Reversed-Phase HPLC Method for Hydrophobic Protein Analysis

Jasmine Wang, Bhargavi Vemulapalli, Susanna Bilbulian, Liu Tie, Huiming Yan, Ziping Wei, and Patricia W. Cash
Analytical Biochemistry Department, MedImmune, Gaithersburg, MD. USA

Abstract

A reversed-phase high performance liquid chromatography (RP-HPLC) method has been developed to measure the concentration of intact hydrophobic protein and the level of protein fragments. It is very difficult to elute the hydrophobic protein from traditional reversed-phase columns. The method described here employs an Intrada WP-RP column packed with a newly developed reversed-phase ligand. The ligand has an optimal surface polarity that increases column efficiency during hydrophobic protein analysis (1). The elution of the protein and separation of fragments was achieved by optimizing the gradient.
The protein concentration in the test sample was determined using regression analysis from a standard curve. The determinations were performed in the linear range of 1 – 10 µg. The method qualification results demonstrate that the method is specific, precise, accurate, and linear. The method is compatible with on-line ESI LC/MS detection for the identification of the protein - related impurities.

Introduction

Reversed-phase high-performance liquid chromatography (RP-HPLC) is one of the most useful techniques for the separation of proteins. In this technique, proteins are separated based on their hydrophobic properties. The retention of proteins with high hydrophobicity is a major concern in this type of protein analysis by HPLC (2, 3).
Here we present a reversed-phase chromatographic method that implements an Intrada WP-RP column packed with a high resolution silica matrix with 3µm particles and 300Å pore size. The method allows us to separate, identify, and quantitate a hydrophobic protein in cell culture medium and in-process samples.

Experimental Parameters

- System: Agilent 1100/1200 HPLC
- Analytical Column: Intrada WP-RP (4.6 x 250 mm, 3 µm 300Å)
- Column Temperature: 35°C
- Mobile Phase:
  - A: 0.1% TFA/HPLC Water
  - B: 0.1% TFA/Acetonitrile
- Flow Rate: 0.75 ml/min
- Autosampler Temperature: 4°C
- Sample Load: 10 µg
- UV Detection: 280 nm
- Gradient: 0-100% B in 18 minutes

Protein Quantification: Specificity

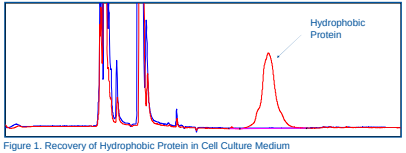


Figure 1. Recovery of Hydrophobic Protein in Cell Culture Medium

Purified protein was spiked into the medium and analyzed following the above procedure. The spike recovery was determined to be 91% of the theoretical value.

Protein Quantification: Linearity

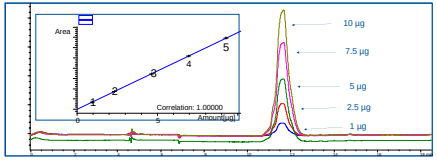


Figure 2. Linearity Evaluation

The UV responsed are linear in the range of 1-10 µg. Correlation coefficient was 1.0.

Protein Quantification: Repeatability

Test Sample	Measured Concentration (µg/ml)		% CV
	Value	Mean	
Hydrophobic Protein	926.4	914.0	1.4
	925.7		
	919.9		
	912.6		
	905.8		
	893.8		

Table 1. Results of Repeatability Evaluation

The repeatability of the method was evaluated by analyzing the test sample in six replicates. The % CV was 1.4.

Protein Quantification: Accuracy

Hydrophobic Protein (µg/ml)	Measured Concentration (µg/ml)		% CV
	Value	Mean	
300	290.3	300	3.5
	298.4		
	311.1		
600	576.6	588	3.8
	573.9		
	613.8		
900	878.4	886	1.8
	875.7		
	904.7		

Table 2. Results of Accuracy Evaluation

The accuracy of the method was evaluated by analyzing the test sample in triplicates at three different levels. The % CV between the three replicates at each level was 1.8-3.8.

