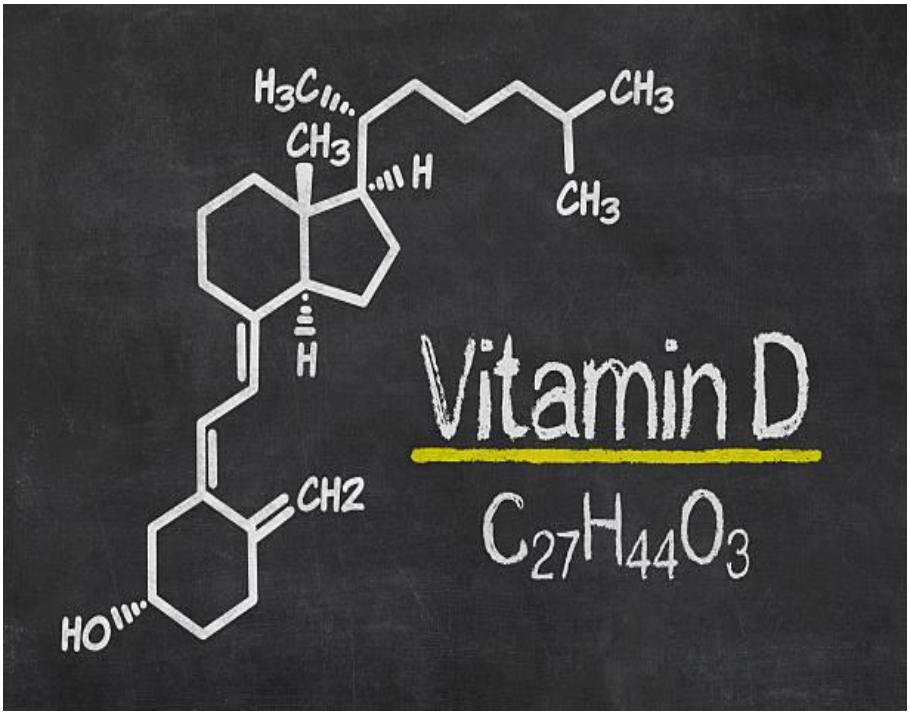

ANALYTICAL REPORTS FOR QUALITATIVE AND QUANTITATIVE DETERMINATION OF VITAMIND'S IN THE SAMPLE.

NAME:PRIYANKA
KALIDAS SHET
ROLL NO :20P0490085

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1. INTRODUCTION

Vitamin D is a group of fat soluble secosteroids responsible for increasing intestinal absorption of calcium, magnesium and phosphate and many other biological effects. In humans, the most important compound in this group is vitamin $_{D3}$ and vitamin D_2.[1] Both are also naturally occurring forms that are produced in the presence of the sun's ultraviolet -B rays hence its nick name 'the sunshine vitamin, but D2 is produce in the plant fungi and d3 is animals, including humans Vitamin D supplements are commonly used to treat and prevent it is important to maintain healthy levels of vitamin D. This can be done by taking 400-1000 IU of vitamin D daily or spending 15-30 min in the sun each day.[2] Vitamin d is produced in humans from its precursor 7- vitamin d deficiency. People who don't get enough sun and people who are 65 years or older are at the risk for deficiency. People also use vitamin d for weak bone and brittle bones, heart disease, asthma, high fever, and many other. There is no evidence to support using vitamin D supplements for covid -19.[3] Vitamin D has a significant role in calcium homeostasis and metabolism.[4]Its discovery was due to effects to find the dietary substance l lacking in the children with rickets. Vitamin D supplements are given to treat or to prevent osteocalcin and rickets.[5] Vitamin D is not only important for bone health but can also affect the development of several nonbone diseases. which is converted to vitamin D by the temperature dependent process. dehydrocholesterol by the action of sunlight. the photoconversion of the precursor yield provitamin D.[6]

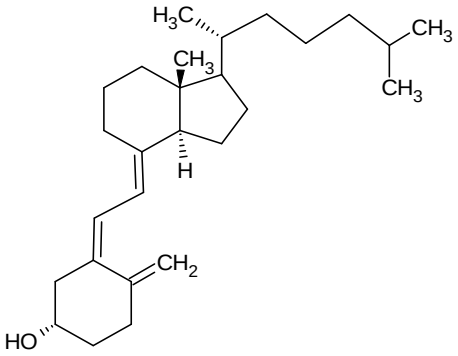
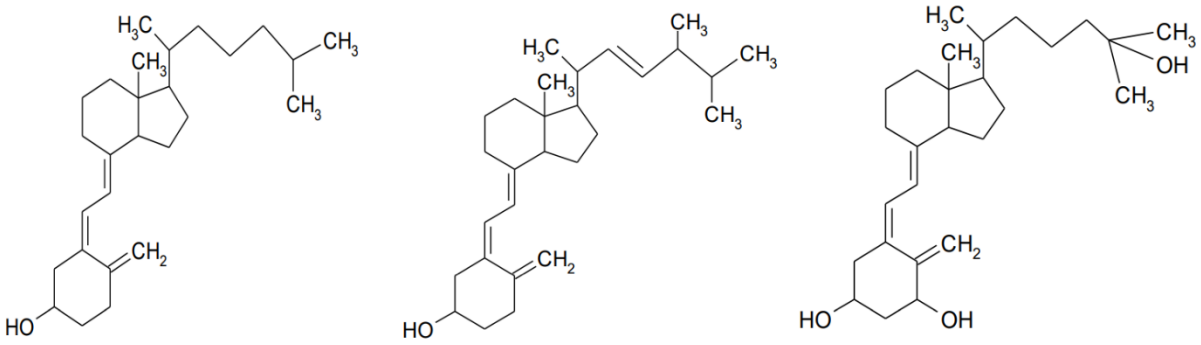


Figure 1;structure of vitamin D

2. VITAMIN D CHEMISTRY.

Vitamin D exist in a number of form, where the major physiologically relevant forms are vitamin D_2 and vitamin d_3 .[7]Vitamin d 2 is produced by ultraviolet irradiation of plant and steroid ergosterol. vitamin d_3 is synthesis in the skin of vertebrates through the action of UVB and 7-dehydrocholesterol, Vitamin d 2 is the less common form of vitamin d and has potentially lower bioavailability than vitamin d3. [8] Vitamin d from sunlight or dietary source is biologically inactive and is hydroxylated via a two steps process to 1,25 dihydroxy vitamin D active.[9]



Vitamin D_2 (ergocalciferol) | Vitamin D_3 (cholecalciferol) | Activated vitamin D (calcitriol)

Figure 2;types of vitamin D

3. SOURCES OF VITAMIN D

Figure 3;different sources of Vitamin D

i. SUNLIGHT

Most vitamin d is produced to exposure of the skin to UVB through sunlight exposure. In winter however, oral intake of vitamin d may be the primary source as the UVB related synthesis in the skin is limited.[10]Similarly, oral intake of vitamin d is the primary source all the year round for people not exposed to sunlight. for example, due to wearing of clothing that prevent skin exposure.[11]

ii. FOOD SOURCE

Only a limited number of foods naturally contain vitamin d. The main source of dietary vitamin d were fish/fish product followed by egg, fats ,oils, bread/bakery products ,and milk and milk products. The other source of vitamin D are salmon, liver, cod liver cheese[9]

iii. DIETARY SUPPLEMENT

Vitamin D may be sourced from dietary supplement. A widely available source is cod liver the vitamin d present in supplement can be in both vitamin D_3 however vitamin D_{2i}s rarely used as the fortification in supplement. Dietary supplements are regulated under the dietary supplement regulation 1985 and are administered medsafe.[7]

4. USES OF VITAMIN D

Vitamin d decreases cell proliferation and increase cell differentiation, stop growth of new blood vessels, and has significant anti-inflammatory effects. Vitamin d contribute to decrease in muscle strength, cause cancer, cardiovascular disease.[12] The vitamin d is responsible for wide range of function of the body.[9] A bone disorder that occur in the people with kidney disorder taking the specific form of vitamin d called calcitriol by mouth help to manage low calcium level and prevent bone loss on the people with kidney failure. Its strength the immune system.[13] Its boosts your mood. It can aid in the weight loss. [14]It's can lower the risk of rheumatoid arthritis. It may improve the brain function. It can aid in weight loss. It lowers the risk of the type 2 diabetes. It can help lower blood pressure. Reducing the risk of multiple sclerosis.[15]

Figure 4; Deficiency of vitamin D

5. ESTIMATION OF VITAMIN D

IN this the vitamin was detected after chemical derivatization using MALDI-TOF mass spectroscopy based on the perylene matrix chip.

