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Original Research Article

Extraction of Vitamin D_3 from Oil Injection Solution and Analysis of Its Content by HPLC/MS

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Abstract: This paper describes a practical method for extracting cholecalciferol from oil solutions followed by HPLC/MS analysis. This original analytical method was developed for small (less than 1 ml) volumes.

Keywords: Vitamin D3, HPLC/MS, Extraction, Grapeseed Oil.

INTRODUCTION

Vitamin D3 (Figure 1) is a fat-soluble compound responsible for increasing the absorption of calcium and phosphate in the intestines, as well as performing numerous other biological functions (Bikle DD. 2025). It is available commercially as an oil-based injection solution, drops, and soft gel capsules.

Cholecalciferol degradation yields various products, mainly isomers like *trans-vitamin D3*, *tachysterol*, and *isotachysterol* (due to light/heat/acid), and oxidation products such as *1-hydroxy-vitamin D3*, *1-keto-vitamin D3*, and fatty acid esters (from lipid environments), driven by light, heat, oxygen, metal ions, and pH changes, which can complicate the analysis of the chromatogram due to the many side peaks.

The most important step in the vitamin D3 analysis using HPLC/MS is its extraction from the oil. We have previously encountered the problem of efficient extraction of an analyte from an oily solution, and this problem was solved for oily solutions of altrenogest (Yefimov SV., Gil P. 2022). In this study, we developed a more efficient and versatile method for extracting oil-soluble analyte followed by HPLC/MS analysis. Furthermore, the presented method is equally suitable both for sample volumes of 5 ml or more, as well as for volumes less than 1 ml.

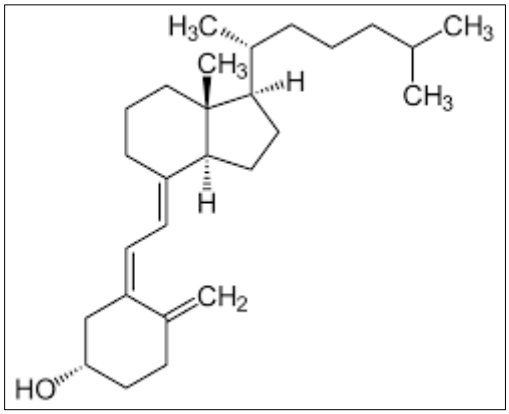

Figure 1: Vitamine-D3 (Cholecalciferol). $C_{27}H_{44}O$, 384.648g·mol^{-1}, m/z+=385, soluble in ethanol

MATERIALS AND METHODS

Chemicals

All liquid reagents including water, acetonitrile, and methanol were HPLC grade. Formic acid, 98–100% analytical grade, was from Merck. Vitamin D3 was USP analytical standard. Mobile phase (chloroform/methanol): 0.1% formic acid in $CHCl_3$/ Meth-OH 30/70.

Agilent LC/MS Instrument

(Agilent Single Quadrupole LC/MS instrument 2019) consists of the following components: Single quadrupole (SQ) mass selective detector (MSD) with electrospray ionization (ESI) and 150 V fragmentor, plus Diode Array Detector (DAD). Gas temperature is 300°C, capillary voltage 4000 V and nebulizer 15psi. Column - C18 250x4.6 mm, 2.7µm; DAD - 274 nm; run time - 8min; temperature - 25°C; flow rate - 1mil/min.

Preparation of Standard Stock Solution

Weigh 10mg of D3 USP standard, dissolve it in 10 ml ethanol and filtrate. Prepare, if it is necessary, 4 calibration solutions in range 0.1 – 1.0 g/l diluting the standard. The previously found linearity range is 0.1 to 2.0 g/L.

Samples Preparation

0.2, 0.4, 0.5, and 1.0 ml of the grapeseed oil solution (25000 IU/ml) were mixed with ethanol in a 10 ml flask. The flasks shook and vortexed for 5 minutes to extract vitamin D3. The solutions were left undisturbed overnight, after which the extract was separated, filtered through a 0.45 µm filter and analyzed by HPLC/MS. The sample volume and extract volume were adjusted by multiplying by a factor so that the sample volume was equal to one (Table 1).

