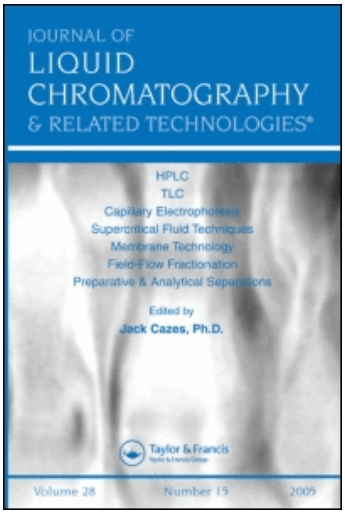

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Vitamins D_2 and D_3 in Feedingstuffs by High Performance Liquid Chromatography

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To cite this Article Hung, George W. C.(1988) 'Determination of Vitamins D_2 and D_3 in Feedingstuffs by High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 11: 4, 953 — 969
To link to this Article: DOI: 10.1080/01483918808068357
URL: http://dx.doi.org/10.1080/01483918808068357

JOURNAL OF LIQUID CHROMATOGRAPHY, 11(4), 953-969 (1988)

DETERMINATION OF VITAMINS D_2 AND D_3 IN FEEDINGSTUFFS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A sensitive and reliable HPLC method for complete separation and quantitation of vitamins D_2 and D_3 in complicated biological mixtures such as feedingstuffs has been developed and described. The method has been applied to the quantitative determination of D_2 and D_3 in feedingstuffs and related matrices at both high premix levels and low feed levels ranging down to a detection limit near 100 IU/lb or 0.22 IU/g sample. The procedures consist of the initial step of sample preparation by extraction; four sample cleanup stages: Sep/Pak/Silica Cartridge Cleanup, Millipore-Teflon Cleanup, Gel Permeation/Sephadex LH-20 Column Cleanup, and HPLC/Partisil-PAC Column Cleanup; and the final step of Reverse-Phase HPLC Separation, Identification, and Quantitation. The analytical Column used was Rainin Accupak 20 cm-3 um C-18 & Guard Columns. The Waters Associates Model 440 Fixed Wavelength UV Detector at 254 nm was used for all measurements. All separations and quantitations were carried out isocratically at room temperature and under subdued lighting. By these procedures, the sensitivity for both D_2 and D_3 is about the same (20 ng), and the resolution is excellent. Normally, the D_2 peak eluted at 30-31 min. and the D_3 peak followed at 2-3 min. later. By using the standard calibration and standard addition methods,

the percent recovery ranges from 90.0%-104.8% with the mean value of 97.4%, whereas the accuracy is from 85.3% to 108.9% with the average of 97.8%. The standard deviation is ±5.2% and the coefficient of variation is 5.3%.

INTRODUCTION

Vitamin D, particularly vitamins D_2 and D_3, plays a vital role in the normal biological or biochemical functions. Consequently, the development of analytical methods for assaying biological functions or biochemical activities of vitamins D_2 and D_3 has been a continuous interest for several decades. The earliest developed methods of biological assays for vitamins D_2 and D_3, which uses animals for direct testing, is an approximate, time-consuming, and expensive method (2). Besides, it is not overly precise. Among others, a large number of physical-chemical methods have been proposed and developed that have limited applications for identifying and quantitating D_2 and D_3 in various systems. For examples, colorimetric method (1), nuclear magnetic resonance spectroscopy (3), infrared spectrophotometry (4,5), and recent gas-liquid chromatography (8-15), have been tried on a variety of matrix systems with partial successes.

