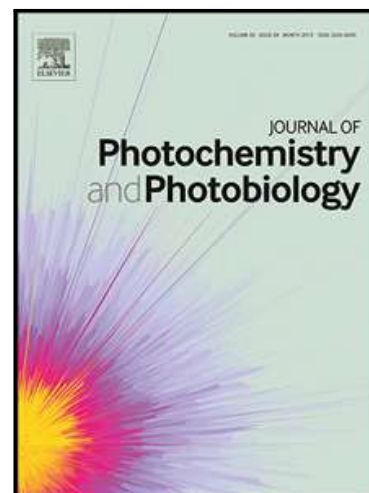


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Highlights

- Vitamin D₃ conversion is most efficient under shorter UVB/UVC wavelengths
- Vitamin D₃ conversion rate increases at higher temperatures
- UV absorbing compounds reduce vitamin D₃ conversion

An interplay of light and temperature: Vitamin D₃ formation *in vitro*, a model for *in vivo* plant studies

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Abstract

Vitamin D deficiency is a global issue that requires attention, given its essential functions in the human body. The synthesis of vitamin D₃ in the human skin is subject to limitations related to the availability of UV radiation, which can be particularly limited at higher latitudes, especially during the winter months. Additionally, Vitamin D₃ can be acquired through diet. Given that most vitamin D sources are animal-based, the discovery of vitamin D₃ in plants is of particular interest to those following vegan or vegetarian diets. While the characteristics of vitamin D biosynthesis in the human skin are well established, there is a lack of knowledge regarding biosynthesis in plants. This study aimed to evaluate the influence of several factors, including light, temperature, and plant matrix compounds, on the vitamin D₃ conversion reaction. The formation of previtamin D₃ from 7-dehydrocholesterol (7-DHC) was demonstrated to be dependent on UVC and UVB light, while the subsequent formation of vitamin D₃ from previtamin D₃ was shown to be dependent on temperature. Exposure to longer UV wavelengths led to a relative increase in lumisterol content. Furthermore, a concentration-dependent effect of UV-absorbing compounds was observed. These novel insights into the formation of vitamin D₃ will underpin future strategies aimed at optimising vitamin D₃ content in crop species.

Keywords: UVB, lumisterol, tachysterol, cholecalciferol, photochemical reactions, model system

1. Introduction

The issue of adequate human nutrition remains unsolved, with the prevalence of undernourishment increasing from 8.0 to 9.8% of the world population between 2019 and 2021 [1]. Malnutrition can result not only from a lack of food, but also from an insufficient supply of essential nutrients. Vitamin D deficiency is a global issue, with approximately 40% of the European population being deficient in this vitamin [2].

Vitamin D is the only vitamin that can be synthesised by the human body and is therefore classified as a pseudo-vitamin. It is vital for the normal functioning of human physiology, specifically impacting the processes of calcium and phosphate metabolism [3, 4]. Hypovitaminosis can result in rickets (a condition affecting children) and osteomalacia (a condition affecting adults), as well as the decalcification of bones [5]. Vitamin D

hypervitaminosis can also occur, but only following excessive intake of vitamin D (mostly through supplements). Such hypervitaminosis can lead to hypercalcaemia, osteoporosis and renal failure [2].

Vitamin D exists in several chemical forms, the most common of which are vitamin D₃ (cholecalciferol) and vitamin D₂ (ergocalciferol) [6]. The structural difference between the two forms of vitamin D is a double bond and a methyl group on the carbon chain moiety of vitamin D₂ which is lacking in vitamin D₃. These structures originate from different precursor molecules. Vitamin D₃ is synthesised from 7-dehydrocholesterol (7-DHC), whereas vitamin D₂ is synthesised from ergosterol [3].

Ultraviolet (UV) light is essential for the conversion of vitamin D₃ precursor molecules into vitamin D₃ and is categorised into three distinct wavelength zones: UVC (200-280 nm), UVB (280-325 nm) and UVA (325-380 nm). While all UV radiation has the potential to be harmful to human and plant cells, the shorter UV wavelengths contain more energy per photon and are more harmful to organisms. To date, UVB light is reported to be the most effective wavelength-band for driving the conversion reaction of provitamin D₃ to vitamin D₃ [6].

In humans, the synthesis of vitamin D₃ occurs when UVB light from the sun reaches the epidermal layer of the skin, inducing the synthesis of vitamin D₃ [7]. Following the conversion of 7-DHC to vitamin D₃, the latter undergoes further hydroxylation to yield the bioactive forms calcifediol and calcitriol. The efficacy of the reaction in the skin is contingent upon a number of factors, including the skin phototype and age of the individual, as well as environmental variables such as latitude, seasonality and UV intensity and dose [8]. For example, during the winter months the UVB intensity at latitudes above 35° (north and south) is inadequate for the endogenous conversion of vitamin D₃.

Vitamin D₃ can also be obtained from animal-based foods including fatty fish, eggs, and cod liver oil, which all have a high content. Additionally, vitamin D₂ is biosynthesized in mushrooms as a result of UV exposure. Moreover, consumption of fortified foods, the best known of which is vitamin D fortified milk, can also help to avoid/treat Vitamin D deficiency [9]. The recommended daily vitamin D intake in the absence of endogenous vitamin D synthesis is 20 µg day⁻¹, as outlined in the New Reference values for Vitamin D by the German nutrition Society (DGE) [10]. The No Observed Adverse Effect Level (NOAEL) is 250 µg day⁻¹ as outlined by the EFSA Panel on Dietetic Products, Nutrition and Allergies (EFSA, 2021) [5].

