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RESEARCH ARTICLE

# Development and Validation of Analytical Methods by RP-HPLC for Estimation of Vitamin $D_3$ and Biotin in Calpond Gold Gel Suspension and its Stability at Accelerated Conditions

Sunil Shukla[1,*], Chanchal Tiwari[1], Naveen Kumar[1], Shipra Shukla[1], Adrija Chowdhury[1], Kamal Rukhaya[1], Aditi Mishra[1], Simran Narang[2] and Tanuj Hooda[3,*]

[1]*Research and Development Centre, Carus Laboratories Pvt, Ltd., Karnal, Haryana - 132022, India;* [2]*Guru Jambheshwar University of Science & Technology, Hisar, Haryana - 125001, India;* [3]*M.M. College of Pharmacy, Maharishi Markandeshwar (Deemed to be University), Mullana, Ambala, Haryana, India*

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**Abstract:** ***Introduction*:** In the current research, simple and isocratic analytical methods were developed and validated by ICH Q2 (R1) guidelines for the accurate quantitative measurement of Vitamin $D_3$ and Biotin in Calpond Gold Gel suspension.

***Methods*:** The analytical methods were developed using RP-HPLC with the best chromatographic conditions. These methods were validated for system suitability, specificity, linearity, LOD, LOQ, accuracy, precision, and robustness. Moreover, an accelerated stability study was carried out for Vitamin $D_3$ and Biotin to estimate the rate of degradation at accelerated conditions (Temp. 40°C ± 2°C and RH 75% ± 5% for 6 Months).

***Results*:** Methods showed good suitability (tailing factor < 2%, Theoretical plates > 2000), robustness and specificity without any significant interference, exhibited good linearity ($R^2$ > 0.990) over concentration ranges (3200 – 9600) IU for Vitamin $D_3$ and (12.5 – 37.5) µg/ml for Biotin, precision (RSD < 2%), recovery rates (> 99%), and LOD, LOQ were found to be 608.79 IU, 1844.82 IU and 1.72 µg/ml, 5.22 µg/ml for Vitamin $D_3$ and Biotin. Additionally, the % Assay complied with the in-house acceptance criteria (< 90%) of label claim from 0-6 months.

***Discussion*:** The developed methods were found in accordance with ICH Q2 (R1) guidelines. The validation parameters remained within the acceptable limits, indicating that the methods are reliable for the quantitative determination of Vitamin $D_3$ and Biotin in Calpond Gold Gel suspension. Moreover, Vitamin $D_3$ and Biotin were found to be stable during the accelerated stability study.

***Conclusion*:** The simple and isocratic RP-HPLC methods were successfully developed and validated according to the current ICH guidelines. The developed methods might be useful for the estimation of vitamin $D_3$ and Biotin in feed supplements, veterinary, and pharmaceutical formulations.

**Keywords:** RP-HPLC, validation, vitamin $D_3$, biotin, ICH guidelines, stability study.

## 1. INTRODUCTION

In pharmaceuticals, it is crucial to analyze bulk drug materials, intermediates, contaminants, drug formulations, degradation products, and associated metabolites [1-5]. Compared to the analysis of drugs and their metabolites in plasma & biological samples like blood, urine, or hair, pharmaceutical analysis techniques are significantly simpler [6-10]. However, as pharmaceutical product quality control is closely linked to patient health, the clear identification of medications in pharmaceutical formulations is equally crucial. Chemical analysis is essential to pharmaceutical control & drug development to guarantee patient safety and effectiveness [11-15].

For this reason, the pharmaceutical sector places a high priority on adequate and genuine quality control procedures.

*Address correspondence to this author at the Research and Development Centre, Carus Laboratories Pvt, Ltd., Karnal, Haryana - 132022, India; E-mail: sun.shukla5@gmail.com (S.S.) and M.M. College of Pharmacy, Maharishi Markandeshwar (Deemed to be University), Mullana, Ambala, Haryana, India;
E-mail: tanujhooda2010@gmail.com (T.H.)

The creation of highly complex compounds and therapeutic formulations as a result of pharmaceutical research and development necessitates the use of highly selective and innovative analytical techniques to facilitate their separation as well as purification. Therefore, suitable analytical techniques need to be created to regulate the caliber of pharmaceutical analysis. Pharmaceutical medications are identified and quantitatively analyzed using a variety of methods, including spectrophotometry, fluorimetry, electro-analytical techniques (primarily voltammetry), titrimetric and chromatographic techniques like HPLC, TLC, GC, & CE [16-25].

Calpond Gold Gel suspension is a feed supplementary product for cattle, containing a blend of Vitamin $D_3$, Biotin, and others as active constituents for maintaining higher lactation persistency, supporting sustained milk production throughout the lactation cycle, and thus, improving their udder health. Biotin is also known as Vitamin H (Figure 1). It is crucial for cattle as it acts as a cofactor for several key enzymes involved in gluconeogenesis, lipogenesis, and protein synthesis. Biotin plays a significant role in the metabolic processes necessary for maintaining optimal health and productivity in dairy cows. Research studies have consistently shown that biotin supplementation can enhance milk production in dairy cattle [26-33]. Vitamin $D_3$, as shown in Figure 2, was found to increase the milk yield in lactating cows [34, 35]. It is crucial for managing calcium absorption and metabolism [36-38]. It was mentioned that Vitamin $D_3$ helps stimulate cattle's immunity [39-42]. Vitamin $D_3$ was proven to be effective in preventing milk fever [43, 44].

Several High-Performance Liquid Chromatography (HPLC)-based methods have been developed for analyzing Biotin and Vitamin $D_3$ in pharmaceutical products. While these techniques are commonly used, they face notable challenges that limit their efficiency. These issues include decreased method stability, limited selectivity, and longer analysis times. Additionally, these methods often require expensive solvents, generally for gradient elution, which raises the overall analysis cost. The sample preparation steps for these techniques can also be complex and time-consuming, often involving advanced procedures such as supercritical fluid extraction or solid-phase extraction before HPLC separation [45-55].

Despite these challenges, there is a growing need for an alternative analytical approach that can overcome these limitations. The ideal method should offer higher sensitivity, greater selectivity for vitamin detection, and reduced analysis time. Furthermore, it should be cost-effective, minimizing the use of expensive solvents and simplifying the sample preparation process. Ultimately, such a method would streamline the overall procedure, making it more practical and accessible for routine analysis of both pharmaceutical and biological samples [56].

