. The reactional mixture contained PEP 4 mM in Tris-HCl buffer (100 mM, pH 8) containing Mg^{2+} 5 mM, KCl 20 mM, $NaHCO_3$ 10 mM, NADH 0.35 mM, MDH 30 units/ml and PEPcase 0.015 units/ml in a final volume of 1 ml.

The substrate used for the MDH activity assay was oxaloacetate 1

mM in Tris-HCl buffer (100 mM, pH 8) containing NADH 0.35 mM and MDH 0.03 units/ml. Disappearance of NADH is followed at 340 nm.

Malic enzyme activity is followed at 340 nm (reduction of $NADP^+$) with L-malate 4 mM as substrate in Tris-HCl (100 mM, pH 8) containing $NADP^+$ and malic enzyme 0.02 units/ml.

Interferent elimination : Protein solutions (25 µg/ml) were prepared in MilliQ water, salt solutions 100 mM (Tris; phosphate; NaCl, KCl) and detergents 1 % (Triton X-100, Tween 20, CHAPSO). 100 µl of a protein solution, put down in a microconcentrator Microcon, were centrifuged at 13 000 g, 4°C, 5 min. After centrifugation protein were solubilised in 100 µl of MilliQ water to be centrifuged again. Centrifugation steps are stopped when absorbance of the supernatant was equal to the absorbance of protein solution in water. Absorbance was measured with a Hewlett Packard 8453 spectrophotometer.

RESULTS AND DISCUSSIONS

Elimination protocol for interferents in protein solutions

The absorbance value of some proteins with or without salts have been measured at 190 nm (Table 1). These interferents presented a very high absorbance at 190 nm. For example, NaCl (100 mM) presented an absorbance value of 2.2345. This high value make the signal saturating in presence of proteins. Thus lactoglobuline 25 µg/ml in water show an absorbance value of 1.6799 at 190 nm. With NaCl, this value gets to 2.3496. This saturation phenomenon was

TABLE 1 : Absorbance at 190 nm of different proteins solutions

		A (190 nm) buffer + protein (25 µg/ml)				
Buffer	A (190 nm)	Lactoglo-bulin	Cytochro-me	Chymo-trypsin	Trypsin	Glycin
Water	0	1.6799	2.2716	2.471	2.431	2.4566
Tris (100 mM)	2.3168	2.4159	2.5052	2.5257	2.5086	2.5086
Phosphate (100 mM)	2.2808	2.3512	2.5381	2.4939	2.3987	2.489
NaCl (100 mM)	2.2345	2.3496	2.4262	2.4582	2.4835	2.4585
KCl (100 mM)	2.2771	2.3261	2.4371	2.4823	2.5381	2.4571

observed with all the proteins tested. Spectra presented in figure 1 illustrate saturation observed at 190 nm for lactoglobuline in the presence of salts.

During proteins purification protocols, the presence of interferents is necessary. So the direct quantification of proteins at 190 nm needs the elimination of these substances.

In order to eliminate these salts we have elaborate a process using a combination of microconcentrators Microcon and centrifugation. The technical index of the Microcon membranes indicate a centrifugation between 3000 and 14 000 g. In order to reduce the time treatment, we have fixed the centrifugation speed at 13 000 g during 5 min. With this rotation speed, the same value of absorbance is observed before and after centrifugation, so that there is no denaturation of proteins.

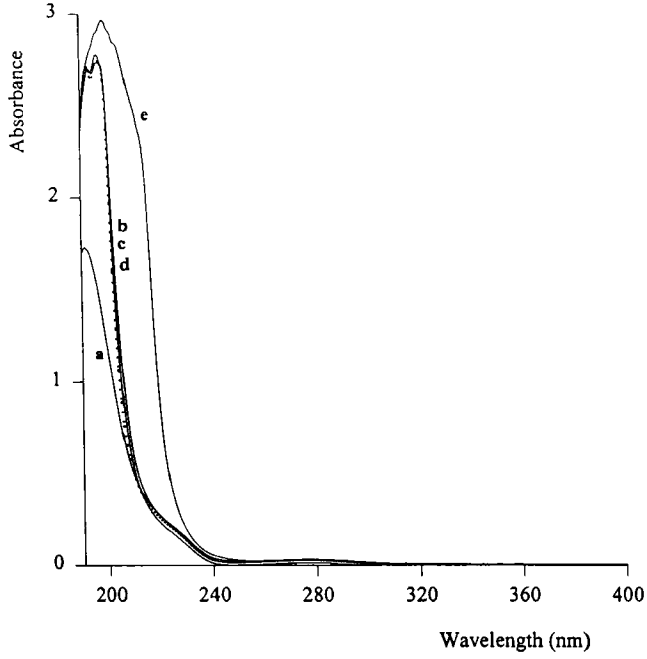

Figure 1 : Lactoglobulin (25µg/ml) UV/visible spectra
Proteins solutions were prepared in :

a - Milli Q water.
b - Na-phosphate (100 mM) buffer.
c - NaCl (100 mM).
d - KCl (100 mM).
e - Tris-HCl (100 mM) buffer.