As interest in vegetarian and vegan diets continue to grow, the importance of plant sources of vitamin D is becoming increasingly apparent. It has been demonstrated that plants can accumulate and contain vitamin D₃, vitamin D₅ (sitocalciferol) and vitamin D₇ (campecalciferol) [11]. So far, especially plants of the *Solanaceae* family, such as tomatoes, have been shown to contain vitamin D derivatives [6]. Biosynthetically, 7-DHC is produced as part of phytosterol metabolism, specifically in the pathway from cycloartenol to cholesterol [6]. During exposure to UV light, 7-DHC is not only enzymatically converted to cholesterol, but also photochemically converted to vitamin D₃ [6]. The precise relationship between the enzymatic and photochemical reactions during UV treatment is not yet fully understood. A study conducted by Li et al., (2022) [12] demonstrated that genetically modified tomatoes with higher 7-DHC content exhibited enhanced vitamin D₃ content following UV treatment.

The synthesis of vitamin D₃ from 7-DHC in humans is relatively well understood [7, 13]; however, the location of vitamin D₃ synthesis in plants, and its potential function, remain poorly understood [6]. It is also largely unknown how vitamin D₃ synthesis is affected by other metabolic changes in UV exposed plants. For example, in response to UV light, a plant accumulates UV-absorbing and antioxidant compounds to prevent UV penetration and damage. UV absorbing compounds, such as flavonoids, are localised in the leaf epidermis and chloroplast membrane [14]. Thus, it can be speculated that plant UV protection will, in turn, negatively affect the synthesis of vitamin D₃ in plants. Similarly, compounds with antioxidative properties are distributed throughout plant cell compartments [15], rendering them highly probable to interact with the vitamin D synthesis.

From a chemical perspective, the vitamin D₃ conversion reaction can be divided into two distinct phases. The initial step is the B-ring-opening reaction of 7-DHC to previtamin D₃, which is photochemically driven by UV radiation (Fig. 1) [16]. Subsequently, the [1,7]-sigmatropic hydrogen shift of previtamin D₃ to vitamin D₃ is thermally driven [16]. The yield of vitamin D₃ is contingent upon the efficiency of both steps of the conversion process. Once a balance between previtamin D₃ and vitamin D₃ is reached, the content of vitamin D₃ is stable. Furthermore, the occurrence of reaction by-products cannot be ruled out. 7-DHC can be photochemically converted to lumisterol₃ and previtamin D₃ can be thermally converted to tachysterol₃, which are the *trans*- and *cis*-isomers of 7-DHC and previtamin D₃, respectively [16, 17]. The quantity of by-products generated can influence the yield of vitamin D₃. These photo- and thermochemical conversion reactions are critical in determining vitamin D₃ content yet have only been characterised for human skin. It is currently unknown how these reactions determine *in planta* vitamin D₃ accumulation.

The aim of this study was to investigate the kinetics of vitamin D₃ synthesis reactions using varying levels of UV light and temperature. In addition, the influence of UV absorbing, and antioxidant compounds was evaluated using an *in vitro* system. The characterisation of vitamin D₃ biosynthesis reaction provides novel insights that can be used as a starting point to understand vitamin D₃ synthesis and accumulation in plants.

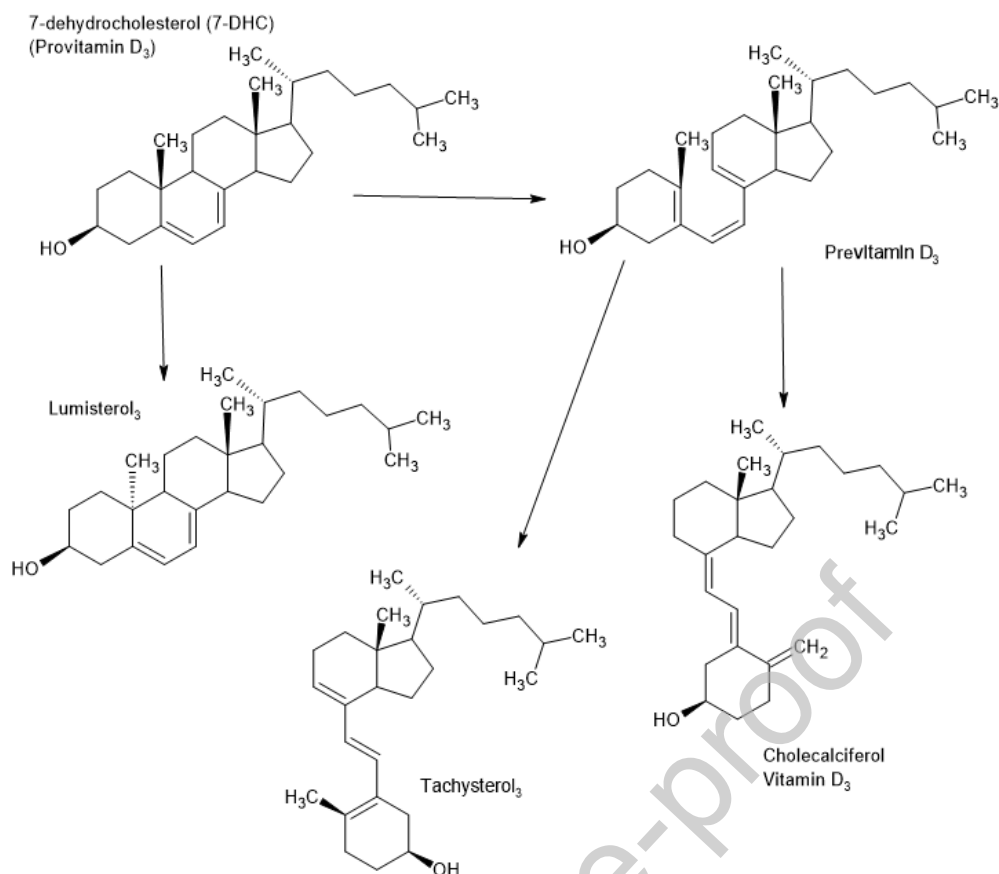


Fig. 1 Vitamin D₃ conversion reaction adapted from Okamura *et al.*, (1993) [16].

