Significance of Thermal Isomerisation on the Quantitation of Total Vitamin D_3 in Foods

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Abstract

Sample preparation techniques for the analysis of vitamin D_3 in food matrices typically utilise a saponification step, either at room temperature or at elevated temperatures. A calciferol (vitamin D_2 or isotope labelled vitamin D_3) is generally chosen as the internal standard to compensate for changes of previtamin D_3-vitamin D_3 isomerisation during analysis, as well as to correct for analyte loss through complex sample preparation steps. Manufacturing practices and processing parameters contribute to previtamin D formation in food products. A significant proportion (5.6–8.3%) of the total vitamin D3 in premixes was found as previtamin D_3, indicating that it is likely, depending upon storage temperature and the time since manufacture, that a vitamin D_3-fortified food product will contain a similar proportion of previtamin D_3 prior to analysis. Conversely, freshly-prepared internal standard solutions have low previtamin D levels (< 1%). In lieu of direct measurement, this discrepancy in previtamin D content between the internal standard and analyte forms of vitamin D will lead to analytical bias. To mitigate this as a source of potential error, it is recommended that sample pre-treatment steps are appropriately set and controlled. Based on this work, saponification times greater than 300, 120, or 60 min for temperatures of 60, 70, or 80 °C respectively should be employed and that saponification at room temperature be avoided.

Keywords

Vitamin D, Previtamin D, Saponification, Isomerisation

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Introduction

Vitamin D as ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), is a secosterol that plays a major role in bone health. Both forms are available through the diet, and vitamin D_3 is also formed in the skin via irradiation of 7-dehydrocholesterol to previtamin D3 with subsequent thermal isomerisation (Figure 1). The main biological function of vitamin D is controlling the absorption, transport, and deposition of calcium and phosphorus as part of bone mineralisation. In addition to rickets in children and osteomalacia in adults, vitamin D deficiency has been associated with increased rates of cancer, diabetes, and cardiovascular disease (DeLuca 2004; Hewavitharana 2013; Higashi et al. 2010).

The accurate analysis of vitamin D_3 in foods is challenging; aside from irreversible loss of vitamin D_3 through oxidative degradation, the reversible isomerisation of vitamin D_3 to previtamin D_3 is thermally induced, changing the relative proportion of each until equilibrium is reached (Mackay et al. 1979; Mulder et al. 1971). This isomerisation is not affected *in vitro* by solvent, pH or UV light (Keverling Buisman et al. 1968), although *in vivo*, biological macromolecules may play a catalytic role (Tian & Hollick 1995). An important consequence of this phenomenon for nutritional purposes is that the measurement of total vitamin D_3 (the sum of vitamin D_3 and previtamin D_3) is necessary to obtain complete, reliable and consistent results (de Vries 1979).

During analysis of vitamin D_3 in foods, alkaline hydrolysis is the preferred technique to remove triglycerides prior to organic solvent extraction of the non-saponifiable fat-soluble vitamins. Such saponification procedures can be characterized as "high temperature–short time" or "low temperature–long time", with either strategy commonly used in numerous methods (Eitenmiller and Landen 2008; Perales 2005). The time/temperature for "hot" saponification is typically in range 60–80 °C for 30–60 min, whereas "cold" saponification is generally performed at room temperature for up to 24 hours. Cold saponification has the advantage of limiting further isomerisation to < 5% of previtamin D, whereas methods that utilise hot saponification during analysis yield a significant increase in the formation of previtamin D (Gomes et al. 2013; Perales 2005), leading to an underestimation of the results for methods measuring vitamin D_3 only (Schadt et al. 2012).

To overcome the problem of thermally induced changes in previtamin D and vitamin D concentrations, many methods use calciferol internal standards, either vitamin D_2 for liquid chromatography-ultraviolet (LC-UV) methods or isotopically enriched vitamin D_3 for liquid chromatography–mass spectrometry (LC-MS) methods (Eitenmiller and Landen 2008; Perales 2005).

Every technique applied to the analysis of total vitamin D in foods is vulnerable to various factors that need to be considered to ensure an accurate measurement. Because of their inherent UV molar

absorptivities, different instrument responses for previtamin D_2, vitamin D_2, previtamin D_3, and vitamin D_3 are generated in LC-UV methods (Hanewald et al. 1968). LC-MS methods that involve extraction without further clean-up will obtain complete recovery of both vitamin D_3 and previtamin D_3 (Huang and Winters 2011; Kwak 2014; Schadt et al. 2012). However, the equivalence of ionisation efficiencies of vitamin D and previtamin D cannot be assumed and would need to be demonstrated. Both LC-MS and LC-UV methods that use some form of clean-up, such as semi-preparative chromatography or solid-phase extraction, may have a discriminatory effect on vitamin D and previtamin D forms (AOAC 2016a; Heudi et al. 2004; Sliva et al. 1992; Strobel et al. 2013). Recent LC-MS methods using 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) derivatisation quantitate total vitamin D by detecting the vitamin D-PTAD adduct ions only; the contribution of previtamin D-PTAD adducts ions to total vitamin D is putatively accounted for by the internal standard (Abernethy 2012; AOAC 2016b; Gill et al. 2015; Gomes et al. 2015).

