Differential Thermal Isomerization: Its Role in the Analysis of Vitamin D_3 in Foods

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Abstract

Background: For nutritional purposes, the measurement of vitamin D_3 (defined as the sum of vitamin D_3 and previtamin D_3) is required to obtain an accurate and reliable estimate of its content in foods. An often neglected aspect in the development of methods for the analysis of vitamin D_3 is accounting for any potential analytical bias in the results associated with differential thermal isomerization between previtamin D and vitamin D. *Conclusions:* For LC-UV methods using a vitamin D_2 internal standard, cold saponification, or direct lipid extraction techniques should be avoided, unless chromatographic separation of vitamin D_2, vitamin D_3, and their previtamin forms is achieved so that UV absorbance corrections can be made. For both LC-UV and LC-MS methods using calciferol internal standards, the simplest solution to avoid analytical bias due to the presence of previtamin D is to utilize heating conditions (typically during saponification) such that previtamin D and vitamin D in the sample and the internal standard reach an equivalent equilibrium state prior to instrumental analysis. Only under such circumstances is the integration of previtamin D unnecessary to obtain accurate results for vitamin D_3. *Highlights:* A detailed discussion of the quantitation of vitamin D_3 in food with concise recommendations for avoiding measurement bias as a consequence of differential thermal isomerization.

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Introduction

Vitamin D has many health benefits and is pivotal in the formation of bone, and in maintaining calcium and phosphate homeostasis (1, 2). It also plays a role in non-bone related functions including cell differentiation, is associated with a lower risk of Alzheimer's disease, and may be used to delay the onset of different dementia diseases such as Parkinson's. A deficiency of vitamin D classically leads to rickets in infants and osteomalacia in adults and is further associated with an increased risk of cancers, insulin resistance, cardiovascular problems, and autoimmune pathologies (3–9).

In humans, vitamin D is acquired either via UVB (290–315 nm) light-induced biosynthesis of vitamin D_3 (cholecalciferol) from provitamin D_3 (7-dehydrocholesterol) in live skin epidermal cells or by the ingestion of vitamin D_3, vitamin D_2 (ergocalciferol), or to a lesser extent, vitamin D_4 (22,23-dihydroergocalciferol) in foods, either present naturally or supplemented (10). Vitamin D_4 is not common and its metabolite has only 60% of the bioactivity of the metabolite of vitamin D_3 in rats (11). Once generated *in vivo* or absorbed, vitamin D is transported to the liver by serum vitamin D binding protein, where it is hydroxylated to 25-hydroxyvitamin D. It is in this form that vitamin D is transported systemically before further hydroxylation in the kidney to form the hormonally bioactive metabolite 1,25-dihydroxyvitamin D.

Foods that contain naturally occurring vitamin D_3 include oily fish (12), meat and offal (13), eggs (14), and dairy products (13). Vitamin D_2 is produced in phytoplankton, terrestrial plants, yeasts, and other fungi by UVB irradiation of ergosterol (11, 15, 16). Vitamin D_2 and vitamin D_4 can be found in mushrooms that have been grown under exposure to UVB light (11, 17).

Vitamin D, as monitored by systemic 25-hydrovitamin D levels, is increasingly deficient in certain human populations because of reduced exposure to UVB light, caused by various factors such as latitude, age, skin pigmentation, time spent indoors, or covered skin because of religious beliefs or melanoma prevention (2, 18). In some countries, particularly those in northern latitudes, where significant levels of vitamin D are not likely to be formed endogenously, foodstuffs are typically fortified. The USA fortifies breakfast cereals, milk, and selected dairy products; in Canada and Scandinavia, butter, milk, and margarine are fortified. Germany and the UK have fortified breads and the UK also has fortified orange juice (19–22). Food was initially fortified with vitamin D_2; however, after it was discovered that 7-dehydrocholesterol could be isolated inexpensively from sheep lanolin, fortification with vitamin D_3 has been typically used in preference to vitamin D_2.