2. Material and methods

2.1 Chemicals

L-ascorbic acid (99%), apigenin ($\geq 95\%$), caffeic acid, cholecalciferol (vitamin D₃), *p*-coumaric acid, 7-dehydrocholesterol (7-DHC), *trans*-ferulic acid, gallic acid, isopropanol (for HPLC, 99.9%), reduced L-glutathione, and quercetin (95%, HPLC grade), were purchased from Sigma-Aldrich (Schnelldorf, Germany). Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Fisher Scientific (Dublin, Ireland). Tachysterol₃ (80%) and lumisterol₃ ($\leq 90\%$) were purchased from Toronto Research Chemicals (North York, Canada).

2.2 Experimental design: *in vitro* study

An *in vitro* system was developed to study how the vitamin D₃ conversion reaction is influenced by various factors namely, UV exposure conditions, PAR background, temperature and plant matrix compounds. This system monitored the conversion of 7-DHC into vitamin D₃ using a stock solution of 7-DHC in isopropanol (5 mg ml⁻¹) as a substrate for the reaction. The experiments were performed in two stages. Firstly, the reaction was performed in open, square 25-well plates (Fisher Scientific, Dublin, Ireland) for the UV exposure stage and secondly in closed 2 mL microtubes (Fisherbrand, Fisher Scientific, Dublin, Ireland) for the

temperature incubation stage. In all experimental setups, UV exposure was performed using broadband TL12 lamps (BB) (40 W, Phillips, Amsterdam, The Netherlands), except for the narrowband UV exposure, which was performed with TL01 lamps (NB) (315 nm, 40 W, Phillips, Amsterdam, The Netherlands). The experiments were conducted without a UVC blocking filter, unless otherwise stated. The light emission spectrum as well as the intensity of both UV lamps were measured with a FLAME-T-UV-VIS-ES spectroradiometer (Ocean Insights, Duiven, The Netherlands) (Fig. S1a, c). Each experiment was performed at least twice and with four to five replicate reaction vessels per experiment. After the UV exposure, samples were incubated at 40°C for 2h, unless otherwise stated.

2.2.1 Influence of UV dose

The samples containing the 7-DHC standard were exposed to four different UV intensities (UV1, UV2, UV3 and UV4) for three irradiation durations, resulting in twelve UV doses (Table 1). The set-up was such that the distance between the samples and the UV lamps varied, therefore the temperature was also measured and was found to vary between UV1 (25°C) and UV4 (31°C).

Table 1 UV intensities and dose for TL12 lamps.

	Irradiation duration [min]	UV intensity [mW m^{-2}]	UV dose [kJ m^{-2}]
UV 1	120	4.5	32.4
	240	4.5	64.8
	360	4.5	97.2
UV 2	120	7	50.4
	240	7	100.8
	360	7	151.2
UV 3	120	11.4	82.08
	240	11.4	164.16
	360	11.4	246.24
UV 4	120	15.8	113.76
	240	15.8	227.52
	360	15.8	341.28

2.2.2 Influence of UV spectrum and PAR background

Samples were exposed to UV light of different spectra emitted from TL12 lamps by means of different UV filtering films. The cellulose acetate filter (95 μm thickness; Kunststoff Folien Vertrieb GmbH, Hamburg, Germany) blocks mainly UVC light and emission is referred to as UVAB; the mylar (MY) filter (125 μm thickness; Tocana Ltd., Dublin, Ireland) blocks UVC and UVB light and emission is referred to as UVA; and the LEE filter (80 μm thickness; 226R LEE filter U.V., from QLX Lighting Ltd., Dublin, Ireland) blocks all UV light and emission is referred to as noUV (Fig. S1 and S2). The transmission of the filters was measured using a spectrophotometer (Genesys 50, Fisher Scientific, Dublin, Ireland) (Fig.

S2). Spectra, of TL12 lamps with the different filters were measured with a FLAME-T-UV-VIS-ES spectroradiometer (Fig. S1b, d, e). The UV intensities for the no filter (UVABC), cellulose acetate (UVAB), mylar (UVA) and noUV covered lamps were 11.1 mW m^{-2} , 10 mW m^{-2} , 6 mW m^{-2} and, 0 mW m^{-2} , respectively, with a radiation duration of 2 h (UV dose: 130 kJ m^{-2} , 125 kJ m^{-2} , 66 kJ m^{-2} and 0 kJ m^{-2}). To study the influence of the Photosynthetic Active Radiation (PAR) background, the samples were exposed to UV with and without additional PAR light. PAR intensities were measured using a PAR meter (SKR100, Skye instruments, Wales, UK) and were as follows: UVABC, $169 \mu\text{mol m}^{-2} \text{ s}^{-1}$; UVAB, $165 \mu\text{mol m}^{-2} \text{ s}^{-1}$; UVA, $155 \mu\text{mol m}^{-2} \text{ s}^{-1}$; and noUV, $160 \mu\text{mol m}^{-2} \text{ s}^{-1}$.

2.2.3 Comparison of narrowband UVB vs. broadband UV light

To further investigate the influence of the UV spectrum, the samples were exposed to UV using either a narrowband (NB) TL01 UVB lamp (315 nm) or a broadband UV (BB) TL12 (Fig. S1a, c). The UV intensity for both lamps was set to 10 mW m^{-2} (72 kJ m^{-2}) and the samples were exposed for 2 h. The temperature was monitored during UV exposure and was found to be: $23.3 \pm 0.2^\circ\text{C}$ for NB and $23.8 \pm 0.5^\circ\text{C}$ for BB.

