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# Significance of Previtamin D Chromatographic Resolution in the Accurate Determination of Vitamin D<sub>3</sub> by HPLC UV

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## Abstract

Conventional methods using HPLC with UV detection have used vitamin D<sub>2</sub> as an internal standard with the expectation that this fully compensates for the heat-dependent equilibrium of vitamin D<sub>3</sub> with its previtamin. Previtamin D has a different spectral absorptivity from vitamin D and may be present in different proportions in samples and standards. Therefore, vitamin D<sub>2</sub> and vitamin D<sub>3</sub> and their previtamin forms must be chromatographically resolved to achieve accurate quantitation of total vitamin D. This study identified four chromatographic columns (ACE C<sub>18</sub>, ACE C<sub>18</sub> AR, Vydac 201 TP C<sub>18</sub> and Polaris C<sub>18</sub>-Ether) with adequate selectivity that should be applied for food testing and further confirmed that both parent vitamins isomerise at the same rate under thermal conditions.

## 1. Introduction

HPLC for vitamin D testing of foods has been used in routine analytical work for over three decades and although increasingly superseded by LC-MS/MS (Huang et al., 2009; Gilliland et al., 2013; Gill et al., 2015; AOAC 2016a), many laboratories continue to use UV-based methods (Vanhaelen-Fastré & Vanhaelen, 1981; Indyk & Woollard, 1985; Sertl & Molitor, 1985; O'Keefe & Murphy, 1988; Agarwal, 1989; Perales et al., 2005; Eitenmiller et al., 2008; AOAC 2016b). To achieve satisfactory analytical outcomes LC-UV methods rely on vitamin D<sub>2</sub> (ergocalciferol) as an internal standard to determine vitamin D<sub>3</sub> (cholecalciferol), and *vice versa* (Hagar et al., 1994; Salo-Väänänen et al., 2000), where a minimum chromatographic requirement is that vitamin D<sub>2</sub> and vitamin D<sub>3</sub> are resolved. The degree of separation is dependent on the intrinsic nature of the underlying silica and the surface bonding, and

is traditionally accomplished using C<sub>18</sub> or C<sub>30</sub> columns (Indyk & Woollard, 1985; Sliva et al., 1992; Blanco et al., 2000; Staffas & Nyman, 2003; Huang et al., 2012). A C<sub>30</sub> column has greater hydrophobicity and provides stronger retention of fat-soluble vitamins (Huang et al., 2009).

The temperature-dependent formation of previtamin D, as illustrated in Fig. 1, has been demonstrated by several researchers (Keverling Buisman et al., 1968; Mulder et al., 1971; de Vries et al., 1979). Endogenous levels of previtamin D<sub>3</sub> in a food product, in contrast to the absence of previtamin D<sub>2</sub> in the internal standard, will contribute to a small, but significant quantitative error due to different spectral absorptivities (Gill et al., 2019). Using vitamin D<sub>2</sub> as the internal standard therefore requires not only adequate chromatographic resolution of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> but also chromatographic resolution of both previtamins. Optimum vitamin D<sub>2</sub>–vitamin D<sub>3</sub> separation alone does not imply effective quantitation, as previtamin D<sub>3</sub> often co-elutes with vitamin D<sub>2</sub> and cannot be differentiated using UV detection, therefore potentially contributing to minor analytical bias.

Provided that saponification conditions are sufficient for vitamin D and previtamin D from both sample and internal standard to reach equilibrium, it is not necessary to quantitate previtamin D peaks in the analysis of total vitamin D<sub>3</sub> (sum of previtamin D<sub>3</sub> and vitamin D<sub>3</sub>). However, few reported analytical methods accomplish this requirement, resulting in unequal proportions of previtamin D forms from sample and internal standard (Gill et al., 2019). Under such conditions it is important to ensure that the previtamin forms are chromatographically resolved from the parent vitamin forms.

The principle aim of this study therefore, was to evaluate a wide range of analytical chromatographic columns and to identify those with the capability to achieve baseline resolution of the four vitamin D forms.

