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# Quantitative Analysis of Fat-Soluble Vitamins in Feed Additives Using an In-House Developed and Validated HPLC Method

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**Abstract**

Quantification of vitamins in complex matrices such as feed additives is a time-consuming analytical procedure. In this study, a simple and precise in-house High Performance Liquid Chromatography (HPLC) method was developed and validated for the simultaneous detection and quantification of four fat-soluble vitamins such as vitamin A, $D_3$, E, and $K_3$ in feed additives. The HPLC method was developed and validated using reversed-phase column chromatography. The chromatographic separation of the vitamins was carried out at 25°C temperature on a reverse-phase C18 column using a binary gradient pump mode. Mobile phase constituents were solvent (a): deionized water and (b) methanol. Detection was performed with HPLC ultraviolet/visible detection set at 325, 265, 230, and 254 nm wavelength for vitamin A, $D_3$, E, and $K_3$ respectively. The flow rate was 1.0mL/min and the total run time was 20min. The method was validated according to the guidelines of the International Conference on Harmonization (ICH) and Food and Drug Administration (FDA), USA, and acceptance criteria for system suitability, specificity, linearity, accuracy, and precision were met in all the cases. The Relative Standard Deviation (RSD) for system suitability and precision was <2% for all the studied vitamins. The linearity of the calibration curves was excellent ($R^2$>0.999) at concentrations of 2.5, 5.0, 7.5, 10.0, 15.0, and 20.0 µg/mL for all vitamins, and the range of linearity of this method was 0.0-50.0 µg/mL with R2 value greater than 0.999. The limits of detection values were 0.0022, 0.0012, 0.0022, and 0.0020 µg/mL for vitamin A, $D_3$, E, and $K_3$, respectively, and the limits of quantification values were 0.0066, 0.0038, 0.0066, and 0.0061 µg/mL for vitamin- A, $D_3$, E, and $K_3$ respectively. The recovery percentages ranged from 85% to 103%, and the robustness of the method is also high with excellent reproducibility. The overall parameters of the proposed method met the validation criteria and this method could be a precise and highly desirable analytical procedure for accurate quantification of four fat-soluble vitamins such as A, $D_3$, E, and $K_3$ in feed additives using a single chromatographic run.

**Keywords:** Fat-soluble vitamins; Feed additive; HPLC; Method development & Validation

## Introduction

Vitamins are a heterogeneous group of organic compounds and an essential component of feed additives that impact the feed presentation, hygiene, digestibility, intestinal health, and are required for the normal functioning of the animal body [1-5]. As a component of feed additives, fat-soluble vitamins such as vitamins- A, $D_3$, E, and $K_3$ are extensively used in livestock production, especially in the modern poultry farm and livestock industry [3,6]. The deficiency of these vitamins leads to a serious malfunction in various organs and systems in the body that could seriously affect animal performance by decreasing the growth rate or increasing the incidence of reproductive failures and other abnormalities [7-9]. Therefore, the body needs a continuous supply of well-balanced fat-soluble vitamins to prevent deficiency symptoms and maintain normal body function. Therefore, the control of fat-soluble vitamins in feed formulations is really important.

Quantitative analysis of vitamins from composite sample like feed additives is still a challenge and cumbersome analytical procedure for the analysts due to the highly reactive nature of vitamins and quantitative isolation of each component [10-12] in the mixed formulation. Several methods have been reported such as ultraviolet/visible (UV/Vis) spectrophotometry, fluorimetry, chemiluminescence, capillary electrophoresis, thin-layer chromatography, and liquid chromatography with different conditions for the determination of each component of fat-soluble vitamins in different matrices [13,14].

The liquid chromatography technique has been widely applied for the analysis of fat-soluble vitamins in different matrices with some drawbacks like longer retention time, low reproducibility, lower accuracy, and sophisticated mobile preparation with longer column equilibration, and the total run time of the analysis [14]. Because of the intricate composition and unstable nature of vitamins, the extraction and analysis procedure of the fat-soluble vitamins in feed additive




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is a boring and troublesome act; sometimes, a special procedure is required to get the analyte contained in the sample matrix, and their experimental procedures are also time-consuming and cost value is high due to a large number of reagents and organic solvents [15]. However, a few validated methods [16] available for the for combined detection and quantification of all fat-soluble vitamins feed additives. Hence, there is a need for simple, rapid, fit-for-purpose methods that can easily be used for routine quality analysis of the feed additives.