2.2.4 Influence of temperature post UV exposure

To study the influence of the temperature on the conversion of previtamin D₃ to vitamin D₃, samples containing 7-DHC were exposed to broadband UV at 15.0 mW m^{-2} (108 kJ m^{-2}) for 2 h (mean temperature 29°C) and then incubated at one of five temperatures: 4°C , room temperature (RT), 30°C , 40°C , and 50°C . RT was measured throughout the experimental period and averaged 19°C . Samples were collected at eight time points over a 6-day experimental period at 0h, 1h, 4h, 6h, 24h, 48h, 72h and 144h.

2.2.5 Influence of temperature during UV exposure

To study the influence of temperature during UV exposure, samples were exposed to broadband BB UV (7 mW m^{-2} for 2 h, 84 kJ m^{-2}) in a heated water bath. A control, without an elevated temperature (7.7 mW m^{-2} for 2 h, 92.4 kJ m^{-2}), was set up with the same UV lamps. Temperature was measured both in the heated water bath ($38.6 \pm 0.4^\circ\text{C}$) and the unheated control ($29.2 \pm 1.3^\circ\text{C}$). To account for potential differences in evaporation from the open vessels at the two temperature regimes, the experiment was repeated without UV light exposure and a reference factor was calculated for the control vs. heated water bath (39°C). Additionally, after the UV exposure the samples were incubated at 40°C for 2 h. During this incubation the samples were sealed and thus no evaporation occurred. Samples were collected both before and after the incubation period (before IC, after IC respectively).

2.2.6 Influence of UV absorbing compounds

To study the influence of plant UV-absorbing compounds on the vitamin D₃ conversion reaction, four different UV absorbing compounds (quercetin, QCT; caffeic acid, CA; *p*-coumaric acid, pCoA and, ferulic acid, FA) were added to the *in vitro* system. The compounds were added in five different concentrations ranging from 0.75 mg mL^{-1} to

7.5 mg mL⁻¹, while the 7-DHC concentration was kept at 5 mg mL⁻¹. As a control, 7-DHC (5 mg mL⁻¹) was added to the system without any added UV-absorbing compound. The samples were exposed to 15 mW m⁻² (110 kJ m⁻²) BB UV radiation for 2 h. The levels of 7-DHC, previtamin D₃ and vitamin D₃ were measured before and after UV exposure.

To study the influence of plant antioxidant compounds on the vitamin D₃ conversion reaction, four different compounds with antioxidant activity (ascorbic acid, AC; dehydroascorbic acid, DHA; gallic acid, GA; L-glutathione reduced, GSH) were added to the *in vitro* system. AC, DHA, and GSH were freshly dissolved in water and added to the 7-DHC solution, resulting in a concentration of 1.5 mg mL⁻¹ for the antioxidant compounds and a concentration of 3.3 mg mL⁻¹ for 7-DHC. The control contained the same water/isopropanol ratio (30% v/v) and 7-DHC concentration, and no antioxidant was added. Gallic acid was added in five different concentrations increasing from 0.75 mg mL⁻¹ to 7.5 mg mL⁻¹, while the 7-DHC concentration was 5 mg mL⁻¹. GA was dissolved in isopropanol and 7-DHC was directly added to this solution. The samples were exposed to BB UV light for 2h at 12 mW m⁻² (87 kJ m⁻²). The 7-DHC, previtamin D₃ and vitamin D₃ contents were measured before and after UV exposure

2.3 Determination of sterols

All samples were filtered through a PTFE filter (Fisherbrand, Fisher Scientific, Dublin, Ireland) and then transferred to HPLC vials and measured immediately. The measurement was performed on an Agilent 1290 Infinity II HPLC equipped with a DAD detector (Agilent Technologies, Schnelldorf, Germany). The chromatographic separation was performed using an Ascentis C18 column (150 mm x 4.6 mm, 3 µm; Supelco, Sigma Aldrich Intl GmbH, Schnelldorf, Germany) and a mobile phase containing 95% A: ACN (+0.1% H₂O) and 5% B: MeOH. The flow rate was set to 1.1 ml min⁻¹ and an isocratic elution was used. 7-DHC was quantified at 282 nm and vitamin D₃ and pre-vitamin D₃ at 265 nm. Identification and quantification were based on an external calibration with authentic standards of 7-DHC, vitamin D₃, tachysterol₃ and lumisterol₃ (Fig. S3).

2.5 Statistical Analysis

Statistical differences were tested using either a student's t-test, a 1-way ANOVA or a 2-way ANOVA followed by the appropriate post-hoc test (indicated in each figure) ($p \leq 0.05$) applied to the data. Correlation analyses were performed using Pearson's method. Data are presented as means $\pm$ SEM, unless otherwise stated.

3. Results

3.1 Influence of light on the vitamin D₃ conversion reaction

3.1.1 Influence of UV dose

The influence of the UV dose (intensity $\times$ duration) on the vitamin D₃ conversion reaction was determined. Firstly, 7-DHC was exposed to four different UV intensities for three different irradiation durations. The findings revealed a decreasing 7-DHC content with an increasing UV dose, whereas the levels of pre- and vitamin D₃ content demonstrated a more

complex dose-response curve (Fig. 2). Previtamin D₃ and vitamin D₃ contents increased up to a UV dose of 82 kJ m⁻² and 65 kJ m⁻², respectively. However, UV doses above 151 kJ m⁻² led to a decrease in vitamin D₃ and previtamin D₃ content. Overall, correlation analysis revealed a negative correlation between the UV dose and the content of the three compounds (Fig. S4a-c). While the decrease of 7-DHC content with an increasing UV dose can be explained by the conversion reaction, vitamin D₃ content was shown to be lowered by high doses of UV exposure (Fig. S4). Lumisterol₃ content increased at UV doses up to 164 kJ m⁻² and then decreased rapidly, whereas tachysterol₃ was only present at UV doses from 228 kJ m⁻² upwards (Fig. S4d).