## 2. Materials and methods

### 2.1. Apparatus

Analytical chromatography was performed on an Agilent HPLC system (Santa Clara, CA, USA), with G1322 A degasser, G1311 A quaternary pump, G1329B autosampler, G1316-A column compartment and G1315 A diode array detector, or on a Shimadzu Prominence HPLC system (Columbia, MD, USA), with LC-20AD pump and DGU-20 A degasser unit, SPD-M20 A diode array detector, CTO-20AC column oven and sample injection by a Shimadzu Nexera SIL-30AC autosampler at 10 °C.

Other equipment used were a three-decimal place balance for weighing samples (Ohaus Explorer Pro, Parsippany, NJ, USA) and four decimal place balances for standards (Sartorius, Göttingen, Germany).

## 2.2. Columns

Separations were performed on 16 reversed-phase columns in 5  $\mu\text{m}$  fully porous and 2.6  $\mu\text{m}$  superficially porous (core-shell) formats, to identify those with sufficient chromatographic separation of vitamin D<sub>2</sub>, vitamin D<sub>3</sub>, previtamin D<sub>2</sub> and previtamin D<sub>3</sub>. Columns selected for further studies were Acclaim C<sub>30</sub> (Thermo Fisher, Waltham, MA, USA), ACE C<sub>18</sub> and ACE C<sub>18</sub>-AR (Advanced Chromatography Technologies, Aberdeen, Scotland), Vydac 201 TP C<sub>18</sub> (Hichrom, Reading, England), Microsorb 300-5 and Polaris C<sub>18</sub>-Ether (Agilent, Santa Clara, CA, USA); all were 5  $\mu\text{m}$  fully porous and 4.6  $\times$  250 mm.

## 2.3. Reagents

Vitamin standards ( $\geq 98\%$ ), cholecalciferol (vitamin D<sub>3</sub>) and ergocalciferol (vitamin D<sub>2</sub>), were obtained from Sigma Aldrich (St. Louis, MO, USA). Methanol and acetonitrile of HPLC grade were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Standards were prepared in acetonitrile and UV exposure minimised by using amber glassware under UV-filtered lighting.

## 2.4. Standards

Following preliminary temperature studies, all columns were operated at 30 °C under the same isocratic conditions with an acetonitrile mobile phase. The 5  $\mu\text{m}$  columns were operated at 1.0 mL/min and the 2.6  $\mu\text{m}$  core-shell columns at 0.7 mL/min, and under these conditions, standard solutions were monitored for changes in vitamin D and previtamin D. The primary monitoring wavelength used was 265 nm with spectral data in the range 200–350 nm acquired for peak identity and purity assessments. Previtamin D peak identifications were achieved by heating standard solutions for 1 h at 60 °C and monitoring proportional changes in peak areas.

## 2.5. Isomerisation experiments

Freshly prepared solutions of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> standards were subjected to time trials at ambient temperature ( $18 \pm 2$  °C) for the appearance of their respective previtamins. At each time point, extracts were placed in a  $-18$  °C freezer until ready for analysis. Peak identities were confirmed by the changes in area with time and by spectral scanning, in which previtamin D isomers have maxima at 262 nm, i.e. slightly lower than their parent calciferols. Peak areas were normalised using published extinction coefficients ( $E^{1\%}$ ), with previtamin D<sub>2</sub> and previtamin D<sub>3</sub> peak areas multiplied by 2.28 (475/208) and 2.22 (485/218) respectively (Hanewald et al., 1968).

### 3. Results and discussion

Incomplete chromatographic resolution of the four calciferol vitamers will lead to compromised quantitation and an accurate estimate of total vitamin D<sub>3</sub> content can only be obtained when there is complete resolution of the previtamin D peaks. It is therefore of concern that the majority of C<sub>18</sub> columns do not facilitate complete resolution of all four relevant vitamin D forms.