The detection was difficult due to the presence of hydrophobic characteristic of the vitamin d due to absence of functional group and having plasma in low concentration. from many different types of vitamin d metabolites ,25(OH)D3 has been selected as the biomarker for various disease. By using the derivatization method of vitamin D BA was selectively reacted to the hydroxyl group of the 25(OH)D3 along with the vitamin d to form hemiacetal salt with the charge labelling effect by using the nucleophilic addition reaction. By using conventional organic matrix called CHCA vitamin d3 can also be dectedd and the mass peak from the fragments removed using MALD-TOF mass spectrometer based on the perylene matrix chip. when both the mixture of BA derivatized 25(OH) B3 and BA derivatized vitamin D3 was analyzed using perylene matrix chip, both was simultaneously detected without the mass peak from the fragment's matrix molecules.[6]

2.Solvents, Standards and Samples All solvents and diluents used were HPLC-grade, including reagent alcohol (ethanol with 5% isopropyl alcohol as denaturant). All vitamin standards were obtained from Sigma-Aldrich® Inc. (Allentown, PA). These included ergocalciferol (D2), cholecalciferol (D3), ± α-tocopherol (E), DL α-tocopherol acetate (E acetate), phylloquinone (K1), menaquinone (K2), retinyl acetate (A acetate), and retinyl palmitate (A palmitate). The structures of these vitamins are shown in Figure 1.8 All secondary standard dilutions were made using a diluent of 80% ACN in water. As the concentration of individual vitamins in dietary supplements and food/beverage products varies considerably, a working standard was prepared to cover the wide range of concentrations. The working standard (WS) was prepared by weighing out 20 - 2000 µg/mL of each of the eight vitamins, depending on the vitamin's relative absorptivity and expected concentration in typical dietary

supplements. Subsequently, calibration standards were prepared via serial dilution of WS.1 In preparing the WS, the eight vitamins were

improve solubility of the K1, K2, D2, and D3 vitamins, reagent alcohol was added to each volumetric flask, adding 30% of flask volume, and then filled to the mark with the 80% ACN/water diluent. Vitamins A (acetate and palmitate), E, and E acetate were also initially dissolved in reagent alcohol, though, for these, the volumetric flasks were then filled to mark with reagent alcohol. Each volumetric flask was then sonicated for five minutes. No visual precipitate was observed after sonication. Equal volumes of each of the eight individual stock standards were then added together to produce the WS solution, and the volumetric flask was filled to volume with diluent. Within the WS, the vitamins ranged in concentration from 0.05 to 260 µg/mL, depending on the vitamin. For calibration, all serial dilutions were prepared from the WS. All standards were filtered through 0.45 µm PTFE filters prior to injection. All individual stock solutions and working standards were stored at 4.0 °C when not in use.[11]

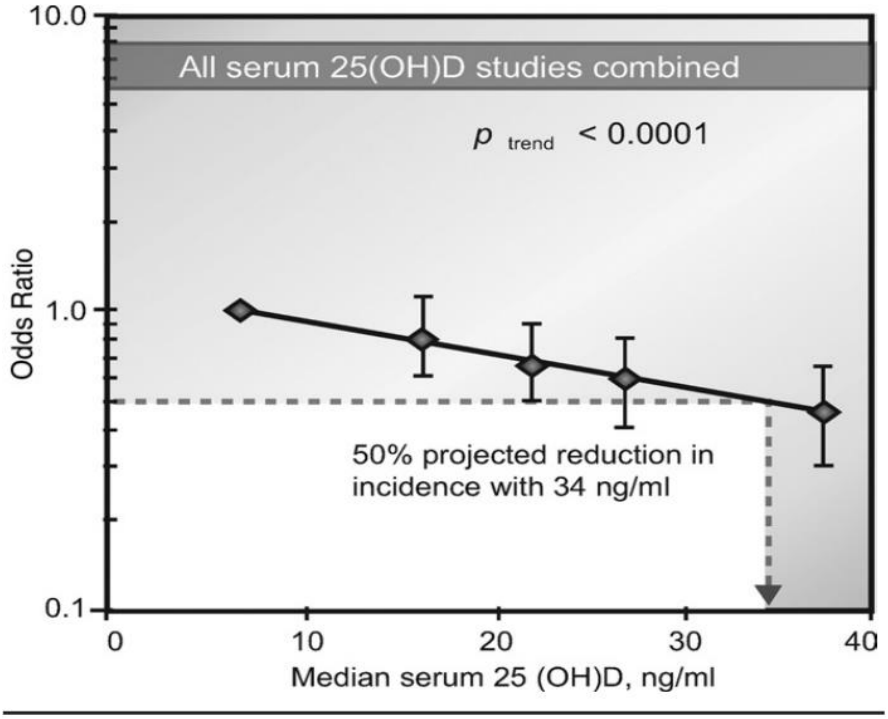


Figure 5; graph of odds ratio vls median serum 25(OH)D/ml

3.collection of the sample. The age, sex was noted. and the venous blood sample were collected from the fasting people of 12 hours overnight. The sample has been protected from the sunlight centrifuged and stored at 80c until analysis.by using three different techniques like HPLC, CLIA and EIA vitamin d is analyzed.by using HPLC 25 hydroxyvitamin D in the plasma was determine by using the HPLC by using c18 reverse phase column with the gradient elution. To prepare the sample 2ml of precipitation reagent along with the internal standard was taken in a test tube and the 1.0ml of serum was added to the tube without the mixing of the sample.to avoid the boiling of the protein. after addition of the sample, it was allowed tom settle down for 5 min at room temperature than after it is set it was mixed in vortex for 10 second to obtain flocculent precipitate after 5 min it was again vortex mixed and centrifuged at 2000g for 10 min. The clear supernatant was decanted into a disposable glass test tube which was protected from exposure to natural sunlight to prevent degradation of analytes. Then 1.0 ml of water was added to each extract and transferred 3.5 ml of extract mixture. Then the DEC was rinsed with 2.0 ml of 35:65 CH3CN–water and was eluted the Strata-X cartridge in the DEC zone with 2.0 ml of CH3CN. The eluate was dried at 35˚C under a stream of nitrogen and the dry extract was and vortex-mixed for 5 seconds. Water was then added to the tube, and the contents were vortex mixed for 5 seconds. The

sample was centrifuged at 2000 g for 10 min to settle the precipitate. The clear liquid was transferred to an amber coloured vial. The sample was finally capped. The extract was stable for 3 days at room temperature. The processor software calculated relative retention time for peak identification and peak-height ratio for quantification.[12]

Age	45.37 ± 8.51
Number of subjects	n = 216
Sex:	
Male	129
Female	87
Demographic data:	
Urban background	123
Rural background	93

Value are mean ± SD; n = number of cases.

Table 1; personal profile of the subject

Methods of blood vitamin D estimation	Concentration of vitamin D in blood (nmole/L)
HPLC	69.34 ± 8.02
CLIA	68.46 ± 8.01
EIA	67.18 ± 8.21
HPLC vs CLIA	CI = 0.6525 - 1.1068
HPLC vs EIA	CI = 1.8916 - 2.4232

Values are mean ± SD; CI = confidence interval.

Table 2; Comparison of mean value if three methods of blood vitamin D estimation

By using automated chemiluminescent immunoassay technology 25(OH)D serum was measure. CV for inter-assay analyses is 5.8% at a 25- hydroxyvitamin D level of 39.5 nmol/L and 3.1% at 121.25.

USING HPLC ABALYSIS.

4.A high-performance liquid chromatograph Series 1100/1200 equipped with a diode array detector and a Chem Station data acquisition system, was used. The chromatographic separation was performed on a reversed-phase Gemini C18 100 × 3.0 mm, 3 µm particle size column (Phenomenex, USA) at 40°C using acetonitrile–water (99:1, v/v) as a mobile phase at a flow rate of 1 mL/min. The injection volume was set between 3 and 50 µL, depending on the type USING LC-MS ANALYSIS[12]

USING LC-MS ANALYSIS.

5. Identification of vitamin D3 degradation products was carried out on [16]an Agilent Infinity 1290 LC attached to a 6460 QQQ mass spectrometer using electrospray ionization in positive scan mode from m/z 200 to 500 to confirm the stability-indicative nature of the developed HPLC–UV method. In addition, MS–MS product ion scans for precursor ions were performed at a collision energy of 15 eV. The operating conditions for MS were as follows: drying gas temperature 275°C, drying gas flow 5 L/min, nebulizer 45 PSI, sheath gas temperature 320°C, sheath gas flow 11 L/min, capillary entrance voltage 4,000 V, nozzle voltage 1,000 V and delta EMV 200 V. The chromatographic conditions were the same as for HPLC analysis, except for the mobile phase. The mixture of methanol and 0.1% formic acid was used instead, as it provides better ionization of vitamin D3 and its related compounds at comparable retention times. Additionally, a diode array detector was coupled to the LC–MS f the preparation. The detect [on on was carried out at 265 nm.[12]