Therefore, the present study aimed to develop and validate a simple and precise HPLC technique for the simultaneous detection and quantification of four fat-soluble vitamins like vitamin-A (retinol), vitamin-$D_3$ (cholecalciferol), vitamin-E (alpha-tocopherol), and vitamin-$K_3$ (menadione) in the complex compound like feed additives.

## Materials and Methods

### Chemicals and reagents

Methanol (HPLC grade), concentrated ammonia, and authorized reference standard of Vitamin $D_3$ (cholecalciferol) (Lot: LRAC6497) employed in this study were purchased from Sigma-Aldrich (Darmstadt, Germany), Vitamin A acetate (retinol) (Code: DRE-CA17923820, Lot: G1127532) was purchased from LGC, Germany, and Vitamin $K_3$ (Menadione) (Code: M0300000, Batch: 1.4, ID: 00 GhBU), and Vitamin-E(alpha-tocopherol acetate) (Code: T1600000, Batch: 10.0, ID: 0077yJ) were purchased from European Pharmacopoeia Reference Standard, Council of Europe, EDQM CS 30026F-67081, Strasbourg, Cedex. Double deionized (DI) water utilized in this study was obtained from a water deionization plant (ePure-D4642-33, Thermo Fisher Scientific, USA). All solutions were sonicated and filtered through a 0.45µm filter employing a vacuum filtration unit (Welch, Pall Scientific, USA) before use.

### Instrumentation and Chromatographic Conditions

The high-performance liquid chromatographic system (Prominence, Shimadzu, Japan) equipped with UV-VIS detector, binary gradient pump mode, and Lab Solution data processing software was used in this study. HPLC column Shim-pack GIST (5µM C18, 4.6 X 150 mm), P/N: 227-30017-07, S/N: 18L09240 (Shimadzu, Japan) was used for the separation of vitamins. The optimized chromatographic conditions used in this study were shown in Table 1. The whole chromatography was performed at ambient temperature.

### Preparation of standard solution

Stock standard solution of 1000µg/mL was prepared by weighing 10mg of the vitamin-A, vitamin-$D_3$, vitamin-E, and vitamin-$K_3$ in a 10mL amber color volumetric flask separately, and diluted to volume with HPLC grade methanol. These solutions were used as reference stock standard solutions and kept in a refrigerator at -20°C for further use. Intermediate standard solutions of 100µg/mL of each vitamin were prepared from stock standard solution in methanol. Mixed working standard solutions of the studied vitamins were prepared daily from intermediate standard solutions. Before injecting into the liquid chromatography system, the standard solutions were filtered through a 0.45µm Polytetrafluoroethylene (PTFF) syringe filter.

### Preparation of sample solution

Weighed accurately about 8.0gm of sample in a 50mL amber color volumetric flask. Added 6.0mL of concentrated ammonia and heat at 80°C in a hot water bath for 2minutes with shaking. Allowed to cool to at 20°C temp in cold water bath and volume with methanol and sonicated for 1.0min at room temperature. Then the sample solution was filtered through Whatman no.1 filter paper. Further diluted 5mL of the filtrated solution to 50mL methanol and mixed well. Again, filtered the sample solution with a 0.45µM PTFE syringe filter and transferred to the sample vial and immediately stored the sample vial in the refrigerator for 5min before analysis.

### Method validation parameters

Method validation of the present study was performed by measuring the essential parameters of the validation process like system suitability, specificity, linearity, range, accuracy, precision, Limits of Detection (LOD), Limits of Quantification (LOQ), and robustness. The validation parameters were evaluated using recommended guidelines of the International Conference on Harmonization (ICH) [17] and also the US Food and Drug Administration (FDA) [18-19].

***System suitability*:** The system's suitability was evaluated by six replicate (n=6) analyses of an aqueous mixture of all vitamin standards. The acceptance limit of the various parameters for system suitability of the procedure is calculated in step with the rules of ICH and FDA where the acceptance criteria are the percentage of relative standard deviation (RSD) for retention time, Peak Area (PA), and height is <2%, the number of Theoretical Plates (TP) over 2000, Tailing Factor (TF), and Peak Resolution (RS) are >1.5.