Given the nutritional significance of vitamin D, it is important to have accurate and precise methods to estimate its concentration in foods. There are many analytical challenges in the determination of

the vitamin D content in food; multiple forms of the vitamin are present in relatively low concentrations among an excess of structurally similar sterol-type compounds. New analytical techniques and numerous enhancements to established methods have been applied to improve the analytical performance in vitamin D analyses (23–26).

Complications uniquely associated with the effect of vitamin D-previtamin D isomerization on method accuracy have recently been reported (27–29), although this aspect of vitamin D analysis has commonly been neglected in the literature. This review examines the various techniques used in the analysis of vitamin D_3 in foods, identifies potential analytical bias that may result from differential thermal isomerization, and makes recommendations to mitigate them for future method development. Differential thermal isomerization is defined as the propensity of the internal standard and the sample analyte to contain different proportions of previtamin D at the time of analysis that is dependent on their relative thermal histories.

Thermal Isomerization

The interconversion between previtamin D and vitamin D occurs via a [1,7] antarafacial sigmatropic rearrangement. This thermally induced rearrangement is dynamically reversible, such that both forms coexist and interconvert, which makes the accurate analysis of vitamin D_3 in foods challenging due to changes in their relative proportions until equilibrium is reached (30). Although *in vitro* studies have shown that vitamin D-previtamin D isomerization is not affected by solvent, pH, or UV light (31), kinetic and thermodynamic studies have demonstrated that biological macromolecules may play a role in modulating this isomerization *in vivo* (32, 33). In addition, previtamin D_3 exists as two geometric isomers (5,6-s-*cis*-previtamin D_3 and 5,6-s-*trans*-previtamin D_3); however, only the former is in thermal equilibrium with vitamin D_3, with the latter being a precursor to non-antirachitic congeners (34) (Figure 1).

For nutritional purposes, this thermal isomerization necessitates the measurement of the sum of vitamin D_3 and previtamin D_3 to obtain an accurate and reliable estimate of the vitamin D_3 content in foods (35). This need to measure the sum of both forms was expressed by the SPIFAN Nutrients Expert Review Panel in its development of the vitamin D SMPR (36).

Internal standardization has been used almost exclusively in recent years to quantitate the vitamin D_3 content of foods, utilizing either vitamin D_2 for LC-UV methods or stable isotope labelled vitamin D_3 for LC-MS methods. The use of calciferol internal standards offers the advantage of both correcting for manipulative losses throughout the complex extraction process and compensating for losses caused by thermal isomerization to previtamin D (27, 37). However, it has been implicitly assumed

that the proportions of previtamin D in the internal standard and the sample are equivalent at the time of analysis, or that any proportional differences have little impact on the measured results. If this assumption is in error, a small but significant bias will result (28).

The impact of differential thermal isomerization on accurate quantitation in vitamin D analyses arises from a number of contributing factors: (i) the predisposition of the analyte and standards to undergo thermal isomerization; (ii) different contributions of isomers in the standards and the analyte at the time of analysis; and (iii) each isomer possessing distinct detection characteristics.

Analytical Techniques

Since the late 1970s, numerous methods for the analysis of vitamin D in foods have been published (23, 24, 38). The relatively low concentration levels of vitamin D in the presence of many potentially interfering compounds necessitate complex sample preparation procedures to isolate the vitamin D from the sample matrix. When coupled to highly specific and sensitive detection techniques, sample cleanup steps can be minimized. However, as vitamin D has neither a useful chromophore, natural fluorescence, nor strong electrochemical properties, various derivatization techniques have been applied prior to chromatographic determination.