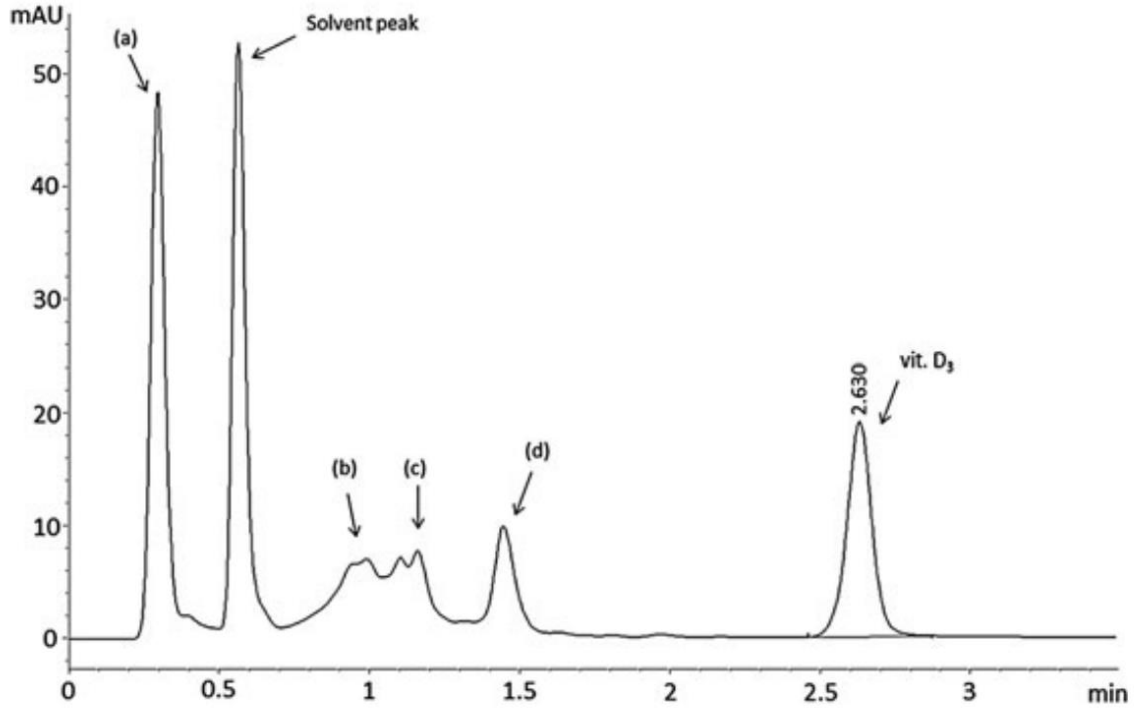


Figure 6; A chromatogram of vitamin D sample after thermal degradation in water.

6.The HPLC used in this method consisted of Quaternary pump, sample injector with 20ulsample loop; degasser and photodiode array detector. The chrome lion software version which controls the whole liquid system are used for the evaluation and quantification. The reverse phase is used as the column. the acetonitrile of PH5.19 or methanol with PH 4.7 and methanol with 0.1% formic acid is used as two mobile phase during the estimation. flow rate of the sample is adjusted to 0.4ml min per sec and the mobile phase with a time window of 10 min. The temperature of the column is kept constant that is 40 degrees. The result was noted at 265nm wavelength for the vitamin D The chemical and solvent used are methanol and acetonitrile. The stock solution mixture of vitamin D was prepared and diluted using the acetonitrile. standard was prepared in methanol and stored at -20. 350ul of methanol and 2-propanol in the ration of 80:20 was added to 0.5ml of sample matrix. The vitamin D was extracted by mixing two times with 1ml of hexane. The centrifugation technique was used to separate. The phases the upper organic was decant to a conical tube and nitrogen was used to dried and the residue was dissolved in appropriated volume of mobile phase. A stock solution of 1mg mL−1 Vit D3 was prepared by dissolving 1mg Vit D3 lyophilized standard in 1mL of methanol and then

solutions of different concentrations for construction of calibration plots were prepared from this stock solution. The mobile phase was filtered through a 0.45 µm membrane filter and passed through column. at 0.4 mL min−1 for column equilibration; the baseline was monitored continuously during this process. Detection was carried out at λmax265 nm. The prepared dilutions were injected in series, peak area was calculated for each dilution, and concentration was plotted against peak area. Standard addition method was used to determine the accuracy. Matrix) was spiked with 3,5 and 15 ng mL-1 standard and the mixtures were analysed by the proposed method. The experiment was performed in triplicate.

