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Quantification of vitamin-B2, B6 in *Spinacia oleracea* by HPTLC

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Abstract
A new HPTLC (High Performance Thin Layer Chromatography) method was developed for the quantification of vitamin-B2, B6 in *Spinacia oleracea.* Separation of vitamins was achieved by using mobile phase as methanol: benzene: 0.1% formic acid (5: 4: 1 v/v), stationary phase as TLC silica gel 60 F_{254} (Aluminium sheets). The sample volume sprayed was 30µL, with dosage speed of 20 µL/sec. The determination was carried out by using the densitometric absorbance mode at 254nm and 365nm. R_f values & area of vitamin-B2, B6: 0.854, 0.784 & 1095, 780 respectively. The developed method was validated as per ICH guidelines and it was found to be reproducible and convenient for quantitative analysis of vitamin-B2, B6 in *Spinacia oleracea* (spinach). The method was linear in the range of 2000 ng to 10000 ng with correlation of 0.9992, 0.9993 & R_f values are 0.874, 0.780 for vitamin-B2, B6 respectively, other parameters like area was quantified and % RSD was calculated which was found to be NMT 2.0%. The method was simple, accurate, precise and successfully applied for the routine quantitative analysis.

Keywords: *Spinacia oleracea* (spinach), HPTLC, quantification, Vitamin-B2, B6

Introduction
Vitamin-B2 (Riboflavin) is 7,8-dimethyl-10-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]-2H, 3H, 4H, 10H benzo [g] pteridine-2, 4-dione. Molecular formula is $C_{17}H_{20}N_4O_6$, molecular weight is 376.3639 g/mol. It is used for the treatment of ariboflavinosis (vitamin B2 deficiency), cosmetic colorant; hair dyeing; skin conditioning, food additives [1]. Vitamin-B6 (Pyridoxine) is 4, 5-bis (hydroxymethyl)-2-methylpyridin-3-ol. Molecular formula is $C_8H_{11}NO_3$, molecular weight is 169.1778 g/mol. It is indicated for the treatment of vitamin B6 deficiency and for the prophylaxis of Isoniazid-induced peripheral neuropathy. It is also approved by Health Canada for the treatment of nausea and vomiting in pregnancy in a combination product with Doxylamine (as the commercially available product Diclectin) [2]. From literature review [3-12], no method was reported for quantification of vitamin-B2 & B6 in *Spinacia oleracea* by HPTLC. The main aim is to isolate and quantify the vitamin-B2, B6 present in *Spinacia oleracea* extract by using HPTLC. The main objective of the study is to develop a new HPTLC method for separation of vitamin-B2, B6 present in *Spinacia oleracea* extract, to validate the developed HPTLC method as per ICH guidelines and application of developed HPTLC method for the routine quantitative analysis.