***Specificity*:** The specificity of the assay method was investigated by injecting the standard of the target concentration (10µg/mL) in the extracted placebo to demonstrate the absence of interference with the elution of target the analyte.

***Linearity and range*:** To evaluate the linearity, six mixture standards of vitamin-A, vitamin-$D_3$, vitamin-E, and vitamin-$K_3$ at 25, 50, 75, 100, 150, and 200% of target concentration (10µg/mL) were prepared and a linear equation was established for each vitamin by plotting the peak area versus the concentrations. Three calibration curves were acquired on three consecutive days with a specified standard concentration of the studied vitamin. Linearity was calculated by running six standard mixtures of each vitamin, at final concentrations of 2.5, 5.0, 7.5, 10.0, 15.0, and 20.0 µg/mL. The range of the linearity of this method was 0.0, 0.5. 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 30.0, and 50.0 µg/mL at final concentrations of each vitamin.

***Accuracy*:** The accuracy of the study was performed by estimating the percent of the recovery. To carry out the recovery spiked samples were prepared at three concentrations (50%, 100%, and 150% of the target analyte) of each vitamin in the pre-analyzed formulation blank matrix. Three individually prepared replicates at each concentration were analyzed and the recovery percent of each vitamin was calculated using the formula: recovery (%) = (amount obtained/amount spiked) ×100.

***Precision*:** The precision of the method was estimated based on repeatability and intermediate precision. The repeatability was calculated on the results obtained on the same day for six independent standard mixer solutions of the target concentration (10µg/mL) of the analyte and the intermediate precision was assessed by calculating the repeatability at three concentration levels (50%, 100%, 150%) of the

**Table 1:** Optimized chromatographic conditions.

| Parameters | Conditions |
|---|---|
| Stationary Phase (column) | Shim-pack GIST (5μM C18, 4.6 X 150 mm), |
| Mobile Phase | Methanol: Water (98:2) |
| Pump Mode | Binary gradient |
| Flow Rate (mL/min) | 1.0 ml |
| Run time (minutes) | 20 |
| Auto Sampler Temperature (°C) | 4 |
| Column Temperature (°C) | 25 |
| Injection Volume (μL) | 20 |
| Detection Wavelength (nm) | Vitamin-$K_3$: 254 |
| | Vitamin-A: 325 |
| | Vitamin-$D_3$: 265 |
| | Vitamin-E: 230 |
| Peak Identification | Vitamin-$K_3$: 1st |
| | Vitamin-A: 2nd |
| | Vitamin-$D_3$: 3rd |
| | Vitamin-E: 4th |

target concentration (10μg/mL) of the analyte by two analysts on two different days. The percentage of RSD was calculated for evaluating the precision of this study.

***Limit of detection* (LOD) *& Limit of quantification* (LOQ):** The lowest qualitative and quantitative concentrations of the obtained linearity range were calculated for every vitamin in keeping with the guidelines of ICH- 2000. The LOD and LOQ of this study were determined using the formula: k x S.D/b, where k=3.3 for the LOD and 10 for the LOQ, S.D = the standard deviation of the intercept, and b=slope of the calibration curve.

***Robustness*:** The robustness of the method was evaluated by analyzing the same standards (15μg/mL) with small but deliberate variations in method parameters such as column temperature, injection volume, and flow rate and performed in triplicate. The percentage of Relative Standard Deviation (RSD) was calculated for estimating the robustness of this study, and the acceptance criteria are the percentage of RSD for the peak area is <2%.

***Statistical analysis*:** The data obtained in this study were analyzed with the software Lab Solution and Statistical Package for the Social Sciences version 16 statistical package by one-way analysis of variance, and in regression analysis, the least square method was performed.