In various studies, biotin has been quantified using a C18 column by HPLC method [45, 57-59]. In this study, we utilized the RP-HPLC method using a C8 column for biotin quantification, resulting in excellent peak separation and shorter retention time than the C18 column in our formulation. Since biotin is a highly polar, water-soluble molecule with limited hydrophobic interaction, it often exhibits poor peak shape, excessive tailing, and variable retention on longer-chain C18 phases. To address these limitations, a C8 column was selected because its shorter alkyl chain provides a milder hydrophobic environment and better compatibility with polar analytes, resulting in improved peak symmetry and more consistent elution behavior compared with the C18 column. In contrast, the highly lipophilic vitamin $D_3$ requires a stronger hydrophobic phase, such as a C18 column, for effective retention and resolution [60–63].

In the previous research, acetonitrile and orthophosphoric acid were used as mobile phases with the C8 column for biotin analysis [64]. However, no study has yet utilized a C8 column as a stationary phase with sodium perchlorate as the mobile phase for biotin estimation. Sodium perchlorate was selected because perchlorate ions act as effective chaotropic agents, reducing secondary interactions of polar analytes with residual silanol groups. This results in improved peak symmetry and enhances reproducibility, particularly for polar compounds like biotin, which exhibit poor retention in other buffer systems. In our method, sodium perchlorate was found to reduce peak tailing and provide better separation with shorter retention times [63, 65].

Additionally, the current study utilized an isocratic elution technique, which is simple and time-efficient, as it required only one mobile phase. Moreover, previous research has reported lower recovery rates for Vitamin $D_3$ during method validation [66]. However, in our study, methanol was used as the mobile phase for Vitamin $D_3$, and the recovery rates were found to be higher than those previously reported. Hence, in the current research, simple and isocratic analytical methods utilizing Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) have been developed and thoroughly validated by ICH Q2 (R1) guidelines for the accurate quantitative measurement of Vitamin $D_3$ and Biotin in the Calpond Gold Gel suspension. These methods ensure the precise determination of the two key active ingredients, Vitamin $D_3$ and Biotin, in the product, and undergo a rigorous validation process to confirm their reliability, sensitivity, and accuracy for the intended analytical purposes. Furthermore, an accelerated stability study was carried out for Vitamin $D_3$ and biotin using the developed RP-HPLC methods to ensure the sustainability of ingredients at accelerated conditions (Temp. 40°C ± 2°C and RH 75% ± 5% for 6 Months).

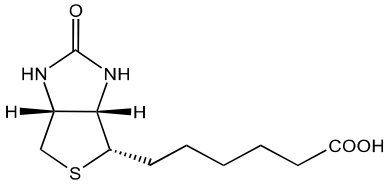



**Fig. (1).** Chemical structure of biotin.

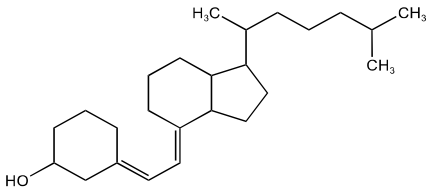



**Fig. (2).** Chemical structure of vitamin $D_3$.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and Reagents

All the chemicals and reagents used in the analysis were of analytical grade. Acetonitrile, Methanol, and HPLC water were purchased from Qualigens; Orthophosphoric Acid was purchased from SD Fine Chem Limited. Sodium Perchlorate Monohydrate and Sodium Hydroxide (NaOH) pallets were purchased from Loba Chemie Pvt Ltd. Biotin and Vitamin $D_3$ were used as working standards and obtained from Sigma Aldrich.

### 2.2. Instrumentation

RP-HPLC of Shimadzu LC-2050 series liquid chromatography was utilized for the development and validation of methods and stability study.

## 3. ANALYTICAL METHODS DEVELOPMENT

### 3.1. Chromatographic Conditions

The mobile phase for Vitamin $D_3$ consists of Methanol (100%) with a flow rate maintained at 1.0 ml per minute. A stainless steel column 250 mm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5µm), was utilized. 50 µl of the solution was injected. The detection was completed at a 254 nm wavelength.

The mobile phase of Biotin is composed of 1.0 g of Sodium Perchlorate Monohydrate dissolved in 900 ml of distilled water and mixed with 85 ml of Acetonitrile and 1 ml of Orthophosphoric Acid. Volume makeup (1000 ml) was done with water, followed by mixing and filtering. The flow rate was maintained at 1.0 ml per minute. A stainless steel column 150 mm × 4.6 mm, packed with octadecylsilane bonded to porous silica (5µm), was utilized. 50 µl of the solution was injected. The detection was done at a 200 nm wavelength. The column temperature was maintained at 25°C. The total run time was 20 minutes for Vitamin $D_3$ and 25 minutes for biotin.

### 3.2. Preparation of Standard Solution

The standard solution of Vitamin $D_3$ was prepared by dissolving 16 mg of Vitamin $D_3$ in 100 ml of methanol, followed by sonication for 2-3 minutes at 25°C. Further, 2 ml of the above-prepared Vitamin $D_3$ solution was transferred to a 100 ml volumetric flask and diluted with methanol up to the mark. Further, 5ml of the above-prepared Vitamin $D_3$ solution was transferred to a 100 ml of volumetric flask and diluted with methanol, followed by sonication for 2-3 minutes. After sonication, the volume was made up to the mark with methanol and filtered through a 0.20 µm syringe filter. The final solution was then transferred to the amber-coloured HPLC vials. The final concentration of the Vitamin $D_3$ standard solution was 6400 IU.

A standard solution of biotin was prepared by dissolving 25 mg of biotin in 100 ml of 0.1N of NaOH solution at 25°C. Further, diluted 10 ml of the above solution to 100 ml with mobile phase, followed by sonication for 2-3 minutes for its complete dissolution, and filtered through a 0.20 µm syringe filter. The final solution was then transferred to the amber-coloured HPLC vials. The final concentration of the biotin standard solution was 25 µg/ml.