Microcon is used to concentrate, remove salt and purify tiny quantities of proteins. Proteins stay on the upper of the membrane (YM membrane type) and the salts pass in the pellet. Microcon 10 (molecular cut of 10 kDa) have been tested with lactoglobuline (25 µg/ml) in MilliQ water. After the first centrifugation an absorbance

value of 0.8835 was observed for the solution in the pellet when no absorbance was expected in the pellet since it is supposed to be only water. It seemed that substances existing on the membrane before any utilisation was eliminated during the first centrifugation. Table IIa show that after three centrifugations pellet absorbance is close to 0.

When the treatment is performed on protein solution in buffers, we noted an absorbance value of the pellet higher than with water. This is due to the elimination of the salts and of the substances inherent to the membranes. All the interferent was eliminated after four centrifugations (Table IIa).

This protocol (4 centrifugations of 5 min at 13 000 g) allowed the recovering of lactoglobulin without any salts with 93 % average yield (Table IIa).

The same treatment has been applied for other proteins with Microcon 30 (molecular cut of 30 kDa) (Table IIb). Similar treatment efficiency was observed. Indeed four centrifugations allowed the recovering of thyroglobulin without any salts with 96 % yield.

Microcon 10 have been tested in the presence of detergents too. Results obtained for lactoglobulin and trypsin with detergents, CHAPSO, Tween 20 and Triton X-100 are presented in table III. These detergents have been chosen because of their general use in protein purification processes. Absorbances values are measured at 190 nm and 277 nm, because, as it was demonstrated in a precedent work (Baret *et al.*, 1997), these wavelengths presented the best correlation factors between absorbance and protein concentrations for the whole proteins tested. Spectra (with and without detergents)

TABLE 2 : Protein yield after centrifugation on Microcon

TABLE 2a : centrifugations on Microcon 10 of lactoglobulin (25 µg/ml)

	Before centrifugation	Centrifugation 1	Centrifugation 2	Centrifugation 3	Centrifugation 4	Centrifugation 4	Yield %
Buffer		Culot	culot	culot	culot	surpernatant	
Water	1.6799	0.8835	0.2091	0	0	1.5097	90
Tris (100 mM)	2.4159	2.2634	1.422	0.1894	0.0911	1.5862	94
Phosphate (100 mM)	2.3512	1.6826	0.3391	0.0954	0.0815	1.4263	85
NaCl (100 mM)	2.3496	2.1368	1.1322	0.2299	0.0901	1.6152	96
KCl (100 mM)	2.3261	2.1495	1.197	0.2827	0.1044	1.6709	99

TABLE 2b : centrifugations on Microcon 30 for 4 protein s (25 µg/ml)

	Before centrifugation	Centrifugation 1	Centrifugation 2	Centrifugation 3	Centrifugation 3	Yield %
water + protein		Culot	culot	culot	surpernatant	
Phosphorylase b	1.1246	1.1045	0.2256	0.0767	0.902	80
β-galactosidase	2.2036	1.4864	0.2091	0.0756	1.7748	81
β-amylase	1.858	1.2269	0.1351	0.0098	1.4584	78
thyroglobulin	2.2408	1.2077	0.1649	0.0842	2.14	96

TABLE 3 : centrifugations on Microcon 10 of lactoglobulin and trypsinogen (25 µg/ml)i n the presence of detergents absorbance was measured at 190 nm

	Before centrifugation	Centrifugation 1	Centrifugation 2	Centrifugation 3	Centrifugation 4	Centrifugation 4	Rendement %
Detergent		Culot	culot	culot	culot	surnageant	
LACTOGLOBULIN							
Water	1.5706	0	0	0	0	1.2936	82
CHAPSO 1%	2.5927	2.3745	2.3938	1.206	0.1672	1.2618	80
Tween 20 1%	2.9233	0.9209	0.1168	0	0	2.1273	135
Triton X-100 1%	4.2192	2.3377	3.2302	0.6566	0	2.3614	150
TRYPSINOGEN							
Water	1.6028	0.8568	0	0	0	1.394	87
CHAPSO 1%	2.8484	2.3864	2.4578	1.1308	0.2145	1.3032	81
Tween 20 1%	3.0707	0.1328	0	0.1423	0	2.1889	136
Triton X-100 1%	3.9027	1.7796	1.4904	0.1679	0	3.1093	194

are presented in figure 2. The same saturation phenomenon could be observed with detergent than with salts.