A fundamental assumption of all of these methods is that the proportions of previtamin D in the internal standard and in the sample are equivalent (Gill et al. 2016). Any differences in the previtamin D: vitamin D ratio between the internal standard and the sample may introduce quantitative bias. This is especially true for analytical methods that do not resolve and quantitate previtamin D separately as part of the analytical procedure.

The purpose of this research was to evaluate this assumption by (i) determining whether significant levels of previtamin D_3 were present in samples prior to routine analysis; (ii) assessing whether an isomerisation bias is possible, (iii) quantifying the magnitude of any potential bias, and (iv) recommending sample extraction protocols to minimise analytical error associated with previtamin D formation during analysis.

Experimental

Apparatus

A high performance liquid chromatography (HPLC) system consisting of an LC-20AT pump, an SIL-20AHT autosampler, a CTO-20AC column oven, an SPD-M20A photodiode array detector and a DGU 20AS degasser unit (Shimadzu, Kyoto, Japan) was used and incorporated a Cosmocore Cholester column (2.6 µm, 4.6 × 150 mm; Nacalai Tesque, Kyoto, Japan). Lab Solutions software (Shimadzu) version 5.73 was used for instrument control and data processing. Luer-lock syringes (3 mL; Hapool, Shandong, China) and Phenex nylon syringe filters (0.22 µm × 25 mm; Phenomenex, Torrance, CA, USA) were used for standard filtration.

Reagents

Vitamin D_3 (cholecalciferol), absolute ethanol, LC-grade methanol and acetonitrile, reagent-grade sodium chloride and sodium ascorbate, were supplied by Merck (Darmstadt, Germany). Water was purified to 18.2 MΩ resistivity using a Genpure water system (Thermo Fisher Scientific, Waltham, MA, USA).

Standard solutions

A vitamin D_3 stock standard (1 mg mL^{-1}) was prepared by dissolving 100 mg in 100 mL of ethanol; five × 20 mL aliquots of this solution were immediately stored at −80 °C.

Standards at equilibrium

To model the behaviour of food samples that may contain a range of previtamin D_3: vitamin D_3 ratios, a set of vitamin D_3 standards were equilibrated to different temperatures was required. The temperatures 0, 10, 20, and 30 °C were chosen to model food product samples at expected storage temperatures. To determine the length of time needed to equilibrate these standards at each temperature, isomerisation rate constants described previously were used (Hanewald et al. 1968). The theoretical time taken to reach 99% equilibrium fraction ($t_{0.99}$) for pure vitamin D_3 can be estimated (Equation 1) based on these constants (Equations 2, 3). At low temperatures, it can take months or years to reach equilibrium; although, this time can be reduced significantly if the standard is initially equilibrated at a higher temperature followed by cooling to a lower temperature (Equation 4) (Keverling Buisman et al. 1968).

Therefore, from a set of five × 20 mL vials of vitamin D_3 stock standard, one vial remained in storage at −80 °C, three vials were stored at 20 °C, and one vial was stored at 30 °C. After 33 days of equilibration, two of the vials held at 20 °C were transferred to lower temperatures, namely one aliquot was from 20 to 0 °C and the other from 20 to 10 °C; the other three vials remained at −80, 20, and 30 °C (Table 1). All vials remained at the targeted temperatures for a further 6 months to ensure that equilibration was complete prior to the commencement of time/temperature studies described below.

$$\text{vitamin } D_3 \underset{k_{-1}}{\overset{k_1}{\leftrightharpoons}} \text{previtamin } D_3$$

$$t_{0.99} = \frac{2 \times 2.303}{(k_1 + k_{-1})} \quad \text{(Equation 1)}$$

$$\log k_1 = \frac{-4200}{T} + 10.29 \quad \text{(Equation 2)}$$

$$\log k_{-1} = \frac{-5180}{T} + 12.53 \quad \text{(Equation 3)}$$

where:
k_1 = isomerisation rate constant for vitamin D_3→previtamin D_3 (min^{-1})
k_{-1} = isomerisation rate constant previtamin D_3→vitamin D_3 (min^{-1})
T = temperature (K)

$$t_{0.99} = \frac{2.303}{(k_1 + k_{-1})} log\ 100\left(1 - \frac{f_{T_0}}{f_{T_1}}\right) \quad \text{(Equation 4)}$$

where:

$$f_T = \frac{100\ k_1}{k_1 + k_{-1}}$$

, the fraction of vitamin D at temperature T

Time/temperature experimental

Four experiments were performed to simulate typical saponification temperatures (25, 60, 70 and 80 °C) used in many published analytical methods for the analysis of vitamin D in foods (Eitenmiller and Landen 2008; Perales 2005). For each experiment, five sets of 13 test tubes were placed in a rack and 5 mL of ethanol and 1 mL of sodium ascorbate (10% m/v) solution were added to every test tube. Equilibrated stock standard vials were removed from storage and for each equilibrated temperature (−80, 0, 10, 20, and 30 °C), 100 µL aliquots were pipetted into a set of 13 tubes. For the high temperature experiments (60, 70 and 80 °C), the tubes were placed in a water bath at the required temperature, whereas the ambient temperature experiment (25 °C), the tubes were placed in an HPLC column oven (Table 2). At the appropriate time, one test tube from each of the equilibration temperatures was removed and immediately cooled in a sodium chloride-ice water bath, and an aliquot was syringe filtered into an HPLC vial then stored at −18 °C to prevent any further change in vitamin D: previtamin D ratio prior to HPLC analysis.

