# A Simple and Alternative UV Spectrometric Method for the Estimation of Vitamin D3

*Asma Rahman[a], Md. Mahbubur Rahman[b], Mohammad Shahnoor Hossain[c], Md. Sarowar Jahan[d], Noor Jahan Akter[e] and Md. Latiful Bari[a]*

## Abstract

*Background.* A simple, rapid, accurate, precise, and economic spectrophotometric methods for estimation of vitamin D in from food, feed, pharmaceutical and environmental samples have been developed. *Method.* Vitamin D has absorbance maximums at 265.0 nm, so absorbance was measured at the same wave length for the estimation of vitamin D. Absorbance is measured at 275.0 nm. This drug obeys the Beer Lambert's law in the concentration range of 12 to 315 ng/mL. Methods are validated according to ICH guidelines and can be adopted for the routine analysis of vitamin D from food and drug samples. *Results.* This method offers a very simple procedure, avoids aggressive sample treatments, excellent determination coefficient ($r^2$) value of 0.999. The average percentage of relative standard deviation (% RSD) for intra- and inter- day precision was found to be 0.14% and 0.13%, respectively. The average percentage of recovery was found to be 100.88%, hence, simple, cost effective, provided better precision and accuracy. *Conclusion.* Thus, this method could be applicable in accurate estimation of routine analysis of vitamin D from food, feed, pharmaceutical and environmental samples in food and drug industry.

*Significance* | Simple UV spectrometry based method for Vitamin D estimation

*Correspondence:* **Md. Latiful Bari,** PhD, Principal Scientist, Centre for Advanced Research in Sciences (CARS), University of Dhaka, Dhaka-1000, Bangladesh.
Contact no.: +8801971560560;
E-mail: latiful@du.ac.bd

## Introduction

Vitamin D includes a group of steroids molecules which is responsible for numerous cellular processes including calcium and phosphorous homeostasis (Chen et al., 2016; Japelt & Jakobsen, 2013), glucose metabolism (H, O, Da, O, & Ng, 2013; Jamka et al., 2015), endocrine signalling (Al-Hendy, Diamond, Boyer, & Halder, 2016; Santos et al., 2017), cellular proliferation, differentiation and apoptosis process (Arboleda Alzate, Rodenhuis-Zybert, Hernandez, Smit, & Urcuqui-Inchima, 2017; Bartels et al., 2013; Bosetti et al., 2016; Hu & Zuckerman, 2014; Hunten & Hermeking, 2015), and development of immune response (Al-Jaderi & Maghazachi, 2013; Alhassan Mohammed et al., 2017; Boontanrart, Hall, Spanier, Hayes, & Olson, 2016). Even though the relationship of vitamin D deficiency correlates with various diseases, its deficiency or insufficiency is widespread among the children and adults across the world (Holick, 2002; Lopez-Sobaler et al., 2017; Malabanan, Veronikis, & Holick, 1998; Ovesen, Andersen, & Jakobsen, 2003). Due to its immense

**Author Affiliation:**
a. Centre for Advanced Research in Sciences (CARS), University of Dhaka, Dhaka-1000, Bangladesh
b. Department of Biotechnology and Genetic Engineering, Mawlana Bhashani Science and Technology University, Santosh, Tangail-1902, Bangladesh
c. Department of Genetic Engineering and Biotechnology, University of Dhaka, Dhaka-1000, Bangladesh
d. Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka-1000, Bangladesh
e. Institute of Statistical Research and Training, University of Dhaka, Dhaka- 1000, Bangladesh.

physiological importance, vitamin D is currently included in various foods and beverages worldwide. A wide variety of methodologies have been developed for the quantification of vitamin D in food feed environmental, clinical and pharmaceutical samples. The analysis of vitamin D compounds is further complicated by their compound structural similarity of metabolites, the predominantly hydrophobic/ lipophilic nature of vitamin D compounds and the hydrophilic nature of some of the metabolites, and their instability in the presence of heat or UV light. Accordingly, analysis requires selective and rapid methods and among these, there are two main types of methods which are used routinely, namely: competitive immunoassays and methods based on chromatographic separation followed by non-immunological direct detection (HPLC-UV and LC–MS/MS). Immunoassays are frequently used as commercial kits that include RIA, ELISA, CLIA, and ECL methods. Immunoassays are readily automated, suitable for high throughput and do not require high level technical skills to obtain satisfactory results. However, the major disadvantage is that of specificity as immunoassays are not able to detect D2, thus making them unsuitable for monitoring any food, feed, water and environment samples. In recent years, LC-MS/MS has been using for vitamin D analysis because it conveys both high specificity and sensitivity (Qi et al., 2015). LC-MS methods allow the separation of compounds based on their polarities, ionization behaviors, and mass to-charge (m/z) ratios and can offer very low limits of quantitation. However, these LC-MS methods also have challenges include, abundant isobaric and isomeric interferences (Volmer, 2015), low ionization efficiencies for mass spectrometric analyses (Hewavitharana et al., 2014) and matrix effects (Gomes et al., 2013). Therefore, increasing the selectivity and sensitivity for determination of vitamin D metabolites has been the aim of various methods and has focused on sample clean-up/extraction and analyte concentration prior to determination.