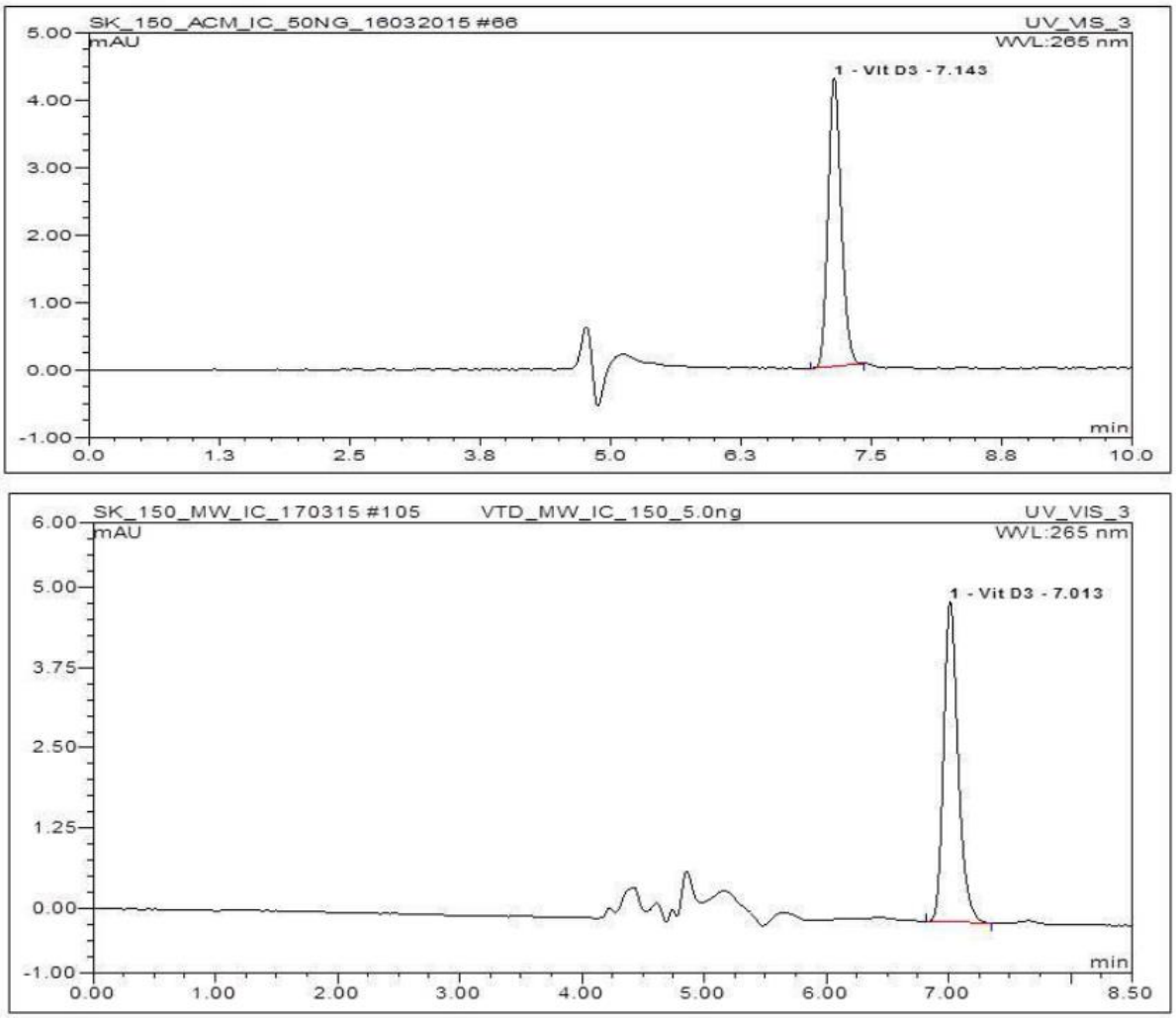


Figure 7 Chromatograms of a standard Vitamin D_3 using mobile phase

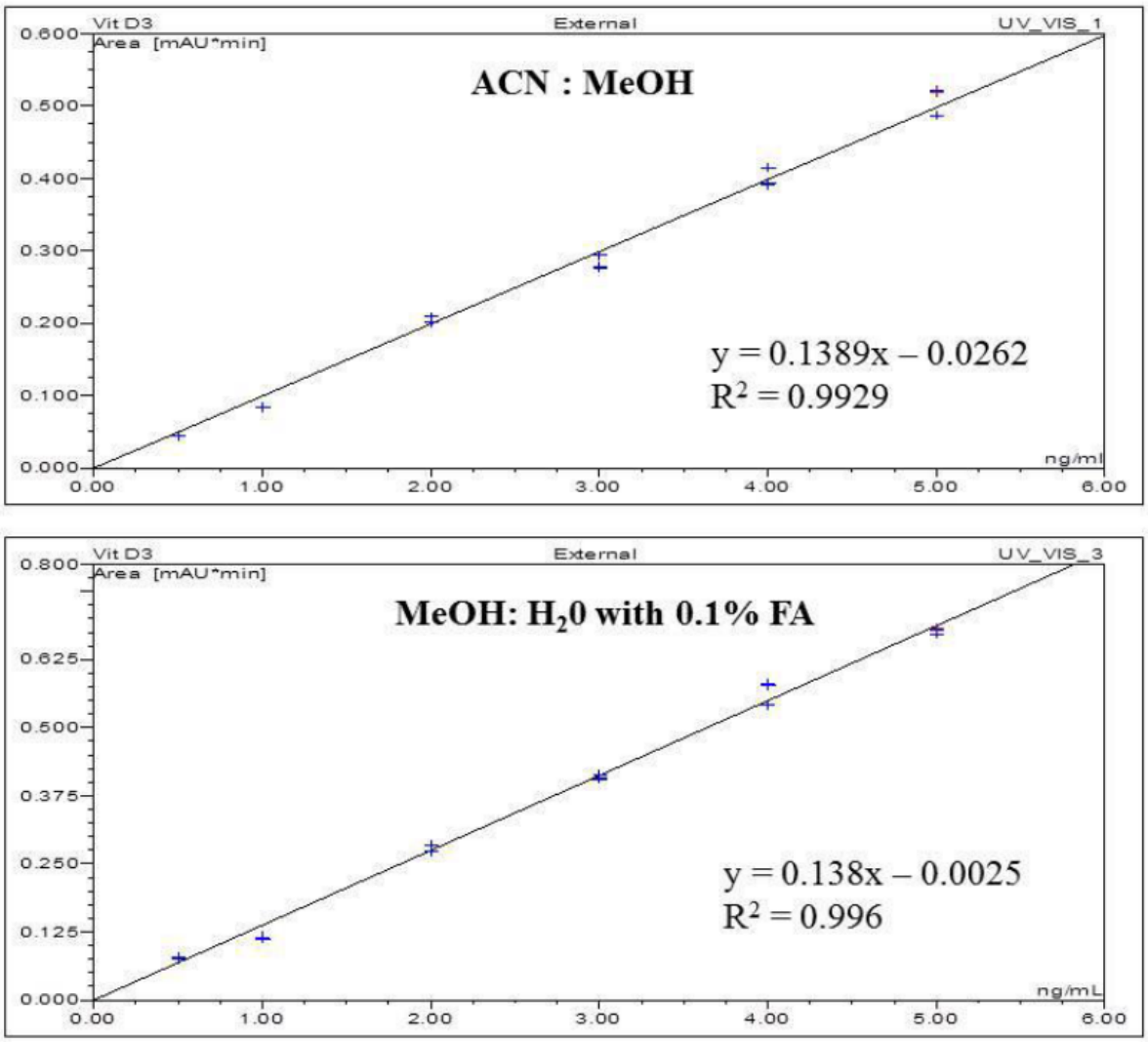


Figure 8calibration of Vitamin D_3 standard

7.All the standards, spikes and the sample are made to 1ppm along with the internal standards. The Vitamin D3-d3 and Vitamin D2-d2 are made to 10 % menthol with the addition methanol. By using 7890/7000 QQQ-GCMS Gc analysis with the tandem mass spectrometric detection was performed capillary column was equipped with 15m*0. 25mm.The column I connected via a T connector to a 30m column of the same time. The oven temperature was kept at 150 ®c which was held for 2 min. After some time, temperature was change to 20®c per min to 315®c at time run of 15.25 min. The spitless mode kept during the time of inlet running at 210®c which was held for the until the end of the run. The mass spectrometry interface was used which kept at 290®c. The helium is used as the carrier gas with the flow rate of 1.2 mL/min on column 1 and 1.4 mL/min on column

2. Scanning was performed over the m/z 25 – 600 mass range. Product ion scans were performed for m/z values 325.3, 337.3, 341.3, 358.9, 367.3, 379.3, 456.3, 463.9, 468.6, 471.6, 484.6, 544.6, and 556.4 with scanning between m/z 40 - 556. The initial collision energy was 15 eV, nitrogen was the collision gas and helium were the quench gas. The final method included specific Multiple Reaction Monitoring settings for each of four Vitamin D compound internal standards as needed. MS1 resolution was set to wide and MS2 resolution was set to widest for all MRMs in order to achieve maximum sensitivity. Compound concentration interpolation was carried out by means of Agilent Mass Hunter software.[17]

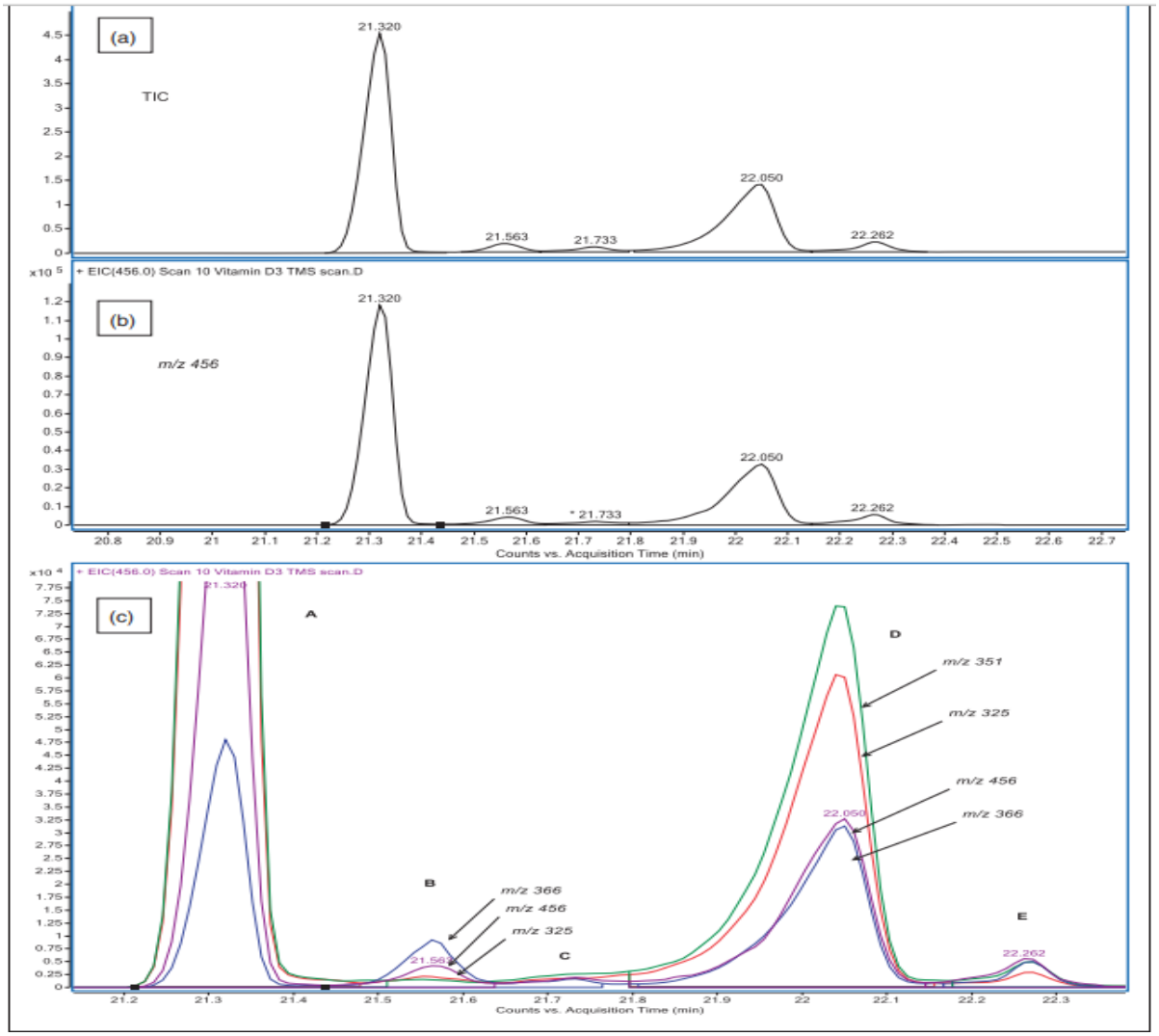


Figure 9;Vitamin D_3 -TMS chromatograms (a) total ion chromatogram,(b) extracted ion chromatogram (c) detailed extracted ion chromatogram.