#### 3.1. Method development and validation

Adequate vitamin D<sub>2</sub> and vitamin D<sub>3</sub> separation ( $R_s > 1.8$ ) could be achieved with all 16 commercial C<sub>18</sub> columns evaluated. However, the capability to also separate previtamin D from their respective vitamin D<sub>2</sub> and vitamin D<sub>3</sub> parents was limited to only four of the columns: ACE C<sub>18</sub>, ACE C<sub>18</sub>-AR, Agilent Polaris C<sub>18</sub>-Ether and Vydac 201 TP. The column evaluations were all performed isocratically in acetonitrile without attempting to optimise with solvent changes and gradient elution. Even though some columns might not have been operating optimally, it was considered expedient to compare column performance under common conditions. If the four vitamin D chromatographic peaks were not resolved under these conditions, the column will not be fit for purpose for more complex food extracts.

Columns that achieved acceptable resolution of all four previtamin D and vitamin D peaks are illustrated in Fig. 2. The ACE C<sub>18</sub> column had a separation capability that was superior to that of the ACE C<sub>18</sub>-AR column; the latter column is designed for enhanced aromatic selectivity, indicating that the vitamin D steroidal structure predictably does not interact with the available aryl functionality. The equivalent ACE C<sub>18</sub>-PFP column had a further reduction in resolution that was commensurate with its increased polarity, leading to co-elution of previtamin D<sub>3</sub> and vitamin D<sub>2</sub>. The Polaris C<sub>18</sub> Ether column, with an end-capped ether group to improve polar retention, assisted vitamin D separations presumably through its hydroxyl functionality. In addition, Polaris silica has 180 Å pores, i.e. larger than the 100 Å ACE silica chemistry. Speculation that pore size is important originates from the ability of the Vydac 201 TP column, with 300 Å pores, to efficiently separate calciferols. However, the selectivity of the Vydac 201 TP column is different from those of both the ACE column and the Polaris C<sub>18</sub>-Ether column, in that previtamin D<sub>3</sub> elutes between vitamin D<sub>2</sub> and vitamin D<sub>3</sub> (Fig. 2). Trials using an equivalent 300 Å C<sub>18</sub> Microsorb 300-5 column exhibited similar tendencies to separate the calciferols, although with less efficiency than the Vydac column. Both 300 Å columns had significant peak tailing when operated in 100% acetonitrile, which could possibly be improved with a methanol:water gradient.

In contrast to these columns, it was noted that for the enhanced hydrophobic Acclaim C<sub>30</sub> column, the previtamin forms eluted after their respective parent vitamins; nonetheless, this did not solve

quantitation problems as previtamin D<sub>2</sub> co-eluted with vitamin D<sub>3</sub>. This reversal of elution order had been noted by Huang et al. (2009) using a YMC S3 carotenoid C<sub>30</sub> column. A core-shell pentafluorophenyl column showed separation in the same order as for the C<sub>30</sub> columns (Wei et al., 2017).

### 3.2. Isomerisation

In its *cis*-configuration, the target measurand vitamin D<sub>3</sub>, will readily interconvert with previtamin D<sub>3</sub>, and the related interconversion also occurs with vitamin D<sub>2</sub>. Measurable levels of previtamin D were absent in freshly prepared vitamin D<sub>2</sub> or vitamin D<sub>3</sub> standard solutions, but were rapidly formed dependent on the temperature of storage. For this reason, standards were kept at –18 °C for up to 6 months and under such conditions, the extent of isomerisation remained negligible. The vitamin D<sub>2</sub> and vitamin D<sub>3</sub> standards were removed from the freezer, divided into 10 vials and kept at ambient temperature for successive days and returned to the freezer at each time point to prevent further conversion. After 10 days, all standards were analysed simultaneously, where the results confirmed that vitamin D<sub>2</sub> and vitamin D<sub>3</sub> isomerised to their previtamin forms at the same rate (Fig. 3).