Establishing an appropriate extraction method for vitamin D is crucial as it cannot be assessed by the validation process (Heijboer et al., 2012). Therefore, the release of the vitamin from the sample matrix is a crucial step that affects the sensitivity and reproducibility of the analytical process. Ideally, the extraction method must be capable of dislodging the entire vitamin content from the matrix before analysis.

A variety of extraction methods was developed in recent year and compared and the results showed high variability illustrating inconsistency in the extent of vitamin D release from the matrix (Heijboer et al., 2012). On the contrary, traditionally, saponification has been used for the extraction of vitamin D from foodstuffs including milk and LLE and SPE also have been used as extraction/sample clean-up procedures in vitamin D assays analyses after the release from matrix components such as protein

and fat. Traditionally, the process of alkaline saponification for the extraction of vitamins and step-wise HPLC analyses has been widely used for analysis of lipophilic vitamins in animal feeds and the most common procedure by which to extract vitamin D compounds from foodstuff (Berg et al., 1986). The hydrolysis reaction attacks ester bonds and releases the fatty acids from the glycerol of glycerides and phospholipids, as well as from esterified sterols and carotenoids (Thompson et al., 1982). This reaction also frees vitamin D from any binding matrix that may exist in the sample matrix. Given the lack of stability of vitamin D, it is common to use antioxidants such as butylated-hydroxytoluene (2,6-di-tert-butyl-4-methylphenol) and ascorbic acid in the saponification process (Demchenko et al., 2011; Japelt et al., 2011; Kienen et al., 2008; Trenerry et al., 2011; Perales et al., 2005), combined with potassium hydroxide in ethanol or water solutions. The importance of potassium concentration in ethanol or methanol in saponification to obtain vitamin D in milk matrix has been reported in various studies. However, there was no significant difference when either methanol or ethanol were used (Paixao & Stamford, 2002). Ethanolic KOH prevents the formation of emulsions and mixes well with fat, but it requires daily preparation (Perales et al., 2005). In contrast, aqueous KOH does not mix well with fat, but is more stable – this probably being the reason why it is more often used (Perales et al., 2005).

Hot saponification consists of treating the sample with ethanolic or aqueous KOH at temperatures between 60–100°C and times range of 20–45 min while cold saponification consists of treating the sample overnight with ethanolic or aqueous KOH at room temperature, under slow constant stirring (Perales et al., 2005). Thermal isomerisation of vitamin D to pre-vitamin D may be avoided in a cold saponification procedure (isomerisation losses of less than 5% under cold conditions versus about 10–20% under hot conditions). Furthermore, this method provides satisfactory extraction and recovery and is simpler to operate with less operator attention (Thompson et al., 1982; Thompson et al., 1977).

Once saponification has been completed, the non-saponifiable fraction is extracted with organic solvents that are not miscible in water. Preferably with hexane instead of di-ethyl ether because di-ethyl ether is more inflammable and unstable than hexane and the latter can be simply removed at low pressure at a temperature below 50°C) (Thompson et al., 1982). In addition, there are various other conditional factors such as sample particle size (mesh), ratio of sample to reagent, extraction time, extraction equipment and pre-purification that can affect extraction efficiency (Qian & Sheng, 1998). Subsequently, evaporation is used to remove the organic solvents. These extraction methods were further simplified in this study to reduce the complexity involved in the process. Since efficient extraction of vitamin D and purification was the main challenge in all the analytical technique, therefore, we use

this extraction method and UV-visible spectrophotometer instead of HPLC, LC-MS/MS to quantify vitamin D in food feed, environmental and pharmaceutical samples.

## Methods

### Materials

Chemicals and solvents including vitamin $D_3$ (C-9756; 1 mg), methanol, acetonitrile, 2- propanol and hexane used in this study were purchased from Sigma-Aldrich Chemie, GmbH, Germany. Food and environmental samples were collected from different market of Dhaka city. Pharmaceutical samples were collected from local pharmacies of Dhaka city and human blood samples were collected from the healthy volunteers after one to one talked and written consent were taken from each volunteer.

### Sample Collection

In total 28 samples of them 14 food samples, 6 environmental samples, 3 feed samples, 3 clinical samples & 2 pharmaceutical samples, were analyzed in this study.