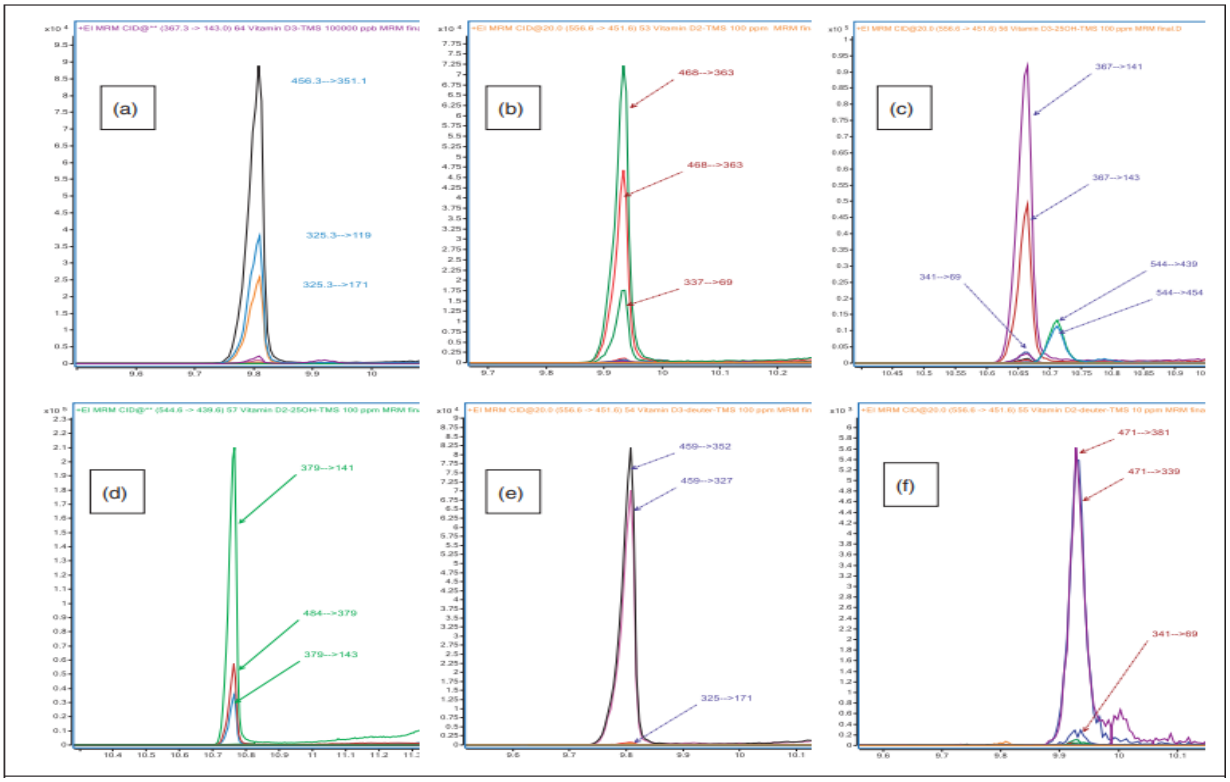


Figure 10;extracted ion chromatogram specific to MRMs of Vitamin

8.The chemical use are methanol, acetonitrile, 2 propenal and hexane. In total 28 samples of them 14 food samples, 6 environmental samples, 3 feed samples, 3 clinical samples & 2 pharmaceutical samples, were analysed in this study. The methanol was prepared as the stock solution. It was diluted to the different concentration ranging from 12mg/ml to 315mg/ml and the absorbance was taken by using the 275 as the wavelength. The food and the solid sample were blended dried well and made in a powered form. from hat 0.1 gm was dissolve into 5ml of methanol and collected in 12ml falcon tube and kept in dark for 2 hours. After 2 hours solid materials were separated from the liquid methanol by using Whatman 1 filter paper. Vitamin D was extracted from methanol by mixing slowly three volumes of hexane 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) 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 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 D3 was analysed using absorbance value from 210 nm to 800 nm to determine the optimum UV absorbance. The proposed methods were successfully applied to the analysis of vitamins D3, in food, feed, pharmaceutical, clinical and environmental samples. 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 D3 and the linearity was evaluated by the least- square regression method, which was used to calculate the regression coefficient value (r2), y-intercept and slope of the regression line.[18]

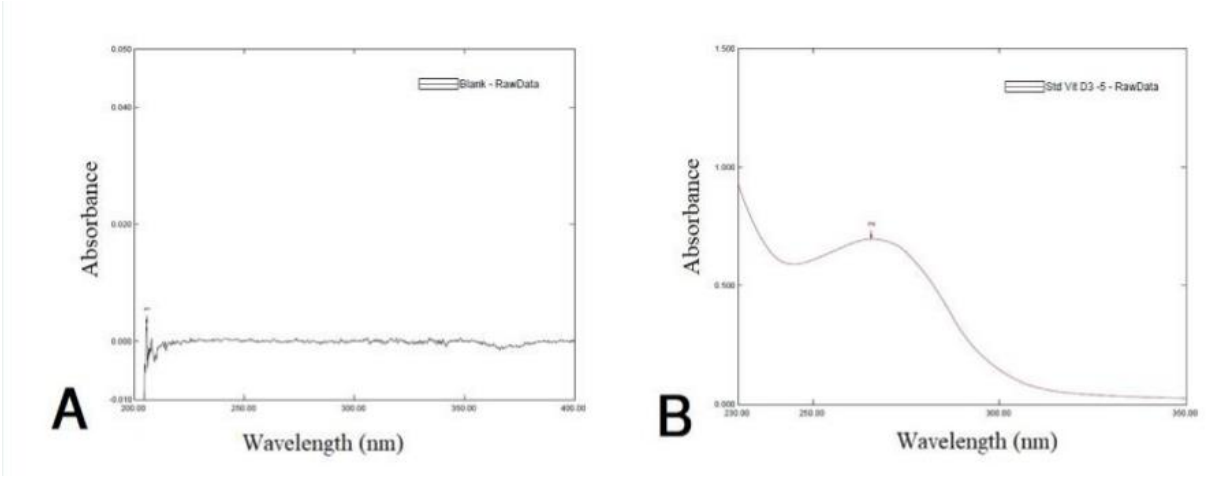


Figure 11;UV spectrum of blank and standard vitamin D3

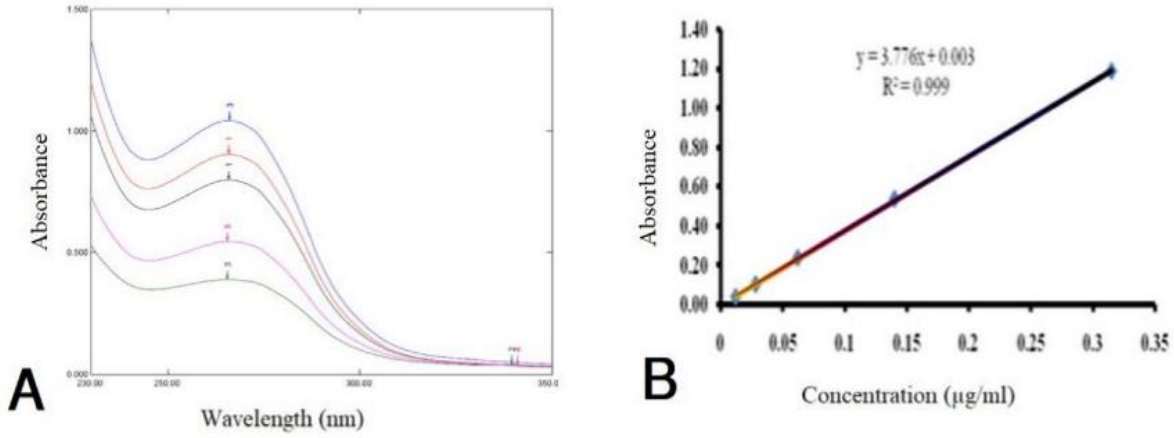


Figure 12; UV absorbance of five concentration of standard solution for calibration

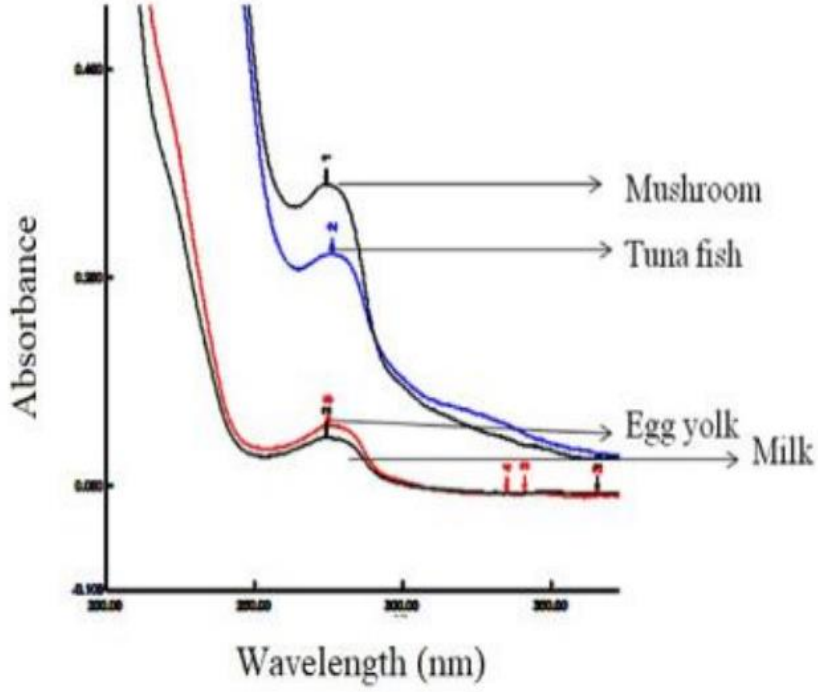


Figure 13 UV spectrum of one representative sample spectrum for mushroom, tuna, egg yolk and milk

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

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;result of method validation