The most recent advance in high performance liquid chromatographic instrumentation (HPLC) and column packing techniques have provided a more reliable method for separation and determination of fat-soluble vitamins. Williams *et al* (16) partially resolved D_2 and D_3 by using reverse phase partition chromatography and a 3 m Permaphase ODS Column. Osadca and Araujo (17) described a simple and rapid qualitative method for determining the presence of D_2 and D_3 in various commercial preparations by reverse phase HPLC. DeVries *et al* (18-20)

reported a series of systematic and comprehensive studies on HPLC methods for D_3 in vitamin D concentrates and in multivitamin preparations. From the results of collaborative study, they have recommended that the HPLC method for the determination of D_3 in multivitamin preparations should be adopted as the official first action for an alternative to the chemical method. Unfortunately, vitamin D_2 was not included in the study, neither was there an indication of the possible separation of D_2 and D_3. The Vydac 201TP54 reverse phase column and acetonitrile/methanol (50/50) mobile phase (21) were used successfully to resolve the D_2 and D_3 standard mixture. However, so far, no reports on practical applications of HPLC techniques to separate and quantitate the amounts of D_2 and D_3 in complicated biological species such as feedingstuffs have appeared in the literature. The present paper describes a quantitative reverse phase HPLC method for separating and determining D_2 and D_3 contents in feedingstuffs. A Rainin's short analytical column (10 cm) packed with small particle size (3 um) C-18 and a mixture of methanol/water (90/10) as mobile phase were used. The procedures consist of the initial sample preparation by extraction, four sample cleanup stages followed by the final step of HPLC separation, identification, and quantitation.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

(a). Standards.--Vitamin D_2 standard: Ergocalciferol, crystalline, 1 g, (40,000,000 IU/g or 40,000,000 U.S.P. Units per gram, Sigma, St. Louis, MO). Vitamin D_3 standard: cholecalciferol, an activated 7-dehydrocholesterol (Sigma, St. Louis, MO). Vitamins D_2 and D_3 standard

solutions. Solution A: 50 mg/100 ml dichloromethane. Solution B: working standards, 0.2 ml of solution A/100 ml HPLC mobile phase.

(b). Solvents.--All solvents used are HPLC grade, distilled in glass (Burdick and Jackson, Fisher Scientific, and Waters Associates, etc.).

(c). Butylated hydroxytoluene (BHT) solution.--1 mg/ml hexane (Fisher Scientific).

(d). Sodium phosphate tribasic solution.--20% aqueous solution (Fisher Scientific).

(e). Mobile Phase.--90/10 Methanol/Water mixture (Fisher Scientific).

(f). Mobile Phase for GPC.--98/2 Chloroform/Isooctane mixture (Fisher Scientific).

(g). Eluting Solvent for Sep/Pak.--Benzene/Ethyl Acetate mixture, 99/1 (Fisher Scientific).

(h). Adsorbents.--Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ).

(i). Sep/Pak/Silica Gel Cartridge (Waters Associates).

(j). Millipore F. H. 0.5 um Teflon Filter Membrane (Millipore Corp., Bedford, MA).

Apparatus

(a). Liquid Chromatograph: Waters Associates, Inc, Model 6000A Pump, U6K Septumless Universal Injector, Model 440 Fixed Wavelength UV Detector at 254 nm wavelength (Waters Associates, Milford, MA). This equipment was used for analytical separation and quantitation.

(b). Gel Permeation Chromatograph: Analytical Bio-Chemical Laboratory, Inc., Model 1002 GPC equipped with a 45 cm x 1.5 cm column slurry packed with Sephadex LH-20 (Analytical Biochemical Lab., Inc., Columbia, MO). It was employed for cleanup purpose.

(c). Cleanup Liquid Chromatograph.-- Varian HPLC equipped with Walco Universal Injector and 100 micro-

liter sample loop (Varian Associates, Inc., Palo Alto, CA).

(d). Column.--Analytical Column, Rainin Accupak 10 cm-3 um C-18 Column and Guard Column (Rainin Instrument CO., Inc., Woburn, MA). Cleanup Column, Whatman 25 cm-10 um Partisil PAC Column with Guard Column (Whatman Chemical Separation, Inc., Clifton, NJ).

(e). Rotary Evaporator: Fisher Scientific, Inc., St. Louis, MO).

(f). Dri-Block Heater: Fisher Scientific, Inc., St. Louis, MO).

