International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.5, No.3, pp 1132-1137, July-Sept 2013

Development and Validation of UV Spectrophotometric Method for Total Protein Estimation in Seeds of Pisum sativum L.

Jitendra Y. Nehete*[1], Sonali R. Gawali[2], Rajendra S. Bhambar[3], Minal R. Narkhede[4]

[1-3] Department of Pharmacognosy,[4] Department of Pharmaceutics, MGV's Pharmacy College, Panchavati, Nashik-422003, India,

***Corres. Author: jynehete@yahoo.com**

Abstract: A simple, accurate, precise and sensitive UV spectrophotometric method was developed for determination of total protein in seeds of *Pisum sativum* L. (Fabaceae). Phosphate buffer used as solvent and wavelength corresponding to maximum absorbance of the protein was found at 278.60nm. Beers law was observed in the concentration range of 200- 1000µg/ml with correlation coefficient 0.9996. The linear regression equation obtained by least square regression method were y=0.0008x + 0.0186, where y is the absorbance and x is the concentration of standard protein solution. The method was validated as per ICH guidelines. The values of relative standard deviation and % recovery were found to be satisfactory, indicating that proposed method is precise and accurate and hence can be used for the routine analysis of total protein estimation in seeds of *Pisum Sativum* L.
Key words: *Pisum Sativum* L., UV spectrophotometer, ICH.

Introduction[1]

Pisum sativum L. is main source of protein, which contained amino acid required for human body. It also used in pharmaceutical industry in microencapsulation, controlled delivery, hydrogels, composites, nanoparticles, microparticle and beads. It consists of storage proteins like globulin, albumin, prolamin, glutelin.

Literature survey revealed that there is no reported method for quantification of total protein in seeds of *Pisum sativum* L. The objective of present work was to develop a simple, sensitive, precise and accurate UV spectrophotometric method for the determination of total protein in seeds of *Pisum sativum* L. as per ICH Guidelines.

Materials and Method

Instrumentation

A Shimadzu UV spectrophotometer model 2450 with 1.4cm matched quartz cells were used for measuring absorbance.

Chemicals and reagents

Egg white albumin standard, Phosphate buffer were obtained from Sigma Aldrich Ltd. All other reagents were of analytical grade. Protein sample was obtained in our laboratory. Pea seeds were obtained from local market and authenticated from Botanical Survey of India, Pune and flour (mesh no.80) was made by hammer mill.

Preparation of protein sample[2]

Separation of pea proteins from pea seed flour (PF) was performed by salt/pH extraction to yield crude protein. 250 g of PF was dissolved in 1.5 L of a K_2HPO_4 buffer (50 mM and 0.5 M NaCl at pH 7.20) (volume–weight ratio of 6 mL buffer per g PF), and then stirred continuously using a magnetic stirrer for 1 h at room temperature (~21–22 °C). The mixture was centrifuged at 18,600×g for 15 min at 4 °C. The supernatant was collected and filtered using glass wool to remove large undissolved particles. The filtered supernatant was then diluted using 5 volumes of cold (4 °C) Milli-Q water, adjusted to pH 4.50 using 2 N HCl, and then left to stand overnight (16 h) within a cold room (4 °C) to facilitate settling of salt-soluble proteins. The supernatant was decanted off and the remaining pellet was collected by centrifugation. The recovered pellet was washed with 200 mL of Milli-Q water and collected and identified by **Ninhydrin** and **Biuret** chemical tests.

Determination of maximum wavelength (λ_{max})[3]

Preparation of stock solution

Standard stock solution of egg white albumin was prepared by dissolving accurately weighed 100mg of albumin in buffer in a 100ml volumetric flask to give a concentration of 1000µg/ml.

From above stock solution, pipette out 2ml and 4ml in to 10ml volumetric flask and finally made up the volume with buffer to produce a concentration of 200µg/ml and 400µg/ml respectively. The samples was then scanned in UV spectrophotometer from a range of 200-400nm against buffer as blank and the wavelength corresponding to maximum absorbance in buffer was recorded.

