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Production of Vitamin D_3 Enriched Designer Chicken Eggs by Direct Ultra Violet Blue (UVB) Light Exposure

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ABSTRACT

The market demand for designer eggs has been increased in the recent past. In the present study, chicken eggs were enriched with vitamin D_3 by UVB exposure @ 3 h/day. The trial was commenced from 29 weeks of age and conducted for 12 weeks on thirty-two number of crossbred layer birds (White Leghorn N strain and Desi). The vitamin D_3 concentration in egg was significantly higher ($p<0.01$) in the treated group compared to the control group. The vitamin D_3 concentration in eggs of UVB light exposed birds was 72.34±1.55 µg/10 g egg yolk and it was significantly higher compared to untreated group (17.92±1.98 µg/10 g egg yolk). These results clearly indicated an enriching influence of UVB radiation on the concentration of vitamin D_3 in eggs and therefore recommend an easy, cheap and safe procedure for producing designer eggs.

HIGHLIGHTS

- The chicken eggs were enriched with vitamin D_3 by UVB exposure @ 3 h/day.
- Ultra Violet Blue (UVB) light exposure would be a better and cheaper choice for enhancing vitamin D_3 content of eggs and to produce designer eggs enriched with vitamin D_3.

Keywords: Designer eggs, Ultra violet blue light, Vitamin D_3

Eggs are vital part of the diet which has been used as a food by human beings since ancient times. The poultry eggs are regarded as inexpensive, convenient and low-calorie sources of high quality protein with several other essential nutrients. Designer eggs are those that have modified contents from standard egg according to the consumers' preference or market demand. Vitamin D_3 is an important vitamin for various metabolic functions of the body viz normal bone formation, calcium-phosphate absorption and immune function. Although vitamin D_3 can be endogenously produced in the skin under sunlight, public consciousness of the adverse effects of sun exposure and the use of skin protection measures led to low vitamin D_3 level in the body. Recent studies in India have shown that in the past decade, 50-94% of apparently healthy individuals seem to have vitamin D_3 deficiency. Deficiency of vitamin D_3 leads to public health problems like rickets, osteomalacia and prostate cancer in human beings.

In addition to fish and milk, egg yolk is considered as a natural source for both vitamin D_3 and 25-hydroxyvitamin D_3 (Browning and Cowieson, 2014). It is an effective practice to supply vitamin D_3 in poultry diet to improve the content of cholecalciferol and 25-hydroxycholecalciferol in egg yolk. Since excess intake of vitamin D_3 leads to toxicity, maximum limits of supplementation have been fixed worldwide for animal feed formulation. Therefore, UVB light exposure to laying hens suggested as an

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alternative source to increase the quantity of vitamin D_3 in eggs, without compromising the health of birds.

MATERIALS AND METHODS

Experimental layout

Thirty-two numbers of 29 weeks old crossbred (White Leghorn N strain X Desi) layer birds procured from All India Coordinated Research Projects (AICRP) on Poultry for Eggs, Mannuthy, KVASU. The birds were divided into two treatment groups, each with four replicates having four birds in each replicate. On completion of 28 weeks of age, the birds acquired an average body weight of 1.28±0.06 Kg. Birds were reared at 24.5 ± 0.5˚C ambient temperature and at relative humidity of 60-80 per cent. A photoperiod of 16 h per day was ensured throughout the period.

Feeding schedule of birds

The total quantity of feed assigned per day was divided into 4 parts and each part was fed just before lighting schedule to ensure that the UV radiation falls on the featherless skin of feet and legs, while all birds were pecking the feed in standing position (Fig. 1). Water was provided *ad libitum.*

Fig. 1: UVB tubes placed in the lower front part of cages at 20 cm distance (***Source:*** Original)

Exposure to UVB light

Birds of treatment group was exposed to UVB (280-315 nm) light (M/s Philips India Ltd., Hyderabad) for 3h daily @ 8.00-8.30 a.m., 11.00 a.m.-12.00 p.m., 2.00-3.00 p.m. and 4.30-5.00 p.m. The UVB radiation dosage at a distance of 20 cm was 76 µW/cm^{2}as claimed by the manufacturer. A 60cm long, 240 V, 50 Hz and 36 W UVB tube equipped with heat protection reflector was placed in the lower front part of the cages as shown in Fig 2, so as to ensure an optimal UVB light exposure on the featherless skin of feet and legs of birds, especially during the feeding time. An opaque paper board was placed from the level of feeder to the top of the cage (Fig. 2) in order to prevent any harmful UV radiation falling on eyes and combs.