(g). Wrist-Action Shaker: Burrell Corp., Pittsburgh, PA.

(h). Hamilton Micro-Syringe: 10 ul, 50 ul, Hamilton Company, Reno, Nevada.

Procedures

The complete procedures consist of the initial step of sample preparation by extraction, four cleanup stages, followed by the final step of HPLC reverse phase separation, identification, and quantitation. All the procedures are applicable to the separation and determination of vitamins D_2 and D_3 in animal feeds and related matrices at both high premix levels and low feed levels. All preparations, separation, and quantitation procedures were carried out under subdued lighting at room temperature.

Sample Preparation: The original feed sample was first ground to about 200 mesh particle size and homogenized. Two different sample sizes were used in the determination. For high premix levels, we used 100 mg, for low feed levels, we used 1-20 g sample sizes depending upon the D_2 and D_3 contents in the sample. All samples were weighed into a 250 ml glass stoppered Erlenmeyer flask, then added 1 ml of antioxidant, butylated-hydroxytoluene

solution, 2 ml of 20% sodium phosphate tribasic solution, and 200 ml of methylene chloride extracting solvent. Sodium phosphate tribasic solution was added to dissolve the protective coating of resin and allowing the D_2 and D_3 to go into the organic phase. The sample mixture and container were wrapped in aluminum foil and shaked for one hour. After that, the extraction mixtures were filtered through fluted paper into a 250 ml volumetric flask, and brought to the volume with methylene chloride solvent. An aliquot containing about 100 IU each of D_2 and D_3 was then taken and transferred into a Rotary Evaporator over 50°C water bath for drying. The residues was dissolved in 5 ml benzene for next step of separation.

Sep/Pak/Silica Cartridge Cleanup: This step removes much of the coloration from the sample and retains some of polar constituents found in the feed. It is a quick and easy filtration step. In operation, all sample residues in benzene and in the washings from the previous step were transferred into a 10 ml glass syringe attached to a Sep/Pak/Silica Cartridge, and filtered through the cartridge. The first eluted benzene layer was discarded. We collected all eluted portions of 1% ethyl acetate in benzene and let them dried again over 50°C water bath by using Rotary Evaporator.

Millipore F. H. 0.5 um Teflon Filter Cleanup: The operational principle for this step is similar to the Sep/Pak/Silica Cartridge Cleanup. The residues from the previous step was dissolved in 5 ml methylene chloride and filtered through the 10 ml glass Syringe-Swinney-Millipore-0.5 um Teflon Membrane filtration system. The filtrate was collected into a glass Scintillation Vial, then dried over a Dri-Block at 50°C with N_2 gas. We then reconstituted the residue with

1 ml of chloroform-isooctane mixture (98/2) for further cleanup by high pressure Gel Permeation Chromatography (GPC).

Gel Permeation Chromatography/Sephadex LH-20 Column Cleanup: This step is a size exclusion-preparative-filtration cleanup. By this step, most of the heavier oils, higher molecular weight particles, and pigmentation would be removed or eluted before vitamins D_2 and D_3. The equipment used was Model 1002 GPC manufactured by Analytical Biochemical Lab., Inc., Columbia, MO. The column used was a 45 cm x 1.5 cm column, slurry packed with Sephadex LH-20. The mobile phase was chloroform-isooctane mixture in the ratio of 98/2. The column was first equilibrated with mobile phase at a flow rate of 4.0 ml/min, then injected 1 ml of 1 ug each of D_2 and D_3 working standards, and monitored the column eluate with Waters Associates Model 440 fixed wavelength UV detector set at AUFS = 0.005 and at 254 nm wavelength. Vitamins D_2 and D_3 fractions were eluted between 29 and 56 min. By the same operation, we also injected the sample residue in the 1 ml mobile phase into the column, and collected the fractions between 29 and 56 min. The fractions were again evaporated with the Rotary Evaporator to the volume of 10-15 ml and filtered through the Swinney-Millipore-Membrane filtration system into the glass Scintillation Vial. The filtrate was evaporated to dryness with N_2 gas over Dri-Block at 50°C, and reconstituted with 100 to 500 microliters of methylene chloride for HPLC/Partisil-10 PAC Column final cleanup.