As expected, previtamin D peaks increased in area at approximately half the rate of the concomitant decrease in parent vitamin, due to their different spectral absorptivities. This supports the applicability of vitamin D<sub>2</sub> as a surrogate for vitamin D<sub>3</sub>, compensating both for manipulative losses during difficult multi-stage sample preparations and for heat-induced isomerisation, and is consistent with previous literature (Hanewald et al., 1968). However, as discussed, this attribute is applicable only when both previtamin D forms are chromatographically resolved from each other and their parent forms under HPLC-UV conditions.

## 4. Conclusions

When HPLC-UV analysis is employed for the analysis of vitamin D<sub>3</sub>, incorporating vitamin D<sub>2</sub> as an internal standard, it is important to fully separate the four vitamin D forms. In an acetonitrile mobile phase, only four of sixteen C<sub>18</sub> columns were found to achieve this, with the Vydac 201 TP C<sub>18</sub> column demonstrating a selectivity that was different from that of the other columns. Columns capable of adequate previtamin resolution in model standard solutions should be favoured for future vitamin D determinations in food matrices to avoid potential bias due to inadequate resolution of previtamin D forms. Also, it was confirmed that the rate and extent of thermal isomerisation of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> to their previtamin forms were equivalent, supporting the utility of ergocalciferol as an internal standard.

## Declarations of interest

None.

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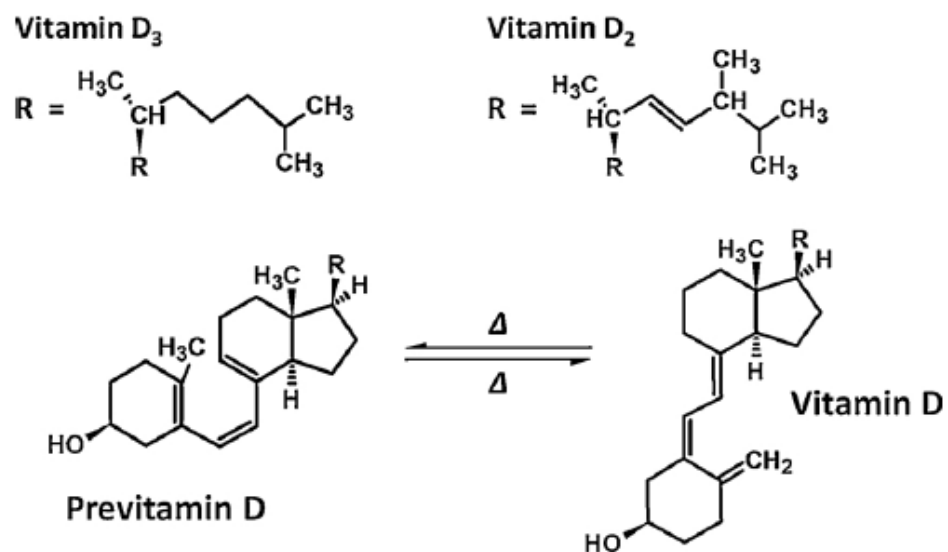