Fig. 2: UVB exposure to featherless shank of birds during feeding time by placing opaque paper board placed from the level of feeder to top of the cage (***Source:*** Original)

Standards for LC-MS

Stock solution of cholecalciferol (vitamin D_3) was prepared in amber coloured 100 mL volumetric flasks by dissolving 20 mg of vitamin D_3 (M/s Sigma Aldrich, Sweden) in 100 mL of HPLC grade n-Hexane. Composite working standard solutions of 125, 250, 500, 750 and 1500 µg/mL were prepared by diluting the stock solution. The preparation of samples and standards were carried out in subdued light and stored in amber coloured vials at 4˚C to avoid photodegradation.

Mobile phase for LC-MS

A mixture of HPLC grade methanol with 0.1% formic acid (pH 3.0) and distilled water with 0.1% formic acid

(pH 2.83) at 95: 5 was used as mobile phase (Kumar *et al.* 2015). After preparation, it was sonicated and filtered through solvent filtration unit under vacuum.

Saponification and extraction of sample

Homogenized egg yolk sample (10 g) was dispersed in 10 mL of ascorbic acid solution (10% w/v, in distilled water). To this mixture, 25 mL of potassium hydroxide solution (50%, w/w, in distilled water) and 50 mL ethanol (99%) were added. Saponification process was conducted at room temperature overnight, with continuous stirring using a magnetic stirrer (Mattila *et al.* 1992).

Saponified contents were transferred into a 250 mL separating funnel and extracted twice for 2 min with mixture containing 57.5 mL each of petroleum ether (bp 40-60°C) and diethyl ether at 1:1 ratio. The combined extract was washed with distilled water (50 ml) until pH 7 was attained and dehydrated with anhydrous sodium sulphate (5 g). The extract was lyophilised and dissolved in 10 mL of n-Hexane.

Final clean up technique of sample

A chromatography column with sintered glass disc (30 cm length and 20 mm diameter) was used for Purification of the extract. It was prepared with 2 g silica gel suspended in 10 mL n-Hexane and allowed for the stabilization. n-Hexane was drained out from the suspension, before loading the sample. The extracted sample (10 mL) was passed twice through the column and eluted @ 6 drops/ min and finally eluted with 15 mL of n-Hexane-isopropanol solvent mixture (99.5: 0.5). It was freeze dried and reconstituted with 1 ml of n-Hexane and filtered through Millex HV membrane filter (0.45 µm, Millipore, France) on the day of LC-MS analysis.

Detection and quantification of vitamin D_3

The vitamin D_3 concentration in the samples was detected by using single quadruple MS detector and quantification was done by Chromeleon software interfaced to the computer.

Chromatographic and mass spectrometric conditions

Standard chromatogram and mass spectra were generated for each injected standard and test material using (Diode-Array Detection) DAD of chromatograph with run time of 13 min and mass spectrometry respectively. The column temperature was held constant at 40˚C. Twenty microliter of blank (mobile phase), standards, control and test samples were injected separately into the C18 column using autosampler. The samples were separated by reverse phase column elution using mobile phase flow @ 0.5 mL/ min. The UV-VIS detection was carried out at 265 nm wavelength which was specific for vitamin D_3. The MS detector was set with vapourizer temperature of 40°C and a pressure of 42-45 bar. The ions were monitored through single ion monitoring (SIM) method at mass/charge (m/z) ratio of 385.41 which was specific for vitamin D_3.

Precision of method by intra-day variation of spiked concentrations

Precision and accuracy of the method employed were calculated by intra-day and inter-day variation in percentage recovery of vitamin D_3 from egg yolk. The egg yolk samples were spiked with 1000 µg/mL of vitamin D_3 before the extraction procedure and recovery was estimated thrice a day and precision was expressed by coefficient of variation.

Accuracy of method based on recovery percentage

Accuracy of the method was calculated by standard addition method. Yolk samples were spiked with 750, 1500 and 2000 µg/mL of vitamin D_3 before the extraction procedure and the recovery was estimated.

Robustness

The robustness of the method was determined to assess the effect of small but deliberate variation of the chromatographic conditions of the analyte and was estimated by changing the flow rate and concentration of mobile phase up to 1 per cent.

The limit of detection (LOD) and Limit of quantification (LOQ)

Limit of detection is the lowest concentration in a sample that can be detected, but not quantified, from background noise. Limit of quantification is the lowest concentration

of analyte with a signal to noise ratio of at least 10 and can be determined with acceptable accuracy and precision.

LOD = 3.3 X (standard deviation of intercept /slope)

LOQ = 10 X (standard deviation of intercept /slope).

STATISTICAL ANALYSIS

Results were expressed as mean (±S.E.). The statistical significance of difference or relation between the two treatments were analysed by CRD using the software Statistical Product and Services (SPSS) version 24.0 and the differences were considered statistically significant at 5% level ($p<0.05$) (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

Preparation of calibration curve

Chromatogram of different concentration of vitamin D_3 standards 125, 250, 500, 750 and 1500 µg/mL were generated and a representative figure of the concentration 1500 µg/mL is shown in the Fig. 3.