## Results and Discussion

This study reveals the development and validation of a definite analytical method where the validation criteria [16-18] are met in all the cases. Typical chromatograms of the standard solution and the actual feed additive sample are shown in Figure 1a and b, respectively. The elution of fat-soluble vitamins through the analytical column occurs in an exceedingly specific order and in groups that depend on their chemical properties [20]. The retention time of those four fat-soluble vitamins was as follows: 5.18±0.01 min (Vitamin-A l), 8.10±0.01 min (Vitamin-$D_3$), 15.32 ±0.01 min (Vitamin-E), and 2.33±0.01 min (Vitamin-$K_3$). The system suitability parameters (Table 2) show that the percentage of RSD for retention time, peak area, and height is <2%, the number of TP is over 2000, and TF and RS are >1.5, indicating that the values are within the required limits of the validation process [17-19]. The specificity test results (Figure 1 and Table 3) of the assay method demonstrates the absence of interference with the elution of vitamin-A, vitamin-$D_3$, vitamin-E, and vitamin-$K_3$ in the extracted placebo. From the linearity of Figure 2 and Table 4, it is found that all of the vitamins maintain excellent linearity (R2>0.999) within the concentration range of 2.5-20.0 μg/mL. The range of linearity of this method was 0.0-50.0 μg/mL with an R2 value greater than 0.999. The accuracy of the method was determined by recovery percentage and the values of all the vitamins are between 85% and 103% (Table 5), which suggests that the method is accurate and also indicates that the commonly used excipients present in the feed additive formulations are not interfering the proposed method. The precision for the method and analyst was evaluated which are shown in Tables 6a and 6b. The results demonstrate that the RSD value for both cases is <1%, which indicates that the proposed method has excellent reproducibility.

The LOD for vitamin-A, vitamin-$D_3$, vitamin-E, and vitamin-$K_3$

**Table 3**: Specificity test report.

| Sample Name | | Retention Time (min) | Peak area |
|---|---|---|---|
| Reagent Blank Solution | | Nil | Nil |
| Matrix blank solution | | Nil | Nil |
| Vitamin-A | Std-10μg/mL | 5.18 | 1788496 |
| | Std-spiked-10μg/mL | | 1673090 |
| Vitamin-$D_3$ | Std-10 μg/mL | 8.1 | 657231 |
| | Std-spiked-10μg/mL | | 614431 |
| Vitamin-E | Std-10μg/mL | 15.33 | 182731 |
| | Std-spiked-10μg/mL | | 183321 |
| Vitamin-$K_3$ | Std-10 μg/mL | 2.33 | 1539365 |
| | Std-spiked- 10μg/mL | | 1492475 |

**Table 2:** System suitability parameters of the proposed method.

| Vitamin | Theoretical plates | Tailing factor | Resolution | Reproducibility | | |
|---|---|---|---|---|---|---|
| | | | | % RSD of RT | % RSD of Peak Area | % RSD of Height |
| Vitamin-A | 28618 | 1.198 | 6.747 | 0.068 | 0.149 | 0.208 |
| Vitamin-$D_3$ | 29903 | 1.185 | 5.316 | 0.05 | 0.139 | 0.195 |
| Vitamin-E | 31903 | 1.201 | 10.538 | 0.012 | 0.183 | 0.176 |
| Vitamin-$K_3$ | 20779 | 1.291 | 1.667 | 0.126 | 0.148 | 0.197 |

**RSD**=Relative standard deviation

**Table 4**: Regression parameters of analyzed fat-soluble vitamins (regression coefficient, $R^2$) in calibration curves.

| Vitamin | Regression coefficient | | |
|---|---|---|---|
| | 1st day | 2nd day | 3rd day |
| Vitamin-A | $R^2$=0.9994 | $R^2$=0.9995 | $R^2$=0.9995 |
| Vitamin-$D_3$ | $R^2$=0.9996 | $R^2$=0.9996 | $R^2$=0.9996 |
| Vitamin-E | $R^2$=0.9995 | $R^2$=0.9995 | $R^2$=0.9994 |
| Vitamin-$K_3$ | $R^2$=0.9996 | $R^2$=0.9996 | $R^2$=0.9996 |