In summary, a higher UV dose does not automatically lead to a higher vitamin D₃ content, the most efficient UV dose was found to be between 82 kJ m⁻² and 151 kJ m⁻².

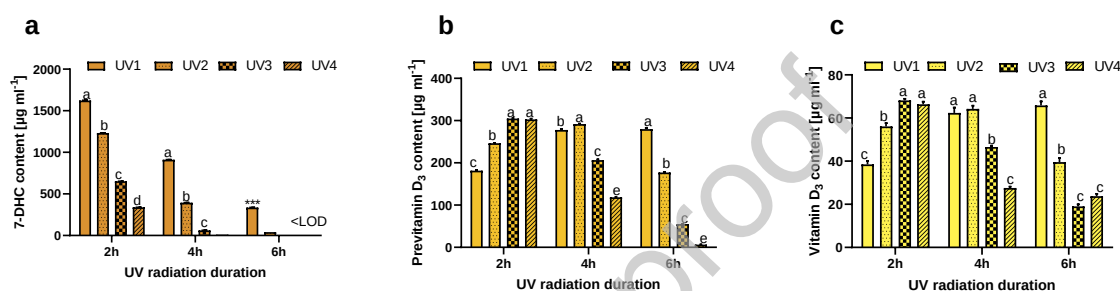


Fig. 2 Influence of UV dose on the vitamin D₃ conversion reaction. (a), 7-DHC content; (b), previtamin D₃ content; (c), vitamin D₃ content. Bars represent mean ± SEM, n = 5; letters indicate significant differences between the different intensities at one irradiation duration in alphabetical order from highest to lowest (1-way ANOVA followed by a post hoc Tukey's test, p ≤ 0.05, *** ≤ 0.001). UV doses (UV1, UV2, UV3 and UV4) are listed in **Table 1**.

3.1.2 Influence of UV light spectra altered using filters

In addition to the UV dose, the UV spectrum was hypothesised to be an important factor for the conversion of 7-DHC into vitamin D₃. To investigate the influence of the UV spectrum on the vitamin D₃ conversion, three UV-blocking filters (cellulose acetate – UVAB; mylar – UVA and UV blocking LEE filter – noUV) were used as well as no filter treatment (UVABC) (Fig. S1 and S2). UVABC showed the lowest content of 7-DHC, corresponding to the highest content of previtamin D₃ and vitamin D₃ (Fig. 3). This was followed by UVAB treatment, which showed a higher content of 7-DHC compared to UVABC, but a lower 7-DHC content compared to UVA and noUV treatments. The lower 7-DHC content of the UVAB treatment was reflected in higher previtamin D₃ and vitamin D₃ contents. UVA and noUV treatments showed almost no pre- and vitamin D₃ content, while UVA showed a significantly lower 7-DHC content than noUV.

In summary, the highest content of pre- and vitamin D₃ can be found when no filters were applied (UVABC). UVA and noUV treatments proved to be ineffective for vitamin D₃ conversion.

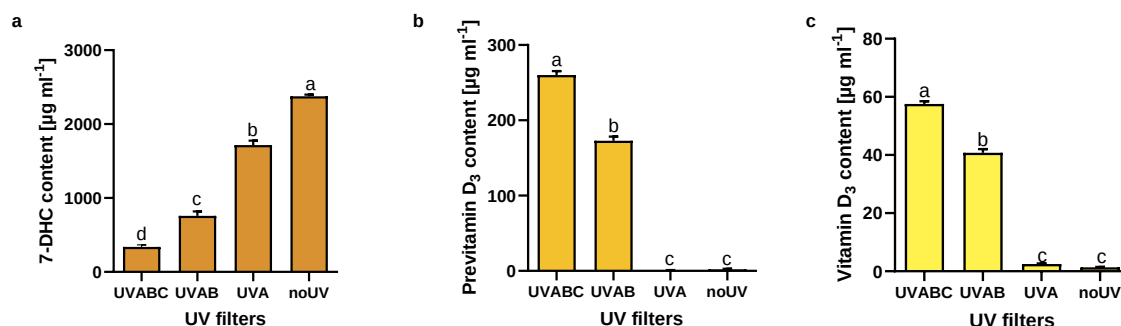


Fig. 3 Influence of UV filters that alter the UV lamp spectrum. (a), 7-DHC content; (b), previtamin D₃ content; (c), vitamin D₃ content. Bars represent mean $\pm$ SEM, $n = 5$; letters indicate significant differences between the filters in alphabetical order from highest to lowest (1-way ANOVA followed by a post hoc Tukey's test, $p \leq 0.05$). UVABC, no filter; UVAB (cellulose acetate filter); UVA (mylar filter); noUV (UV-blocking filter). UV transmission of the filters is shown in Figure S2. UV doses: UVABC, 130 kJ m^{-2} ; UVAB, 125 kJ m^{-2} ; UVA, 66 kJ m^{-2} ; noUV, 0 kJ m^{-2} .

3.1.3 Influence of background PAR light

To explore the influence of background PAR light on vitamin D₃ conversion, the same experimental setup as used for determining the effect of UV spectra was repeated with and without PAR light (-PAR, +PAR). To account for the heat generated by the additional light sources, the temperature was measured over the experimental period for both setups (Fig. S6). The 7-DHC content was higher without additional PAR (-PAR) light for all filters (Fig. 4a). Since the content of pre- and vitamin D₃ was very low under UVA and noUV treatments, no significant differences were observed between -/+ PAR. Under UVABC and UVAB treatments, higher previtamin D₃ contents were found in -PAR and this was matched by a higher vitamin D₃ content under UVABC (Fig. 4c, d). The lumisterol₃ content was increased under additional PAR light at UVAB (Fig. 4b).