### Preparation of standard vitamin $D_3$

A stock solution of standard vitamin $D_3$ (0.5 μg/ml) was prepared in methanol and diluted to different concentrations ranging from 12 ng/mL to 315 ng/mL and UV absorbance was taken at 275 nm.

### Extraction of vitamin $D_3$ from samples

Food and pharmaceutical solid samples (capsules, carrot, algae, mushrooms, fishes), were first grounded with blender and dried well, and 1.0 gm of grounded powder was dissolved into 5 ml methanol in a 15 mL falcon tube for 2 hours in the dark with occasional vortex and the solid material was separated from the liquid methanol with Whatman 1 filter paper. Vitamin D was extracted from methanol by mixing slowly three volume of hexane (3 x 2 ml) with the interval of 60 seconds.  In case of serum, and milk samples, 1.0 mL of each sample was transferred in a 15 ml falcon tube, and mixed with methanol and isopropanol (80:20) for deproteination. Egg yolk was taken directly from the egg using a pipette and known concentration of the egg yolk was mixed with methanol and isopropanol (80:20) for deproteination. The contents were mixed using a vortex mixer for two minutes and vitamin D was extracted by mixing slowly three volume of hexane (3 x 2 ml) with the interval of 60 seconds. For all types of samples, the phase separation was done by centrifugation (4000 rpm for 15 min) and 4 ml upper organic phase was transferred to a small beaker and dried under liquid nitrogen gas. The dried extract was solubilized in methanol.

### UV Spectrophotometer reading

UV absorbance reading for each sample was taken and the baseline was monitored continuously during this process. Standard vitamin $D_3$ was analyzed using absorbance value from 210 nm to 800 nm to determine the optimum UV absorbance.

### Statistical Analysis

The proposed methods were successfully applied to the analysis of vitamins $D_3$, in food, feed, pharmaceutical, clinical and environmental samples. These data were subjected to ANOVA test to see any significant difference between the data sets.

### Method validation

**Specificity**: Specificity was determined by testing standard substance against potential interferences. This method was found to be specific because complete separation of vitamin $D_3$ was achieved without any interference. The peak attained was well separated at the baseline as shown in Figure 1.

**Linearity and calibration curve**: The calibration curve was constructed by plotting the absorbance of vitamin D against the respective concentrations, ranging from 12 ng/ml to 315 ng/ml of standard vitamin $D_3$ and the linearity was evaluated by the least-square regression method, which was used to calculate the regression coefficient value ($r^2$), y-intercept and slope of the regression line.

**Precision**: Intraday precision was determined by the % RSD [% RSD = (Standard deviation, SD/ Mean) × 100] of a single solution of a particular concentration by performing six times on the same day. Intermediate precision was investigated by determining the %RSD for a solution of single concentration by injecting three times on three different days.

**Accuracy**: Accuracy of the method was determined by recovery experiments which were carried out by spiking solutions of known concentration of the vitamin D with pre-analyzed sample. The data of the experiment were statistically analyzed using the formula [% Recovery = (Recovered concentration /Injected conc.) x 100] to determine the recovery and the validity of the proposed method.

**Limit of detection (LOD) and Limit of quantification (LOQ)**: LOD and LOQ were determined by the standard deviation (σ) method. LOD and LOQ were determined from the slope, S, of the calibration plot, S y/x, by use of the formulae:

LOD = 3.3 × σ/S and

LOQ = 10 × σ/S.

σ = Standard deviation, S = Slope

**Application of this method:** After validation of this method, it was applied for the estimation of vitamin $D_3$ from food, feed, clinical, pharmaceutical and environmental samples.

**Measurement uncertainty:** Measurement uncertainty is a property of measurement result, not of the method, equipment or laboratory and therefore it is expected to assay only once the result is obtained. Measurement uncertainty is estimated according to the internationally and multidisciplinary harmonized Guide to the Expression of Uncertainty in Measurement (GUM) issued in 1993, corrected in 1995 ("BIPM/IEC/IFCC/ISO/IUPAC/IUPAP/OIML: Guide to the expression of uncertainty in measurement.," 1995.).

## Results

### Validation of the Method

The validity of the method for the examination of vitamin D was investigated by determining specificity, linearity, precision and accuracy. In this study, we validated the protocol for UV-visual spectrophotometer. Validation was determined by analyzing three replicates of each sample within the Beer's law limits.

### Specificity

The specificity of the method was assessed from the chromatogram where vitamin D showed distinct absorbance pattern without any interference (Fig. 1). We also took the absorbance of blank sample (standard vehicle) which represents absence of standard vitamin D (Fig. 1).