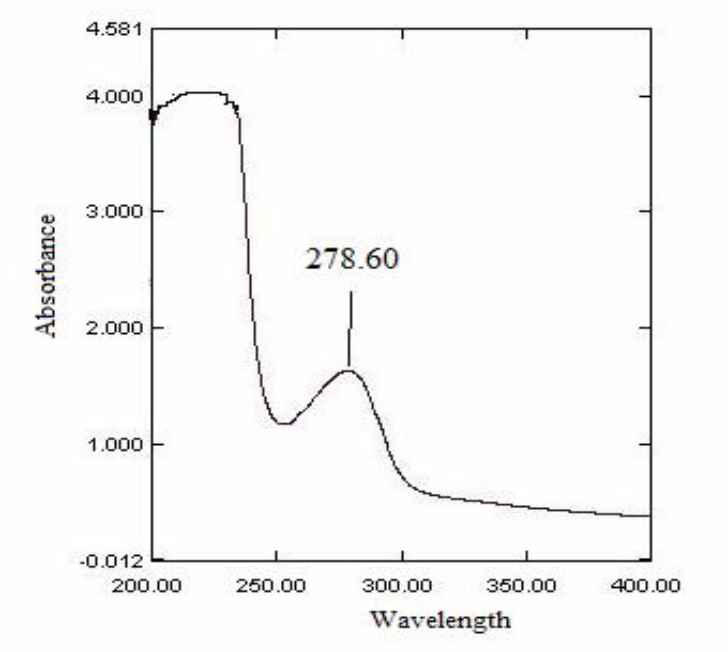

Figure 1: UV Spectrum of Albumin in Buffer

Preparation of standard calibration curve

For the preparation of standard calibration curve, concentration of 10-60µg were prepared by pipetting out 2, 4, 6, 8, and10 ml from 1000µg/ml solution in to a 10ml volumetric flask and made up the volume with buffer. The absorbance of each solution was measured at 278.60nm against buffer as blank. Calibration curve of the drug was then plotted by taking the absorbance obtained on y-axis and the concentration of the solution on x-axis (Figure 2). The curve showed linearity in 200-1000µg/ml with correlation coefficient 0.9996.

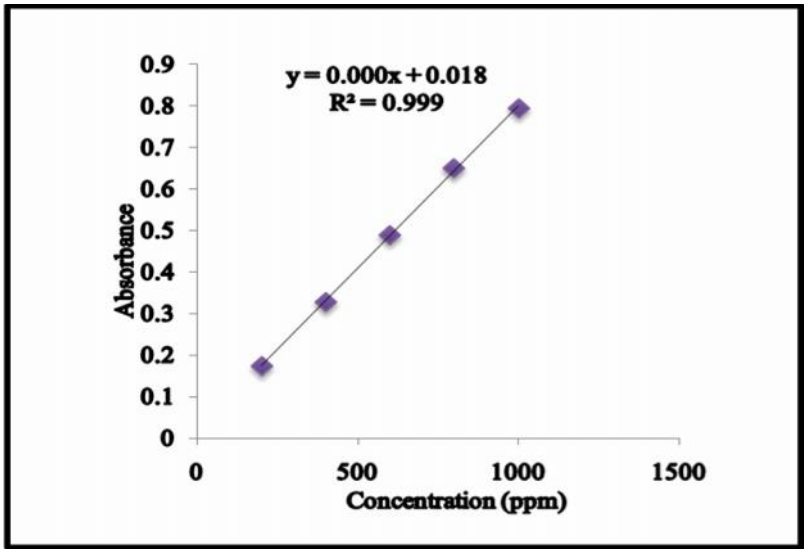

Figure 2: Calibration curve of Albumin

Absorbance of crude protein sample is 0.554

Total Protein Concentration: - y = mx + c

$$x = \frac{0.554 - 0.0186}{0.0008}$$

$$x = 669.25\ \mu g/ml$$

Here, y is absorbance, m- slope, c – intercept, x – concentration.

VALIDATION

Validation can be defined as (ICH) Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics[4, 5, 6].

The method was validated for several parameters like linearity, accuracy, precision, Ruggedness, Robustness, Limit of detection (LOD), and Limit of quantification (LOQ) according to ICH guidelines[3].

Linearity

The linearity of the analytical method was its ability to elicit test results which are directly proportional to analyse concentration in samples within a given range. To establish the linearity of the proposed method, various aliquots of standard solution of the drug were prepared from stock solution and analysed. The drug showed linearity in range of 10-60µg/ with correlation coefficient 0.9997. Linearity data are shown in Table 1.

Precision

Precision studies were carried out to ascertain the reproducibility of the proposed method. Repeatability was determined by preparing six replicates of same concentration of the sample and the absorbance was measured. Intraday precision study was carried out by preparing drug solution of same concentration and analyzing it at three different times in a day. The same procedure was followed for three different days to determine interday precision. The results were reported as %RSD. The precision result showed a good reproducibility (Table 2) with percent relative standard deviation less than 2. The results of intraday and interday precision studies are shown in (Table 3 and Table4).

Accuracy

Accuracy of the proposed method was determined using recovery studies. The recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of the standard albumin to one of the dilution (600µg). The solutions were prepared in triplicates and the % recovery was calculated. The results are shown in (Table 5).

Ruggedness

Ruggedness was determined by carrying out analysis by two different analysts and the respective absorbance was noted and results were indicated as % RSD (Table 6).

Robustness

Analysis was carried out at two different temperatures, room temperature and at 18^0c to determine robustness of method and the respective absorbance was measured. The result was indicated as %RSD (Table7).