Extinction coefficients

A number of extinction coefficients are available for vitamin D_3, however few values for previtamin D_3 have been published. In this study, vitamin D_3, $E^{1\%}$ (265 nm) = 485 g $dL^{-1}cm^{-1}$ and previtamin D_3, $E^{1\%}$ (265 nm) = 218 g $dL^{-1}cm^{-1}$ were used (Hanewald et al. 1968). Peak areas were normalized by correcting the previtamin D_3 peak area by multiplying by 2.22 (485/218).

Extraction of vitamin D_3 from premixes

Four fat-soluble vitamin premixes and a vitamin D_3 ingredient used for infant formula were each evaluated for their inherent previtamin D_3 content. These premixes were within manufacturer recommended use-by dates and had been stored at room temperature in accordance with the manufacturer's instructions for 5–12 months at the time of testing. Vitamin D_3 was extracted using dimethyl sulfoxide (DMSO), as previously described (Pastore et al.1997). Briefly, 2 mL of 10% v/v aqueous DMSO was added to 0.3 g of premix, mixed, and stood for 10 min prior to the addition of 20 mL of isooctane with 10 min shaking. A 10 mL aliquot of isooctane was evaporated to dryness, reconstituted in 2 mL methanol and 0.2 mL water was added, before syringe filtering into an HPLC vial.

Chromatography

The column oven was set at 40 °C with gradients formed by low pressure mixing of the mobile phases water (A), methanol (B), and acetonitrile (C) at a flowrate of 1.5 mL min^{-1}; with separation of previtamin D_3 and vitamin D_3 was achieved using the conditions described (Table 3). The photodiode array detector acquired spectral data between 200 and 300 nm with peak integration performed at 265 nm. To overcome any re-equilibration by delays in the autosampler tray each HPLC vial was removed from −18 °C storage immediately prior to instrumental analysis. The increase in previtamin D_3 and corresponding decrease in vitamin D_3 peaks area upon heating a pure vitamin D_3 standard is illustrated (Figure 2).

Results and discussion

The analysis of four fat-soluble vitamin premixes and a vitamin D_3 ingredient showed a significant fraction of previtamin D_3 is present in premixes prior to manufacture of the finished food product (Table 4). Vitamin premixes were analysed as a reliable proxy for supplemented foods in order to simplify the extraction requirements and thereby minimise changes in previtamin D caused by the analytical process. The results obtained indicate that depending on storage conditions and the time post-manufacture, a supplemented food, in either solid or liquid form, will likely contain a significant proportion of previtamin D_3 prior to analysis. Additionally, food manufacture is generally a heat intensive process and it is probable that more previtamin D will form from vitamin D during production.

Time/temperature model

A preliminary *ab initio* evaluation of potential analytical bias was performed using the published isomerisation rate constants, calculating theoretical initial and equilibrium previtamin D_3 and

vitamin D_3 proportions. A linear interpolation was used to determine whether any bias was possible under typical extraction conditions used in the quantitative estimation of the vitamin D_3 content of food samples. These calculations showed that the presence of previtamin D_3 in a sample prior to analysis is significant, in that a difference in previtamin D_3 fraction between the sample and the internal standard will result in an analytical bias of approximately 5–7%, irrespective of the saponification temperature used (Table 5). This prediction was of particular interest, as many methods use room temperature saponification as a means of limiting isomerisation during analysis (Perales et al. 2005).

Vitamin D_3 time/temperature study

A model system using authentic vitamin D_3 standards was used to evaluate possible bias due to differences in previtamin D: vitamin D ratios between internal standard and sample. Standards can be analysed simply without the need for complex extraction techniques, and provide a clear insight into the magnitude of any potential bias.

Calciferol internal standards, either vitamin D_2 for LC-UV methods or stable isotope-labelled vitamin D_3 for LC-MS methods, have been used almost exclusively in recent years to quantify vitamin D_3. The advantage of these compounds is that they correct for manipulative losses throughout complex analytical procedures. Additionally, calciferol internal standards also potentially correct for losses of vitamin D_3 via thermal isomerisation to the previtamin form. However, these analytical methods generally operate with an underlying, and usually undeclared, assumption that the previtamin D: vitamin D ratios for the internal standard and the sample are identical.