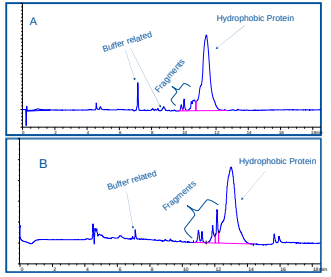
Protein Quantification: Intermediate Precision

Hydrophobic Protein		Measured Concentration (µg/ml)		% CV
		Value	Mean	
Day	1	520.4	523.7	0.5
		527.0		
		519.0		
	2	537.0	527.0	
		526.2		
		518.3		
Analyst	1	537.0	531.6	4.5
		526.2		
		518.3		
	2	580.4	566.8	
		580.6		
		539.5		
Instrument	1	513.7	515.6	0.9
		517.4		
		513.8		
	2	520.4	522.1	
		527.0		
		519.0		

Table 3. Results of Intermediate Precision Evaluation

The intermediate precision was evaluated by different analysts, on different days and on different instruments . The % CV was found to be 0.5 – 4.5.

Protein Identification: Gradient Optimization



Gradient A
With slope at 7
for % B increasing

Resolution = 1.2

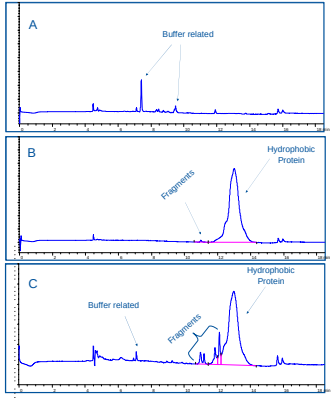
Gradient B
With slope at 6
for % B increasing

Resolution = 1.4

Figure 4. Gradient Optimization

Gradient elution played a key role in the separation of the fragments. The optimal resolution was achieved with gradient B.

Protein Identification: Specificity



A: RP profile of heat-stressed blank buffer

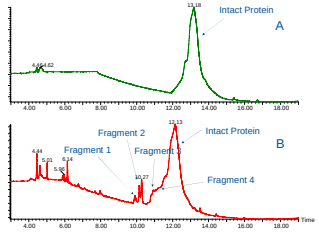
B: RP profile of test sample

C: RP profile of heat-stressed sample

Figure 5. Specificity Evaluation

No interfering peaks were detected in the blank buffer. Additional peaks were observed in the heat-stressed sample. The assay is specific.

Protein Identity Method: Peak Identification by LC/MS



A: RP-UPLC profile of hydrophobic protein

B: RP-UPLC profile of hydrophobic protein heat stressed, 40°C, 3 months

Figure 6. Specificity Evaluation

The fragment peaks and clipping sites were identified by Waters LCT mass spectrometry.

Protein Identification: Repeatability

Test sample	Reported Fragmentation		Std Dev	% CV	Reported Intact Protein		Std Dev	% CV
	Total Peak Area, %	Mean Peak Area, %			Total Peak Area, %	Mean Peak Area, %		
Hydrophobic Protein	0.4221	0.5	0.1	13.3	99.5779	99.5	0.1	0.1
	0.4040				99.5960			
	0.5182				99.4818			
	0.5628				99.4372			
	0.4301				99.5699			
	0.4968				99.5032			

Table 5. Results of Repeatability Evaluation

The repeatability of the method was evaluated by analyzing the test sample in six replicates. The % total fragment peak was determined to be 0.5 and % CV was 13.3.

Conclusion

- An Intrada WP-RP column was implemented for a protein with high hydrophobicity.
- The method allowed us to quantitate the protein concentration and separate the protein related fragments.
- Separation of protein fragments was successfully achieved with the optimized gradient.
- The fragments were identified in heat stressed samples by a RP-UPLC system coupled to a Waters LCT mass spectrometer.
- The qualification results demonstrated that the method was specific, precise, accurate, and linear.

References

1. Imtakt Intrada WP-RP HPLC Column Brochure
   http://www.siswеb.com/lc/imtakt-intrada-wprp.htm
2. C. Guerin, G. Brule, Separation of three proteins from egg white, Sciences des Aliments, 12(4), 705-20 (1992)
3. A. Schafer, the Quality of Egg and eggproducts, Fourth Eur. Symp (1995)

Acknowledgement

The authors would like to acknowledge Dr. Wenjun Mo, Dr. Mark Schenerman, and Dr. Gail Folena - Wasserman for their support of this work.