**Table 5**: Accuracy test report.

| Vitamin | Spike % of the target conc. | Spiked conc. (µg/g) | Obtained conc. (µg/g) | | | Average obtained conc. (µg/g) | Average recovery % |
|---|---|---|---|---|---|---|---|
| | | | 1st Day | 2nd Day | 3rd Day | | |
| Vitamin-A | 50 | 5 | 4.94 | 4.94 | 4.91 | 4.93 | 98.59 |
| | 100 | 10 | 9.5 | 9.54 | 9.48 | 9.5 | 95.04 |
| | 150 | 15 | 12.5 | 12.42 | 13.28 | 12.74 | 84.9 |
| Vitamin-$D_3$ | 50 | 5 | 4.93 | 4.94 | 4.9 | 4.92 | 98.49 |
| | 100 | 10 | 9.46 | 9.49 | 9.44 | 9.46 | 94.63 |
| | 150 | 15 | 13.61 | 13.54 | 13.78 | 13.64 | 90.95 |
| Vitamin-E | 50 | 5 | 5.13 | 5.22 | 5.12 | 5.16 | 103.15 |
| | 100 | 10 | 9.98 | 10.02 | 9.95 | 9.98 | 99.84 |
| | 150 | 15 | 15.5 | 15.47 | 15.29 | 15.42 | 102.8 |
| Vitamin-$K_3$ | 50 | 5 | 5.13 | 5.18 | 5.21 | 5.18 | 103.51 |
| | 100 | 10 | 9.8 | 9.89 | 9.87 | 9.85 | 98.51 |
| | 150 | 15 | 14.77 | 14.79 | 15.29 | 14.95 | 99.66 |

is found to be 0.0022, 0.0012, 0.0022, and 0.0020 µg/mL respectively, and the LOQ for vitamin-A, vitamin-$D_3$, vitamin-E, and vitamin-$K_3$ is found to be 0.0066, 0.0038, 0.0066, and 0.0061 µg/mL respectively (Table 7). The robustness of the method was evaluated which is demonstrated in Table 8. The percentage of RSD for the peak area

**Table 6a:** Precision under repeatability conditions (n=6).

| Vitamin | Retention time % RSD | Area % RSD | Height % RSD |
|---|---|---|---|
| Vitamin-A | 0.068 | 0.149 | 0.208 |
| Vitamin-$D_3$ | 0.05 | 0.139 | 0.195 |
| Vitamin-E | 0.012 | 0.183 | 0.176 |
| Vitamin-$K_3$ | 0.126 | 0.148 | 0.197 |
| **RSD**=Relative standard deviation | | | |

**Table 7**: Limit of detection (LOD) & Limit of quantification (LOQ).

| Vitamin | LOD (µg/mL) | LOQ (µg/mL) |
|---|---|---|
| Vitamin-A | 0.0022 | 0.0066 |
| Vitamin-$D_3$ | 0.0012 | 0.0038 |
| Vitamin-E | 0.0022 | 0.0066 |
| Vitamin-$K_3$ | 0.002 | 0.0061 |
| **LOD**=Limits of detection, **LOQ**=Limits of quantification | | |

of all the studied vitamins is <2%, which indicates that the method is robust.

Some HPLC methods have been published concerning the simultaneous determination of vitamins, but most of the methods are hectic [21] and unable to quantify all the fat-soluble vitamins simultaneously. Although, some stable methods were [22,23] reported for the simultaneous quantification of four fat-soluble vitamins in infant formula and milk powder they are incompetent to analyze feed additives. Few studies mention the methods of MS-MS detector [24,25], which involves specific and expensive equipment that is not available in all laboratories for routine analysis of these four fat-soluble vitamins. The method we developed and validated is more precise with good specificity, linearity, precision, and high accuracy that met all the criteria of the validation parameters (Table 9). The robustness of the method is also good. The easy sample extraction procedure and simultaneous quantification of four water-soluble vitamins in a single chromatographic run make the procedure more convenient. Thus, the developed method could be a useful and precise analytical technique for simultaneous detection and quantification of four fat-soluble vitamins in routine and quality analysis of feed additives that have a wide range of applications in modern livestock production.

## Conclusion

In this study, an accurate analytical method has been developed and validated using a reverse-phase HPLC for the rapid and reliable analysis of fat-soluble vitamins in complex feed additives. Due to the easy sample preparation, short analytical time, and simple mobile phase composition containing water and methanol make the method more economical and suitable for the qualitative and quantitative analysis of fat-soluble vitamins in feed additives. The accuracy, precision, and robustness of the proposed method are also high enough with high excellent recovery and reproducibility; that met the