HPLC/Partisil PAC Column Cleanup: This step is also a preparative-separation cleanup. For samples containing more than 5000 IU of vitamins D_2 and D_3 per pound, the residues after GPC cleanup were sufficiently pure for

direct analysis by HPLC. However, due to the lower levels of D_2 and D_3 in feed sample, to avoid base-line noise, the residues were further purified by using the HPLC/Partisil-10 PAC column. To calibrate the column, we injected 50 microliters of 1 ng of mixed D_2 and D_3 standards into the Whatman 25 cm Partisil-10 um PAC Column through the WALCO Universal Injector and 100 microliter sample loop of Varian HPLC System. The vitamins D_2 and D_3 fractions were eluted between 11-14 min. Consequently, the fractions of D_2 and D_3 in the feed sample were collected during this time interval. These fractions were collected into Scintillation Vial and dried with N_2 gas over the Dri-Block at 50°C. The final residues were reconstituted with 1 ml methanol in a capped vial at 50°C, stored as such and was ready for HPLC reverse phase analysis and quantitation.

HPLC/Reverse Phase C-18 Column Quantitation: In the final analysis and quantitation, the following experimental set-up was employed: (1). Liquid Chromatography Systems: Waters Associates, Model 6000A Pump, U6K Universal Injector, Model 440 Fixed Wavelength Detector at 254 nm; (2). Analytical Column: Rainin Accupak 10 cm -3 μm C-18 Column and Guard Column; (3). Mobile Phase: Methanol/Water (90/10); (4). Flow Rate: 0.8 ml/min--1.0 ml/min.; (5). Pressure: 3500 psi (maximum working pressure); (6). Injection Volume: 1-20 ul; (7). AUFS: 0.005; (8). Chart Speed: 0.25 cm/min.; (9). Detection Limit: 1 ng. At least duplicate runs were made for each analysis. A standard calibration curve: the amount of D_2 and D_3 standards injected (ng) vs. the correspondidng peak height (cm) of the chromatographic response was first made to be used as the basis for the subsequent determinations (Fig. 2). The Rainin Accupak-Microsorb Column, 10 cm long and 3 μm particle size of C-18 column and

Guard Column, were used as the analytical column because it provides faster separation, solvent savings, and high sensitivity, three advantages over others. The flow rates were controlled between 0.8 ml and 1.0 ml per min. so that the column pressure would not go over 3500 psi as recommended by the manufacturer.

RESULTS AND DISCUSSION

The chromatographic responses of both D_2 and D_3 are about the same with the exception of their retention time at the experimental levels. Fig. 1 shows an excellent linearity of standard behaviors for both D_2 and D_3 at nanogram level. Only a single standard calibration curve is therefore present for both D_2 and D_3. The correlation coefficient is 0.9997. In Fig. 2, the well-resolved chromatograms of 20 ng of D_2 and D_3 are given. Generally speaking, the sensitivity for both D_2 and D_3 is about the same. The retention time of D_2 is 30-31 min., and D_3 was eluted 2-3 min. later.

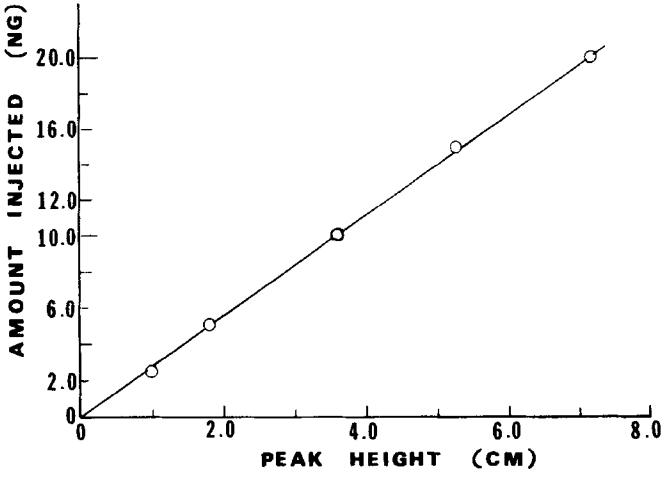

FIGURE 1. Standard calibration curve for quantitation of vitamin D_2 and vitamin D_3 from the HPLC profiles.