### 3.3. Preparation of Sample Solution

For testing of the Vitamin $D_3$ solution, 2 g of the sample was taken in a 100 ml volumetric flask, and 70 ml of methanol was added at 25°C. Then, the complete mixing was carried out with the help of sonication for 2-3 minutes, and volume was made up to the mark with methanol and filtered through a 0.20 µm syringe filter. The final solution was then transferred to the amber-coloured HPLC vials. The final concentration of the Vitamin $D_3$ sample solution was 6400 IU.

For biotin, 2.5 g of sample was taken in a 100 ml volumetric flask, and mixed with 10 ml of 0.1N NaOH. Then, the complete mixing was carried out with the help of sonication for 2-3 minutes. The volume was made up to the mark with the mobile phase and filtered through a 0.20 µm syringe filter. The final solution was then transferred to the amber-coloured HPLC vials. The final concentration of the biotin sample solution was 25 µg/ml.

## 4. ANALYTICAL METHODS VALIDATION: QUANTIFICATION OF VITAMIN $D_3$ AND BIOTIN

The developed methods were thoroughly validated to ensure their reliability and performance. This validation process included evaluating key parameters such as specificity, linearity, LOD, LOQ, accuracy, precision, robustness, and system suitability. These evaluations were conducted in strict adherence to the ICH guidelines for the validation of analytical procedures.

### 4.1. Specificity

The developed methods must exhibit a high degree of specificity, ensuring that only the analyte of interest is detected without any significant interference. To assess this, the blank, placebo solution, and Vitamin $D_3$ and Biotin solutions were injected into the system, and the representative chromatograms were obtained [67-69].

### 4.2. Linearity, LOD, and LOQ

The evaluation of linearity should be conducted by examining the plot that depicts the relationship between the analytical signals and the concentration of the analyte. If the plot reveals a linear trend, the results should then be subjected to appropriate statistical methods to confirm the relationship. In this study, Vitamin $D_3$ and biotin solutions were prepared at five different concentration levels, ranging from 50% to 150% of the working concentration. At each concentration level, the analysis was performed in triplicate to ensure consistency and reliability. The data obtained from the peak areas at each concentration were then analyzed using linear regression. Three separate data sets, each corresponding to one of the triplicate analyses, were constructed and used to evaluate the linearity of the method across the specified concentration range. LOD and LOQ were obtained with the help of linear equations using the formula (3.3 × SD/S) and (10 × SD/S) [67, 69-71].

### 4.3. Accuracy

An accuracy study was conducted by adding known quantities of Vitamin $D_3$ and Biotin into a placebo preparation to assess the method's ability to accurately measure the analyte. The actual concentrations of Vitamin $D_3$ and Biotin were compared with the measured concentrations, and their recovery rates were calculated to evaluate the method's accuracy. The recovery of these methods was assessed at three distinct concentration levels, which represented 50%, 100%, and 150% of the test preparation concentration. For each of these concentration levels, three separate sets were prepared and analyzed to ensure the reliability and consistency of the recovery results at each level [67-70, 72].

### 4.4. Precision

The precision of an analytical procedure refers to the degree of agreement, or the extent of scatter, between a series of measurements obtained from multiple samples taken from the same homogeneous sample, all conducted under the same prescribed conditions. In this study, the precision of the assay methods was evaluated in terms of repeatability by conducting six independent assays (within the same day) of Vitamin $D_3$ and Biotin. To further assess the method's intermediate precision, the same procedure was performed by a different person on a different day and in a different lab, under the same experimental conditions. The precision of the method was acceptable if the %RSD did not exceed a limit of 2% [67, 69, 73].

### 4.5. Robustness

The robustness of an analytical procedure refers to its ability to maintain reliable performance despite small, intentional variations in key method parameters. It serves as an indicator of how consistent and dependable the method will be under typical operational conditions. For this particular study, the factors selected to assess robustness included adjustments to the wavelength (± 5 nm) and flow rate (±10%). These variations were deliberately introduced to evaluate their impact on the analysis of Vitamin $D_3$ and Biotin in samples. The effects of these changes were examined by observing several analytical parameters, including retention time (RT), asymmetry factor, number of theoretical plates, and the assay results [67, 69].

### 4.6. System Suitability

System suitability testing plays a crucial role in determining whether the developed chromatographic system is appropriate and reliable for the intended analysis. In this study, several parameters were evaluated to assess the system's performance, including the %RSD, retention time, area, tailing factors, and the number of theoretical plates. To ensure accuracy and consistency, six replicate samples, each containing Vitamin $D_3$ and Biotin, were analyzed using the newly developed methods. The results of this analysis were carefully examined to verify that the chromatographic system met the requirements for reliable and reproducible performance [67, 69].

### 4.7. Solution Stability

The standard and sample solutions of Vitamin $D_3$ and biotin were injected into the HPLC system at 0 hours, 12 hours, and 24 hours. The difference in %assay results and %RSD values was calculated. The solution was considered stable if the %assay and %RSD values met the acceptance criteria of less than 2.0% [74].

## 5. ACCELERATED STABILITY STUDY OF VITAMIN $D_3$ AND BIOTIN

The accelerated stability testing was performed for 0, 1, 3, and 6 months according to the ICH Stability Guidelines [75]. The samples of Calpond Gold Gel suspension (100 ml) were placed in a stability chamber for 1, 3, and 6 months at accelerated conditions (Temp. 40°C ± 2°C and RH 75% ± 5%). The analysis was carried out through the above validated RP-HPLC method to determine the effect of accelerated conditions on the amount of Vitamin $D_3$ and Biotin in the formulation.

## 6. RESULTS AND DISCUSSION

### 6.1. Optimization of Chromatographic Conditions

For the development of robust and reliable HPLC methods, two different HPLC analytical columns were optimized: a C8 column (150 mm × 4.6 mm, 5 µm) and a C18 column (250 mm × 4.6 mm, 5 µm). Various mobile phase compositions were evaluated, and the optimal combinations were selected based on their ability to produce sharp, well-resolved peaks for Vitamin $D_3$ and biotin using an isocratic elution mode at a flow rate of 1.0 mL/min. The column temperature was maintained at 25°C, with an injection volume of 50 µL.

A Photodiode Array (PDA) detector was utilized for detection. Wavelengths of 254 nm for Vitamin $D_3$ and 200 nm for Biotin were found to be optimal, providing the best sensitivity and peak response for each compound. The total run time was 20 minutes for Vitamin $D_3$ and 25 minutes for Biotin. Retention times were approximately 9 minutes for Vitamin $D_3$ and 16 minutes for Biotin under the optimized conditions.

