High-Performance Liquid Chromatographic Method for the Determination of 25-Hydroxycholecalciferol in Chicken Egg Yolks

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A high-performance liquid chromatographic (LC) procedure was developed for the determination of 25-hydroxycholecalciferol (25-OH-D_3) in chicken egg yolk. The procedure involved extraction with a 2:1 mixture of $CHCl_3$ and CH_3OH, solvent partition between hexane and CH_3CN, adsorption column chromatography on silica gel (230–400 mesh) and on microparticulate silica (20 μm), partition chromatography on a diatomaceous earth support, and quantitation by reversed phase LC on a C_{18} bonded microparticulate silica column. The identity of 25-OH-D_3 in the final extract was confirmed by the ultraviolet spectrum and the mass spectrum of the diheptafluorobutyrate. The endogenous level of 25-OH-D_3 in eggs from chickens on regular diet was 5–8 ng/g. The recovery of 25-OH-D_3 added to pooled egg yolk at the level of 9.5 ng/g was 87.9 ± 6.8% (n = 9, range 75–97%). The endogenous level was elevated to over 100 ng/g 8 days after intramuscular injection of 0.5 or 1 mg of 25-OH-D_3 in corn oil. It was also elevated after the chickens were fed either vitamin D_3 or 25-OH-D_3 at 30 and 45 mg/ton level. The elevation was higher with the 25-OH-D_3 than with the D_3 fed group.

It is now well established that vitamin D_3 is metabolized to 25-hydroxyvitamin D_3 (25-OH-D_3) in the liver and to 1,25-dihydroxyvitamin D_3 in the kidney. Even though other biologically active metabolites have been isolated, 1,25-dihydroxyvitamin D_3 is considered to be the most active metabolite of vitamin D_3. It was reported (Frank, 1974) that 25-OH-D_3 was effective in improving shell calcification of chicken eggs. In order to monitor the concentration of 25-OH-D_3 in the yolk, a sensitive and quantitative method was necessary. To our knowledge, there are no previously reported procedures for 25-OH-D_3 in egg yolk.

METHOD

Solvents. The chloroform, ethyl ether, and methanol were AR grade. All the other solvents were distilled-in-glass quality (Burdick and Jackson, Inc., Muskegon, Mich., or equivalent).

Extraction. The yolk was separated from the white. Residual white from the yolk was easily removed by rolling on a piece of cheesecloth. One yolk or 20 g of pooled yolk was added to a 250-mL beaker. To this was added 0.25 g of sodium ascorbate, 0.5 g of $NaHCO_3$, and 100 mL of a 2:1 $CHCl_3$–CH_3OH mixture and mixed well with a spatula. The slurry was poured into a 500-mL separatory funnel. An additional 200 mL of 2:1 $CHCl_3$–CH_3OH was added using portions to rinse the beaker. The contents were shaken vigorously for at least a minute. Eighty milliliters of water was added to the funnel, and the contents were gently mixed and allowed to separate. The lower organic phase was filtered under suction through a 5.5 cm no. 4 filter paper into a 1-L round-bottom flask. Fifty milliliters of water was added to the aqueous phase which was extracted by gentle shaking with 100 mL of $CHCl_3$. The extract was filtered through the same filter paper into the round-bottom flask. The precipitate on the paper was washed with 15–20 mL of $CHCl_3$. The combined filtrate was evaporated under vacuum on a rotary evaporator in a water bath at ≃40 °C until most of the solvent was removed.

Solvent Partition. The residue was dissolved in 100 mL of hexane and transferred to a 250-mL separatory funnel. It was extracted with 4 × 50 mL of acetonitrile using each portion to first rinse the round-bottom flask. The solvents were mutually saturated with each other. The extract was collected in a 500-mL round-bottom flask and evaporated to dryness under vacuum on a rotary evaporator in a water bath at ≃40 °C. The residue was transferred to a 8-mL test tube using 5 × 3 mL rinsings with pentane and evaporating the solvent under N_2 between each rinsing. The viscous residue was dissolved in a small volume of 1:1 hexane–ether.

Silica Gel Column Chromatography. The column was 12 mm i.d. × 38 cm long. It was fitted with a coarse sintered glass disc and a Teflon stopcock at the bottom and a 24/40 standard taper joint at the top to fit a 250-mL solvent reservoir. Ten grams of silica gel 60, 230–400 mesh (E. M. Laboratories, Elmsford, N.Y.), was slurry packed with hexane–ether (1:1). Approximately 0.5 g of powdered Na_2SO_4 was added at the top.

