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Method Development and Validation for Estimation of Casein in Milk

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Casein; Milk; UV-spectrophotometric method; ICH guidelines; Validation.

ABSTRACT: Milk contains 3.3% total protein and also contains 9 essential amino acids required by humans. Casein is one of the major categories of protein in milk. There are several spectrophotometric methods were carried out for the determination of total proteins in milk samples. Here we developed and validated a simple, accurate, specific and precise spectrophotometric method by using a UV spectrophotometer. In the proposed method 0.1N NaOH was used as a solvent. The λmax of standard casein was found to be 219nm. It exhibited the linearity in the concentration range of 10-50µg/ml with a correlation coefficient of 0.999. The method was validated according to the ICH guidelines for the estimation of casein in milk. The validation studies of the method showed percentage Relative Standard Deviation (% RSD) values less than 2 % indicating that the developed method is precise. The percentage recovery of the casein for the proposed method was found to be 99%. So the developed method can be used for the estimation of casein in the milk sample.

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INTRODUCTION

The milk phosphoprotein, casein (pronounced KY-see) is present in the milk from all mammals. It is found in dairy products such as cheese, milk, and yogurt and is also used independently as an emulsifying and binding agent in countless processed foods, including “vegetarian” cheeses, "vegetarian" meats, cereals, bread, and supplements [1].

The total protein component of milk is composed of numerous specific proteins. The primary groups of milk proteins are the caseins. There are 3 or 4 caseins in the milk of most species; the different caseins are distinct molecules but are similar in structure. All other proteins found in milk are grouped together under the name of whey proteins. The major whey proteins in cow milk are β-lactoglobulin and α-lactalbumin. The major milk proteins, including the caseins, β-lactoglobulin, and α-lactalbumin, are synthesized in the mammary epithelial cells and are only produced by the mammary gland [2].

Many spectrophotometric methods have been evaluated for total protein determination in milk samples in the range of 220- 280 nm due to their tryptophan content, which shows an absorption maximum at this wavelength [3]. However, among them, the Bradford assay was used for the determination of total proteins in skim milk powder and whole milk powder instead of the Kjeldahl method [4]. Another spectrophotometric method was based on the alkaline solution-induced changing the spectrum of tyrosine to higher wavelength values in the UV region. In the range between 248 and 256 nm, the absorbance was found to be a linear function of the wavelength and the slope coefficient is directly proportional to the protein concentration [5].

Based on the above study we have developed and validated cheap and accurate methods as per ICH guidelines. For the estimation of casein present in milk, 0.1N NaOH was used.

MATERIALS AND METHODS

All spectral measurements were carried out in ELICO SL 244 double beam UV-VIS spectrophotometer using 1.00 cm quartz cuvettes. The weighing process was carried out in SHIMADZU AUX 220.

1. MILK SAMPLES AND SOLUTIONS

A. Milk samples: The sample of milk purchased from the local market.

B. Milk solutions: The milk sample was prepared in the following concentration of 0.125 g/l for the UV-219nm

C. Extractor solution of chloroform and methanol: A solution of chloroform-methanol (2:1 v/v) was prepared and used for the extraction of casein in milk samples.

2. STANDARD SOLUTIONS

Casein: Casein (Riedel) solutions were prepared in NaOH 0.10 M and used as a standard in the following concentration, 0.125 g/l for the UV-219.

3. ASSAY METHODS FOR ESTIMATION OF CASEIN IN MILK

i. Extraction of casein

A 5ml of sample milk, 18.0 ml of the solution of chloroform-methanol were added. The tubes were sealed and shaken vigorously for 5 min. The solutions were filtered using a quantitative filter paper and the organic phase was discarded. Then, 6.0 ml of chloroform and 6.0 ml of water were added to the solid samples and they were shaken for 5 min and filtered [3].

ii. Selection of Analytical Wavelength

10 µg/ml solutions of casein were prepared in 0.1M NaOH by appropriate dilution and the spectrum was recorded between 200-400 nm. The maximum wavelength of casein was found to be 219nm.