### 6.2. Methods Validation

#### *6.2.1. Specificity*

The developed methods were found to be specific without any significant interference. The specificity of Vitamin $D_3$ and Biotin was determined by comparing the chromatograms of the sample solution with the standard solution, placebo, and the blank, ensuring that the peak observed in the chromatograms corresponds to only one component. The chromatograms for blank, placebo, standard, and sample solutions for Vitamin $D_3$ and biotin are demonstrated in Fig. (**3**).

#### *6.2.2. Linearity, LOD, and LOQ*

During the linearity test, we found a regression coefficient ($R^2$) of 0.998 for Vitamin $D_3$ and 0.997 for biotin, demonstrating that the analytical procedure exhibits an excellent linear relationship between analyte concentration and peak area, well within the acceptance criteria of 0.990 to 1.000, respectively. The results obtained for linearity, LOD, and LOQ are given in Table **1**. These results confirmed the reliability of the methods for quantifying Vitamin $D_3$ and biotin in the sample.

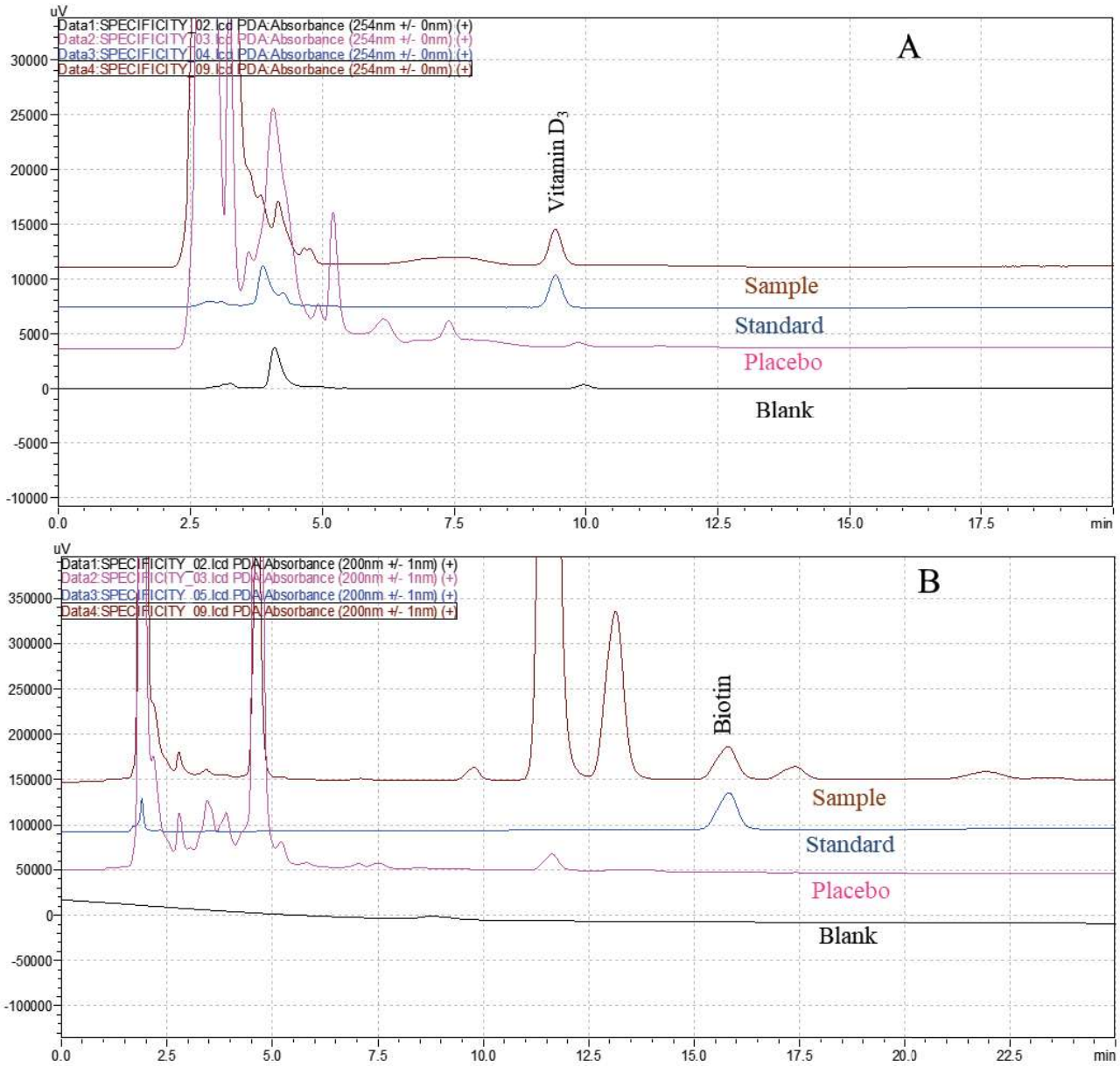



**Fig. (3).** Representative Chromatograms of Specificity (**A**) Vitamin $D_3$ (6400 IU), and (**B**) Biotin (25 µg/ml). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

**Table 1. Linear regression data.**

| Parameters | Vitamin $D_3$ | Biotin |
|---|---|---|
| Slope | 5.88 | 55757.28 |
| Intercept | 982.60 | 21274.80 |
| Standard Error | 443.06 | 12983.62 |
| Regression Coefficient ($R^2$) | 0.998 | 0.997 |
| Range | 3200 – 9600 IU | 12.5 – 37.5 µg/ml |
| LOD | 608.79 IU | 1.72 µg/ml |
| LOQ | 1844.82 IU | 5.22 µg/ml |

### *6.2.3. Accuracy*

The results were found to be accurate at three distinct concentration levels. The recovery percentages for Vitamin D3 and Biotin were found to be 98.29% to 99.86% and 99.39% to 99.61%, respectively, at concentration levels of 50% to 150%. However, the %RSD for Vitamin $D_3$ and biotin was found to be 0.36% and 1.09%, respectively. The %RSD values were within the acceptance criteria of less than 2.0% as given in Table **2**. The respective accuracy chromatograms (50%, 100%, and 150%) for Vitamin $D_3$ and Biotin are shown in Fig. (**4**).