In summary, additional PAR has an influence on the vitamin D₃ conversion in such a way that more 7-DHC is converted in the presence of PAR, while effects on pre- and vitamin D₃ content are modest.

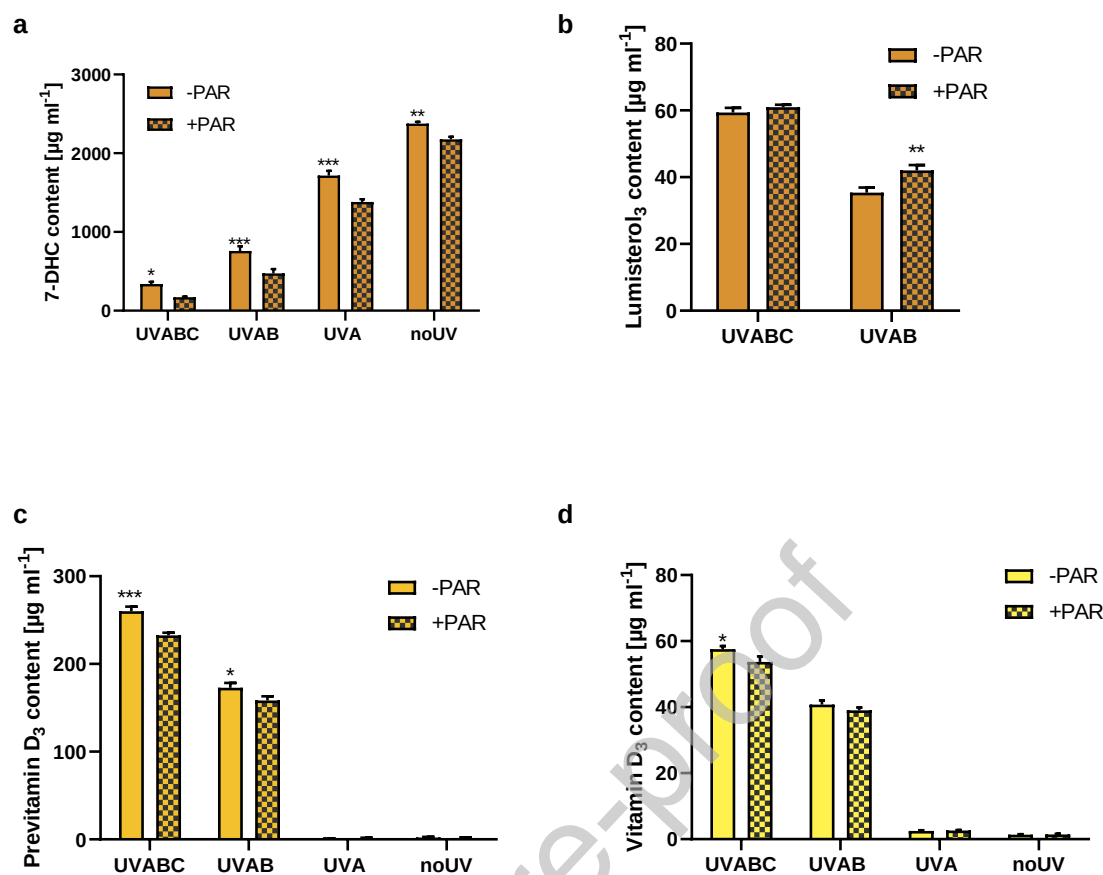


Fig. 4 Influence of PAR background light on vitamin D₃ conversion reaction with different UV blocking filters. (a), 7-DHC content; (b), lumisterol₃ content; (c), previtamin D₃ content; (d), vitamin D₃ content. Bars represent means $\pm$ SEM, $n = 5$. Asterisks indicate significant differences between with and without background PAR light (2-way ANOVA followed by a post hoc Šidák correction, $*\leq 0.05$, $**\leq 0.01$, $***\leq 0.001$). UVABC, no filter; UVAB (cellulose acetate filter/CA); UVA (mylar filter/MY); noUV, (UV-blocking filter).

3.1.4 Comparison of narrowband vs. broadband UV light

To study the differences between broadband and narrowband UV, samples were exposed to either a NB UV lamp (max. ABS: 315 nm) or a BB UV lamp (max. ABS: 300 - 325 nm). The content of previtamin D₃ and vitamin D₃ was 5.3- and 6.4-fold higher under the BB lamps compared to the NB lamps (Fig. 5c, d). This was also reflected in the yield, with the reaction under BB converting 4% of 7-DHC into vitamin D₃, 21% into previtamin D₃, 17% into lumisterol₃ and 58% into unknown reaction products. The reaction under NB UV converted only 1% of 7-DHC into vitamin D₃, 4% into previtamin D₃, 10% into lumisterol₃, but into 85% of unknown reaction products (Fig. 5e).

In conclusion, the conversion of vitamin D₃ from 7-DHC was less effective under NB UV light than under BB UV light.