The residue from the solvent partition was transferred quantitatively onto the column with additional rinsings of the 8-mL test tube with hexane–ether (1:1). The column was eluted under N_2 pressure with 75 mL of hexane–ether (1:1) at ≃1 mL/min and the effluent was discarded. It was then eluted under N_2 pressure with methylene chloride–ether (1:1) at ≃1 mL/min. The first 20 mL was discarded and the next 60 mL collected in a 150-mL round-bottom flask and evaporated to dryness under vacuum on a rotary evaporator in a water bath at ≃40 °C. The residue was immediately dissolved in pentane and transferred quantitatively to a 5-mL tapered tube using 5 × 2 mL rinsings of the flask. It was evaporated to dryness under N_2 with the tube immersed in water at ≃35 °C and reconstituted in exactly 150 μL of 2.5% isopropyl alcohol in hexane. (There was an orange-red precipitate in the solution.)

Microparticulate Silica Column. The column was 6 mm i.d. × 15 cm long. It was fitted with a 10/18 standard taper female joint at the top to fit a solvent reservoir. The lower end of the column was drawn out to a bore of ≃1 mm diameter. The solvent reservoir was constructed from a 25-mL volumetric pipet whose stem was cut off below the bulb and fitted with a 10/18 standard taper female joint. The packing material was 0.5 g of a 20-μm microparticulate silica (Partisil 20, Whatman, Inc., Clifton, N.J.) and the mobile phase was 2.5% isopropyl alcohol in hexane.

A wad of glass wool was inserted into the tapered end of the column and pressed into a tight plug. One-half gram of the Partisil 20 was added to the column and packed by tapping with a glass rod. A pinch of powdered anhydrous

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Na_2SO_4 was placed on top of the packing. The column was equilibrated by passing 25 mL or more of the mobile phase. A known aliquot (usually 125 μL) of the extract from the previous column was applied onto the column head with a 100-μL syringe and forced down with N_2 pressure. Then the reservoir was attached and filled with the mobile phase. The column was eluted at a flow rate of ≃0.75 mL/min under N_2 pressure. The first 7 mL was discarded and the next 7 mL collected. It was evaporated to dryness in a 50-mL round-bottom flask under vacuum in a water bath at ≃40 °C and quantitatively transferred to a 2.5-mL tapered test tube using 5 × 2 mL pentane rinsings of the flask. The solvent was evaporated under N_2 in between rinsings with the tube immersed in water at ≃35 °C. The residue was redissolved in ≃100 μL of pentane.

Celite Partition Column Chromatography. The column was constructed as for the microparticulate silica except that the length was 30 cm and the reservoir was made from the bulb of a 15-mL pipet. Alternately, we have used as the column a 5-mL disposable serological pipet (Kimble Products, Toledo, Ohio). A glass tube of the same dimensions which could be attached to it by a Teflon adapter was used as a reservoir to hold about 8–10 mL of solvent.

Two hundred and fifty milligrams of sodium ascorbate was dissolved in 20 mL of water, mixed with 80 mL of methanol in a 500-mL tintered glass separatory funnel, and shaken with 400 mL of pentane to effect mutual saturation. The methanol–water was the stationary phase and the pentane, the mobile phase. The methanol–water discolored with age, but was usable for at least a week.

One and a half grams of Celite 545 (Johns Manville Product Corp., New York, N.Y.) was transferred to a 15-mL test tube and mixed well with 1.3 mL of 80:20 methanol–water using a spatula. A small wad of glass wool was placed in the outlet of the column. The wide end was inserted into the test tube containing the wet Celite and a small portion of the absorbent was collected in the column and tamped very gently with a glass rod. In this manner, all the Celite was packed in the column. A thin layer of powdered anhydrous Na_2SO_4 was placed on top of the Celite. The extract from the Partisil 20 column was transferred to the top of the Celite with a 100-μL syringe using two or more 100-μL washings of the test tube. Next the reservoir was attached to the column and filled with the mobile phase. The column was eluted with a small N_2 pressure to obtain a flow rate of ≃0.75 mL/min. The first 5 mL was discarded. The next 7 mL containing 25-OH-D_3 was collected in a graduated test tube. The eluate was transferred in small portions with additional rinsings of the tube to a 2.5-mL glass-stoppered tapered test tube and evaporated to dryness under N_2 with the tube immersed in water at ≃35 °C. The residue was reconstituted in exactly 100 μL of ethyl acetate or acetonitrile for the high-performance liquid chromatographic analysis. Note: Adjust the amount of stationary phase and the flow rate so that there is no stripping of the stationary phase during the run.