### *6.2.4. Precision*

The relative standard deviation of the assay of six preparations was calculated and found within the acceptance criteria. Intra-day and Inter-day precision studies of Vitamin

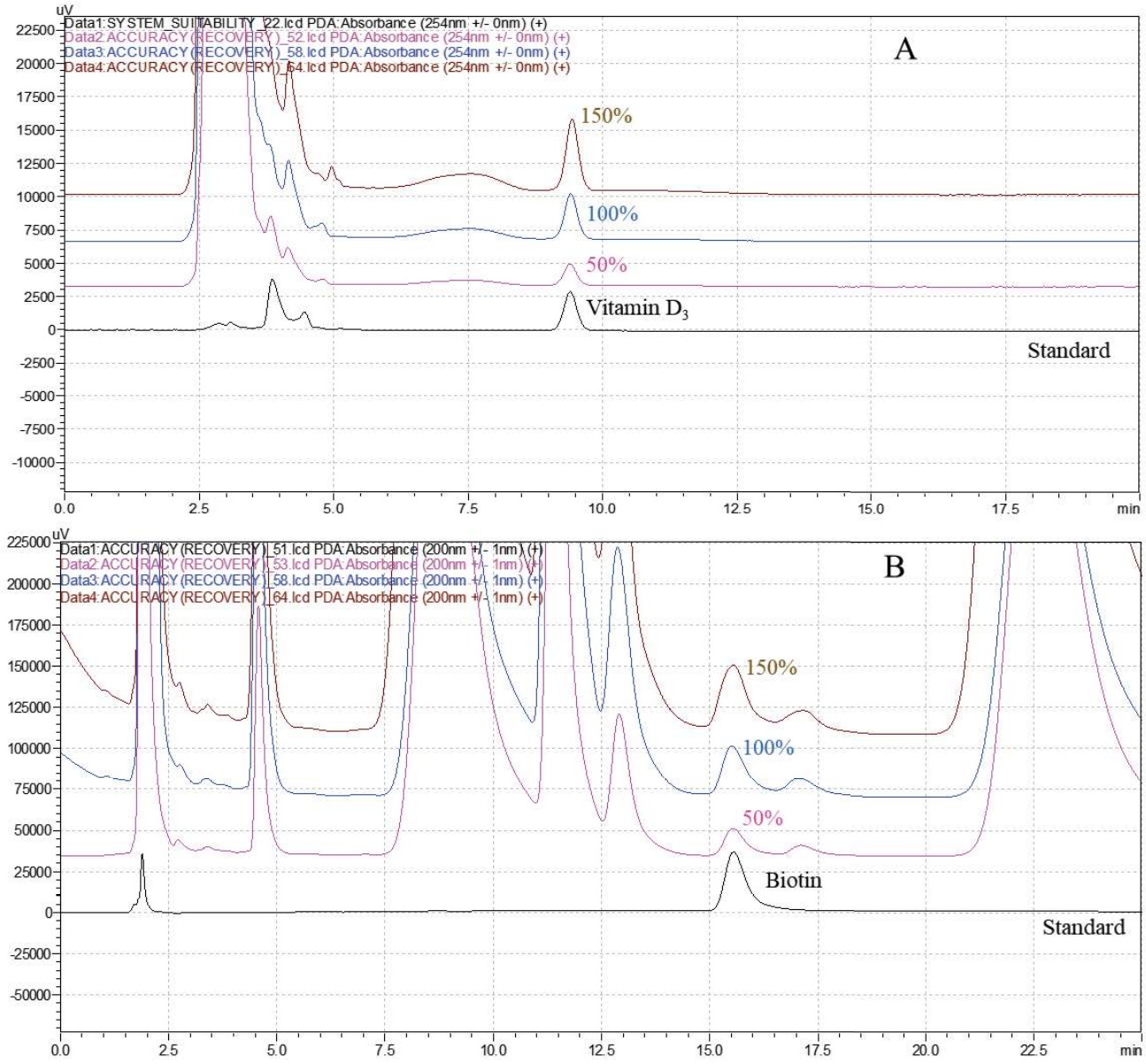



**Fig. (4).** Representative chromatograms of accuracy (**A**) vitamin $D_3$, and (**B**) biotin. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

$D_3$ showed % RSD of 1.15 % and 0.60 %, respectively. For biotin, the %RSD of precision studies was found to be 0.79 % and 0.43 %, demonstrating that the methods were precise and within the acceptance criteria of not more than 2.0% as given in Table **2**. The chromatograms of precision studies of Vitamin $D_3$ and biotin are shown in Fig. (**5**).

### *6.2.5. Robustness*

In this study, no significant changes in the analytical parameters such as retention time, peak area, tailing factor, number of theoretical plates, and percentage assay were observed with the change in wavelength (± 5 nm) and flow rate (±10%). It was observed from Table **3** that the percentage relative difference between assay values did not exceed the acceptance criteria of 2.0%. This ensures that the assay is consistently reliable and unaffected by minor variations in conditions. The respective chromatograms of the robustness study of Vitamin $D_3$ and biotin are shown in Fig. (**6**).

### *6.2.6. System Suitability*

Each chromatogram was processed, and peak areas were calculated. The %RSD of six replicate injections of Vitamin $D_3$ and biotin was found to be within the acceptance criteria of less than 2.0%. The tailing factor was found to be less than 2.0%. Theoretical plates were found to be more than 2000, which fulfill the acceptance criteria for Vitamin $D_3$ and biotin. The results of system suitability for Vitamin $D_3$ and biotin are given in Table **4**. The respective chromatograms are shown in Fig. (**7**).