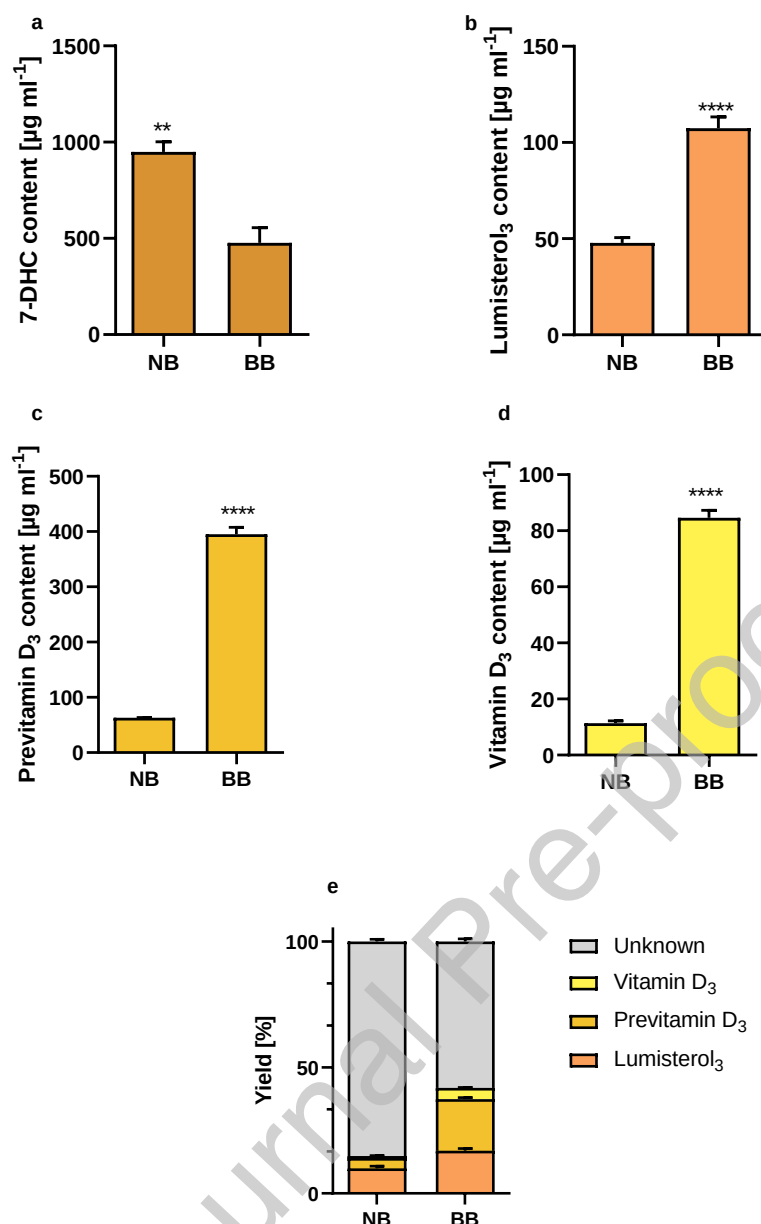


Fig. 5 Effect of narrowband and broadband UV lamps on the vitamin D₃ conversion reaction. (a), 7-DHC content; (b), lumisterol₃ content; (c), previtamin D₃ content; (d), vitamin D₃ content; (e), reaction yield. Bars represent mean $\pm$ SEM, n = 5. Asterisks indicate significant differences between narrowband and broadband lamps (unpaired t-test, two-tailed, $** \leq 0.01$, $**** \leq 0.0001$).

3.2 Influence of temperature on the conversion reaction

3.2.1 Effect of temperature post UV radiation on the conversion of previtamin D₃ to vitamin D₃

To test the influence of temperature on the conversion of previtamin D₃ to vitamin D₃, previously UV-exposed 7-DHC samples were incubated at different temperatures for a period of up to 144 hours. The results showed that the previtamin D₃ content decreased with

increasing temperature and time, corresponding to the increase in vitamin D₃ content (Fig. 6b, c). Depending on the temperature, the equilibrium of the reaction was reached after a certain time point. The higher the temperature, the faster the equilibrium was reached. At both 40°C and 50°C, the highest content of vitamin D₃ was reached after 48 h. The greatest differences in vitamin D₃ content were found between 4°C and 20°C, with up to 3.5-fold higher vitamin D₃ content at 20°C.

In summary, a higher temperature increases the conversion rate of previtamin D₃ to vitamin D₃ (Table S1).

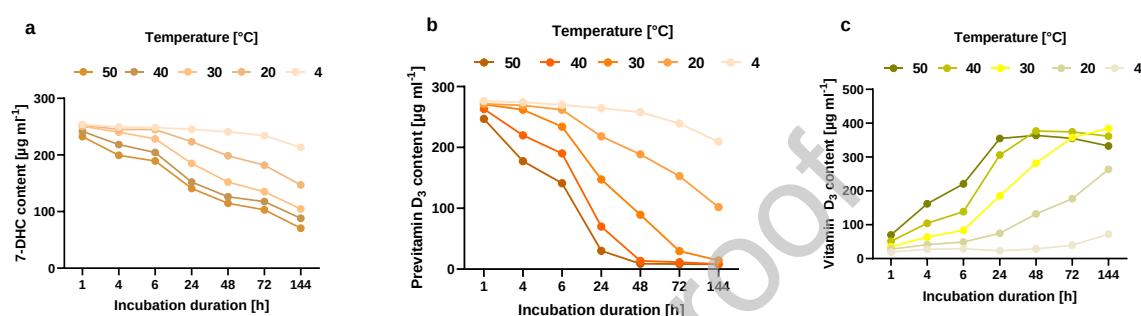


Fig. 6 Influence of temperature on conversion of previtamin D₃ to vitamin D₃. (a), 7-DHC; (b), previtamin D₃; (c), vitamin D₃ contents. Points represent mean $\pm$ SEM.