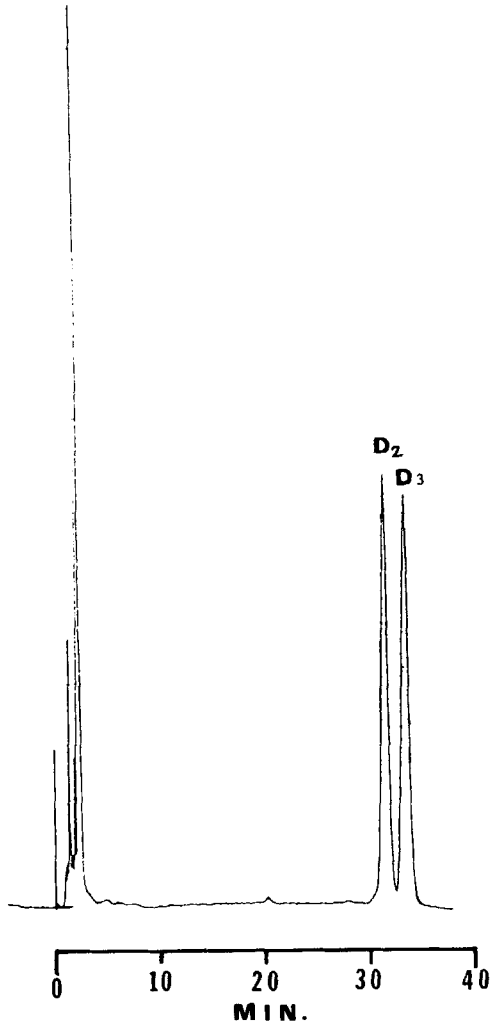

FIGURE 2. HPLC profiles for 20 nanograms of vitamin D_2 and vitamin D_3 standard mixtures.

The analytical chromatographic profiles of a high level premix sample containing about the same potency of 2.3 million IU/lb of both D_2 and D_3 after the stage of GPC cleanup are given in Fig. 3-A and 3-B. Fig. 3-A is the chromatogram of the original sample without adding any standard samples. It was used for identification and quantitation of D_2 and D_3 contents in the premix sample and also served as a blank for recovery study. Fig. 3-B is the chromatograms of the original premix sample in Fig. 3-A spiked with 125 ug of both D_2 and D_3 standards and served as a controlled sample for recovery study. After a long procedure of many stages of cleanup, an excellent recovery of 99.9% was still obtained.

The chromatograms for a very low level feed sample after the final stage of HPLC/Partisil PAC Column cleanup are shown in Fig. 4-A and 4-B. Fig. 4-A is for the blank sample containing about 60 IU/lb of D_2 (below the detection limits, estimated) and 2100 IU/lb of D_3, while Fig. 4-B is for the spiked sample containing both original sample and 33% of equivalent to the expected level of the original sample of D_2/D_3 standards. They demonstrate a neat and much clearer base line as compared to the results of the GPC cleanup stage in Fig. 3-A and 3-B. They also strongly suggest that the performance of HPLC cleanup step is critical and absolute necessary for obtaining a better chromatographic profile of D_2 and D_3 from a complicated and very low-level feed sample. The percent recovery for this sample is 94.30% as can be seen from Table 1 Summary of the Experimental Results.