The majority of the published methods for the analysis of vitamin D in foods do not make specific reference to previtamin D, and it can be difficult to assess whether it was aggregated in the results. Further, the majority of reported methods have omitted to identify the possible impact of previtamin D and its thermal isomerization upon the quantitative results obtained. Those few publications that do specifically refer to previtamin D have recognized the need to: (i) separate previtamin D forms from the parent vitamin D (39); (ii) pre-equilibrate the internal standard (40) or external standard (41) to room temperature; and (iii) ensure that the saponification conditions are sufficient to reach equilibrium (42), such that quantitative bias can be either corrected for or eliminated.

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Sample Preparation

Sample Extraction

Saponification is the most common technique that is used to remove lipids from foods and release vitamin D. The time/ temperature combinations for "hot" saponification are typically reported to be in the range 60–100 °C for 30–60 min, whereas "cold" saponification is generally performed at room temperature for up to 15–18 h. Both alternatives have commonly been applied to the analysis of vitamin D_3 in foods, and have been considered to be equivalent in performance (23, 24, 43). Due to the complex nature of vitamin B_{12} and the form in which it presents in milk and infant formula, sample preparation requires considerable care to ensure the precision of methods. Conventional preparation procedures generally include denaturation of the binding protein, conversion into the most stable form (CN Cbl) by reaction with cyanide ion, and concentration and separation by a clean-up technique. Conversion to CN-Cbl is a common sample preparation step regardless of what other determination techniques are employed; however, the continued utilisation of cyanide raises health and safety concerns in the workplace.

The cold saponification period of 15–18 h generally occurs overnight and tends to be more operationally convenient as it requires less equipment and its labor time is similar to that of hot saponification. Depending upon the total analysis time, approximately 2–3% of a pure calciferol internal standard that is added to a sample will isomerize to previtamin D. Most food matrices will have a significant proportion (> 5%) of previtamin D at the time of analysis, particularly vitamin D fortified foods that are stored at room temperature. The modest previtamin D content of the internal standard stored at low temperature is insufficient to correct for the higher endogenous previtamin D content in samples (28). Cold saponification has often been advocated to minimize changes in the previtamin D concentration in both the sample and the internal standard (44–46); however, the lack of a heating step to drive the equilibrium to a common endpoint will result in a significant difference in the ratio of previtamin D to vitamin D between the analyte and the internal standard at the time of analysis (28).

The hot saponification technique offers a reduced total test turnaround time compared with cold saponification. The increased thermal profile of this approach does increase the proportion of previtamin D in the sample and, where applicable, the calciferol internal standard. However, this will also result in a reduction in the differential contribution of previtamin between the internal standard

and the sample and, provided equilibrium at a given temperature is reached, this source of bias will be eliminated. Specific recommendations for time/temperature combinations to mitigate this bias have been reported (28) and, in general, temperatures above 80 °C for a minimum of 60 min should be used, with lower temperatures requiring an extended time.

Many methods recommend refluxing the sample for 30 min (39, 40, 47, 48) or 45 min (41, 49). However, such conditions are insufficient to establish thermal equilibrium, as boiling solutions of ethanolic hydroxide may not reach sufficiently high temperature (28, 50). Those methods that reflux aqueous hydroxide solutions (51, 52) will boil at higher temperatures, such that 30 min is sufficient for equilibrium to be attained (28).

Direct lipid extraction without saponification has rarely been reported in the analysis of vitamin D in foods (53, 54). As this extraction is performed rapidly at room temperature, the ratio of previtamin D to vitamin D in the sample and in the internal standard will scarcely change during analysis and any difference in the previtamin D fraction between the sample and the internal standard will be preserved into the instrumental phase.

Sample Cleanup

The complex nature of vitamin D analysis in food typically necessitates further sample clean-up prior to instrumental analysis. Both polar solid-phase extraction (39, 45, 49, 55–57) and/or normal phase semi-preparative chromatography (47–49, 55, 56) are commonly used techniques to fractionate sample extracts prior to final analysis.