Sample Category	Sample name	Sources	Liquid/solid	No. of samples	Vitamin D
Food	Milk	Shikderbari, Abdullahpur, Bansree, Basabo, Jatrabari	Liquid	5	12 (ng/ml) 11 (ng/ml) 13 (ng/ml) 10 (ng/ml) 12 (ng/ml)
	Carrot	Dhaka city market	Solid	3	ND ND ND
	Egg yolk (ng/gm)	Tongi bazar, Abdullahpur, Faidabad, Jatrabari	Semi-solid	4	15 (ng/g) 14 (ng/g) 16 (ng/g) 15 (ng/g)
Feed	Poultry Feed	Savar, Tongi and Old Dhaka	Solid	3	ND 0.10 (ng/g) 0.12 (ng/g)
Environmental	Mushrooms	Mashroom culture center, Savar Dhaka	Solid	3	65 (ng/g) 61 (ng/g) 62 (ng/g)
	Algae	Bay of Bengal Cox Bazar	Solid	3	ND ND 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) 21 (ng/ml) 23 (ng/ml)
Tuna	Tuna fish	Imported from USA	Solid	2	59 (ng/g) 52 (ng/g)

Table 4; vitamin D concentration in various food sample

9.The chemical and reagent used are acetic acid glacial, methanol ethanol triethylamine isopropyl alcohol. The chemical and reagent are analytical grade. In these two systems are used HPLC and UHPLC. In which HPLC are equipped with ACE 5 C18 column was used were as UHPLC was equipped with the ACE EXCEL 2C18 column. Diode array detector was used in

both the system along with the online degasser. Both the systems were equipped with autosampler (SIL-20AXR) with injection volume ranging between 0.1 and 50 μL. The two mobile phase are used one is MeOH while other is IPC and both are pumped at the ration of 50:50 at the Ph 6.0 which are adjusted using the ACOH The silanol is used as the blocker in 0.5%.. 5.0 μL and 1.0 μL injection volume were injected and flow rate was set at 1.0- and 0.6-mL min−1 for HPLC and UHPLC, respectively. The detection was carried out at 25∘ C and 40∘ C, respectively, for HPLC and UHPLC with best selected wavelength of 265 nm by i-DReC Shimadzu LC program was used to record chromatograms, peak quantification, and integration. Mobile phase, standard solutions, and samples were filtered through nylon filter before injection into chromatographic system.[19]

Column	Analyte	R_s	T_f	α	N
		HPLC			
Hypersil ODS (250 × 4.6 mm, 5 μm)	Vitamin D_3	—	1.11	—	4132
	MK-7	13.14	1.36	5.64	8329
Venusil XBP C18 (250 × 4.6 mm, 5 μm)	Vitamin D_3	—	1.14	—	3763
	MK-7	12.54	1.31	5.31	7296
ACE 5 C18 (250 × 4.6 mm, 5 μm)	Vitamin D_3	—	1.11	—	5867
	MK-7	15.92	1.12	6.83	9683
Purespher® RP-18 (250 × 4.6 mm, 5 μm)	Vitamin D_3	—	1.37	—	3923
	MK-7	13.45	1.09	5.91	8209
		UHPLC			
ACE Excel 2 C18-PFP (100 × 2.1 mm, 2 μm)	Vitamin D_3	—	0.94	—	23289
	MK-7	27.61	0.98	4.61	28521
Waters ACQUITY 1.7 BEH C 18 (100 × 2.1 mm, 2 μm)	Vitamin D_3	—	1.32	—	19342
	MK-7	24.23	1.15	4.25	25029
Agilent Poroshell 2.7 120 EC C18 (100 × 2.1 mm, 2 μm)	Vitamin D_3	—	1.23	—	15687
	MK-7	21.54	1.15	3.71	22143
Phenomenex Kinetex 2.6 C18 (100 × 2.1 mm, 2 μm)	Vitamin D_3	—	1.07	—	11981
	MK-7	19.21	1.12	3.31	19814

Table 5;result of tested stationary phase

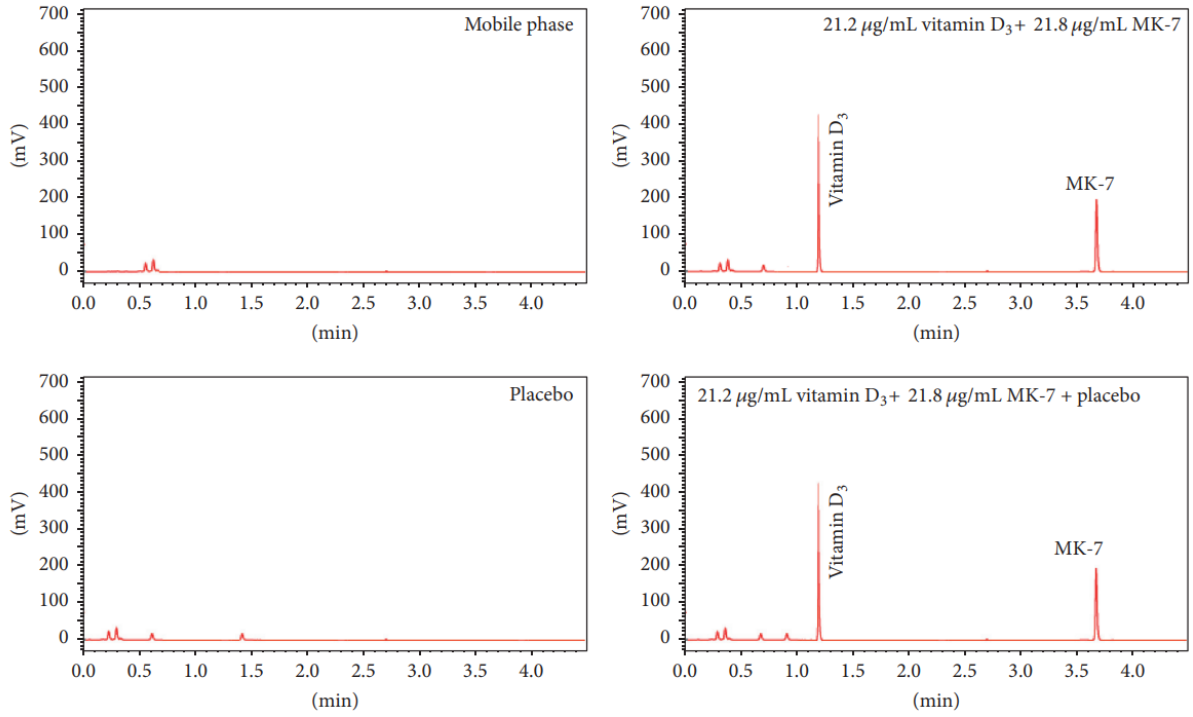


Figure 14; typical UHPLC chromatograms of Vitamin D_3 and Mk-7 and without placebo

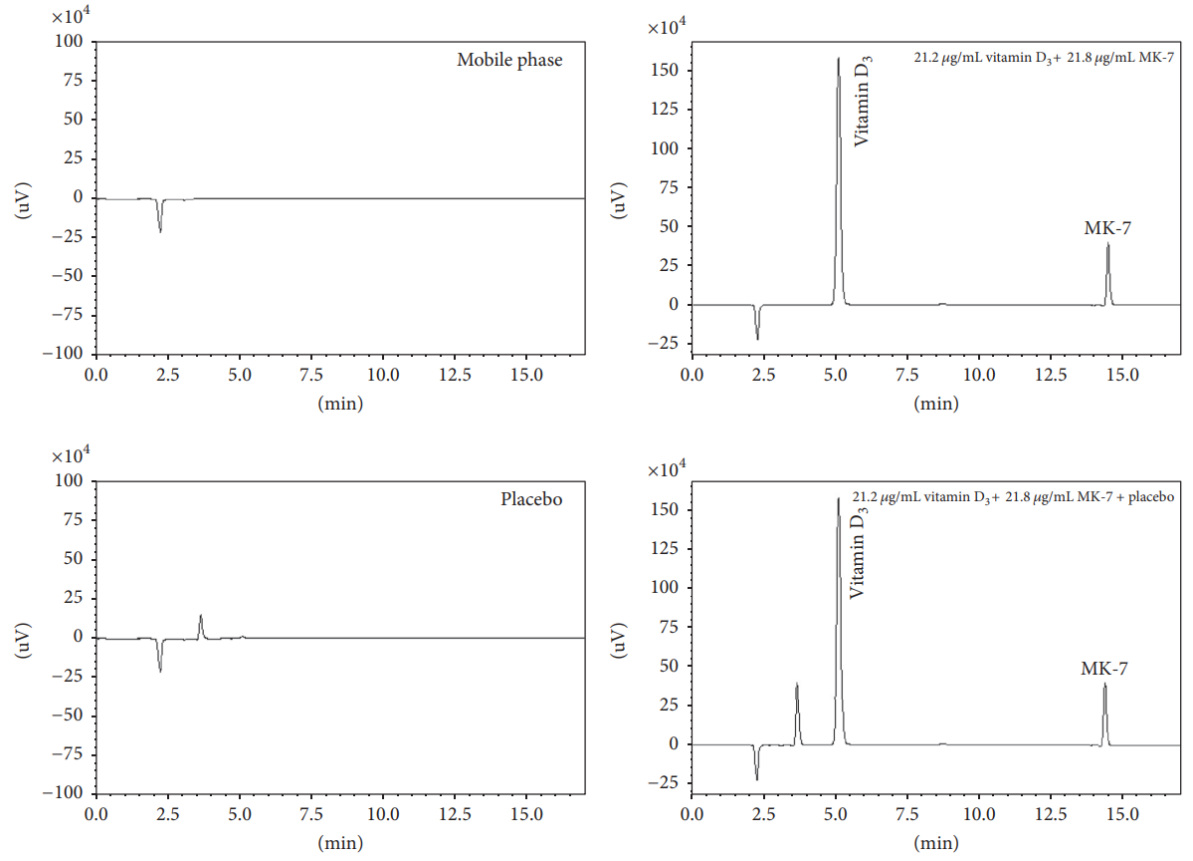


Figure15;typical HPLC chromatograms of Vitamin D_3 and MK-7 with and without placebo