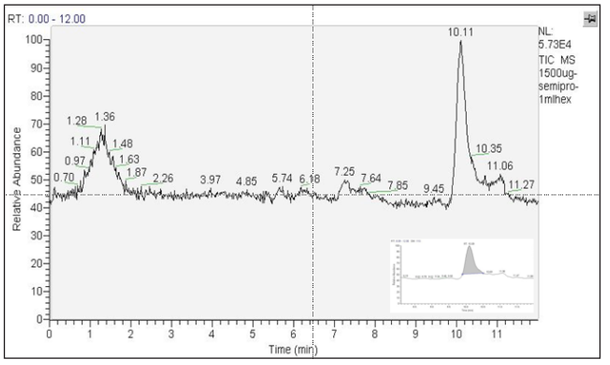

Fig. 3: Chromatogram of vitamin D_3 standard 1500 ug/mL (***Source:*** Original)

The retention time (RT) of vitamin D_3 standards was found to be between 10.09 and 10.26 minutes. The height and area of the peaks were found to be increased with increasing concentration of standards (Table 1).

Calibration curve was prepared by plotting peak area (Y axis) versus vitamin D_3 concentrations (µg/mL) (X axis). The regression line (R^2 >0.99) showed excellent relationship between peak area and vitamin D_3 concentrations over a range of 125 to 1500 µg/mL as shown in the Fig. 4.

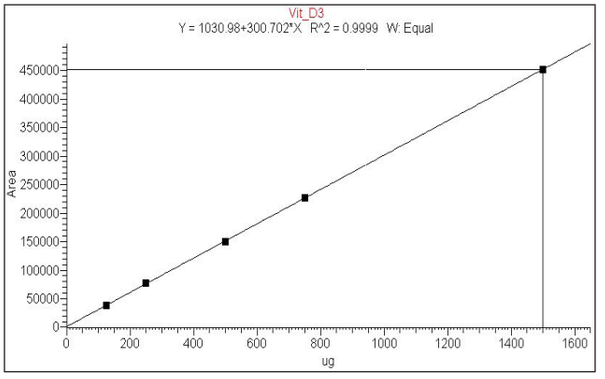

Fig. 4: Calibration curve of vitamin D_3 with different standards (***Source:*** Original)

A representative spectrum of vitamin D_3 is shown in Fig. 5, which was applicable to all samples, since the detection was carried out in MS detector with SIM method (m/z ratio of 385.41).

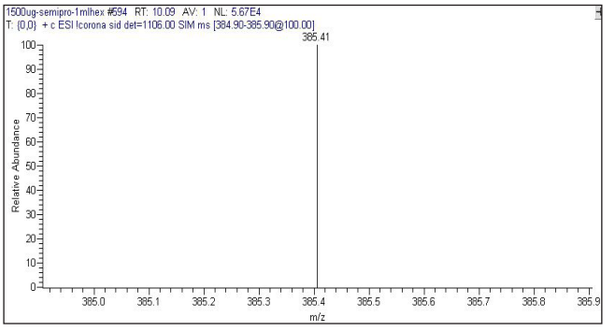

Fig. 5: MS spectrum of vitamin D_3 standard 1500 ug/mL (***Source:*** Original)

Vitamin D_3 content in egg samples

The data shown in Table 2 clearly indicated that the effect of UVB light treatment was highly significant ($p<0.01$) for vitamin D_3 concentration in egg samples during the experimental period. Vitamin D_3 concentration noticed in egg samples of UVB light treated group was 72.34 µg/10 g egg yolk, while Vitamin D_3 level was significantly lower ($p< 0.01$) in the non-exposed group (17.92±1.98 µg/10 g egg yolk).

Table 1: LC-MS Retention time (RT), area, height, expected amount and calculated amount of vitamin D_3 standards (***Source:*** Original)

Sl. No.	Standard concentration(µg/mL)	RT (minute)	Area of the peak (min × mAu)	Height of the peak (mAu)	Expected amount (µg/mL)	Calculated amount (µg/mL)
1	125	10.26	38915.95	3179.95	125.000	125.988
2	250	10.20	76958.66	6066.99	250.000	252.501
3	500	10.20	149381.34	9648.86	500.000	493.346
4	750	10.23	227427.14	12778.37	750.000	752.891
5	1500	10.09	452166.57	27715.72	1500.000	1500.273

Table 2: Mean (±S.D. /S.E.) values of retention time, area, height and amount of each treatment group (***Source:*** Original)

Treatment groups	No of samples	Mean value of Area (min*mAu)	Mean value of amount (µg/10 g egg yolk)	p-value
		Mean ± S.D.	Mean±S.E.	
Control group	12	6434.38 ± 2069.75	17.92^{a}±1.98	0.000**
Treatment group	12	22809.76 ± 1652.46	72.34^{b}±01.55	

Means bearing different superscript within a column differ significantly; ** Highly significant ($p<0.01$).