High-Performance Liquid Chromatography (LC). The LC conditions were: instrument, Varian Model 8500 (Varian Instrument Division, Palo Alto, Calif.); column, C_{18} bonded microparticulate silica (Zorbax ODS, Dupont), 5 μm, 2.1 mm × 25 cm; mobile phase, CH_3CN–CH_3O-H–H_2O (94:3:3); flow rate, 0.5 mL/min; detector, 254 nm UV (Model 440, Waters Associates, Inc., Milford, Mass.); sample size, 5 μL; sensitivity, 0.01 aufs or other appropriate setting. Standard solution of 25-OH-D_3 was prepared from a 100 μg/mL stock solution in ethanol. Appropriate aliquots were evaporated to dryness under N_2 and then reconstituted in 100 μL of ethyl acetate or acetonitrile. The concentration of this solution was usually 5 ng/μL. The concentration of 25-OH-D_3 in the samples was calculated from the peak height responses of the samples and standard injected onto the column. The response of the standard was linear to concentration over a wide range.

RESULTS AND DISCUSSION

It is generally known that 25-OH-D_3 is not a very stable compound. Because of this and the fact that the concentration of 25-OH-D_3 in egg yolk was usually less than 10 ng/g, the whole procedure should be carried out with care. Glassware cleanliness is important as is the avoidance of unnecessary exposure to excessive heat, light, and air.

The procedure is lengthy, but the egg yolk is such a concentrated mass of fats, steroids, carotenes, xanthophylls, and other pigments that all the steps were necessary to obtain an extract that gave an adequately clean chromatogram on the LC. The three column chromatographic purification steps have been successfully used with appropriate modifications for the analysis of 25-OH-D_3 in cow and human serum (Koshy and VanDerSlik, 1974, 1977a, 1978) and in cow liver, kidney, and muscle (Koshy and VanDerSlik, 1977b). The 10-g silica gel column was effective in removing the bulk of the oily residue in the extract. The 25-OH-D_3 did not move on this column with 75 mL of the first solvent which was hexane–ether (1:1). Most of the less polar pigments and lipids were removed in this portion. The first 20 mL of the next solvent, which was methylene chloride–ether (1:1), removed more of the less polar impurities. A colored band eluted with the 25-OH-D_3 fraction. When this extract was subjected to LC on the C_{18} bonded reversed phase column, there were peaks with longer retention times than 25-OH-D_3, suggesting the presence of less polar impurities. Most of these were removed on the 20-μm microparticulate silica column with the 2.5% isopropyl alcohol in hexane as the mobile phase. The extract at this point was fairly clean, but still had some impurities which were more polar than 25-OH-D_3 (shorter retention time on the LC) and the chromatogram showed a wavy pattern in the 25-OH-D_3 region. The Celite partition column with the methanol–water (80:20) as the stationary phase and pentane as the mobile phase was very effective in improving the appearance of the chromatogram. In our experience with the analysis of 25-OH-D_3 in biological samples, we have found this column to be very useful. Depending upon the nature of the sample, columns ranging in size from 0.25 to 2 g of Celite have been successfully used. It may be pointed out that the elution profiles reported here for the three columns may vary slightly depending on the quality of the packing materials and the operational technique. So, it is important that the profiles be established independently and minor modifications made if necessary.

We found 25-OH-D_3 in egg extracts to be stable overnight in hexane and pentane in the refrigerator. Therefore, if the extracts could not be processed during the day, they were stored overnight in these solvents. An analyst could analyze four samples in a normal working day.

Figure 1 shows typical chromatograms of 25-OH-D_3 standard and an egg yolk extract. The standard represented 20.5 ng on the column. Under the LC conditions, 25-OH-D_3 had a retention time of 6 min with no interference from related compounds. Vitamin D_3 had a retention time of about 13 min and the more polar metabolites, 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, and *t*-25-OH-D_3, had very short retention times, eluting immediately after the solvent peak.