The results are presented in Table III only for the measures at 190 nm. Only CHAPSO could be eliminated by this technic; protein yield is then 80 % for lactoglobulin and trypsinogen. It seemed difficult to eliminate Tween 20 and Triton X-100 from protein solutions. These detergents might form micelles which stay on the membrane and interfere in the centrifugation course.

The protocol described above has been tested for lactoglobulin with very weak concentrations (10 and 3 µg/ml) in different buffers. An absorbance value of 0.45068 is observed with lactoglobulin in MilliQ water at 3 µg/ml concentration. With the same protein in Tris buffer (100 mM) an absorbance value of 2.4337 was measured. After the four centrifugations on Microcon 10, the solution absorbance dropped to 0. 42499. The lactoglobulin only is recovered with 94 % yield. Lactoglobulin spectra registered before and after centrifugation at 10 µg/ml (results not shown) and 3 µg/ml (figure 3) showed that interferents is really eliminate during the protocol, the spectrum obtained after the four centrifugations match with the spectrum of the protein in MilliQ water.

The protocol proposed is resumed in figure 4. After four centrifugations, corresponding to a treatment of 20 min, on microconcentrators Microcon there is a protein recovering without any interferents with 80 to 99 % yield.

Application to enzymatic solutions

The process described in figure 4 has been tested for three enzymes : PEPcase, MDH and malic enzyme. Results are presented in table IV.

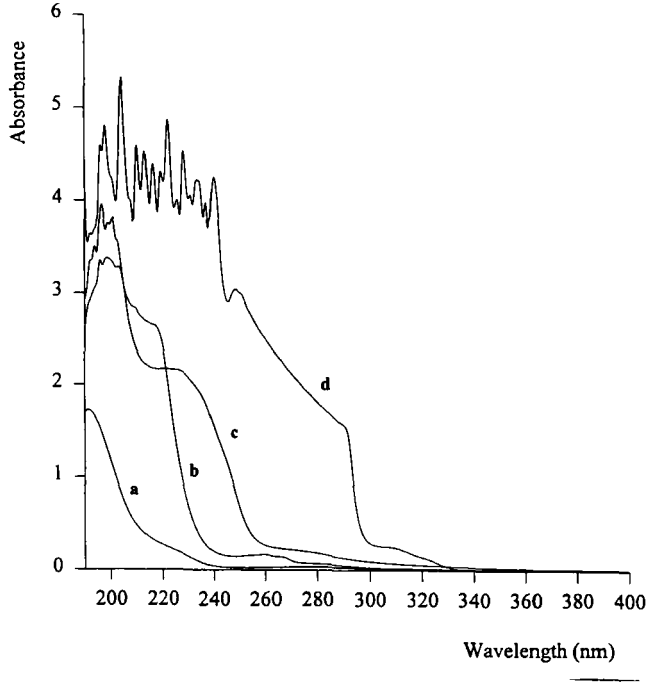

Figure 2 : Lactoglobulin (25µg/ml) UV/visible spectra
Proteins solutions were prepared in :

a - Milli Q water.
b - CHAPSO (1%).
c - Tween 20 (1%).
d - Triton (1%).

For these enzymes the protocol has been modified so as to preserve the enzymatic activity. We have shown above that there were substances on Microcon membranes which were eliminated during the first centrifugation step. In order to limit the number of centrifugations in the case of enzymes, the first centrifugation has