### Linearity

To determine the linearity of the developed method, baseline was monitored continuously during this process. Detection was carried out at 210 nm to 800 nm, maximum signal was found at $\lambda_{max}$ 275 nm for vitamin $D_3$. The linearity for the vitamin D3 was determined using standard solutions having five concentrations. The linear regression data for the calibration curve revealed that the response is linear over the experimental concentration range 12 to 315 ng/mL with coefficient of correlation ($r^2$) value as 0.999. Chromatograms and linearity curve are shown in Figure 2 and results are summarized in Table 1.

### Precision

Precision of this method was verified by intra- and inter-day precision studies. Intra-day precision was performed by analysis of a single concentration for six times on the same day. The intermediate precision of the method was checked by investigation on three different days. Results are summarized in Table 1. Intra-day and inter-assay precision were 0.14% and 0.13% respectively in our study.

### Accuracy

Accuracy of the method was verified by studying recovery experiments which were performed by spiking solutions of known concentration of vitamin $D_3$ with pre-analyzed sample. To evaluate the accuracy of the method, successive analysis (n=3) of standard solutions of vitamin $D_3$ was carried out and the results are presented in Table 2.

### Sensitivity

The LOD and LOQ were separately determined on the basis of standard calibration curve. The residual standard deviation of the regression line or the standard deviation of y-intercepts of regression lines was used to calculate LOD and LOQ. Sensitivity of the proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ). The results of LOD and LOQ were found to be 0.004 ng/mL and 0.01 ng/mL respectively which are shown in Table 2.

### Investigation of the vitamin D concentration of the samples

Spectra of samples are shown in Figure 3 and data are presented in Table 4. Absorbance peak at 275 for vitamin D was detected in tuna, milk, egg, mushroom, feed, clinical and pharmaceutical samples. Vitamin D concentrations were very low in milk and relatively high in mushrooms and tuna fish. We were unable to detect vitamin D specific signal in carrot and sea algae with this experimental condition. We also studied the animal feed and pharmaceutical preparations which contain predetermined level of vitamin D and found nearly similar amount in our assay conditions. We found relatively high signal for clinical sample, which might be due to possible interference with vitamin E and other components which have similar absorbance spectra.

### Estimation of uncertainty measurement

It is a parameter that relates to the values of a measurement that reveals how the observations are spread out on each side of the centre. This parameter can be range, variance, or standard deviation. In our study, we have two sources of variations, namely intra-day variation (or, between days' variation) and inter-day variation (or, within days' variation). These measurement of uncertainties was calculated by means of analysis of variance (ANOVA) (De Beer, 2003) which is presented in Table 3. From the Table 3, it is observed that there is no difference among mean response for different days at 5% level of significance for our proposed UV spectrometry-based method. In other words, UV spectrometry-based method has provided precise estimates.

## Discussion

Vitamin D deficiency becomes pandemic worldwide. The person who is vitamin D deficient is more sensitive to develop numerous diseases. Currently available methods of vitamin D estimations are very costly and most of the people in the third world countries can't afford this. Therefore, it is essential to develop a simple, reliable and cost-effective method of vitamin D estimation. It is very well documented that analytical methods are sensitive and cost-effective in comparison to RIA or ELISA based method (Turpeinen, Hohenthal, & Stenman, 2003). In this study, we developed a simple and low-cost UV based method for vitamin D estimation from food, feed, pharmaceutical and environmental samples. The optimum UV absorbance was selected based on analyzing standard vitamin $D_3$ from 210 nm to 800 nm. We have analyzed various samples for vitamin D estimation in this study using UV-VIS spectrometry at 275 nm. This is in consistent with previous report where this wavelength has been found to show optimum absorbance for vitamin D (Wacker & Holick, 2013). Intra-day and inter-assay precision were 0.14% and 0.13% respectively in our study. The other reported methods developed for vitamin $D_3$ estimation showing within-batch and between-batch precision ranged from 0.83 to <10% and 1.8 to <12% respectively (A.K., 2015; Glendenning et al., 2006; Mata-Granados, Quesada Gomez, & Luque de Castro, 2009).
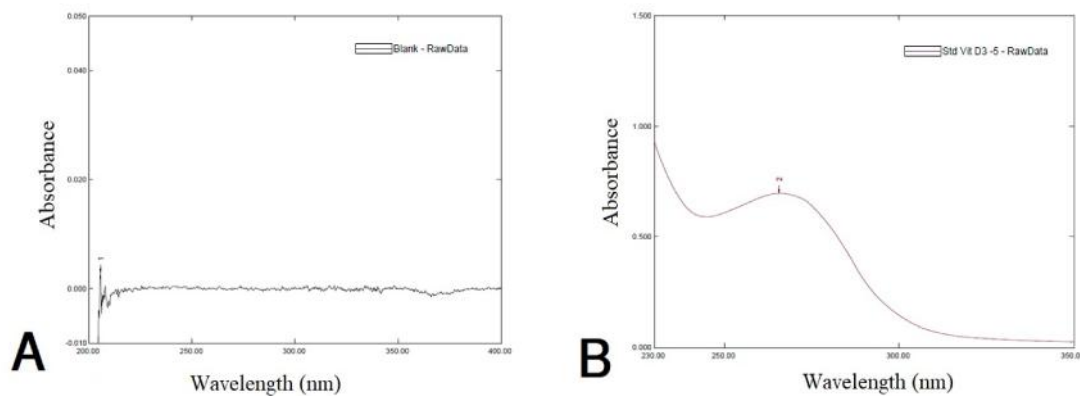
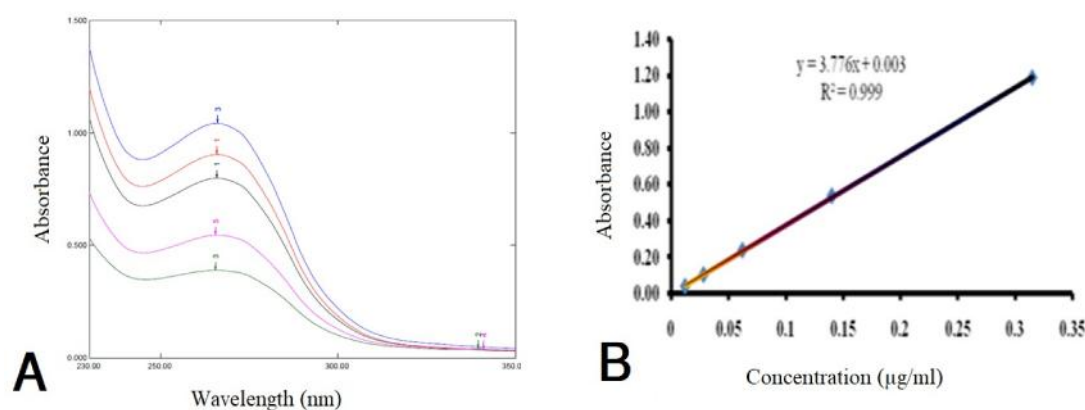Figure 1I UV spectra of A) Blank and B) Standard vitamin D3.