In lieu of direct measurement of previtamin D, this assumption is not valid unless both the sample and the internal standard have had sufficient time, at a given temperature, to reach equilibrium with respect to previtamin D. The presence of previtamin D_3 in premixes and ingredients as well as the high temperatures used during product manufacture, indicate that a supplemented food sample will contain a significant contribution of previtamin D_3 prior to analysis whereas, in contrast, the vitamin D internal standard contains negligible previtamin D at the time of spiking into the sample extract. It is therefore important that exposure to heat during extraction is of sufficient time to allow both the internal standard and the sample to establish equivalent proportions of previtamin D.

Typically, sample preparation techniques for the analysis of vitamin D_3 in food matrices utilise a saponification step, either at room temperature for 12–15 h or at elevated temperatures from 60 to 80 °C for 1 h.

To simulate conditions equivalent to cold saponification, a pure vitamin D_3 standard stored at −80 °C and vitamin D_3 standards previously equilibrated at 0, 10, 20 and 30 °C were warmed in a column oven at 25 °C over a period of 12 days with aliquots analysed for previtamin D_3 and vitamin D_3 content every 24 h. In this way, the solutions commenced re-equilibration to the new temperature from different initial previtamin D concentrations (Figure 3). After 12 h, the results indicate that the −80 °C standard (as a proxy for a calciferol internal standard) contained approximately 98.8% vitamin D_3, whereas the 20 °C standard (as a proxy for vitamin D_3 in a solid or liquid sample) contained 91.5% vitamin D_3. For many analytical methods this difference will result in a measurement bias of up to −7.4% and will report only 92.6% of the true result (Table 6). A larger bias will be expected in samples that have equilibrated during storage to temperatures higher than 20 °C.

Similarly, to simulate the conditions used in hot saponification, a pure vitamin D_3 standard and vitamin D_3 thermally equilibrated standards were heated in a water bath at 60, 70, or 80 °C over a period of 720, 360, or 180 min, with aliquots analysed for previtamin D_3 and vitamin D_3 every 60, 30, or 15 min respectively (Figures 4–6). After 60 min saponification time at 60, 70, or 80 °C respectively, approximately 93.9, 87.6 or 81.2% of the −80 °C standard remained in the vitamin D form, whereas the 20 °C standard contained 88.6, 84.8 or 80.7% vitamin D3. These differences in previtamin D content indicate that for many analytical methods the measured results for samples stored at 20 °C and heated to 60, 70, or 80 °C during saponification will have maximum possible bias of −5.6, −3.2 or −0.6% and will report only 94.4, 96.8 or 99.4% of the true result, respectively (Table 6).

For cold saponification at 25 °C, 8 days was required before the difference between each of the pre-equilibrated standards was less than 1% which far exceeds the typical saponification time of 12–15 h. For hot saponification at 60, 70, and 80 °C respectively, the differences in the vitamin D_3 proportions for each pre-equilibrated standard were less than 1% after 300, 120, and 60 min.

Although these bias values are relatively small and in some cases within analytical precision for most methods, a systematic bias is a fundamental component of analytical error and should be identified and where possible removed. Therefore, to eliminate differential thermal conversion to previtamin D as a source of analytical error, it is recommended that saponification times be greater than 300, 120, or 60 min for temperatures of 60, 70, or 80 °C respectively, and that room temperature saponification be avoided.

Conclusions

Depending on post-manufacture storage conditions, it is plausible that food samples, in either solid or liquid form, containing vitamin D will have a significant contribution of previtamin D prior to analysis.

Analytical methods that use calciferol internal standards are therefore susceptible to bias, due to differences in the proportion of previtamin D between the sample and the internal standard.

To remedy this problem, methods should incorporate a heat treatment during saponification that ensures that both the internal standard and the sample have reached equivalence with respect to their previtamin D contribution prior to instrumental analysis.

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Compliance with Ethical Standards

Conflict of Interest

Brendon Gill declares that he has no conflict of interest. Donald Gilliland declares that he has no conflict of interest. Harvey Indyk declares that he has no conflict of interest. Jackie Wood declares that she has no conflict of interest. David Woollard declares that he has no conflict of interest.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent

Not applicable.

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Table 1. Predicted storage times for vitamin D_3: previtamin D_3 equilibrium

	Equilibrated standards				
Storage	−80 °C	0 °C	10 °C	20 °C	30 °C
Equilibration temperature/ time	−80 °C 0 d[a]	20 °C 32.9 d	20 °C 32.9 d	20 °C 32.9 d	30 °C 10.8 d
	—	0 °C 93.0 d	10 °C 12.7 d	—	—
Total time	0 d	125.9 d	45.6 d	32.9 d	10.8 d
f_E[b]	100%	96%	94%	93%	91%