**Table 2. Accuracy and precision studies data.**

| Parameters | Accuracy (Percentage of Recovery, %RSD) | |
|---|---|---|
| | **Vitamin $D_3$** | **Biotin** |
| At 50% | 98.29%, 0.36% | 99.39%, 1.09% |
| At 100% | 98.45%, 0.36% | 98.89%, 1.09% |
| At 150% | 99.86%, 0.36% | 99.61%, 1.09% |
| Precision (%RSD) | | |
| Inter-day Precision Data | 0.60% | 0.43% |
| Intra-day Precision Data | 1.15% | 0.79% |

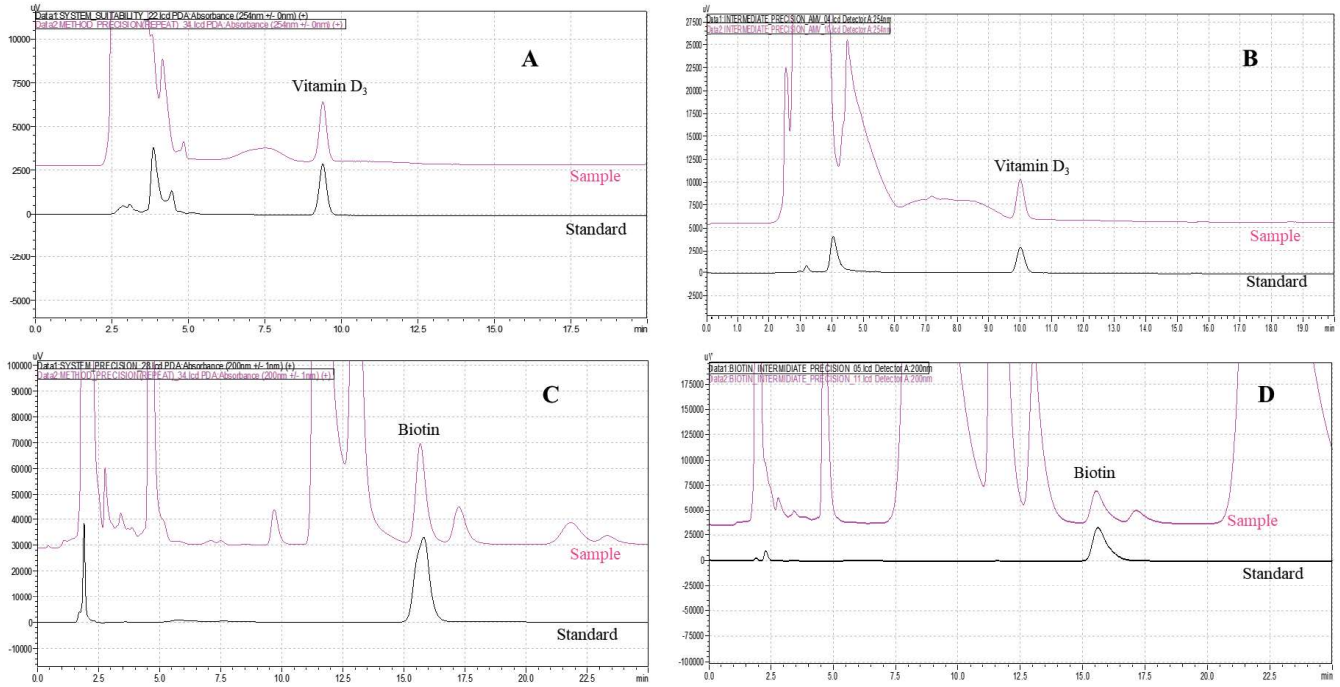



**Fig. (5).** Representative Chromatograms for Method Precision **(A)** Intra-day Precision of Vitamin $D_3$ (6400 IU), **(B)** Inter-day Precision of Vitamin $D_3$ (6400 IU), **(C)** Intra-day Precision of Biotin (25 µg/ml), and **(D)** Inter-day Precision of Biotin (25 µg/ml). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

**Table 3. Robustness study data based on flow rate and wavelength.**

| Samples | Parameters | | % Assay | % Assay Difference |
|---|---|---|---|---|
| Vitamin $D_3$ | Normal condition | | 134.84 | - |
| | Flow Rate | 0.9 ml/min. | 134.54 | 0.30 |
| | | 1.1 ml/min. | 135.39 | 0.55 |
| | Wavelength | 249 nm | 135.74 | 0.90 |
| | | 259 nm | 134.51 | 0.33 |
| Biotin | Normal condition | | 107.61 | - |
| | Flow Rate | 0.9 ml/min. | 108.41 | 0.80 |
| | | 1.1 ml/min. | 109.47 | 1.86 |
| | Wavelength | 195 nm | 109.47 | 1.86 |
| | | 205 nm | 107.57 | 0.04 |