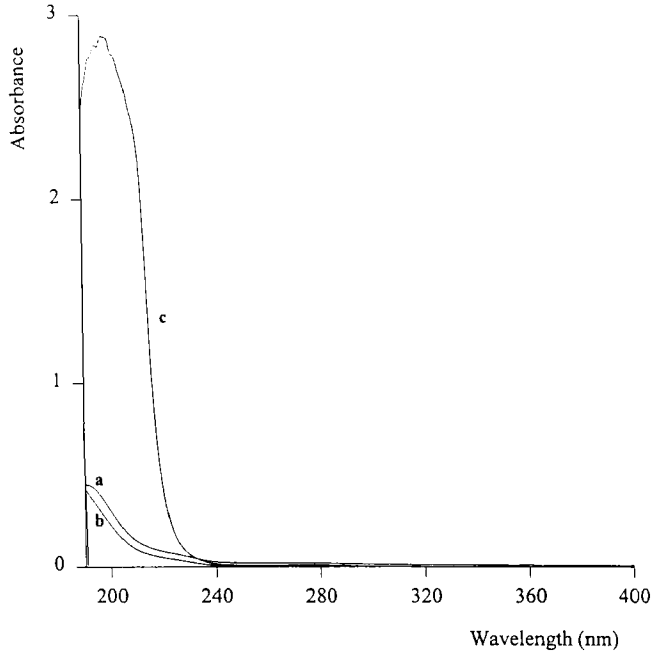

Figure 3 : Lactoglobulin (3µg/ml) UV/visible spectra

Proteins solutions were prepared in :

a - Milli Q water before centrifugation.

b - Milli Q water after four centrifugations.

c - Tris-HCl 100 mM buffer.

been realised in the presence of water alone. With the enzymes three centrifugations have been realised.

Initial enzymatic activity is preserved with 71 and 64 % yield for PEPcase and malic enzyme respectively. For MDH, the yield obtained after treatment is lower (23%) than with the previous two enzymes.

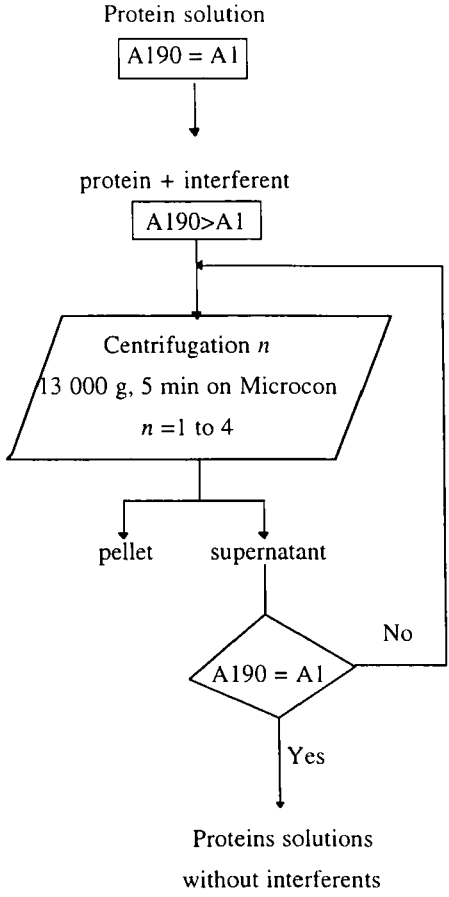

Figure 4 : Elimination of interferents from protein solutions

Table IV : Enzymatic activity before and after centrifugations on Microcon

	before centrifugation	after 3 centrifugations	yield
	activity (nkat)	activity (nkat)	%
PEPcase	40	28.4	71
MDH	44,8	10.4	23
Malic enzyme	253.5	167.7	64

CONCLUSION

We have shown that interferents such as salts or detergents could be easily eliminated from a protein solution. The association of microconcentrators and successives centrifugations allowed a protein recovering without any interferents. As shown in a precedent paper (Baret *et al.*, 1997) it is possible to establish a standard curve with lactoglobulin for concentrations in the range of 0 to 5 μg/ml (and 0-35 μg/ml). So, this method could be used for a direct quantification of proteins.

The protocol proposed in this paper allowed a direct and reliable microquantification of proteins at 190 nm. Every protein solution, wether in the presence of interferents or not, can be quantified. The microquantification by UV spectrophotometry needs elimination of any part of interferents by the use of microconcentrators combined with four centrifugations (13 000 g, 5 min). Thanks to this treatment the quantification of proteins will not take more than 20 min and will not make use of chemical products hence reducing costs.

This protocol could be applied to enzymatic proteins too with weak denaturation. Towards UV quantification, enzymatic activity is preserved and the denaturation is weak. This method is very interesting at the end of the enzyme purification procedure when there is tiny proteins left. There is weak loss, so it is possible to carried out kinetic studies with these proteins, contrary to the other destructive methods.

Acknowledgements

This work was supported by a grant from the Conseil Régional of La Réunion.

REFERENCES

Baret P., Angeloff A., Rouch C., Pabion M. and Cadet F. (1997), "Microquantification of proteins by spectrophotometry. Part I : From 190 nm to 1100 nm; selection of wavelengths", *Spectroscopy Letters*, submitted.

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

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

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

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