[a] d = equilibration time in days

[b] f_E = theoretical proportion of vitamin D_3:total vitamin D_3 at equilibrium

Table 2. Time/temperature intervals for simulation of saponification

	Simulated saponification temperature			
	25 °C	60 °C	70 °C	80 °C
Standards	13 × −80 °C 13 × 0 °C 13 × 10 °C 13 × 20 °C 13 × 30 °C	13 × −80 °C 13 × 0 °C 13 × 10 °C 13 × 20 °C 13 × 30 °C	13 × −80 °C 13 × 0 °C 13 × 10 °C 13 × 20 °C 13 × 30 °C	13 × −80 °C 13 × 0 °C 13 × 10 °C 13 × 20 °C 13 × 30 °C
Initial time (t_0)	0	0	0	0
Interval (t_I)	1 day	60 min	30 min	15 min
Final time (t_{13})	12 days	720 min	360 min	180 min

Table 3 Gradient procedure for chromatographic separation

Time (min)	Mobile phase composition (%)		
	A	B	C
0	15	75	10
16	0	90	10
17	15	75	10
25	15	75	10

A = water
B = methanol
C = acetonitrile

Table 4 Ratio of previtamin D_3 to vitamin D_3 in vitamin premixes and ingredients

Sample	Previtamin D_3	Vitamin D_3
Fat-soluble vitamin premix–1	8.3%	91.7%
Fat-soluble vitamin premix–2	8.1%	91.9%
Fat-soluble vitamin premix–3	5.6%	94.4%
Fat-soluble vitamin premix–4	5.9%	94.1%
Vitamin D_3 ingredient	8.0%	92.0%

Table 5 Theoretical bias for vitamin D_3 measurement in samples at 20 °C

Saponification		Sample equilibrated at 20 °C			Internal standard [a]			Ratio [b]	Potential bias [c]
		Initial	End sap [d]	Equilibrium	Initial	End sap	Equilibrium		
25 °C	*T* [e]	20 °C	25 °C	25 °C	−80 °C	25 °C	25 °C	0.949	−5.1%
	k_1 (min^{-1}) [f]	9.03×10^{-5}	—	1.57×10^{-4}	3.38×10^{-12}	—	1.57×10^{-4}		
	k_{-1} (min^{-1}) [g]	7.09×10^{-6}	—	1.40×10^{-5}	4.90×10^{-15}	—	1.40×10^{-5}		
	t (min) [h]	0	900	24747	0	900	26653		
	f [i]	93%	93%	92%	100%	98%	92%		
60 °C	*T*	20 °C	60 °C	60 °C	−80 °C	60 °C	60 °C	0.929	−7.1%
	k_1 (min^{-1})	9.03×10^{-5}	—	4.76×10^{-3}	3.38×10^{-12}	—	4.76×10^{-3}		
	k_{-1} (min^{-1})	7.09×10^{-6}	—	9.43×10^{-4}	4.90×10^{-15}	—	9.43×10^{-4}		
	t (min)	0	60	404	0	60	492		
	f	93%	91%	83%	100%	98%	83%		
70 °C	*T*	20 °C	70 °C	70 °C	−80 °C	70 °C	70 °C	0.947	−5.3%
	k_1 (min^{-1})	9.03×10^{-5}	—	1.11×10^{-2}	3.38×10^{-12}	—	1.11×10^{-2}		
	k_{-1} (min^{-1})	7.09×10^{-6}	—	2.68×10^{-3}	4.90×10^{-15}	—	2.68×10^{-3}		
	t (min)	0	60	187	0	60	216		
	f	93%	89%	81%	100%	94%	81%		
80 °C	*T*	20 °C	80 °C	80 °C	−80 °C	80 °C	80 °C	0.953	−4.7%
	k_1 (min^{-1})	9.03×10^{-5}	—	2.47×10^{-2}	3.38×10^{-12}	—	2.47×10^{-2}		
	k_{-1} (min^{-1})	7.09×10^{-6}	—	7.17×10^{-3}	4.90×10^{-15}	—	7.17×10^{-3}		
	t (min)	0	60	88	0	60	98		
	f	93%	82%	77%	100%	86%	77%		

a Either vitamin D_2 or stable isotope labelled vitamin D_3
b Ratio = f(sample) / f(internal standard) at end of saponification
c Bias = (1−(f(sample) / f(internal standard))) × 100
d End of saponification
e T = temperature
f k1 = isomerisation constant for reaction vitamin D_3→previtamin D_3 (min^{-1})
g k−1 = isomerisation constant for reaction previtamin D_3→vitamin D_3 (min^{-1})
h t = time (min)
i f = theoretical proportion of vitamin D3:total vitamin D3

Table 6 Potential maximum bias due to isomerisation of a sample equilibrated at 20 °C

Saponification temperature		25 °C	60 °C	70 °C	80 °C
Saponification time		12 h	60 min	60 min	60 min
$f_{(sample)}$ [a]	t_0 [b]	91.8%	91.8%	91.8%	91.8%
	t_S [c]	91.5%	88.6%	84.8%	80.7%
$f_{(internal\ standard)}$ [d]	t_0	100%	100%	100%	100%
	t_S	98.8%	93.9%	87.6%	81.2%
Ratio [e]		0.926	0.944	0.968	0.994
Bias [f]		−7.4%	−5.6%	−3.2%	−0.6%