Table 1 lists the vitamins D_2 and D_3 contents of high-level premix samples (1-5), high levels (sample 6-9) and low levels (sample 10-13) of feed samples. Columns 2 & 3 list the D_2 & D_3 contents of specific samp-

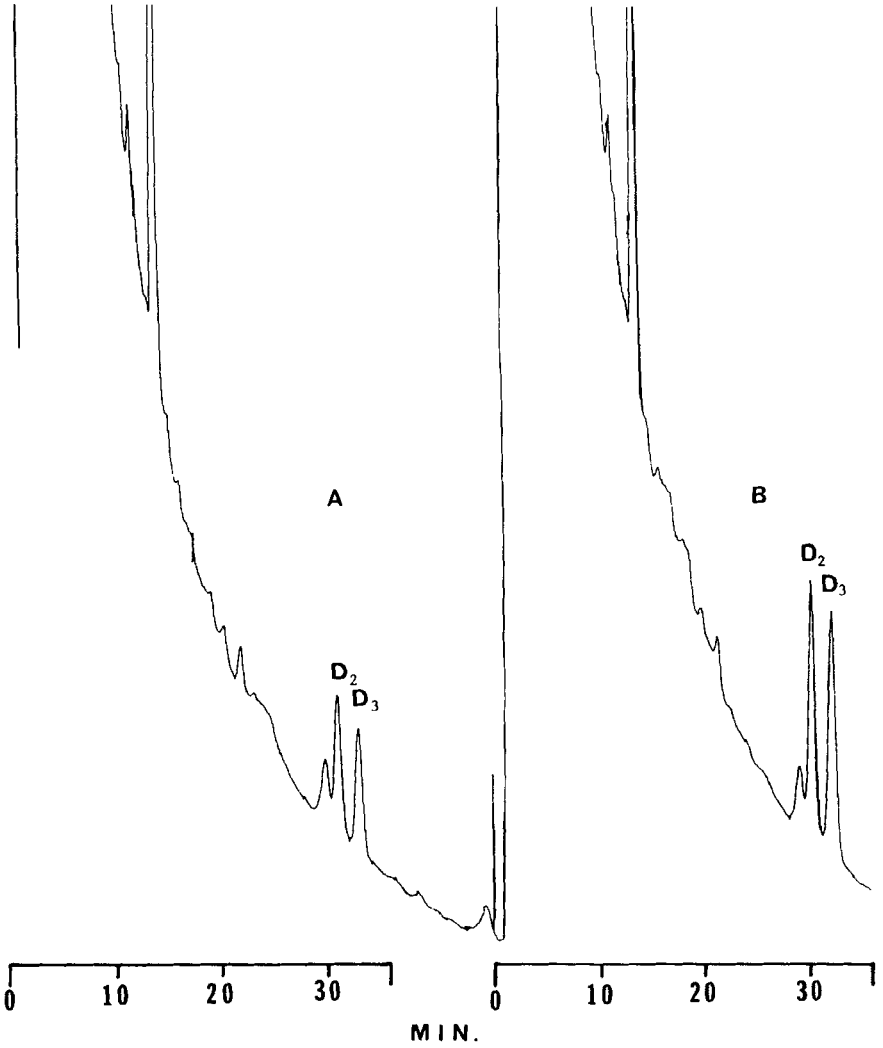

FIGURE 3. HPLC profiles for a high level premix sample. (A). The original sample without adding any standard sample. (B). The original premix sample as in (A) spiked with 125 micrograms of both vitamins D_2 and D_3 standards.