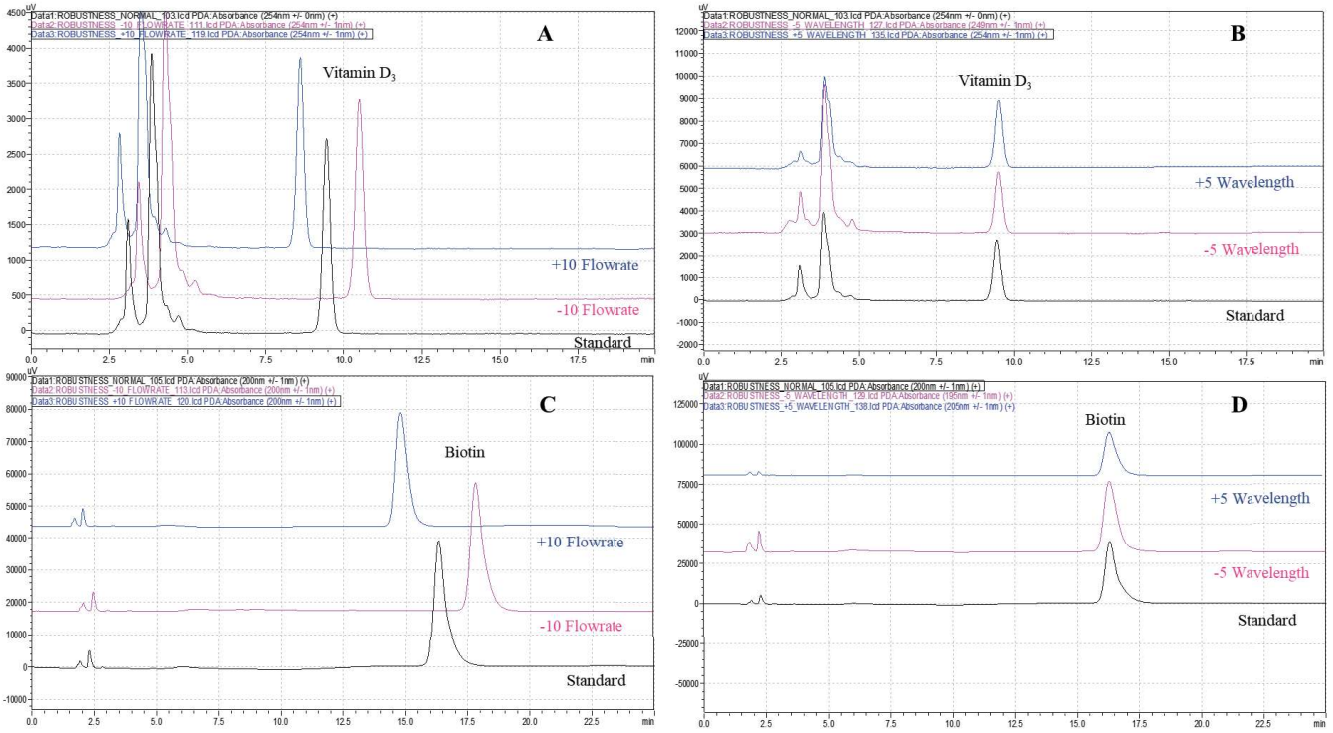
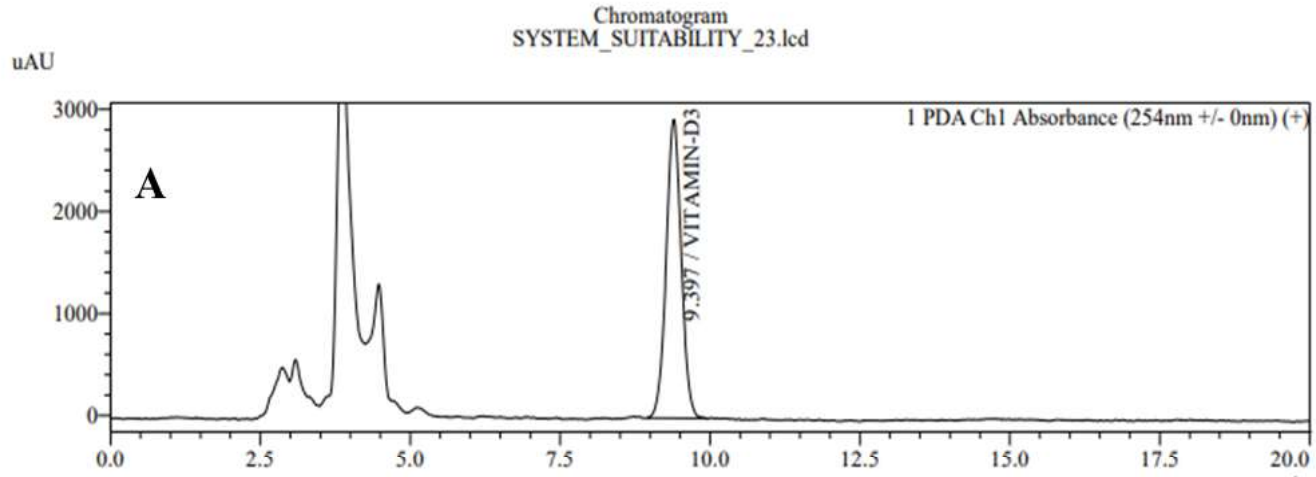



**Fig. (6).** Representative Chromatograms for Robustness: **(A)** Vitamin $D_3$ at ±10% Flow Rate, **(B)** Vitamin $D_3$ at ±5 nm Wavelength, **(C)** Biotin at ±10% Flow Rate, and **(D)** Biotin at ±5 nm Wavelength**.** (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

**Table 4. Data of system suitability study.**

| Parameter | Vitamin $D_3$ | Biotin |
|---|---|---|
| Retention Time | 9.39 | 15.81 |
| Tailing factor | 1.014 | 0.967 |
| Theoretical plates | 43601 | 25429 |
| %RSD | 0.24 | 1.85 |

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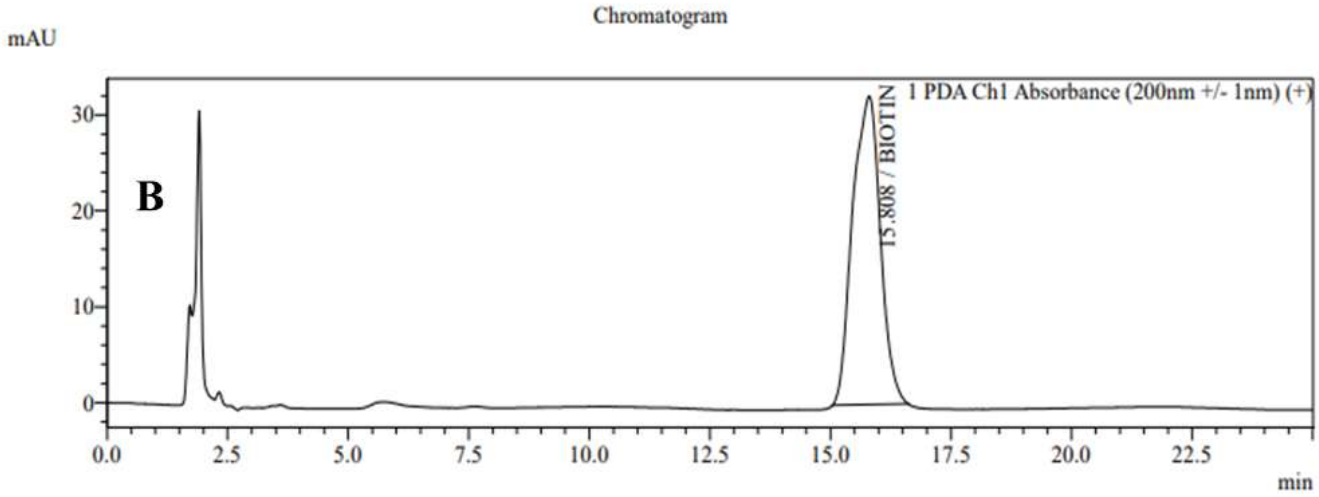


**Fig. (7).** Representative chromatograms of system suitability **(A)** Vitamin $D_3$, and **(B)** Biotin. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