[a] $f_{(sample)}$ = measured proportion of vitamin D_3:total vitamin D_3 in sample

[b] t_0 = initial time

[c] t_S = saponification time

[d] $f_{(internal\ standard)}$ = measured proportion of vitamin D_3:total vitamin D_3 in internal standard

[e] Ratio = $f_{(sample)} / f_{(internal\ standard)}$ at end of saponification

[f] Bias = $(1-(f_{(sample)} / f_{(internal\ standard)})) \times 100$

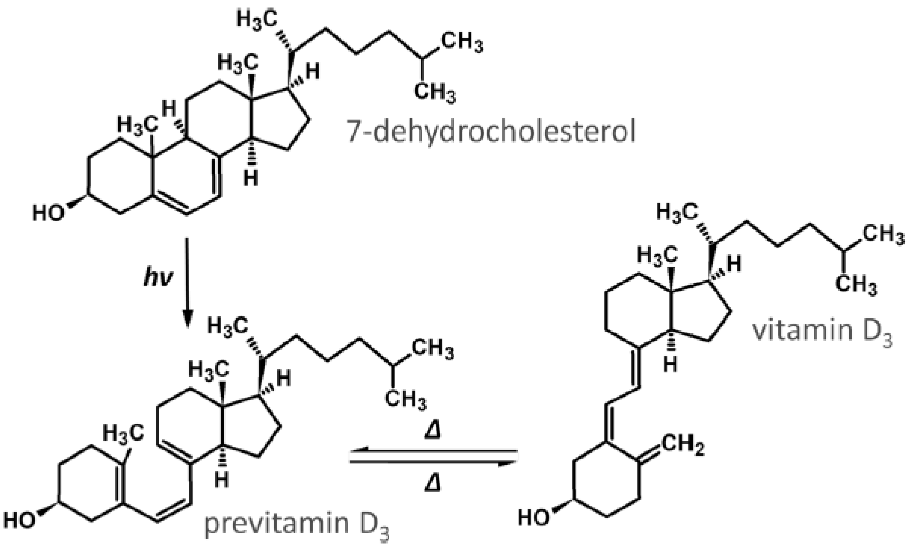

Figure 1. LC MS/MS MRM chromatograms of a mixed nucleotide and nucleotide

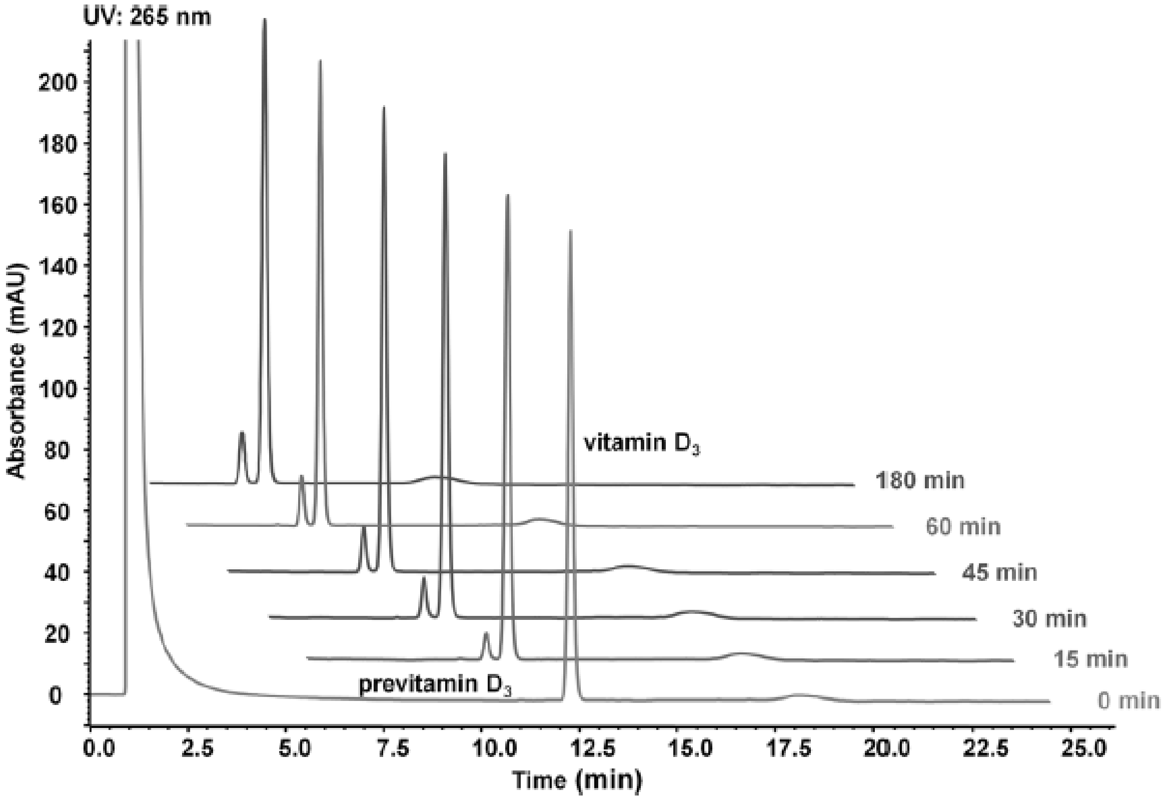

Figure 2. Chromatographic analysis of previtamin D_3 and vitamin D_3 in a standard heated to 80 °C