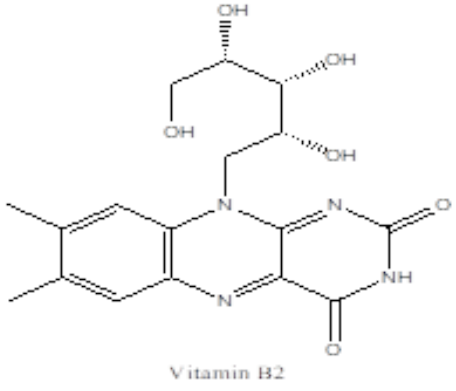

Fig 1: Structure of vitamin-B2

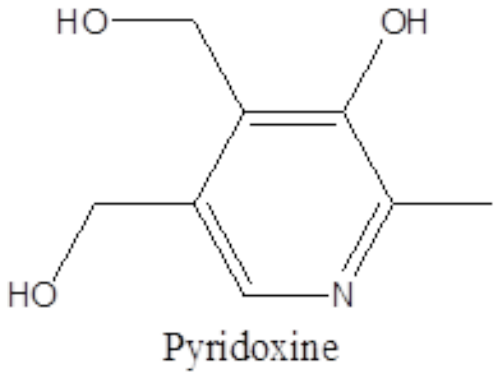

Fig 2: Structure of vitamin-B6

Materials and Methods
Standard vitamin-B2, B6 procured from sigma Aldrich, chemicals from Thermo fisher scientific India Pvt. Ltd., The HPTLC system manufactured by AETRON consists of following components: AETRON HPTLC model containing Linomat – 5 sample applicator, variable wavelength programmable AETRON TLC Scanner – 3 by using sprayl in software, AETRON Twin-trough chambers, Hamilton syringe (100 µL), Chromatographic analysis was performed on aluminum sheets i.e., TLC silica gel 60 F_{254} HPTLC plates (Merck, Darmstadt, Germany), cam port which is, Elite-mini luminous consists of visible light, UV- 254nm, 365nm with EOS utility, quantification of plates is done by using JUST TLC or AETRON IDS software. 1mg sensitivity balance manufactured by ESSAE was used for weighing the compounds.

Mobile Phase Preparation
For better separation of vitamin-B2, B6 - methanol: benzene: 0.1% formic acid was selected as mobile phase in the ratio of 5: 4: 1 %v/v.

Preparation of Standard Stock Solutions

10mg of standard vitamin-B2, Vitamin-B6 was weighed and transferred into different volumetric flasks and dissolved in HPLC water by using cyclomixer. The flasks were shaken and volume was made up to the mark with HPLC water to give a solution of 1000µg/mL i.e., 10mg/mL [Stock solution I].

Preparation of Working Standard Stock Solutions of Vitamin-B2, Vitamin-B6

The working standard solutions of vitamin-B2, Vitamin-B6 was prepared by taking 1mL of stock solution-I and made up to 10 mL with HPLC water (working standard 100µg/mL). From this 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, 1.0 mL was pipetted out in 10mL volumetric flasks separately and the volume was made up to 10 mL with HPLC water. So, application of 2 to 10µg/ mL volume was given a series of spots covering the range of 2000 to 10000ng/spot.

Sample Preparation for Determination of Vitamin B2, Vitamin B6 From Crude Spinach Extract

Dried leaves (up to 75%) of spinach was taken in clean, dried mortar, sand and methanol and HPLC water was added in the ratio of 1:1. Grind with pestle to form paste & then filtered and taken into separating funnel. Benzene was added, mixture was shaken and set aside for 10 min to remove chlorophyll layer. Bottom water, methanol was extracted, 10ml of triturated extract of *Spinacia oleracea* was transferred into volumetric flask (stock). From this, 1mL was pipetted out and diluted with 10mL HPLC water (working standard). From that 0.6mL was pipetted out and made up to 10mL HPLC water. So, application of 6µL volume was given a spot of 6000ng.

Preparation 0.1% Formic Acid

0.1 mL of formic acid was pipetted out transferred into 100mL volumetric flask and made up to 100mL with HPTLC water.

Method Development

After several trials with different combinations and ratios of solvents, chromatographic conditions, the method was optimized.

Table 1

Stationary phase	4.5 cm × 10 cm TLC silica gel 60 F_{254} Aluminium sheets.
Mobile phase	Methanol: Benzene: 0.1% formic acid (5: 4: 1% v/v)
Dosage speed	20µL/sec
Band length Band space	8mm 7mm
Sample volume	30 µL
Detection wavelength	254nm, 365nm

Standard

Fig 3: Chromatogram at 254nm

Fig 4: Chromatogram at 365nm

Sample

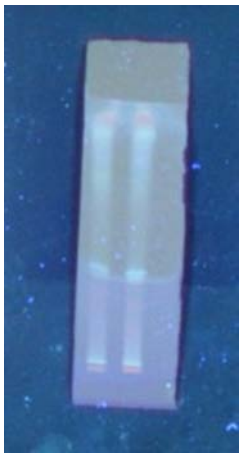

Fig 5: Chromatogram at 365nm

Fig 6: Chromatogram at 254nm

R_f values & area of standard vitamin-B2, B6: 0.854, 0.784 & 1095, 780. R_f values & area of sample vitamin-B2, B6: 0.836, 0.762 & 1036, 684 respectively.