**Table 5. Data of solution stability study.**

| Samples | Time Interval | %Assay | %Assay Difference | Overall Mean % | Overall SD% | Overall RSD% |
|---|---|---|---|---|---|---|
| **Vitamin $D_3$** | Initial | 120.50 | - | 121.35 | 0.91 | 0.75 |
| | After 12 hours | 121.23 | 0.73 | | | |
| | After 24 hours | 122.31 | 1.81 | | | |
| **Biotin** | Initial | 109.01 | - | 108.86 | 0.19 | 0.18 |
| | After 12 hours | 108.93 | 0.08 | | | |
| | After 24 hours | 108.64 | 0.37 | | | |

**Table 6. Accelerated stability study results.**

| Stability Months | Appearance | pH of Sample | Specific Gravity | % Assay | |
|---|---|---|---|---|---|
| | | | | Vitamin $D_3$ | Biotin |
| 0 | Cream coloured suspension | 5.30 | 1.2835 | 185.24% | 197.79% |
| 1 | Cream coloured suspension | 5.28 | 1.2978 | 177.60% | 195.50% |
| 3 | Cream coloured suspension | 5.22 | 1.3079 | 168.04% | 192.12% |
| 6 | Cream coloured suspension | 5.21 | 1.3079 | 157.91% | 186.70% |

### *6.2.7. Solution Stability*

The standard and sample solutions of Vitamin $D_3$ and biotin were found to be stable for 24 hours. The difference in % assay results and % RSD values was found within the acceptance criteria of less than 2.0%, shown in Table 5.

### 6.3. Results of Accelerated Stability Study

The stability of Vitamin $D_3$ and biotin on accelerated conditions showed that the assay results complied with the in-house acceptance criteria of less than 90% of the label claim from the initial month to six months. The results of the percentage assay of Vitamin $D_3$ showed a reduction from 185.24% to 157.91% in six months, and the results of the percentage assay of biotin showed a reduction from 197.79% to 186.70% in six months, as mentioned in Table **6**. The initial assay values were found above 100% for these vitamins, because overages were included in the formulation (Calpond Gold Gel suspension) to compensate for process loss and environmental stress loss, ensuring that the product meets its labelled potency throughout its shelf life. The graphical representation of the rate of degradation is shown in Fig. (**8**).

## CONCLUSION

The simple and isocratic RP-HPLC methods were successfully developed and validated according to the ICH Q2 (R1) guidelines for the quantitative analysis of Vitamin $D_3$ and biotin in Calpond Gold Gel suspension. The developed methods met the ICH acceptance criteria. Furthermore, the

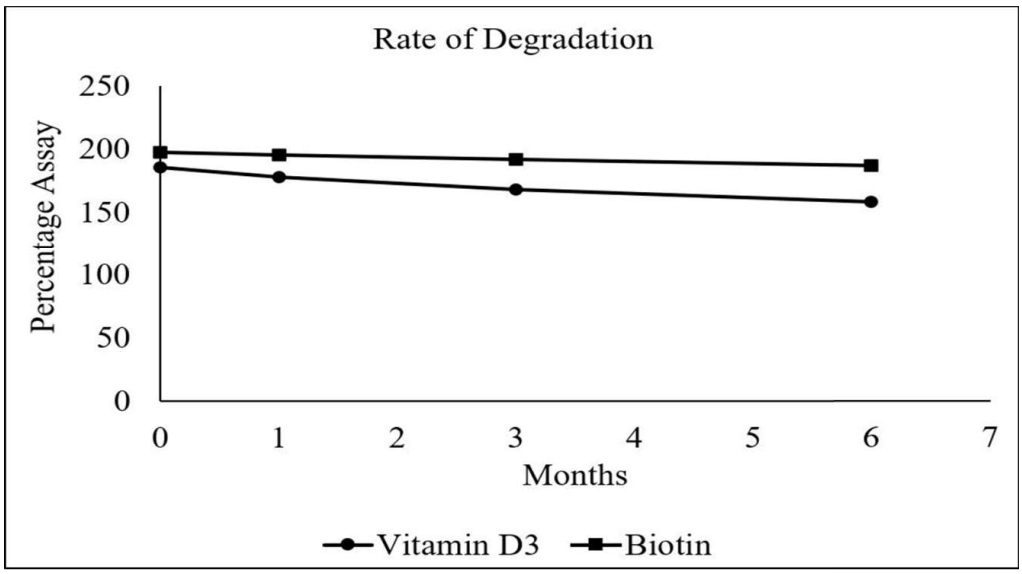


**Fig. (8).** Representation of the rate of degradation of vitamin $D_3$ and biotin. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

accelerated stability study indicated that the concentrations of Vitamin $D_3$ and biotin remained within the in-house acceptance limits throughout the study period. Hence, the developed RP-HPLC methods are suitable for the quantitative determination of Vitamin $D_3$ and Biotin in the Calpond Gold Gel suspension. In addition, the validated methods can be utilized for routine product testing. This successful implementation emphasizes their practical applicability and reliability in supporting day-to-day product quality control activities. While the methods may provide a useful analytical framework for future studies involving other feed supplements, veterinary, or pharmaceutical matrices, such applications would require appropriate method verification or revalidation to ensure accuracy and specificity in different excipient environments.

## AUTHORS' CONTRIBUTIONS

The authors confirm their contributions to the paper as follows: SS was responsible for study conception, design, and data collection, CT carried out the analysis and interpretation of results; NK contributed to conceptualization, AC and KR handled data curation, AM was responsible for visualization, SN conducted the investigation, and TH wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

## LISTS OF ABBREVIATIONS

| | | |
|---|---|---|
| RP-HPLC | = | Reverse Phase High-Performance Liquid Chromatography |
| ICH | = | International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use |
| TLC | = | Thin Layer Chromatography |
| GC | = | Gas Chromatography |
| CE | = | Capillary Electrophoresis |
| RH | = | Relative Humidity |
| SD | = | Standard Deviation |
| RSD | = | Relative Standard Deviation |
| LOD | = | Limit of Detection |
| LOQ | = | Limit of Quantification |
| IU | = | International Unit |

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

Not Applicable.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed during this study are included in this published article.

## FUNDING

None.

## CONFLICT OF INTEREST

The author(s) declare no conflict of interest, financial or otherwise.

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Declared none.

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