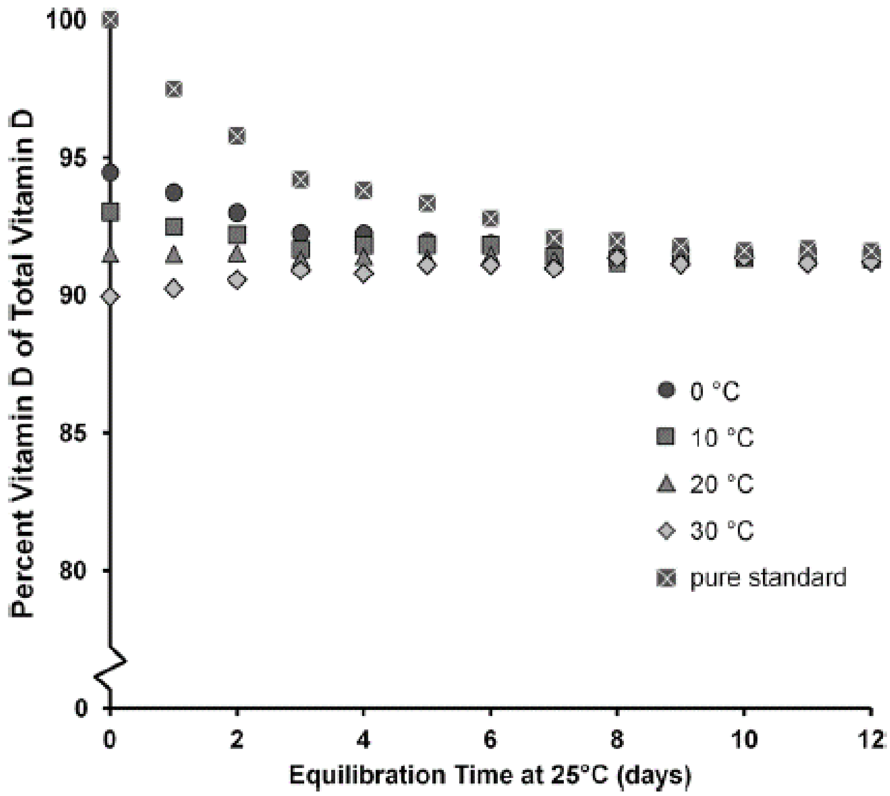

Figure 3. Vitamin D_3 percentage of total vitamin D_3 in equilibrated standards upon heating at 25 °C

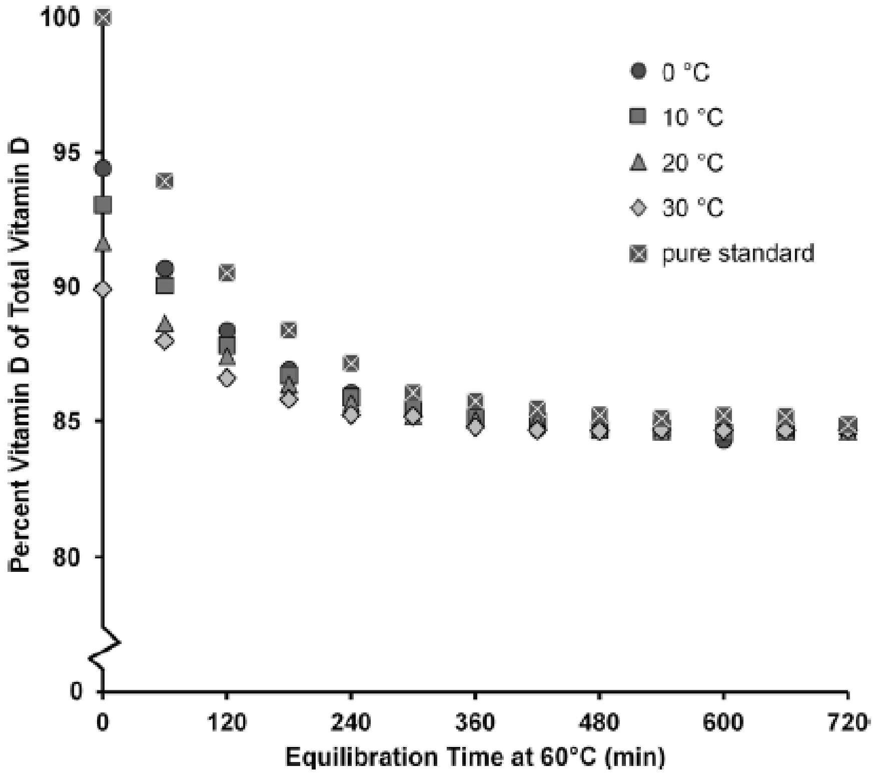

Figure 4. Vitamin D_3 percentage of total vitamin D_3 in equilibrated standards upon heating at 60 °C