Method Validation

Validation of the developed method was done according to ICH Q2(R1) guidelines [13].

Results and Discussion

System Suitability

From the working standard solution 0.6 mL of vitamin-B2, B6 was pipetted out in 10mL volumetric flask separately and the volume was made up to 10 mL with HPLC water. This was sprayed for 6 times i.e., 6 bands.

Table 2: System Suitability Parameters of Standard

Vitamin-B2			Vitamin-B6	
Injection No.	**Retardation factor (R_f)**	**Area**	**Retardation factor (R_f)**	**Area**
1	0.859	1028	0.796	758
2	0.864	1025	0.791	763
3	0.869	1023	0.791	780
4	0.869	1030	0.780	776
5	0.874	1036	0.796	765
6	0.874	1031	0.791	779
Mean		1028.833	770.1667	
Standard deviation (SD)		4.6224	9.3256	
% RSD		0.45	1.21	

Acceptance criteria: The % RSD should be NMT 2.0%.

Specificity

Specificity of the method was established by comparing standard and sample bands for R_f values. From the working standard solution and sample solution, 0.6 mL of vitamin-B2, B6 was pipetted out in 10mL volumetric flask separately and the volume was made up to 10 mL with HPLC water.

Acceptance Criteria: The Retardation factor (R_f) should be identical for both the standard and sample chromatograms.

Linearity

It is mainly used to find out the concentration of unknown samples by comparing it with known standard concentrations. It is plotted for signal response (peak area) versus concentration (ng/mL). From the working standard solution, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, 1.0 mL was pipetted out in 10mL volumetric flasks separately and the volume was made up to 10 mL with HPLC water which gives concentration of 2000 ng/mL, 4000 ng/mL, 6000 ng/mL, 8000 ng/mL, 10000 ng/mL

Table 3: Linearity of standard vitamin-B2, B6

Vitamin-B2			Vitamin-B6	
Concentration (ng/mL)	**Retardation factor (R_f)**	**Area**	**Retardation factor (R_f)**	**Area**
0	0	0	0	0
2000	0.847	345	0.752	260
4000	0.851	691	0.743	520
6000	0.851	1036	0.748	780
8000	0.847	1381	0.739	1040
10000	0.833	1668	0.73	1258
Regression equation	y = 0.1685x + 11.143		y = 0.127x + 8	
Slope (m)	0.1685		0.127	
Correlation coefficient (R^2)	0.9992		0.9993	

Acceptance criteria: Correlation coefficient (R2) should be NLT 0.999.