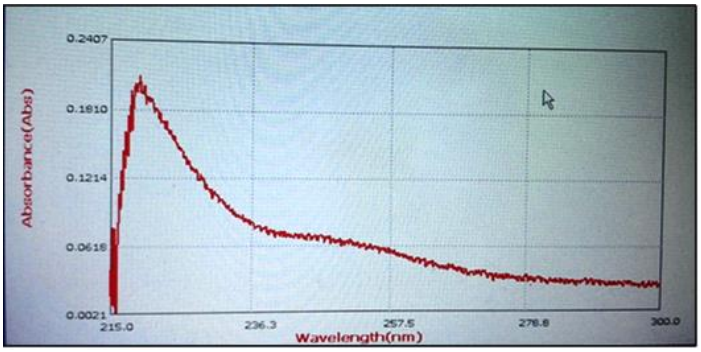

Fig 1: λmax of casein at 219nm by UV-spectroscopy

4. METHOD VALIDATION

The proposed method has been extensively validated in terms of specificity, linearity, accuracy, precision, robustness, ruggedness, limits of detection (LOD) and quantification (LOQ). The accuracy was expressed in terms of percent recovery of the known amount of the standard casein added to the known amount of the milk. The precision was expressed with respect to the repeatability, intra-day and inter-day variation in the expected casein concentrations. The method validation is carried out as per ICH guidelines [6].

A. SPECIFICITY

Specificity is the ability to assess the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s).

B. LINEARITY

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

C. ACCURACY (% RECOVERY)

The accuracy of the method was determined by calculating the recovery of casein by the standard addition method. The accuracy of the analytical method was assessed by determination of recovery for three concentrations corresponding to 50,100 and 150% of test solution concentration. For each concentration, three sets were prepared. The mean recovery of casein was reported.

D. PRECISION

The degree of reproducible results produced by a sample at different conditions

D.1. Repeatability- Repeatability expresses the precision under the same operating conditions over a short interval of time.

D.2. Intermediate precision- Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

D.3. Reproducibility- Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to the standardization of methodology).

E. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The robustness study was carried out by using 20(µg/ml) concentration.

F. RUGGEDNESS

The ruggedness is to expresses within-laboratories variations: different days, different analysts, different equipment, etc. The ruggedness study was by using 20(µg/ml) concentration.

5. DETECTION LIMIT AND QUANTITATION LIMIT

ICH guideline describes several approaches to determine the detection and quantitation limits. These include visual evaluation, signal-to-noise ratio and the use of standard

deviation of the response and the slope of the calibration curve. In the present study, the LOD and LOQ were based on the third approach and were calculated according to the 3.3σ/S and 10σ/S criterions, respectively; where σ is the standard deviation of y-intercepts of regression lines and s is the slope of the calibration curve.

RESULTS AND DISCUSSION

Table 1: Calibration curve of casein data

S.NO	CONCENTRATION(µg/ml)	ABSORBANCE
1	10	0.5175
2	20	0.6113
3	30	0.7223
4	40	0.8201
5	50	0.9131

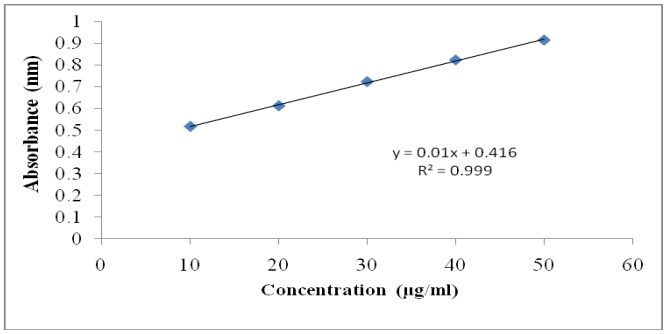

Fig 2: Calibration curve of casein

Table 2: Results of the assay of casein in milk.