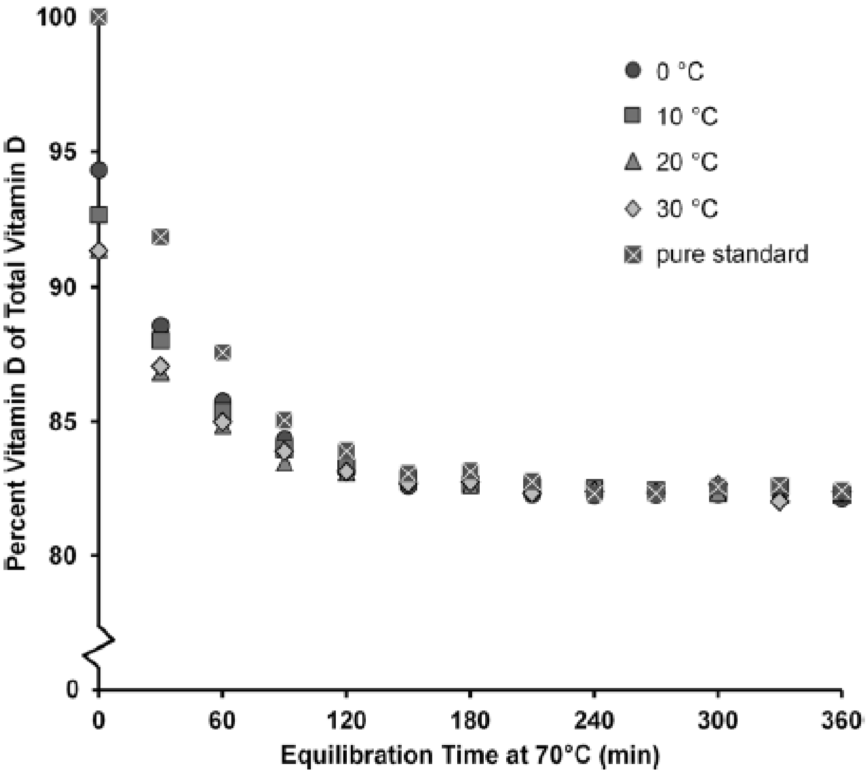

Figure 5. Vitamin D_3 percentage of total vitamin D_3 in equilibrated standards upon heating at 70 °C

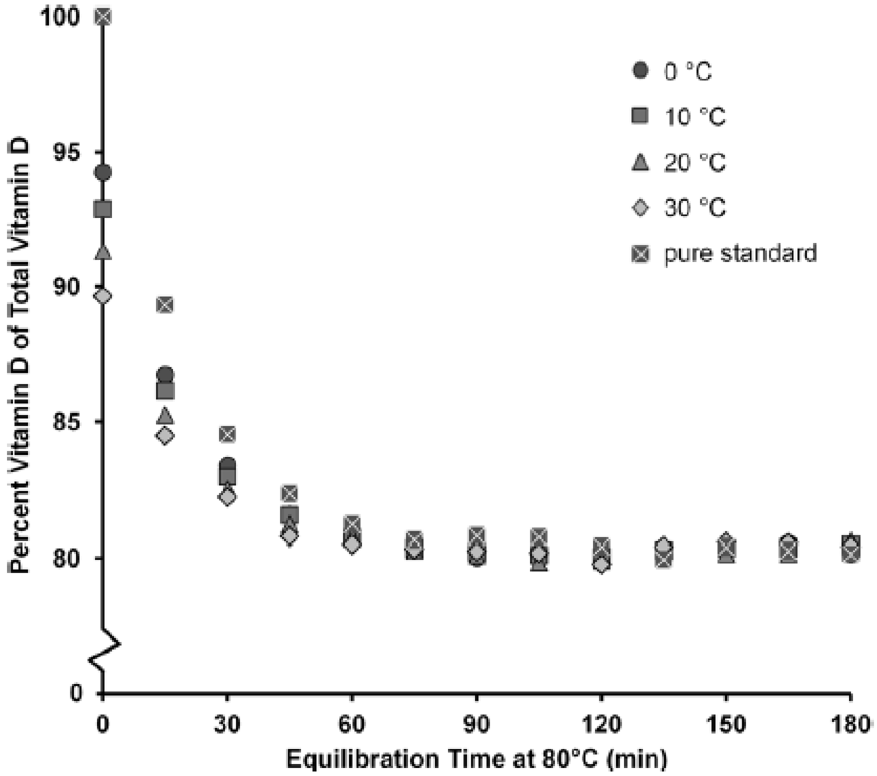

Figure 6. Vitamin D_3 percentage of total vitamin D_3 in equilibrated standards upon heating at 80 °C