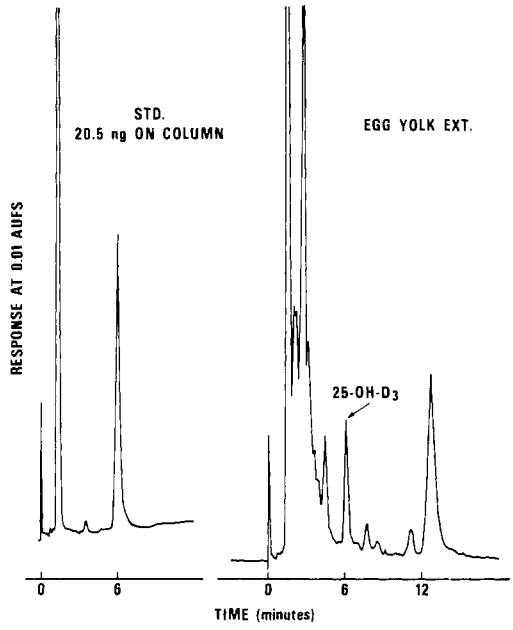

Figure 1. Liquid chromatograms showing 25-OH-D_3 peaks from a standard and an egg yolk extract.

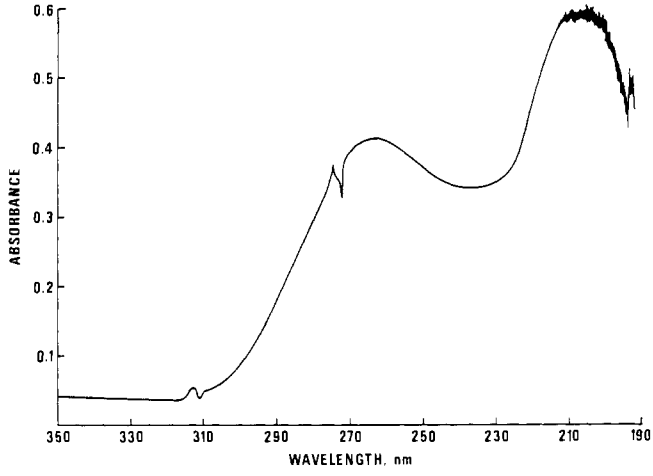

Figure 2. Ultraviolet spectrum of the effluent from the liquid chromatograph of the peak corresponding to 25-OH-D_3.

Confirmation of the Identity of the Peak. Since the UV detector is not selective, it was necessary to prove that the peak from the egg extract with the same retention time as 25-OH-D_3 was 25-OH-D_3. Thirty egg yolks were processed and the extracts combined in 0.5 mL of 2.5% isopropyl alcohol in hexane. Fifty-microliter portions of this extract were repeatedly chromatographed on a 0.25-g Partisil 20 column using 2.5% isopropyl alcohol in hexane as the mobile phase. The first 3 mL from each application was rejected and the next 3 mL collected. After two applications the column was washed with the mobile phase to remove residues from the previous applications. All the ten second 3-mL fractions were combined, evaporated, and redissolved in a small volume of absolute ethanol. Insoluble residues were removed by filtration through a plug of glass wool in a disposable pipet. The solution was concentrated to approximately 20 μL and the whole extract was subjected to LC under conditions described earlier except that the detector was a variable wavelength detector capable of scanning the UV spectrum of the effluent from the column (Variscan, Varian Instrument Division, Palo Alto, Calif.). The spectrum (Figure 2) was characteristic of 25-OH-D_3 with peaks at 263 and 212 nm and the valley

Table I. Recovery of 25-OH-D_3 Added to Pooled Control Egg Yolk

	endogenous level, ng/g	amount added, ng/g	% recovery
	5.4	9.5	97
	7.0	9.5	92
	6.8	9.5	89
	6.0	9.5	84
	5.9	9.5	85
	6.1	9.5	86
	5.3	9.5	87
	5.0	9.5	75
	5.0	9.5	97
mean	5.8 ± 0.7		87.9 ± 6.8