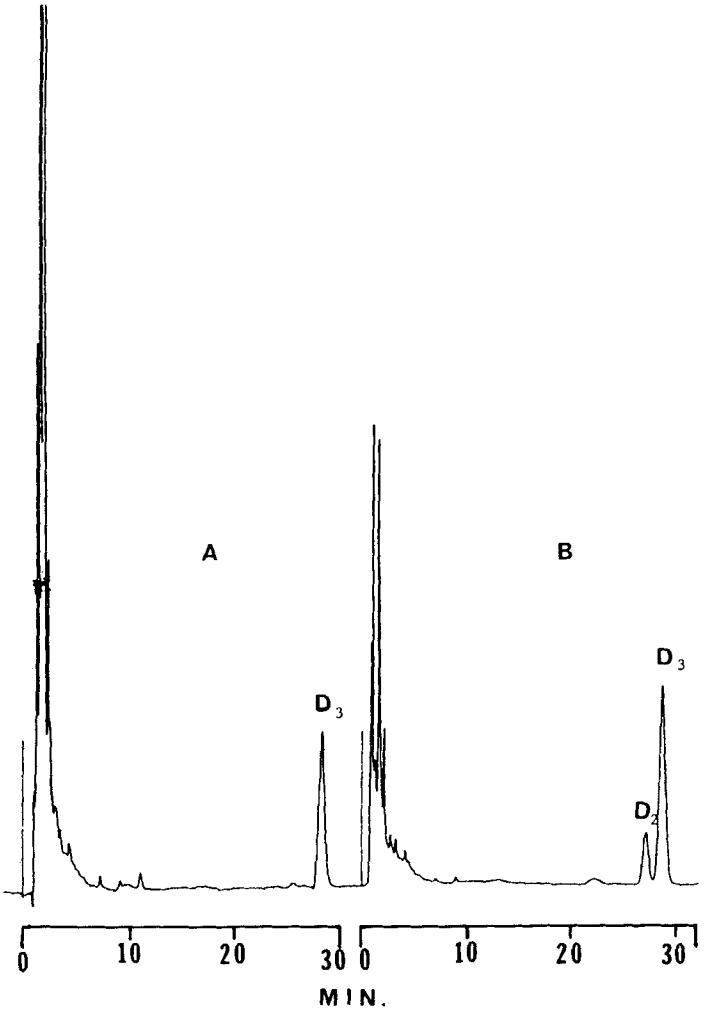

FIGURE 4. HPLC profiles for a very low-level feed sample. (A). The original sample without adding any standard sample. (B). The original feed sample as in (A) spiked with a quantity of vitamins D_2 and D_3 standards equivalent to 33% of the expected level of the original feed sample as in (A).

TABLE 1
EXPERIMENTAL RESULTS

SAMPLE NO.	VITAMIN D_2 (I.U./lb)	VITAMIN D_3 (I.U./lb)	PERCENT RECOVERY (%)
1	2,292,948	2,268,000	99.90
2	11,340	571,536	
3	20,412	544,320	
4	10,900	167,000	
5	10,900	147,000	
6	1,447	5,988	99.79
7	0	2,560	95.57
8	544	2,495	
9	499	2,177	
10	60	2,030	94.30
11	544	1,633	
12	0	1,260	90.00
13	18	73	104.8

les. Column 4 lists the percent recovery of those typically selected samples. Generally speaking, the percent recovery decreases with decreasing amounts of D_2 and D_3 in the samples with the exception of Sample 13. The percent recovery of Sample 13 is 104.8%, the highest one in the 6 samples studied. Since the amount of D_2 and D_3 found in Sample 13 is below the detection limits, the computed results are lower than the expected values and resulting in a higher value of recovery. The average percent recovery, which could be used as a representative of the accuracy of the control study, is 97.4%. The range is from 90.0% to 104.8%. On the other hand,

the accuracy of individual sample, which is not shown in Table 1, ranges from 85.3% to 108.9%. The mean value is 97.8%. The standard deviation for 6 samples studied is ±5.2%, and, the coefficient of variation is 5.3%.

In summary, from the satisfactory results of 13 samples examined, it can be concluded that the HPLC techniques can be considered as a very promising method for separation, identification, and quantitation of vitamins D_2 and D_3 contents in the complicated matrices of biological samples such as feedingstuffs or similar products. The use of correct sample size, the completion of sample cleanup, and the performance of satisfactory recovery study play very important roles in achieving the best results of accuracy and precision of analysis.

ACKNOWLEDGEMENT

The author wish to acknowledge Woodson-Tenent Laboratories, Inc., Memphis, Tennessee 38101 for the permission of using their facilities, equipment, samples, and chemicals to carry out the experimental work.

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