10.The reagent and the chemical used are 30% solution of hydrogen peroxide, sodium hydroxide, hydrochloric acid, formic acid and orthophosphoric acid. The HPLC are used with diode array detector and chem station data acquisition system is used to collect the data. The column is used with the size of 3u particle size and for the chromatographic separation reverse phase Gemini 18 100×3.0mm was used to at 40°C using acetonitrile–water as a mobile phase at a flow rate of 1 mL/min. The injection volume was set between 3 and 50 µL, depending on the type of the preparation. The detection was carried out at 265 nm.

The identification of the vitamin d3 is carried out by using the QQQ mass spectrometer by using electron spray ionization in positive scan mode from m/z 200 to 500 to confirm the stability-indicative nature of the developed HPLC–UV method. In addition, MS–MS product ion scans for precursor ions were performed at a collision energy of 15 eV. The operating conditions for MS were as follows: drying gas temperature 275°C, drying gas flow 5 L/min, nebulizer 45 PSI, sheath gas temperature 320°C, sheath gas flow 11 L/min, capillary entrance voltage 4,000 V, nozzle voltage 1,000 V and delta EMV 200 V. The chromatographic conditions were the same as for HPLC analysis, except for the mobile phase. The mixture of methanol and 0.1% formic acid was used instead, as it provides better ionization of vitamin D3 and its related compounds at comparable retention times. Additionally, a diode array detector was coupled to the LC–MS system to obtain support information about the degradation products.[16]

Abbreviations[a]	Compound	Retention time (min)	UV absorption maxima (nm)	$[M+H]^+$ (*m/z*)	Product ions (*m/z*)[b]
tachy.	Tachysterol D_3	2.28	272, 280, 291	385.4	107.1, 133.1, 159.1, 259.3, 367.4
pre-vit. D_3	Pre-vitamin D_3	2.39	260	385.4	107.1, 133.1, 159.1, 259.3, 367.4
trans.	Trans-vitamin D_3	2.43	273	385.4	107.1, 133.1, 159.1, 259.3, 367.4
vit. D_3	Vitamin D_3	~2.60	265	385.4	107.1, 133.1, 159.1, 259.3, 367.4
lumi.	Lumisterol D_3	3.05	276, 286, 298	385.4	107.1, 135.1, 159.1, 259.3, 367.4
b–d	Hydroxy vitamin D_3	0.9–1.6	250	401.4	109.1, 175.1, 247.1, 365.3, 383.1

Table 6 identified Vitamin D_3 related compound

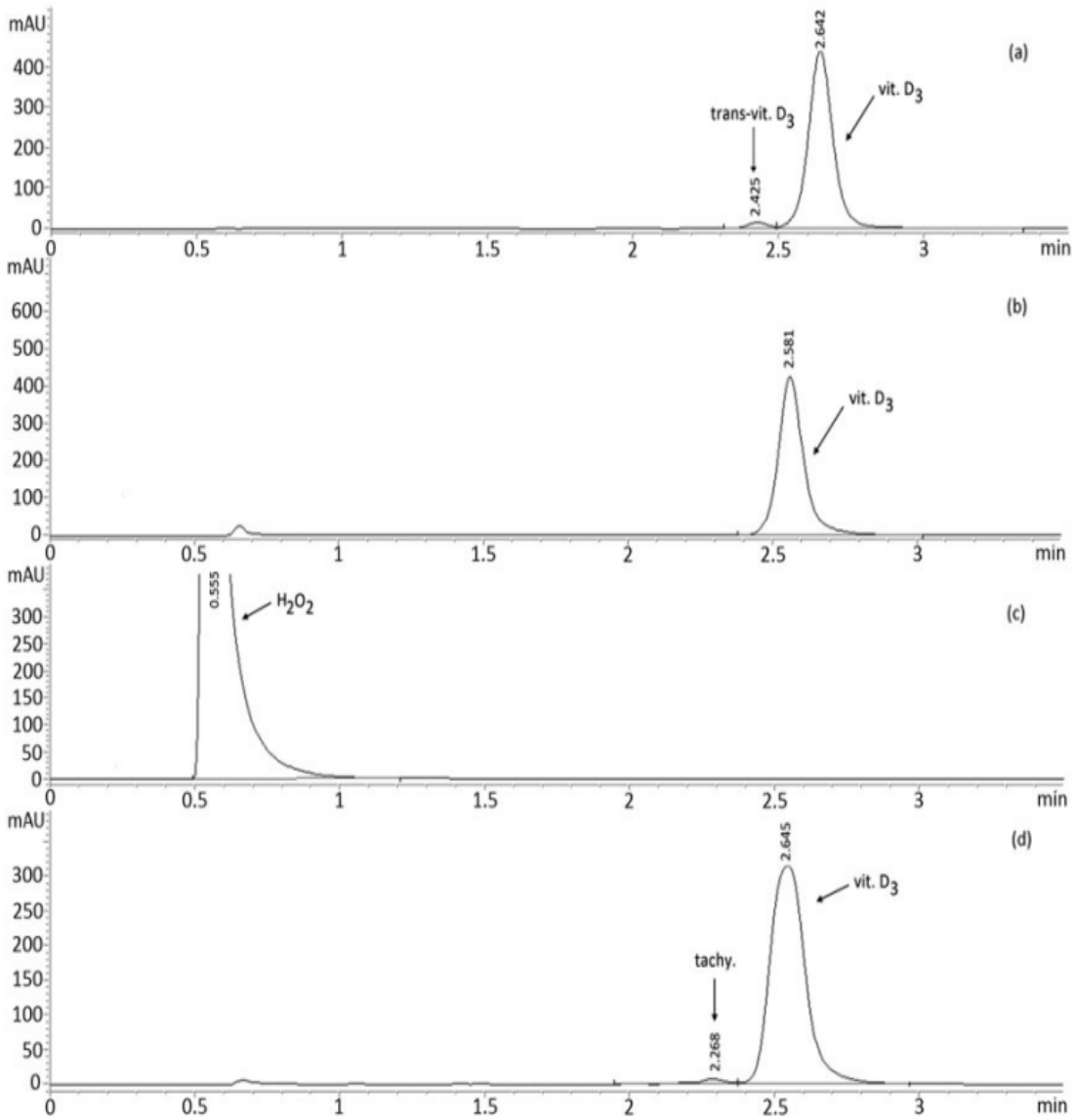


Figure 16; chromatograms of Vitamin D3 stress sample in methanol

11.The solvent use are tetrahydrofuran, methanol and acetonitrile. For the extraction of any solvent extra pure diethyl ether are used. The column used are Hicom C18 stainless steel 250,4.6mm it was filled with 5 µm diameter octadecyl reverse phase packing material. Weigh 0.1 mg of the vitamin D and transferred in 20ml conical flask dissolve these by using 10ml THF should not boil the solution. All other standard solutions of vitamin D3 were prepared from this stock solution by diluting with THF in the further stages of study. Vitamin D2 standard solution was prepared similar to vitamin D3 solution. 0.0132 mg vitamin D2 was accurately weighed in a 20 mL flask and dissolved in 10 mL THF. Calibration curves were prepared by plotting vitamin concentration vs. peak area. For this purpose, 2.02, 4.04, 6.06, 8.08, and 10.10 µg/mL vitamin D3 and 0.65, 1.30, 1.95, and 5.58 µg/mL vitamin D2 standard solutions were prepared by diluting the stock solutions with THF. These standards were injected into the column, respectively, and peak areas were read from the integrator. Particularly, when handling solid samples, extraction yield was important for accuracy of the described method. For this reason, extraction was preceded by a number of steps. In this study, the vitamin D2 sample was extracted in four steps. We accurately weighed a 0.6 g homogenized sample in a 20 mL flask and extracted it with 10 mL extra pure diethyl ether in four steps. Each step was analysed by HPLC. 98.10% Vitamin D2 was recovered in the first and second step. Therefore, a two-step extraction was enough for the sample preparation. The recovery test was carried out parallel to extraction. For this purpose, a proper amount of vitamin D2 standard (10.56, 7.92, 5.58, 2.64 µg) was added to the pharmaceutical preparation containing 35.04 µg/g of vitamin D2. The samples were perpetrated and analysed with HPLC as described below. Recovery percentages were determined to be between 105.48% and 99.65% as shown in Table 1. Because of it being in liquid form, extraction or the recovery test was not necessary for the vitamin D3 sample. The samples were prepared by directly diluting with THF. The vitamin D are generally are in the solid sample by using suitable organic solvent priority of the analysis is extracted by using diethyl ether clearest extracted are obtained. Accurately weigh a 0.6 g homogenised sample in a 20 mL flask. Add 10.0 mL pure diethyl ether into the sample and vigorously shake for 10 minutes. Then the solvent-solid mixture was centrifuged at 2500 rpm for 10 minutes. After 10min the solvent solid mixture was decant into 30ml flask. Total extract volume is 20 mL at the end of extraction steps. Diethyl ether was evaporated with purging nitrogen. The remaining residue was dissolved with 4.0 mL THF. Preparation of the vitamin D3 sample is