Table II. Elevation of 25-OH-D_3 in the Yolk after Intramuscular Administration of 25-OH-D_3 in Corn Oil

chicken no.	dose, mg/mL	25-OH-D_3 concentration, ng/g, days posttreatment 1	4	6	6 & 7	7 & 8
1902	0.25		39.1			
1909	0.5	6.9	36.7	94.1		
1903	1.0			117.3		
1910	1.0		87.1			
1905	0.46[a]				129.0	
1908	0.46[a]					106.0

[a] Mixed with 2.5 mCi [26,27-^{3}H]-25-OH-D_3 (11.3 Ci/mmol).

in the 230–235 nm. The spectrum shows higher absorption below 235 nm than the pure standard. This was partially due to higher background absorption and partially due to other UV absorbing compounds in the extract in this region.

The identity of the LC peak was further confirmed by GLC/mass spectrometry. The effluent from the LC column of the peak corresponding to 25-OH-D_3 was collected in a tapered 5-mL test tube and the solvent removed under N_2. To the residue, 25 μL of pyridine (freshly distilled over KOH) and 20 μL of heptafluorobutyrylimidazole (Pierce Chemical Co., Rockford, Ill.) were added and heated in an oil bath at 65 °C for 0.5 h. The reaction mixture was evaporated under N_2 to an oily residue and extracted with a small quantity of hexane. The extract was transferred to another small tapered tube and reconstituted in about 25 μL of hexane. Four micrograms of pure 25-OH-D_3 was derivatized in a similar manner. The derivatives were subjected to GLC/mass spectrometry (LKB 9000, LKB-Produkter AB, Stockholm, Bromma, Sweden) on a 1% SP-2250 (Supelco, Inc., Bellafonte, Pa.) on Gas-Chrom Q 100/120, 3 mm i.d., 1.5 ft column at 200 °C. The ionizing current was 60 μA and the ionizing voltage 70 eV. There were two GLC peaks characteristic of 25-OH-D_3 due to the pyro and the isopyro forms of the derivative. The first peak was very much larger than the second. Several spectra were obtained from the front, apex, and rear of the two peaks and all were identical with that from the authentic 25-OH-D_3. A typical spectrum (Figure 3) showed a molecular ion with m/e at 578 representing the 25-OH-D_3 diheptafluorobutyrate less a molecule of heptafluorobutyric acid. This loss took place during GLC/mass spectrometry since the parent ion m/e 792 was obtained when the derivative from the pure compound was introduced by the direct probe. Even by direct probe mass spectrometry, the first loss was a molecule of heptafluorobutyric acid, and thereafter the fragments were identical with that by GLC/mass spectrometry. The characteristic ions of 25-OH-D_3 dihepta-

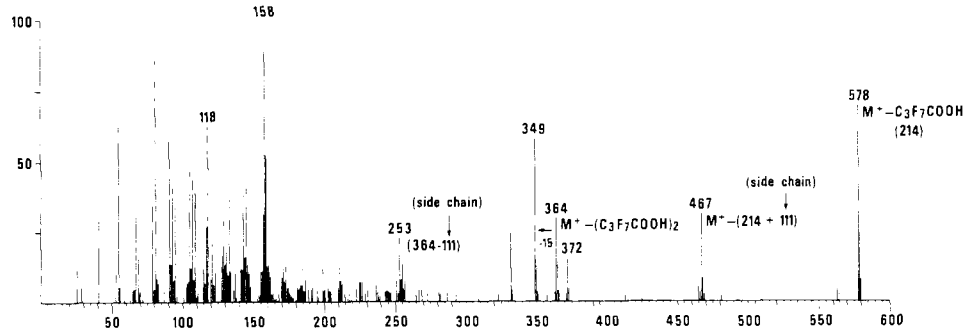

Figure 3. GLC/mass spectrum of 25-OH-D_3 isolated from the egg yolk and derivatized to the diheptafluorobutyrate. Note that the parent ion is $M^+ - C_3F_7COOH$.