Figure 2I A) UV absorbance of five concentrations of standard solution for calibration. B) Calibration curve for standard vitamin D3. Experiments were triplicated.
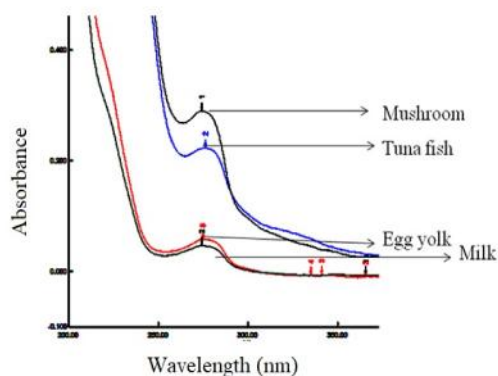


Figure 3I UV spectrum of one representative sample spectrum for mushroom, tuna, egg yolk and milk,

**Table 1**: Results of method validation parameters

| Parameters | Vitamin $D_3$ |
|---|---|
| Linear equation | y= 3.776x+0.003 |
| Coefficient of determination ($r^2$>0.995) | 0.999 |
| Linearity range | 12-315 ng/mL |
| Precision (intra-day, n=6) (% RSD≤2) | 0.14% |
| Precision (inter-day, n=9) (% RSD≤2) | 0.13% |

n= number of determinations

**Table 2**: Accuracy for standard vitamin D3

| Parameters | Vitamin D3 |
|---|---|
| Accuracy (n=3) (avg. % recovery) Standard + spike (ng/ml) | |
| (0.11+0.10) | 101.15% |
| (0.12+0.10) | 100.85% |
| (0.13+0.10) | 100.65% |
| LOD (ng/ml) | 0.004 |
| LOQ (ng/ml) | 0.01 |

n = number of determinations

**Table 3:** Uncertainty calculation from ANOVA table.

| Source of Variation | Degrees of freedom | Sum of Squares | Mean Square | F value | Pr (>F) |
|---|---|---|---|---|---|
| Between days | 2 | 8.3E-07 | 4.15E-07 | 0 | 1.00 |
| Within days | 12 | 2.632 | 0.2193 | | |
| Total | 14 | 2.63200083 | | | |