very simple compared to vitamin D2 sample. For this purpose, accurately measure 1 mL Vitamin D3 preparation into a 20 mL vial and dilute with 10.0 mL THF. All standard and sample solutions were store at + 4°C before analysis bile phase for the analyses of vitamin D2 . The rate of mobile phase was set at 1.0 mL/min. The Vitamin D2 extract was quite clear and did not contain any impurity, which may interfere with the analyte. For this reason, there is no separation problem in vitamin D2 analyses and the isocratic mobile phase composition of methanol and acetonitrile mixture was enough for separation. Commercial vitamin D3 preparation was prepared by dissolving it in herbal oil. Vitamin D3 was highly soluble in the oil fraction. For this reason, when used as a relatively a polar isocratic mobile phase, separation of a vitamin fraction was 978 SARIOGLU, CELEBI, AND MUTLU Recovery Test of Vitamin D2 from the Solid Sample Added Amount of Average Amount of Recovered Average Percentages of Recovered Vitamin D2 (µg) Vitamin D2 (µg) Vitamin D2 10.56 11.14 ± 0.96 105.48 ± €9.09 7.92 8.03 ± 0.98 101.40 ± 12.32 5.28 5.47 ± 0.34 103.66 ± €6.49 2.64 2.63 ± 0.41 99.65 ± 15.58 very difficult. The separation of vitamin D3 could be possible with relatively polar isocratic mobile phases. But polar mobile phase caused prolongation of elution time of the oil fraction. Therefore, gradient mobile phase composition was used in vitamin D3 analyses. The elution was started with 100% of methanol (v/v) for 4 min, followed by the mixture of THF + methanol (13% + 87%, v/v) for 2 min, and finally the mixture of THF + methanol (50% + 50%, v/v) for 5 min at the flow rate of 1 mL/min. [20]

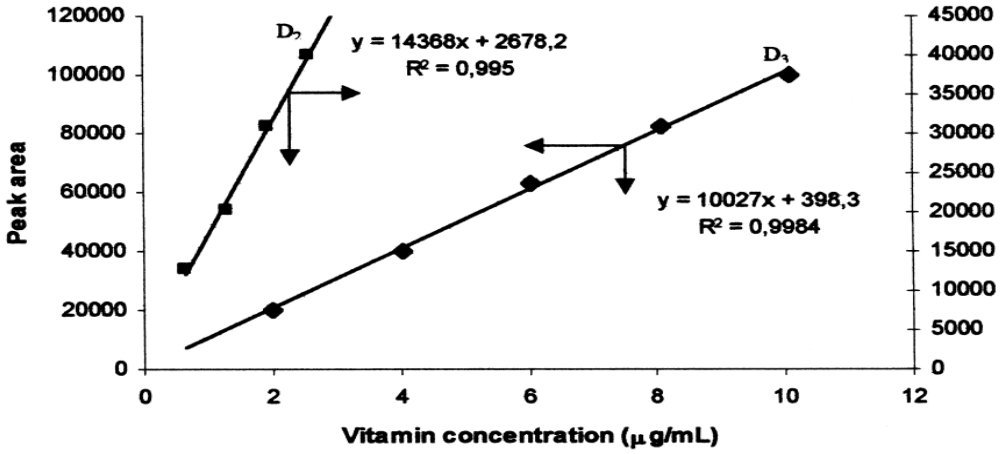


Figure 17 ; graph of vitamin D_2 and D

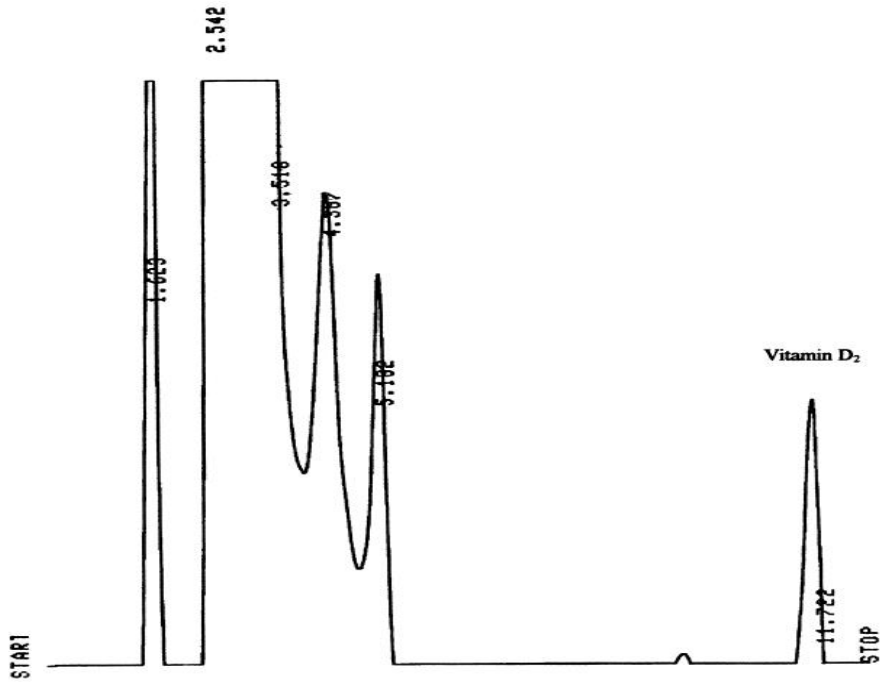


Figure 18;chromatograms of Vitamin D_2 extract

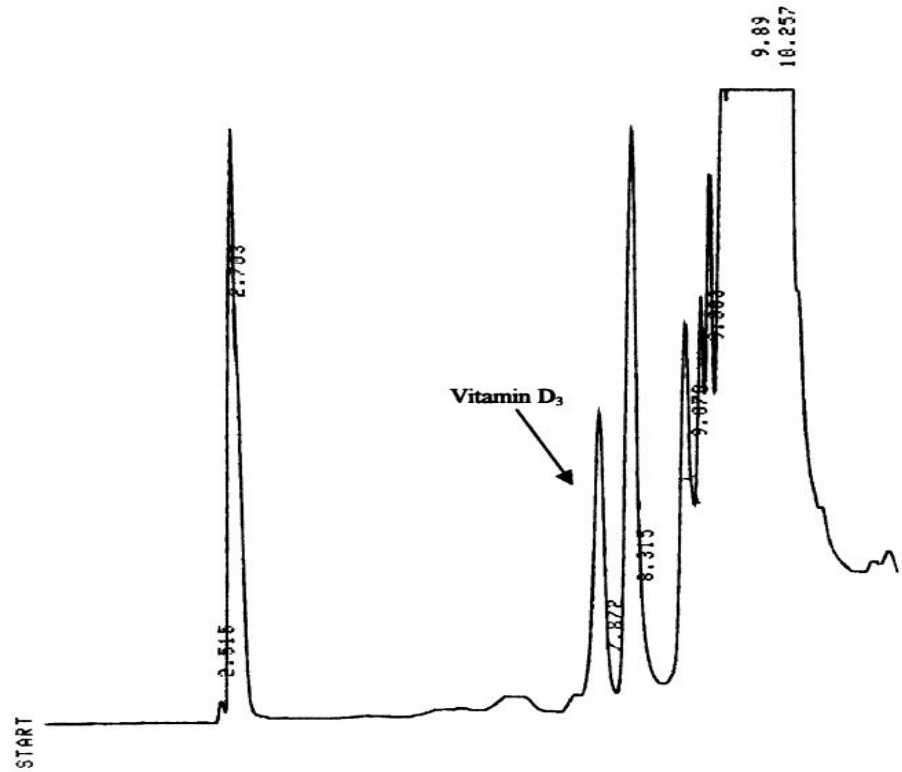


Figure 19; chromatograms of VitaminD_3 in an oil preparation

6. CONCLUSION

Many techniques had been used in the estimation of the vitamin D such as UV spectroscopy, HPLC, GC. But the HPLC techniques is the most preferred one. The advantage of the chromatographic method in comparison with other methods titration, UV-Vis or fluorimetry is that it allows the determination of several various vitamins at the same time. This decreases the amount of time needed for performing analysis and decreases the expenses as well. Even other analytical can be used for the estimation of vitamin D. Even by using the titration method one can determine the estimation of vitamin D in the solution.

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