Fig. 1. Interconversion of previtamin D and vitamin D

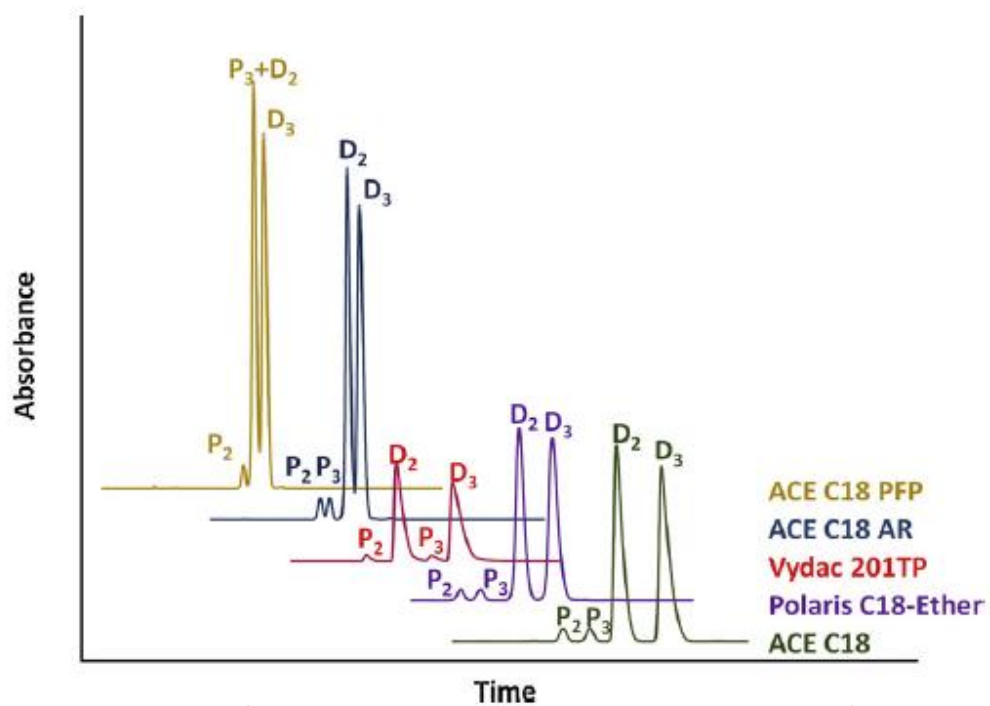


Fig. 2. Separation of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> and their previtamin forms using selected columns (P=previtamin D; D=vitamin D)

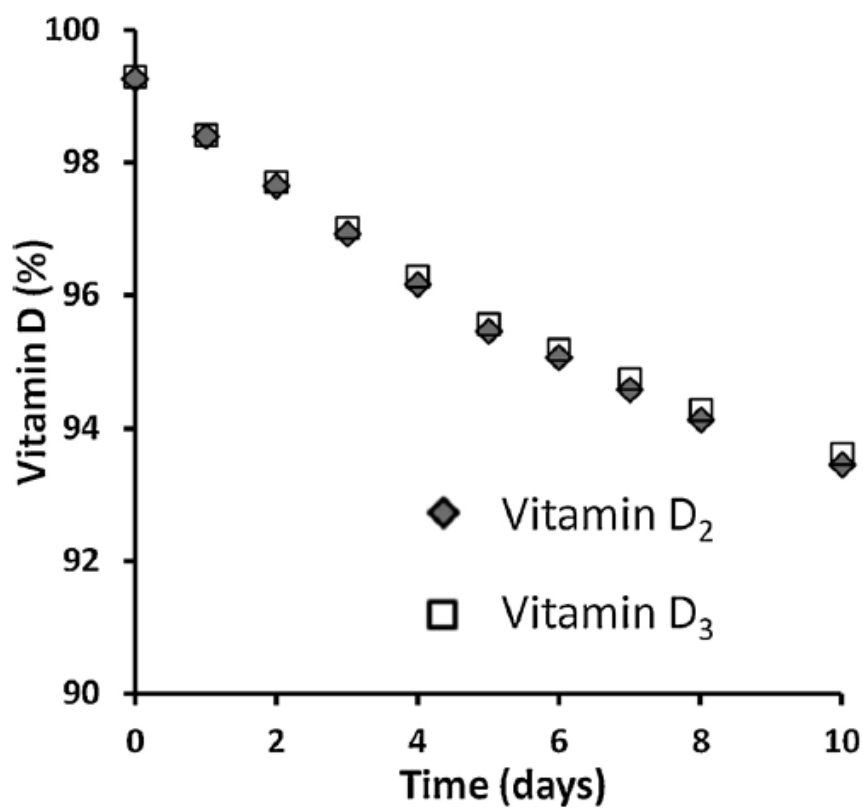


Fig. 3. Decrease in vitamin D<sub>2</sub> and vitamin D<sub>3</sub> as a result of thermal isomerisation at room temperature ( $18 \pm 2$  °C)