**Table 4**: Vitamin D concentrations in various samples (n=28)

| Sample Category | Sample name | Sources | Liquid/solid | No. of samples | Vitamin D |
|---|---|---|---|---|---|
| Food | Milk | Shikderbari, Abdullahpur, Bansree, Basabo, Jatrabari | Liquid | 5 | 12 (ng/ml)<br>11 (ng/ml)<br>13 (ng/ml)<br>10 (ng/ml)<br>12 (ng/ml) |
| | Carrot | Dhaka city market | Solid | 3 | ND<br>ND<br>ND |
| | Egg yolk (ng/gm) | Tongi bazar, Abdullahpur, Faidabad, Jatrabari | Semi-solid | 4 | 15 (ng/g)<br>14 (ng/g)<br>16 (ng/g)<br>15 (ng/g) |
| Feed | Poultry Feed | Savar, Tongi and Old Dhaka | Solid | 3 | ND<br>0.10 (ng/g)<br>0.12 (ng/g) |
| Environmental | Mushrooms | Mashroom culture center, Savar Dhaka | Solid | 3 | 65 (ng/g)<br>61 (ng/g)<br>62 (ng/g) |
| | Algae | Bay of Bengal Cox Bazar | Solid | 3 | ND<br>ND<br>ND |
| Pharmaceutical | Marine Cal-D (200 IU) | Incepta Pharmaceuticals Ltd. | Solid | 1 | 4950 (ng/tablet) |
| | Calbo D (200 IU) | Square Pharmaceuticals Ltd. | Solid | 1 | 4920 (ng/tablet) |
| Clinical | Serum | BSMMU Human | Serum | 3 | 19 (ng/ml)<br>21 (ng/ml)<br>23 (ng/ml) |
| Tuna | Tuna fish | Imported from USA | Solid | 2 | 59 (ng/g)<br>52 (ng/g) |

*ND indicates not detected.

In addition, we found that vitamin A and vitamin E have maximum absorbance at 324 and 298 nm respectively (data not shown). We found that vitamin E has interference at 275 nm and vitamin A does not have any effect at this wavelength. As vitamin E is co-extracted with vitamin D in organic extraction method, presence of vitamin E in serum and some food will show overestimation to a certain extent that should be considered while calculating the total vitamin D concentrations. Simply, value for vitamin E should be deduced from total concentration to present the actual concentration of vitamin D. Since, pharmaceutical preparations, some food and feed are free of vitamin E, this UV-VIS method can directly be applied for estimation of vitamin D by this method.

Total time of analysis was ~3 h for each batch; this time includes extraction, preparation and UV detection. In each batch twelve samples could be analyzed. The required reagents are relatively low cost, easy to find, relatively low toxicity for operators and without excessive problems of waste disposal. The developed method for the determination of vitamin D is simple, precise, accurate, reproducible, cost effective and validated according to ICH guidelines (A.K., 2015).

## Conclusion

In sum, a simple, sensitive, and highly accurate UV-visual spectrophotometric method was developed for the determination of vitamin $D_3$ in food, feed, pharmaceutical, and environmental samples in the present study. It takes shorter time, low cost and less expensive

equipments with great precision. The study indicated that the UV-VIS spectrophotometer can be used for routine estimation of vitamin $D_3$ in an easy and cost-effective way.

## Author Contribution

AR and MMR carried out the experimental works. AR, MSJ and MSH analyzed the data and drafted the manuscript. NJA performed statistical analysis. LB designed and supervised the research, and did meticulous revision of the manuscript. All the authors approved the manuscript.

## Competing financial interests

There is no competing financial interest.

## Acknowledgement

## References

A.K., K. S. C. D. a. T. (2015). An Improved and Sensitive Method for Vitamin D3 Estimation by RPHPLC. *Pharmaceutica Anal. Acta, 6(8)*, 410.

Al-Hendy, A., Diamond, M. P., Boyer, T. G., & Halder, S. K. (2016). Vitamin D3 Inhibits Wnt/beta-Catenin and mTOR Signaling Pathways in Human Uterine Fibroid Cells. *J Clin Endocrinol Metab, 101*(4), 1542-1551. doi:10.1210/jc.2015-3555

Al-Jaderi, Z., & Maghazachi, A. A. (2013). Effects of vitamin D3, calcipotriol and FTY720 on the expression of surface molecules and cytolytic activities of human natural killer cells and dendritic cells. *Toxins (Basel), 5*(11), 1932-1947. doi:10.3390/toxins5111932

Alhassan Mohammed, H., Mirshafiey, A., Vahedi, H., Hemmasi, G., Moussavi Nasl Khameneh, A., Parastouei, K., & Saboor-Yaraghi, A. A. (2017). Immunoregulation of Inflammatory and Inhibitory Cytokines by Vitamin D3 in Patients with Inflammatory Bowel Diseases. *Scand J Immunol, 85*(6), 386-394. doi:10.1111/sji.12547

Arboleda Alzate, J. F., Rodenhuis-Zybert, I. A., Hernandez, J. C., Smit, J. M., & Urcuqui-Inchima, S. (2017). Human macrophages differentiated in the presence of vitamin D3 restrict dengue virus infection and innate responses by downregulating mannose receptor expression. *PLoS Negl Trop Dis, 11*(10), e0005904. doi:10.1371/journal.pntd.0005904