LOQ and LOD

Limit of detection (LOD) is the lowest amount of analyte in the sample that can be detected. Limit of quantification (LOQ) is the lowest amount of analyte in the sample that can be quantitatively determined by suitable precision and accuracy. LOQ and LOD were determined using following equation LOQ-10s/m, LOD-3.3s/m where s is the standard deviation of the response and m is the slope of the related calibration curve. The values of LOQ and LOD were found to be 10.12µg/ml and 3.33µg/ml respectively.

The results of various parameters of the developed method are shown in Table 8.

Table1: Linearity table of Albumin

Concentration(µg/ml)	Absorbance
200	0.174
400	0.328
600	0.490
800	0.650
1000	0.794

Table 2: Precision results showing repeatability

Concentration (µg/ml)	Absorbance	Statistical Analysis
400	0.308	
400	0.307	Mean-0.307
400	0.308	SD- 0.000816
400	0.308	%RSD- 0.0265
400	0.306	
400	0.307	

Table 3: Intraday precision

Concentration (µg/ml)	Absorbance1	Absorbanc2	Absorbance3	Average %RSD
400	0.308	0.307	0.308	0.030
400	0.306	0.306	0.307	
400	0.306	0.308	0.306	
400	0.308	0.306	0.307	
400	0.308	0.306	0.306	
400	0.306	0.307	0.307	
% RSD	0.035	0.026	0.029	

Table 4: Interday precision

Concentration(µg/ml)	%RSD			Average %RSD
	DAY1	DAY2	DAY2	
200	0.066	0.031	0.057	0.051

Table5: Accuracy readings of albumin

Concentration (µg/ml)	Level of Addition (%)	Concentration added in(µg/ml)	% Recovery	Statistical Analysis		
				MEAN	SD	%RSD
600	80	400.8	100.20			
600	80	400.8	99.70	100.23	0.55	0.054
600	80	400.8	100.80			
600	100	600.0	100.70			
600	100	600.0	101.10	100.76	0.30	0.03
600	100	600.0	100.50			
600	120	700.2	99.64			
600	120	700.2	99.99	99.99	0.35	0.03
600	120	700.2	100.34			

Table 6: Results showing Ruggedness

Analyst 1 Concentration(µg/ml)	Absorbance	Statistical analysis
200	0.175	
200	0176	Mean-0.175
200	0.176	
200	0.174	SD-8.366
200	0.176	
200	0.176	% RSD- 0.047
Analyst 2		
200	0.176	Mean-0.174
200	0.174	
200	0.174	SD- 8.162
200	0.175	
200	0.174	% RSD-0.046
200	0.176	

Table7: Results showing robustness

Room temperature Concentration(µg/ml)	Absorbance	Statistical analysis
200	0.174	Mean –0.174
200	0.174	
200	0.176	SD- 0.00081
200	0.175	
200	0.175	%RSD – 0.046
200	0.174	
Temperature 18^0c		
200	0.175	Mean – 0.175
200	0.176	
200	0.176	SD- 0.00075
200	0.175	
200	0.174	%RSD- 0.043
200	0.175	

Table 8: Results of various parameters of developed method

Parameter	Result
Absorption maxima	278.60nm
Beers law range	200-1000µg/ml
Correlation coefficient	0.9996
Regression equation	0.0008x + 0.0186
Slope	0.0008
Intercept	0.0186
Accuracy	99.64-101.1%
Precision (%RSD)	Intraday (0.03), Interday(0.05)
LOD, µg/ml	3.33
LOQ,µg/ml	10.125

Results and Discussion

Identification of proteins can be done by various colour reactions but the reagents used for colour reactions are costly and results obtained are not specific and precise. Simple chromatographic methods are not also effective due to high molecular weight of proteins. More sophisticated methods like gel electrophoresis, size exclusion chromatography are required to detect quantity of protein in sample. Hence an attempt is made to develop simple method for quantization.

The proposed method provides a simple, accurate, economical and convenient method for the analysis of total protein of *Pisum sativum* L. using UV spectrophotometer. The wavelength corresponding to maximum absorbance in buffer was found at 278.60nm. Beers law was obeyed in the concentration range of 200-1000µg/ml with correlation coefficient 0.9996. Accuracy of the proposed method was determined by the recovery studies, and good % recoveries (99.64-101.1%) of albumin obtained indicate that method is accurate. The method was found to be precise as %RSD values for interday and intraday was found to be less than 2. The method was also found to be rugged and robust as the % RSD values were found to be less than 2. The limit of detection and limit of quantification of the proposed method was found to be 3.33 and 10.125 µg/ml indicating that the method developed is sensitive.

Conclusion

The developed method can be concluded to be simple, accurate, and reliable and economical. Hence can be used for the routine analysis of total protein of *Pisum sativum* L. This method could be applied to determine concentration of proteins in food products, herbal drugs and formulations.

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