These chromatographic modes utilizing a silica stationary phase do not separate vitamin D_2 and vitamin D_3 forms, making them ideal for the fractionation of crude sample extracts for subsequent quantitative analysis. However, previtamin D is typically well resolved from vitamin D under normal phase conditions, and thermal equilibration must be achieved prior to clean-up; otherwise the combined vitamin and previtamin fractions must be collected, adding to the complexity of subsequent reversed-phase chromatography.

Standardization

Calibration of Standards

Quantitative chromatographic analysis requires authentic substances to calibrate instrumentation in terms of retention times and detector response. For quantitative work, the purity of the standards needs to be known, which is not an easy undertaking with complex or unstable molecules. Vitamin D

is a well defined molecule and is available in crystalline form but is potentially contaminated with related substances, degradation products, and moisture. Depending upon the declared purity from the manufacturer, optimum accuracy necessitates the evaluation of the concentration and the purity of the standard by the analyst prior to use. Although previtamin D is commercially available for use as an analytical standard, once in solution, a significant proportion will rapidly isomerize to vitamin D, rendering it impractical for quantitative analysis.

UV spectrophotometry is a commonly used method for the calibration of vitamin D solutions. Extinction coefficients ($E^{1\%}$) that have been used to quantitate standard solutions of vitamin D_3 range widely, 450–490 dL/g.cm (58), with many different values reported: 473 dL/g.cm (59), 476 dL/g.cm (26), 485 dL/g.cm (60) at λ_{max} = 265 nm and, 476 dL/g.cm (61) and 485 dL/g.cm (43) at λ_{max} = 264 nm. In AOAC Official Methods 2016.05 (62) and 2002.05 (47), the ($E^{1\%}$) values used were 485 and 480 dL/g.cm, respectively. In contrast, AOAC Official Methods 992.26 and 995.05 (63, 64) used vitamin D_3 sourced from US Pharmacopeia and no correction was made for purity. A partial explanation for the wide range of cited extinction coefficients may be that, when crystalline vitamin D is dissolved in solvent, a proportion will inevitably isomerize to previtamin D, which has a considerably lower absorption coefficient (218 dL/g.cm) than vitamin D at 265 nm (65). Whether any such previtamin D contribution influenced the variability of the reported extinction coefficients is unknown. In routine laboratories, solutions of vitamin D are regularly stored and periodically recalibrated by UV absorbance, and a decrease in absorbance may suggest that degradation has occurred, rather than it being attributable to a continuous isomerization to previtamin D. For this reason, proper handling and storage of the vitamin D material and standards is required. Heating should not be used to facilitate dissolution in the preparation of stock standards, and all standard solutions should be stored at < 0 °C and replaced on a regular basis. Calibration by UV absorbance should be performed only when a standard is initially prepared, and recalibration should be precluded.

External Standardization

The use of external standardization in analytical methods that require complicated extraction techniques should be avoided, as manipulative losses throughout the procedure cannot be accounted for, potentially resulting in low bias. However, external standardization was predominantly used in the 1970s and 1980s for normal phase HPLC methods and, as a consequence, recoveries were typically in the range 80–95% (66–68). Despite this, external standardization has been used more recently in chromatographic methods utilizing electrochemical (69), mass spectrometric (70), and UV (51) detection.

To correct for quantitative recovery and bias caused specifically by the formation of previtamin D during external standard-based analysis, standards were stored at room temperature and were equilibrated until they contained 9% previtamin D (40). An alternative correction technique used with external standardization was to measure vitamin D_3 and make use of a correction factor to allow for losses through the formation of previtamin D3 (41). Although both of these approaches acknowledge the potential bias caused by vitamin D isomerization, and may have potentially mitigated it, other errors may have been introduced.

Vitamin D_2 Internal Standard

As reversed-phase column technology improved, it became possible to reliably separate vitamin D_2 from vitamin D_3. Because of its structural similarities, vitamin D_2 has commonly been used as an internal standard in vitamin D3 analysis by LC-UV (39, 45, 48, 55, 71–74) and LC-MS (75) methods. It has been assumed that the use of vitamin D_2 as an internal standard should eliminate the effect of previtamin D formation, since the isomerization rates of vitamin D_2 and vitamin D_3 are equivalent (27, 37). This assumption is correct when the contributions of previtamin in the sample and the internal standard are the same at the time of chromatographic analysis, which is not the case where the recent thermal histories of the sample and the standard are different (28).