S.NO	CONC. (µg/ml)	STANDARD ABSORBANCE	TEST ABSORBANCE	AMOUNT FOUND	%PURITY
1	20	0.6113	0.6103	19.344	96.7%
2	20	0.6113	0.6109	19.404	97%
3	20	0.6113	0.6104	19.354	96.7%
4	20	0.6113	0.6100	19.314	96.5%
5	20	0.6113	0.6099	19.304	96.5%

MEAN OF ASSAY VALUES	STANDARD DEVIATION	% RSD
0.6103	0.000645	0.064509

Table 3: Linearity

S.NO	CONC. ((µg/ml)	ABSORBANCE (mean,N=6)	STANDARD DEVIATION	%RSD
1	10	0.5169	0.00051	0.0986
2	20	0.6116	0.00040	0.0660
3	30	0.7225	0.00047	0.0653
4	40	0.8220	0.00132	0.1612
5	50	0.9132	0.00060	0.0659

Table 4: Precision

PRECISION		REPEATABILITY		REPRODUCIBILITY		INTERMEDIATE PRECISION	
		MORNING	EVENING	DAY 1	DAY 2	ANALYST 1	ANALYST 2
Absorbance 20µg/ml	S1	0.6115	0.6118	0.6119	0.6110	0.6115	0.6110
	S2	0.6123	0.6125	0.6129	0.6120	0.6123	0.6120
	S3	0.6145	0.6146	0.6145	0.6135	0.6145	0.6156
	S4	0.6134	0.6139	0.6140	0.6145	0.6134	0.6143
	S5	0.6121	0.6120	0.6123	0.6125	0.6121	0.6120
	S6	0.6130	0.6135	0.6134	0.6139	0.6130	0.6135
Mean		0.6128	0.61305	0.61317	0.6129	0.6128	0.61307`
Standard Deviation (SI		0.00107	0.00112	0.001	0.0013	0.00107	0.00171
Relative Standard Deviation(RSD)		0.00175	0.00183	0.00162	0.00213	0.00175	0.00279
%RSD		0.17454	0.18303	0.01623	0.21273	0.17454	0.27943

Table 5: Accuracy

RECOVERY LEVEL	STANDARD CONC. 20(µg/ml)	TEST CONC. 20(µg/ml)	ABSORBANCE			MEAN	%RECOVERY
			S1	S2	S3		
50%	1ml	0.5ml	0.7613	0.7599	0.7625	0.7612	98%
100%	1ml	1ml	0.8112	0.8154	0.8161	0.8142	99%
150%	1ml	1.5ml	0.8612	0.8585	0.8621	0.8606	88%

Table 6: Robustness

ROBUSTNESS		AT ROOM TEMPERATURE (29°C):	ELEVATED TEMPERATURE (35°C)
Absorbance at 20µg/ml	S1	0.6123	0.6134
	S2	0.6133	0.6142
	S3	0.6148	0.6152
	S4	0.6139	0.6146
	S5	0.6129	0.6135
	S6	0.6143	0.6153
Mean		0.61358	0.61437
Standard Deviation (SD)		0.00093	0.00082
Relative Standard Deviation(RSD)		0.00151	0.00133
%RSD		0.15093	0.1329

Table 7: Ruggedness

Ruggedness		Analyst 1	Analyst 2
Absorbance at 20µg/ml	S1	0.6115	0.6110
	S2	0.6123	0.6120
	S3	0.6145	0.6156
	S4	0.6134	0.6143
	S5	0.6121	0.6120
	S6	0.6130	0.6135
Mean		0.6128	0.61307`
Standard Deviation (SD)		0.00107	0.00171
Relative Standard Deviation(RSD)		0.00175	0.00279
%RSD		0.17454	0.27943

Table 8: LOD and LOQ

Limit of Detection	Limit of Quantification
0.178µg/ml	0.542µg/ml

Table 9: Summary of validation parameters for the proposed method

S.NO	PARAMETERS	RESULTS
1.	Absorption maximum (λ_{max})	219nm
2.	Beer's Law limit (µg/mL)	10-50 µg/mL
3.	Slope	0.009463
4.	Intercept	0.4169
5.	Coefficient of correlation	0.999
6.	Accuracy (%recovery)	99%
7.	Precision (% RSD)	0.06123%
8.	Robustness (% RSD)	0.01329%
9.	Ruggedness(% RSD)	0.17454%
10.	Limit Of Detection (LOD)	0.178 µg/mL
11.	Limit Of Quantification (LOD)	0.542 µg/mL

CONCLUSION

The proposed method development provides simple, specific, precise, accurate and reproducible results. The method was validated as per ICH guidelines in terms of specificity, linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ), robustness and ruggedness. The proposed method can be used for routine analysis of casein present in milk.

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DATA AVAILABILITY

Not declared.

CONFLICTS OF INTEREST

The authors declare no conflict of interest in this research article.

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