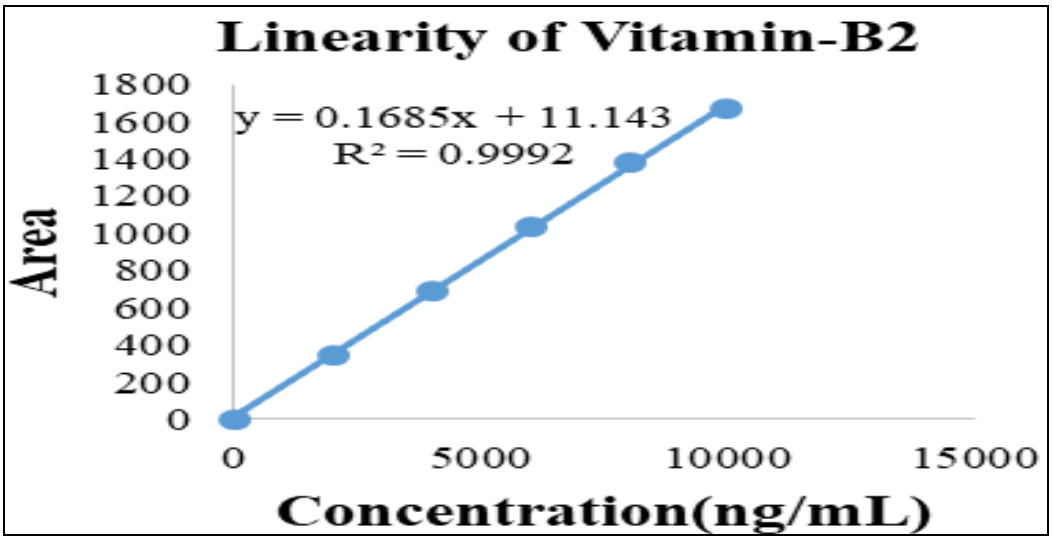

Fig 7: Linearity of vitamin-B2

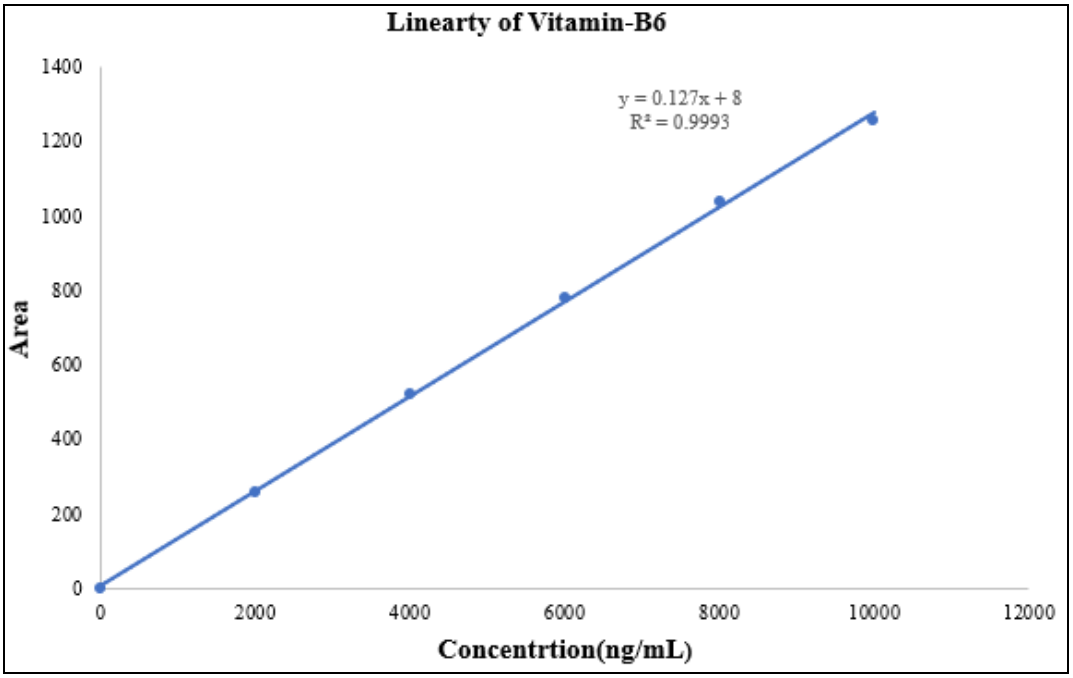

Fig 8: Linearity of vitamin-B6

Accuracy

The accuracy of the method was determined by analyzing three solutions of spinach extract containing vitamin-B2, B6 at approximately 50%, 100% and 150%. From working standard solution, 0.2 mL, 0.6 mL, 1.0 mL was pipetted out and transferred into 10 mL volumetric flasks separately, and volume was made up to 10 mL with HPLC water. Each concentration is spotted for 3 times.

Table 4: Accuracy of vitamin-B2

Concentration (%)	Sample area	Average Sample area	Standard area	%Recovery	%Mean recovery	Overall % Mean recovery
50	694	694.67	1038.67	100.28	100.37	100.06
	698			100.86		
	692			99.99		
100	1029	1033.34		99.13	99.54	
	1032			99.41		
	1039			100.09		
150	1384	1388		99.99	100.28	
	1389			100.35		
	1391			100.50		

Table 5: Accuracy of vitamin-B6

Concentration (%)	Sample area	Average Sample area	Standard area	% Recovery	% Mean recovery	Overall % Mean recovery
50	520	521.33	780	99.60	99.85	99.98
	525			100.56		
	519			99.41		
100	785	785.33		100.24	100.28	
	782			99.86		
	789			100.75		
150	1045	1042.33		100.08	99.82	
	1040			99.60		
	1042			99.79		

Acceptance criteria: The mean % recovery for each level should be not less than 98.0% and not more than 102.0%.