As the molar UV absorbance at 265 nm of previtamin D_3 is approximately half that of vitamin D_3 (65), for accurate results obtained by LC-UV methods, it is necessary that the contributions of both previtamin D and vitamin D are independently determined whenever differential thermal isomerization is a factor. For this reason, it is recommended that vitamin D_2, vitamin D_3, and their previtamin forms be chromatographically resolved to achieve accurate quantitation of vitamin D_3 (27).

For LC-MS methods using vitamin D_2 as the internal standard, chromatographic separation of vitamin D_2 and vitamin D_3 and their previtamin forms may have consequences because matrix effects may suppress or enhance ionization, complicating any corrections of the quantitation of each peak and thereby making the accurate estimation of total vitamin D_3 problematic. However, the co-elution of vitamin D_2 with previtamin D_3, a common problem in LC-UV analyses, is easily avoided as both can be resolved by mass (75).

Non-Calciferol Internal Standards

Non-calciferol internal standards such as dodecanophenone (76) and dihydrotachysterol (77) have been used for the analysis of vitamin D in foods using LC-UV and LC-MS methods, respectively. These offer the usual advantages pertaining to correction for manipulative losses, as well as allowing for the

simultaneous measurement of both vitamin D_2 and vitamin D_3 if both are present in the sample. Quantitative bias due to differential thermal isomerization is avoided, provided that the peak areas for both previtamin D_3 and vitamin D_3 forms are measured independently so that any relative detector response can be corrected.

Stable Isotope-Labelled Internal Standards

LC-MS methods offer several advantages compared with LC-UV methods, although matrix effects contributing to ionization suppression or enhancement are a significant potential disadvantage. The addition of stable isotope-labelled forms of an analyte to the sample as internal standards is used to overcome this limitation. Being chemically identical to the analyte, isotope-labelled standards are ideal internal standards and elute from the column in the same environment as the analyte, which substantially avoids errors associated with ionization effects.

The commercially available tri-deuterated vitamin D (6,19,19-$^{2}H_3$ vitamin D_3) has commonly been used (78–81), including in AOAC methods 2011.11 and 2012.11 (82, 83). However, the location of the deuterium atoms is within the conjugated triene system that undergoes a hydrogen shift during the thermal isomerization between vitamin D and previtamin D. It was demonstrated that this internal standard contributed to significant quantitative bias because of differences in isomerization kinetics between the analyte and the internal standard (29). This problem can be easily avoided by using more recently available alternative stable isotope-labelled internal standards, such hexa-deuterated vitamin D (26,26,26,27,27,27-$^{2}H_6$ vitamin D_3) (53, 84–86) and carbon-13-labeled vitamin D (23,24,25,26,27-$^{13}C_5$ vitamin D_3), in which isotope labels are situated on the side-chain remote from the triene moiety.

Standard Addition

Standard addition has been suggested as an alternative calibration technique to stable isotope internal standardization and has thus been advocated for the analysis of vitamin D using LC-MS (87). However, the spiking of a sample with vitamin D_3 does introduce the need to consider the relative contributions of previtamin D_3 in the spiked standard and the sample. Whether an analytical bias is introduced is also dependent on sample preparation and the instrumental technique.

Instrumental Analysis

LC-UV Methods

Many of the methods used in the early 1980s utilized normal phase chromatography. Previtamin D is often well separated from vitamin D under normal phase conditions and this can be useful for measuring each form separately if needed (88). Reversed-phase chromatography became popular for vitamin D analysis with the development of reliable columns that are capable of the resolution of vitamin D_2 and vitamin D_3 and has become the most common chromatographic technique (40, 47, 49, 51, 56, 57, 77).