**Table 6b**: Intermediate precision.

| Analyte | Day | First analyst | | | Second analyst | | |
|---|---|---|---|---|---|---|---|
| | | Area % RSD (5µg/mL) | Area % RSD (10µg/mL) | Area % RSD (15µg/mL) | Area % RSD (5µg/mL) | Area % RSD (10µg/mL) | Area % RSD (15µg/mL) |
| Vitamin-A | Day-1 | 0.394 | 0.368 | 0.168 | 0.509 | 0.416 | 0.279 |
| | Day-2 | 0.605 | 0.436 | 0.337 | 0.357 | 0.424 | 0.279 |
| Vitamin-$D_3$ | Day-1 | 0.395 | 0.522 | 0.142 | 0.59 | 0.296 | 0.265 |
| | Day-2 | 0.608 | 0.472 | 0.346 | 0.464 | 0.269 | 0.284 |
| Vitamin-E | Day-1 | 0.698 | 0.355 | 0.11 | 0.305 | 0.587 | 0.343 |
| | Day-2 | 0.523 | 0.286 | 0.248 | 4.02 | 0.595 | 0.34 |
| Vitamin-$K_3$ | Day-1 | 0.331 | 0.42 | 0.093 | 0.842 | 0.422 | 0.17 |
| | Day-2 | 0.649 | 0.526 | 0.4 | 0.479 | 0.22 | 0.315 |

**Table 8**: Robustness test report.

| Conditions | Parameters | | | Peak Area % RSD | | | |
|---|---|---|---|---|---|---|---|
| | Temperature (°C) | Injection Volume (µL) | Flow rate (mL/min) | Vitamin-A | Vitamin-$D_3$ | Vitamin-E | Vitamin-$K_3$ |
| NC | 25 | 20 | 1 | 0.412 | 0.528 | 0.243 | 0.479 |
| VC-01 | 29 | 20 | 1 | 0.463 | 0.402 | 0.288 | 0.809 |
| VC-02 | 20 | 20 | 1 | 0.266 | 0.37 | 0.23 | 0.358 |
| VC-03 | 25 | 21 | 1 | 0.326 | 0.389 | 0.469 | 0.243 |
| VC-04 | 25 | 19 | 1 | 0.643 | 0.616 | 0.554 | 0.683 |
| VC-05 | 25 | 20 | 1.1 | 0.428 | 0.126 | 0.431 | 0.57 |
| VC-06 | 25 | 20 | 0.9 | 1.098 | 1.103 | 0.916 | 1.083 |
| **NC=** Normal Condition, **VC=** Varied Condition | | | | | | | |

**Table 9**: Summary of acceptance criteria [17-19] of the validation process and obtained results.

| Parameters | Acceptance criteria | Obtained results | Table/Figure |
|---|---|---|---|
| System suitability | For retention time, peak area, and height: RSD≤1% | RSD<0.2 % | |
| | Theoretical Plates (TP)≥2000 | TP>20000 | |
| | Tailing Factor (TF)≤2 | TF<1.3 | |
| | Resolution factor (Rs)≥2 | Rs>2.0 | Table 2 |
| Specificity | The excipient compounds must not interfere with the analysis of the targeted analyte. | Chromatography shows- | Table 3 |
| | | (i) the existence of peak area in standard solutions and real sample | |
| | | (ii) Absence of peak area in blank and matrix solutions | |
| Linearity | $R^2$> 0.999 | $R^2$> 0.9994 | Table 4 |
| Range | 25, 50, 75, 100, 150, and 200% of target concentration (10µg/mL) | 0.0, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 30.0, and 50.0 µg/mL | - |
| Accuracy | Recovery should be between 80-120 % of the target concentration | 85-103 % | Table 5 |
| Precision Repeatability/Reproducibility precision | RSD≤2 % | Repeatability: RSD<0.2% | Table 6 a, and b |
| | | Intermediate precision: RSD<0.7% | |
| Limit of Detection (LOD) | Concentration of 3:1 signal to nose ratio | Vitamin-A-0.0022µg/mL | Table 7 |
| | | Vitamin-$D_3$-0.0012µg/mL | |
| | | Vitamin-E-0.0022µg/mL | |
| | | Vitamin-$K_3$-0.0020µg/mL | |
| Limit of Quantification (LOQ) | 3.3 x Limit of detection | Vitamin-A-0.0066µg/mL | Table 7 |
| | | Vitamin-$D_3$-0.0038µg/mL | |
| | | Vitamin-E-0.0066µg/mL | |
| | | Vitamin-$K_3$-0.0061µg/mL | |

criteria of the validation process. Therefore, this method could be a validated analytical procedure for accurate quantification of vitamin A, $D_3$, E, and $K_3$ in multi-component feed additives using a single chromatographic run and might be a simple and precise technique to assess the standard and quality of the feed additives intended to be used for increased livestock production.

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