Precision and Accuracy of the method

The intra-day variation was carried out for 1000 µg/mL concentration of vitamin D_3 standard in three replicates at different time intervals in the same day. The precision of procedure was 6.78 % as given in table 3.

Table 3: Recovery of 1000 µg/mL of vitamin D_3 (***Source:*** Original)

Spiked concentration of vitamin D_3	1000 µg/mL
Recovery % at 8.00 a.m.	99.80
Recovery % at 12.00 p.m.	99.00
Recovery % at 4.00 p.m.	88.18
Mean Recovery %	95.66±3.74
Standard deviation	6.49
Co-efficient of Variation %	6.78

The mean recovery of the methods was determined by spiking matrix with different concentration and were found to be more than 95.66 ± 3.74 %. The results, given in Table 4 indicated better accuracy of method.

Table 4: Recovery percentages of vitamin D_3 standards spiked (***Source:*** Original)

Matrix Spiked with vitamin D_3 standard			
Concentrations (µg/mL)	750	1500	2000
% Recovery	99.19	83.76	101.741
Mean ± % RSD	95.66±6.78		

Robustness

There was no significant change in the retention time of analytes found when flow rate of the mobile phase was changed.

Limit of detection and Limit of quantification

It was observed that LOD of this method was 23.99 µg/mL and LOQ of the method was 72.70 µg/mL for vitamin D_3 for vitamin D_3 in egg yolk, with acceptable accuracy and precision.

Since there is a limit for the inclusion of vitamin D3 supplements while formulating the feed. Hence UVB light exposure is an alternate safe method to produce enriched eggs. Vitamin D_3 synthesized in the skin under UVB get coupled with vitamin D binding protein and then transferred to egg. In the ovary, it gets bind with yolk protein (Fraser and Emtage, 1976) for storage.

Council of the European Communities (Council Directive 70/524/EEC) has set a quantitative limit of 3,000 IU per kg feed for laying hens and beyond this limit any further increase in dietary supplementation is not recommended (Schutkowski, *et al.*, 2013). In India vitamin D_3 recommendation for layer birds (21-45 wk) was 1600 IU/kg (BIS 2007).

Studies of Yao *et al*. (2013) reported that usage of vitamin D_3 enriched feeds could increase the vitamin D_3 content in

eggs, to a maximum of 34,815 IU/100 g yolk which could be achieved with a dietary supplementation 1,02,200 IU/kg feed.

Current results showed that an exposure to UVB at a distance of 20 cm with intensity of 76 µW/cm^2 was capable of raising the vitamin D content in egg yolk. These reults agreed with the findings of Schutkowski *et al.* (2013), who demonstrated that both treatment factors (UVB light exposure and vitamin D_3 supplemented feed) were able to increase the vitamin D_3 content in eggs, even though the UVB irradiation was more successful than dietary supplementation. Highest content of vitamin D_3 in eggs could be obtained with a combination of UVB exposure and dietary vitamin D_3 supplementation. Vitamin D_3 level in control eggs was significantly lower ($p<0.01$) than eggs collected from UVB exposed laying hens.

Similarly, report of Kuhn *et al.* (2015) was in accordance with our present study outcomes, where vitamin D enrichment of eggs by daily UVB exposure at the rate of 5 h, could contribute 95% of the maximum attainable vitamin D content in eggs. Mattila *et al.* (1992) observed that two egg samples representing eggs on sale through the retail outlets contained 4.0 and 5.6 µg vitamin D_3 per 100 g. Upon enrichment the egg yolk samples had a vitamin D_3 content ranging from 1.3 to 15.1 µg/100g.

Sivell *et al*. (1982) also found that average vitamin D_3 content of egg yolk samples were 1.2 µg/ 100 g and 0.8 µg/100 g for battery cages and free-range reared eggs respectively. Other than vitamin D_3 active compound, egg yolk also contains other hydroxylated metabolites like 25-hydroxy vitamin D_3 and 1,25 –dihydroxy vitamin D_3 (Koshy and Van Der Slik, 1979; Wei *et al*., 2020).

Designer eggs have a strong market demand due to consumers' readiness to pay more for them due to their nutritional benefits over ordinary eggs. The current study succeeded to produce vitamin D_3 enriched eggs by UVB exposure and the effect of UVB light treatment was highly significant ($p<0.01$) to the concentration of vitamin D_3 in eggs from 29 to 40 weeks of age, without inflicting any toxic side effects on birds' health and was evident from the average hen day and hen housed egg production. The mean vitamin D_3 concentration in eggs of UVB exposed birds was 72.34±1.55 µg/10 g egg yolk. However, the unexposed group of birds had egg vitamin D_3 concentration was 17.92±1.98 µg/10 g egg yolk.

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