In LC-UV methods, optimum separation of vitamin D_2 and vitamin D_3 alone does not imply effective quantitation unless the previtamin peaks are also resolved, as the UV absorbances between each vitamin D and its previtamin form are substantially different (27, 65). It has been reported that the assumption of equivalence in the contributions of previtamin D from the vitamin D_2 internal standard and the sample vitamin D_3 is frequently invalid (28). Additionally, previtamin D rarely co-elutes with its parent form on C_{18} columns and, in an effort to maximize the resolution of vitamin D_2 and vitamin D_3, it is common that previtamin D_3 co-elutes with vitamin D_2. To obtain accurate and unbiased results under LC-UV conditions when using vitamin D_2 as the internal standard requires complete chromatographic resolution of vitamin D_2, vitamin D_3, and their respective previtamin forms, so that the appropriate detector response corrections can be made (27).

LC-MS Methods

In recent years, LC-MS has become increasingly used for the analysis of vitamin D in foods. Atmospheric pressure chemical ionization (APCI) has typically been used for the detection of vitamin D (77–80, 84, 89) since electrospray ionization (ESI) is less effective than APCI in generating ions in low polarity compounds. Although the direct analysis of underivatized vitamin D using ESI has been reported (70), it is most effective when applied following derivatization with a Cookson-type reagent such as 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), facilitating straightforward sample preparation and rapid analysis (53, 81, 85, 90).

Internal standardization is typically used in LC-MS to overcome potential problems with matrix effects, as well as to account for losses through sample preparation. LC-MS methods have the potential for analytical bias, depending on the relative ionization efficiencies for the analyte vitamin D and the internal standard. Internal standards can be isotope-labelled forms of the analyte, non-labelled isomers, or structurally related compounds. The ability of the internal standard to correct for matrix effects is often limited because of inherent differences with the analyte with respect to ionization,

chromatographic retention, and stability, and hence, non-labelled internal standards are typically used when suitable stable isotope-labelled forms are not available, or prohibitively expensive.

Although equivalence of the ionization efficiencies of vitamin D and previtamin D is generally assumed, such equivalence has rarely been demonstrated (84), and any difference in ionization will almost certainly lead to quantitative bias due to differential thermal isomerization (28). The use of a non-labelled internal standard may pose a risk from ionization effects depending upon the particular matrix and the extent of clean-up through analysis. This is particularly true for analytical methods that do not chromatographically resolve and quantitate previtamin D separately as part of the analytical procedure (27).

LC-MS methods using PTAD derivatization accurately quantitate vitamin D by detecting the selected precursor and product ions for the vitamin D adduct only and do not specifically quantitate the previtamin D adduct (53, 62, 81, 85). It is not necessary to quantitate the previtamin D adduct, provided that the heating profile during saponification is sufficient to avoid differential thermal isomerization (28, 42).

Conclusions

Any method for the quantitative analysis of vitamin D in foods will potentially incur a bias due to differential thermal isomerization of the internal standard compared with the analyte vitamin D in the sample. This condition is largely dependent on the initial state of isomerization, and the extent of thermal equilibration associated with the heating profiles during analysis.

For LC-UV and LC-MS methods using calciferol internal standards, the optimum strategy to avoid analytical bias due to the presence of previtamin D is to utilize conditions such that the saponification time is sufficient for the previtamin D and vitamin D in the sample and the internal standard to reach the same equilibrium state. Under such circumstances, the specific integration of the previtamin D peak is unnecessary to obtain accurate results for vitamin D.

Calibration of vitamin D standards using UV absorption should take into account possible thermal isomerization, which will lead to a reduction in absorbance. Standards should not be warmed to facilitate dissolution, the UV absorbance should be measured immediately, and all standard solutions should be stored at < 0 °C and replaced on a regular basis.