Table 1: Samples and extract corrected volume

sampl. volume (ml)	corrected volume (ml)	corrected extract volume V (ml)	1/V
0.2	1	49	0.020
0.4	1	24	0.042
0.5	1	19	0.053
1	1	9	0.111

Calibration Curve and Coefficient of Correlation

The sets of dilutions of standard stock solutions in range of from 0.1g/L to 1.0g/L were tested. Peak areas of the DAD chromatograms and MSD chromatograms used to construct two calibration curves (Figure 2). Calculation of the calibration curve was performed automatically by OpenLAB CDS (Agilent Single Quadrupole LC/MS instrument 2019). The content of vitamin D3 in the samples was determined using these two calibration curves.

Statistical Analysis

Included calculating mean, standard deviation (S.D.), relative standard deviation (RSD), and correlation coefficient (r). Results $p < 0.05$ were considered statistically significant. Least Squares regression analysis was used. In most cases, the calculation was performed automatically by the OpenLAB CDS program.

RESULTS AND DISCUSSION

Let us assume that the oil is slightly soluble in ethanol and therefore the law of distribution between the phases is valid (Patel K., Panchal N., Ingle P. 2019), (ATE Central. 2025), (Rajendraprasad N. 2025). Extraction D3 with ethanol from the grape seed oil and analyzation of the extract.

Distribution Law: $\frac{(N-b)}{v} = \frac{C \times b}{V}$ (1),

Where N- amount of D3 in the oil, b - amount of D3 in the extract, v - volume of the oil sample (1 ml, constant), V - volume of the extract, C- constant. After transformation we have formula (2), which is the linear relationship 1/V from 1/b, where C is intersect, and CN is slope.

$\frac{1}{V} = \frac{1}{b} \times C \times N - C$ (2),

where C is constants.

Calibration Curves

Calibration curves were constructed using a standard and two detectors: DAD and MSD. For MSD, the calibration curve is a quadratic parabola, in accordance with the law derived (Yefimov SV. 2026). Both calibration curves have correlation coefficients acceptable for analysis, greater than 0.999 (Figure 2).

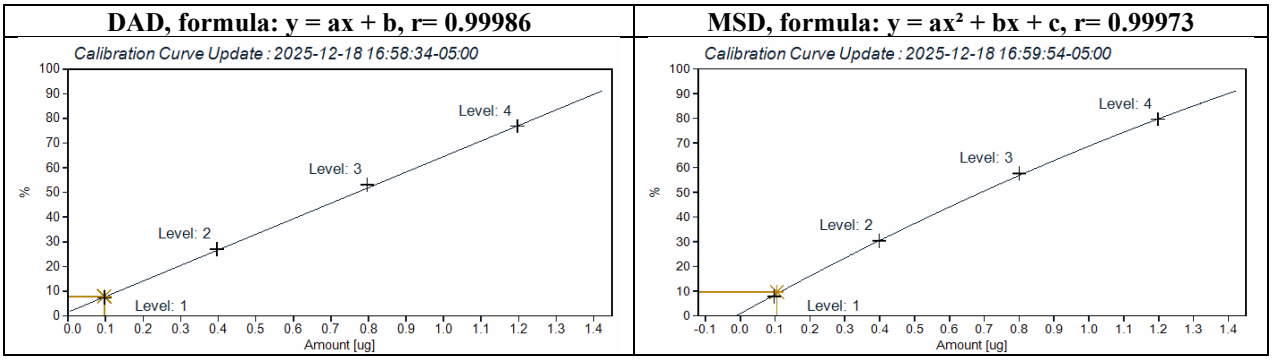

Figure 2: Calibration curves for vitamin D3 determination constructed for DAD (left panel) and MSD (right panel)

Chromatogram

The chromatogram (Figure 3) of the ethanol extract of vitamin D3 from grape seed oil shows a single peak corresponding to vitamin D3. The MS spectrum of the extract is dominated by the signal corresponding to vitamin D3 (m/z+=385.4) and contains a signal (m/z+=338.4).