Table III. Comparative Levels of 25-OH-D_3 (ng/g) in the Yolk from Chicken Fed Vitamin D_3 and 25-OH-D_3[a]

15 mg/ton		30 mg/ton		45 mg/ton	
vit. D_3	25-OH-D_3	vit. D_3	25-OH-D_3	vit. D_3	25-OH-D_3
5.5	4.6	8.9	9.7	8.8	13.2
5.9	3.7	5.5	8.6	8.8	14.1
4.4	6.1	7.2	8.9	11.3	13.6
6.1	6.2	8.0	7.9	8.9	11.7
6.1	4.7	9.8	12.3	8.8	19.1
4.8	5.7	7.7	8.6	8.6	12.3
	6.5	8.2	10.0		18.2
			10.9		12.4
5.5 ± 0.7	5.4 ± 1.0	7.9 ± 1.6	9.6 ± 1.4	9.2 ± 1.0	14.3 ± 2.8

[a] The mean pretreatment value was 5.5 ng/g (n = 16; range, 4.4–6.8 ng; SD, 0.78).

fluorobutyrate are indicated in the spectrum.

Recovery. Extraction efficiency and recovery were determined from the eggs from two chickens that received a single intramuscular injection of 2.5 mCi each of [26,27-H^3]-25-OH-D_3 (11.3 Ci/mmol) together with 0.46 mg each of unlabeled 25-OH-D_3 in corn oil. Eggs from chickens treated at the same time with cold 25-OH-D_3 had elevated levels of 25-OH-D_3 in the yolk 6–8 days after treatment. Therefore, eggs from the sixth and seventh day posttreatment were used in the extraction efficiency and recovery studies. Four solvents were checked for extraction efficiency; 95% ethanol, CH_3CN, ethanol–ether (5:8), and $CHCl_3$–CH_3OH (2:1). Although there was not a significant difference between the four solvents, the $CHCl_3$–CH_3OH (2:1) extraction was chosen because it was easier to purify than the others. The residue after the $CHCl_3$–CH_3OH extraction and the final extract for LC had 2.7 and 82%, respectively, of the total radioactivity in the egg yolk. Determination of the radioactivity in the effluent from the LC column of the peak corresponding to 25-OH-D_3 showed that it was 97% of that injected on the column, thereby confirming that all the radioactivity in the extract was due to 25-OH-D_3. The major loss of radioactivity (8% in the acetone stripping of the silica column) was believed to be due to more polar metabolites of 25-OH-D_3. If this assumption was valid, then the overall recovery was 86%.

Table I shows the reproducibility of the method and the recovery of added 25-OH-D_3 to pooled egg yolk at the level of 9.5 ng/g. In these experiments a known amount of 25-OH-D_3 in ethanol was added to 20 g of the pooled egg yolk at the extraction step and carried through the entire procedure along with the unfortified controls. The analysis was performed by one analyst on different days. The mean for the control yolk was 5.8 ± 0.7 ng/g. The mean recovery of 25-OH-D_3 from the fortified samples was 87.9 ± 6.8%.

Endogenous Level of 25-OH-D_3. A large number of egg yolks from different local poultry farms were analyzed during the past 1.5 years to determine the endogenous level of 25-OH-D_3 in the yolk. Most of these eggs contained 5–8 ng/g. The highest level obtained was 12 ng/g. The endogenous level was elevated by intramuscular injection of 25-OH-D_3. The data obtained from six chickens that were given intramuscular injections of 0.25, 0.5, or 1 mg of 25-OH-D_3 in 1 mL of corn oil are shown in Table II. There was a 20-fold increase in the 25-OH-D_3 concentration in the 0.5 and 1 mg treated chicken within 8 days.

Vitamin D vs. 25-OH-D_3 Fed Chickens. Sixty mature laying hens were allotted evenly into six groups. They were kept on a basal laying mash diet. After the birds were acclimatized and were laying eggs regularly, each group was given a diet containing 15, 30, or 45 mg/ton of either vitamin D_3 or 25-OH-D_3. These diets were mixed weekly for each group to insure drug levels and palatability of feed. After the chickens were on the diet for 28 days, the yolks were analyzed for 25-OH-D_3. The yolks from the chickens in each group were analyzed in the order in which they were laid. The data (Table III) showed no difference between the pre- and posttreatment levels in the 15 mg/ton level feed groups. At the 30 mg/ton level there was slight elevation in both the D_3 and 25-OH-D_3 fed groups. The latter group appeared to have a slightly higher level, but the difference was not significant. The difference was significant in the 45 mg/ton fed chickens. The 25-OH-D_3 concentrations in the yolk were 9.2 ± 1.0 and 14.3 ± 2.8 ng/g in the vitamin D_3 and the 25-OH-D_3 fed chickens, respectively.

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