Bartels, L. E., Jorgensen, S. P., Bendix, M., Hvas, C. L., Agnholt, J., Agger, R., & Dahlerup, J. F. (2013). 25-Hydroxy vitamin D3 modulates dendritic cell phenotype and function in Crohn's disease. *Inflammopharmacology, 21*(2), 177-186. doi:10.1007/s10787-012-0168-y

Berg, V. H., Boshuis, P. G., Schreurs, W. H. (1986). Determination of vitamin D in fortified and nonfortified milk powder and infant formula using a specific radioassay after purification by high-performance liquid chromatography. *J Agric Food Chem*, 34(2), 264-8.

BIPM/IEC/IFCC/ISO/IUPAC/IUPAP/OIML: Guide to the expression of uncertainty in measurement. (1995.).

Boontanrart, M., Hall, S. D., Spanier, J. A., Hayes, C. E., & Olson, J. K. (2016). Vitamin D3 alters microglia immune activation by an IL-10 dependent SOCS3 mechanism. *J Neuroimmunol, 292*, 126-136. doi:10.1016/j.jneuroim.2016.01.015

Bosetti, M., Sabbatini, M., Calarco, A., Borrone, A., Peluso, G., & Cannas, M. (2016). Effect of retinoic acid and vitamin D3 on osteoblast differentiation and activity in aging. *J Bone Miner Metab, 34*(1), 65-78. doi:10.1007/s00774-014-0642-2

Chen, J., Dosier, C. R., Park, J. H., De, S., Guldberg, R. E., Boyan, B. D., & Schwartz, Z. (2016). Mineralization of three-dimensional osteoblast cultures is enhanced by the interaction of 1alpha,25-dihydroxyvitamin D3 and BMP2 via two specific vitamin D receptors. *J Tissue Eng Regen Med, 10*(1), 40-51. doi:10.1002/term.1770

De Beer, J. O., Baten, P., Nsengyumva, C., & Smeyers-Verbeke, J. . (2003). Measurement uncertainty from validation and duplicate analysis results in HPLC analysis of multivitamin preparations and nutrients with different galenic forms. . *Journal of pharmaceutical and biomedical analysis., 32(4-5)*, 767-811.

Demchenko, D. V., Pozharitskaya, O. N., Shikov, A. N., Makarov, V. G. (2011). Validated HPTLC method for quantification of vitamin D3 in fish oil. *J Planar Chromat*, 24, 487-90.

Glendenning, P., Taranto, M., Noble, J. M., Musk, A. A., Hammond, C., Goldswain, P. R., . . . Vasikaran, S. D. (2006). Current assays overestimate 25-hydroxyvitamin D3 and underestimate 25-hydroxyvitamin D2 compared with HPLC: need for assay-specific decision limits and metabolite-specific assays. *Ann Clin Biochem, 43*(Pt 1), 23-30. doi:10.1258/000456306775141650

Gomes, F. .P, Shaw, P. N., Karen, W., Pieter, K., Hewavitharana, A. K. (2013). Recent trends in the determination of vitamin D. *Bioanalysis*, 5(24), 3063-78.

H, P., O, C., Da, U., O, G., & Ng, D. (2013). The impact of Vitamin D Replacement on Glucose Metabolism. *Pak J Med Sci, 29*(6), 1311-1314.

Heijboer, A.C., Blankenstein, M. A., Kema, I. P., Buijs, M. M. (2012). Accuracy of 6 routine 25-hydroxyvitamin D assays: influence of vitamin D binding protein concentration. *Clin Chem*, 58(3), 543-8.

Hewavitharana, A. K., Tan, S. K., Shaw, P. N. (2014). Strategies for the detection and elimination of matrix effects in quantitative LC-MS analysis. *LCGC North America*, 32(1), 54-64.

Holick, M. F. (2002). Sunlight and vitamin D: both good for cardiovascular health. *J Gen Intern Med, 17*(9), 733-735.