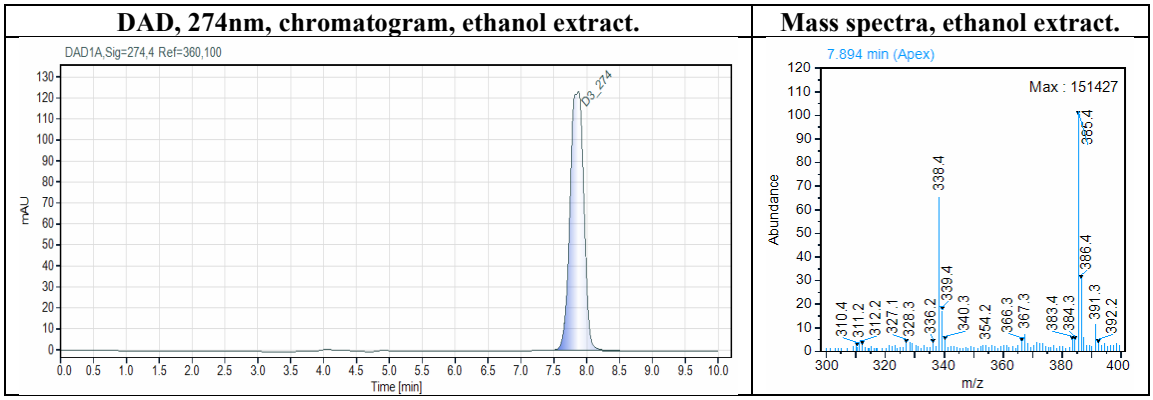

Figure 3: Chromatogram of ethanol extract vitamin D3 from grape seed oil, left, and MS spectra, right

Determination of the vitamin D3 content in grape seed oil based on the dependence of 1/V on 1/b. The concentration of vitamin D3 in a series of extracts (Table 1) was determined, the measurement results were plotted on a graph of 1/V versus 1/b, the obtained points fell on a straight line constructed using the least squares method Figure 4. Based on the slope of the line and the value of the free coefficient (C), the value of N - the content of vitamin D3 in the oil solution sample - was determined; it was 0.168 µg/µL or 6708.7 IU/ml.

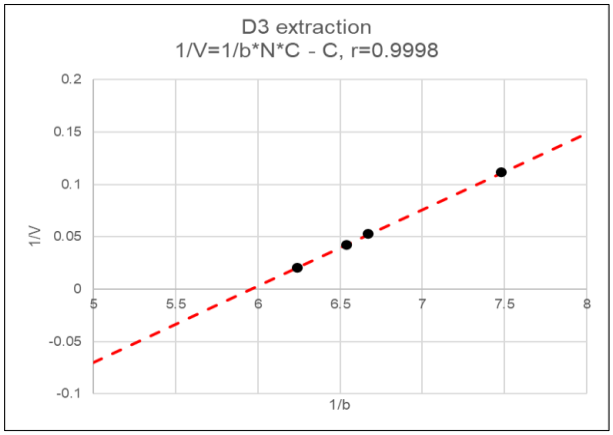

Figure 4: A straight line for determining vitamin D3 content in samples (N). The symbols on the graph represent the results of vitamin D3 determination in various extracts. C=0.43544, and N=0.168µg.

The effectiveness of the proposed method is illustrated in Table 2. Here, the calculated vitamin D3 content (top row) is compared with the measured values in various extracts. All measured values are lower than the calculated value, in accordance with the phase distribution law. The validity of the assumption regarding the negligible solubility of the oil in alcohol is confirmed by a linear relationship with a high correlation coefficient (figure 4).

Table 2: Calculated vitamin D3 content in oil (top line) versus measured values for different extracts.

sampl. volume (ml)	D3 in oil (mg/ml)	Recovery (%)
N/A	**0.168***	**100**
0.2	0.160	95.5
0.4	0.153	91.2
0.5	0.150	89.3
1	0.134	79.7
* - calculated value		

CONCLUSION

The proposed method is effective for analyzing vitamin D3 in small-volume oil solutions when traditional extraction methods using a separatory funnel are inconvenient or unsuitable. The analysis procedure is simple, and the analysis time is no more than 10 hours, including a settling time of 8 hours or less for the two-phase oil-extractor mixture. This method can be generalized for the analysis of any analytes in oil solutions, in particular for the analysis of vitamin A by HPLC.

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