Precision: The precision was determined by system precision and method precision using 100% standard solutions from linearity range i.e., 6000 ng/mL

Table 6: Precision

Injection No.	Area of vitamin-B2		Area of vitamin-B6	
	Method precision	System precision	Method precision	System precision
1	1029	1032	755	758
2	1023	1024	764	763
3	1029	1028	775	780

4	1033	1035	773	776
5	1035	1033	765	765
6	1031	1036	779	779
Mean	1030	1028.833	768.5	770.1667
SD	4.1472	4.6224	8.8034	9.3256
%RSD	0.40	0.45	1.15	1.21

Acceptance criteria: The %RSD should be NMT 2.0%.

Limit of Detection (LOD) & Limit of Quantification (LOQ)

LOD

1mL of standard solutions of vitamin-B2, B6 was pipetted out from linearity solutions of vitamin-B2, B6 2000ng/mL in 10mL volumetric flasks separately and the volume was made up to 10mL with HPLC water which gives concentration of 200 ng/mL

LOQ

1mL of standard solution of vitamin-B2, B6 was pipetted out from working standard solutions of vitamin-B2, B6 in 10mL volumetric flasks separately and the volume was made up to 10mL with HPLC water which gives concentration of 1000 ng/mL

$DL = 3.3\sigma / S$, $QL = 10\ \sigma / S$

Where, σ = Standard deviation, S = Slope

LOD of vitamin-B2, B6 was found to be 8.81 ng, 29.88 ng & LOQ of vitamin-B2, B6 was found to be 26.70 ng, 90.55 ng respectively.

Robustness

From the working standard solution of vitamin-B2, B6 0.6 mL was pipetted out and transferred into volumetric flasks separately and volume was made up to 10 mL with water. Each parameter was sprayed for 2 times and %RSD was calculated.

Table 7: Robustness

Parameter	Area of vitamin-B2	Area of vitamin-B6
Change in mobile phase ratio +0.5 mL formic acid	1040	785
	1046	789
Average	1043	787
Standard deviation	4.2426	2.8284
% RSD	0.41	0.36
-0.5 mL formic acid	1010	722
	1002	725
Average	1006	723.5
Standard deviation	5.6568	2.1213
% RSD	0.56	0.29
Change in band length 10 mm	1080	840
	1082	843
Average	1081	841.5
Standard deviation	1.4142	2.1213
%RSD	0.13	0.25
6mm	995	710
	998	713
Average	996.5	711.5
Standard deviation	2.1213	2.1213
%RSD	0.21	0.30
Change in dosage speed 25µL/sec	1036	778
	1034	780
Average	1035	779
Standard deviation	1.4142	1.4142
%RSD	0.14	0.18
16µL/sec	1035	778
	1033	776
Average	1034	777
Standard deviation	1.4142	1.4142
%RSD	0.14	0.18

Acceptance criteria: The %RSD should be NMT 2.0%.

Conclusion

A new HPTLC method was developed for estimation and quantification of vitamin-B2, B6 in *Spinacia oleracea* and developed method was validated according to ICH Q2 (R1) guidelines. The separation of vitamins was achieved by using mobile phase as methanol: benzene: 0.1% formic acid (5: 4: 1 v/v), stationary phase as TLC silica gel 60 F_{254} (Aluminium sheets). The sample volume sprayed was 30µL, with dosage speed of 20 µL/sec.

The determination was carried out by using the densitometric absorbance mode at 254nm and 365nm. The method was linear in the range of 2000 ng to 10000 ng with correlation of 0.9992, 0.9993 & R_f values are 0.874, 0.780 for vitamin-B2, B6 respectively, other parameters like area

was quantified and % RSD was calculated which was found to be NMT 2.0%.

Thereby, it can be concluded that the developed method was found to be simple, accurate, precise, reproducible and convenient for quantitative analysis of vitamin-B2, B6 in *Spinacia oleracea* (spinach).

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