Hu, X. T., & Zuckerman, K. S. (2014). Role of cell cycle regulatory molecules in retinoic acid- and vitamin D3-induced differentiation of acute myeloid leukaemia cells. *Cell Prolif, 47*(3), 200-210. doi:10.1111/cpr.12100

Hunten, S., & Hermeking, H. (2015). p53 directly activates cystatin D/CST5 to mediate mesenchymal-epithelial transition: a possible link to tumor suppression by vitamin D3. *Oncotarget, 6*(18), 15842-15856. doi:10.18632/oncotarget.4683

Jamka, M., Wozniewicz, M., Jeszka, J., Mardas, M., Bogdanski, P., & Stelmach-Mardas, M. (2015). The effect of vitamin D supplementation on insulin and glucose metabolism in overweight and obese individuals: systematic review with meta-analysis. *Sci Rep, 5*, 16142. doi:10.1038/srep16142

Japelt, R. B., & Jakobsen, J. (2013). Vitamin D in plants: a review of occurrence, analysis, and biosynthesis. *Front Plant Sci, 4*, 136. doi:10.3389/fpls.2013.00136

Japelt, R. B., Silvestro, D., Smedsgaard, J., Jensen, P. E., Jakobsen, J. (2011). LC−MS/MS with atmospheric pressure chemical ionisation to study the effect of UV treatment on the formation of vitamin D3 and sterols in plants. *Food Chemisty*, 129(1), 217-25.

Kienen, V., Costa, W. F., Visentainer, J. V., Souza, N. E., Oliveira, C. C. (2008). Development of a green chromatographic method for determination of fat-soluble vitamins in food and pharmaceutical supplement. *Talanta*, 75(1), 141-6.

Lopez-Sobaler, A. M., Aparicio, A., Gonzalez-Rodriguez, L. G., Cuadrado-Soto, E., Rubio, J., Marcos, V., . . . Ortega, R. M. (2017). Adequacy of Usual Vitamin and Mineral Intake in Spanish Children and Adolescents: ENALIA Study. *Nutrients, 9*(2). doi:10.3390/nu9020131

Malabanan, A., Veronikis, I. E., & Holick, M. F. (1998). Redefining vitamin D insufficiency. *Lancet, 351*(9105), 805-806.

Mata-Granados, J. M., Quesada Gomez, J. M., & Luque de Castro, M. D. (2009). Fully automatic method for the determination of fat soluble vitamins and vitamin D metabolites in serum. *Clin Chim Acta, 403*(1-2), 126-130. doi:10.1016/j.cca.2009.01.029

Ovesen, L., Andersen, R., & Jakobsen, J. (2003). Geographical differences in vitamin D status, with particular reference to European countries. *Proc Nutr Soc, 62*(4), 813-821. doi:10.1079/PNS2003297

Paixao, J., Stamford, T. (2002). A single guide for extraction and quantification of fat-soluble vitamins. Journal of Liquid Chromatography and Related Technologies. *Lacteal matrices*, 25(2), 217-39.

Perales, S., Alegria, A., Barbera, R., Farre, R. (2005). Review: determination of vitamin D in dairy products by high performance liquid chromatography. *Food Science and Technology International*, 11(6), 451-62.

Qian, H., Sheng, M. (1998). Simultaneous determination of fat-soluble vitamins A, D and E and pro-vitamin D2 in animal feeds by one-step extraction and high-performance liquid chromatography analysis. *J Chromatogr A*, 825(2), 127-33.

Qi, Y., Geib, T., Schorr, P., Meier, F., Volmer, DA. (2015). On the isobaric space of 25-hydroxyvitamin D in human serum: potential for interferences in liquid chromatography/tandem mass spectrometry, systematic errors and accuracy issues. *Rapid Commun Mass Spectrom*, 29(1), 1-9.

Santos, J. M., Khan, Z. S., Munir, M. T., Tarafdar, K., Rahman, S. M., & Hussain, F. (2017). Vitamin D3 decreases glycolysis and invasiveness, and increases cellular stiffness in breast cancer cells. *J Nutr Biochem, 53*, 111-120. doi:10.1016/j.jnutbio.2017.10.013

Thompson, J., Hatina, G., Maxwell, W., Duval, S. (1982). High performance liquid chromatographic determination of vitamin D in fortified milks, margarine, and infant formulas. *Journal-Association of Official Analytical Chemists*, 65(3), 624-31.

Thompson, J., Maxwell, W., Labbe, M. (1977). High pressure liquid chromatographic determination of vitamin D in fortified milk. *Journal-Association of Official Analytical Chemists*, 60(5), 998-1002.

Trenerry, V. C., Plozza, T., Caridi, D., Murphy, S. (2011). The determination of vitamin D3 in bovine milk by liquid chromatography mass spectrometry. *Food Chemisty*, 125, 1314-9.

Turpeinen, U., Hohenthal, U., & Stenman, U. H. (2003). Determination of 25-hydroxyvitamin D in serum by HPLC and immunoassay. *Clin Chem, 49*(9), 1521-1524.

Volmer, D. A. (2015). Analysis of vitamin D metabolic markers by mass spectrometry: Current techniques, limitations of the "gold standard" method, and anticipated future directions. *Mass Spectrom Rev*, 34(1), 2-23.

Wacker, M., & Holick, M. F. (2013). Sunlight and Vitamin D: A global perspective for health. *Dermatoendocrinol, 5*(1), 51-108. doi:10.4161/derm.24494