For LC-UV methods using a vitamin D_2 internal standard, cold saponification or direct lipid extraction techniques should be avoided, unless UV absorbance corrections are made following the

chromatographic separation of all four forms. It is important that co-elution of previtamin D_3 with vitamin D_2, a common feature of reversed-phase chromatographic methods, is avoided.

For LC-MS methods in which thermal equilibration of the internal standard and the analyte vitamin D has not been achieved, the equivalence of the ionization efficiencies of vitamin D and previtamin D needs to be demonstrated. In addition, the use of the conjugated triene labelled $^2H_3$-vitamin D_3 as an internal standard should be avoided for quantitative analysis, because its isomerization behavior differs from that of the unlabelled analyte vitamin D_3.

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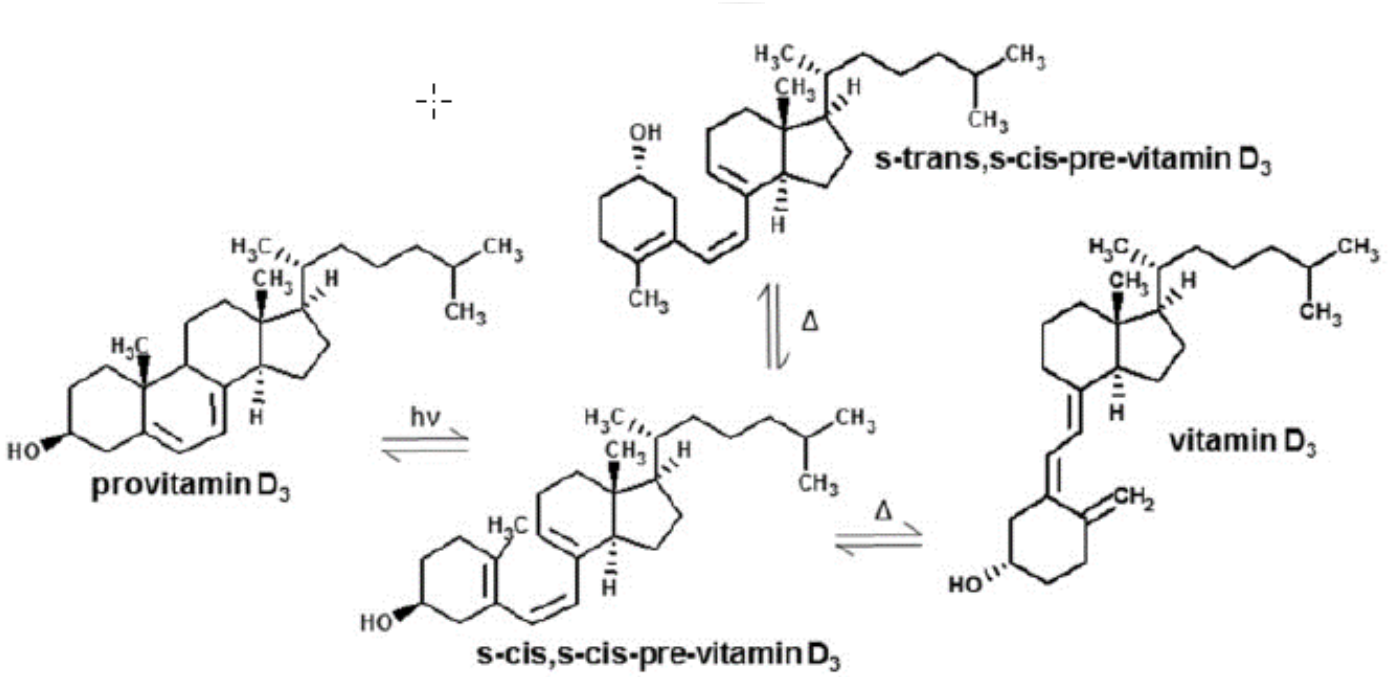

Figure 1. The formation of vitamin D_3 and previtamin D_3 from provitamin D_3