3.2.2 Interaction of UV light and temperature on the conversion reaction

To test the hypothesis that an elevated temperature during UV exposure affects vitamin D₃ conversion, the samples were exposed to UV light at 39°C in a heated water bath. This was compared to the control condition. The results showed no effect of increased temperature on 7-DHC, lumisterol₃ and previtamin D₃ contents either during the UV treatment or the subsequent incubation period (Fig. 7 a, b, c). However, the vitamin D₃ content was increased by 4-fold after UV exposure in a heated water bath (39°C) (Fig. 7 d, before IC). In comparison, the increase of vitamin D₃ content when samples were incubated at 40°C after the UV-treatment, was 2-fold (Fig. 7 d, after IC). This is also reflected in an increased reaction rate at 40°C (Table S2). To exclude any side effects of the temperature, not converted 7-DHC samples (i.e. those that had not been UV exposed) were heated at 40°C and no changes were observed (Figure S7).

In summary, increased temperature increases the reaction rate and therefore the vitamin D₃ content during UV exposure.

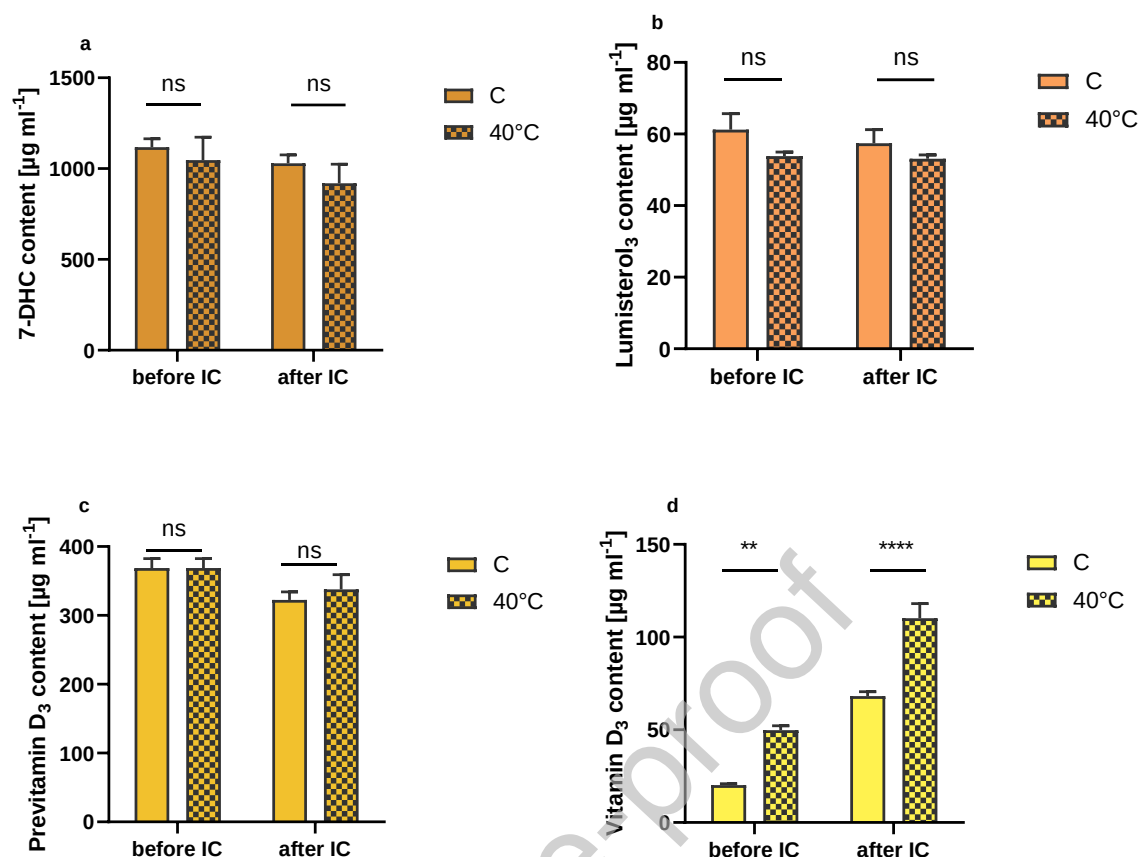


Fig. 7 Influence of temperature on the conversion reaction during UV exposure (before IC) and following an incubation period (after IC). (a), 7-DHC content; (b), lumisterol₃ content; (c), previtamin D₃ content; (d), vitamin D₃ content. Bars represent mean $\pm$ SEM, $n = 5$. Asterisks indicate significant differences between control and 40°C (2-way ANOVA followed by a post-hoc Tukey's test, $** \leq 0.01$, $*** \leq 0.001$).

3.3 Influence of plant matrix compounds on the conversion of vitamin D₃

3.3.1 Influence of UV-absorbing compounds

To explore the influence of UV-absorbing compounds on the vitamin D₃ content, four different UV-absorbing compounds were added to the *in vitro* system in five different concentrations while the initial 7-DHC concentration remained constant. All absorbing compounds influenced the pre- and vitamin D₃ content (Fig. 8). The addition of quercetin increased the pre- and vitamin D₃ content at the lowest concentration but decreased the pre- and vitamin D₃ content at the three highest concentrations compared to the control (Fig. 8a). *p*-Coumaric, caffeic, and ferulic acid decreased the pre- and vitamin D₃ content at all concentrations (Fig. 8b, c, d). Correlation analysis revealed a negative correlation between the concentration of the UV-absorbing compounds and the pre- and vitamin D₃ content (Table S3). Thus, a higher concentration of UV-absorbing compounds leads to a lower vitamin D₃ content. The lumisterol₃ content was reduced compared to the control for all additions of UV-absorbing compounds. For *p*-coumaric, caffeic and ferulic acid additions,

lumisterol₃ was formed only at the two highest concentrations. When quercetin was added, lumisterol₃ was also formed at intermediate concentrations.

In summary, UV-absorbing compounds influence the vitamin D₃ conversion depending on their concentration in the *in vitro* system.

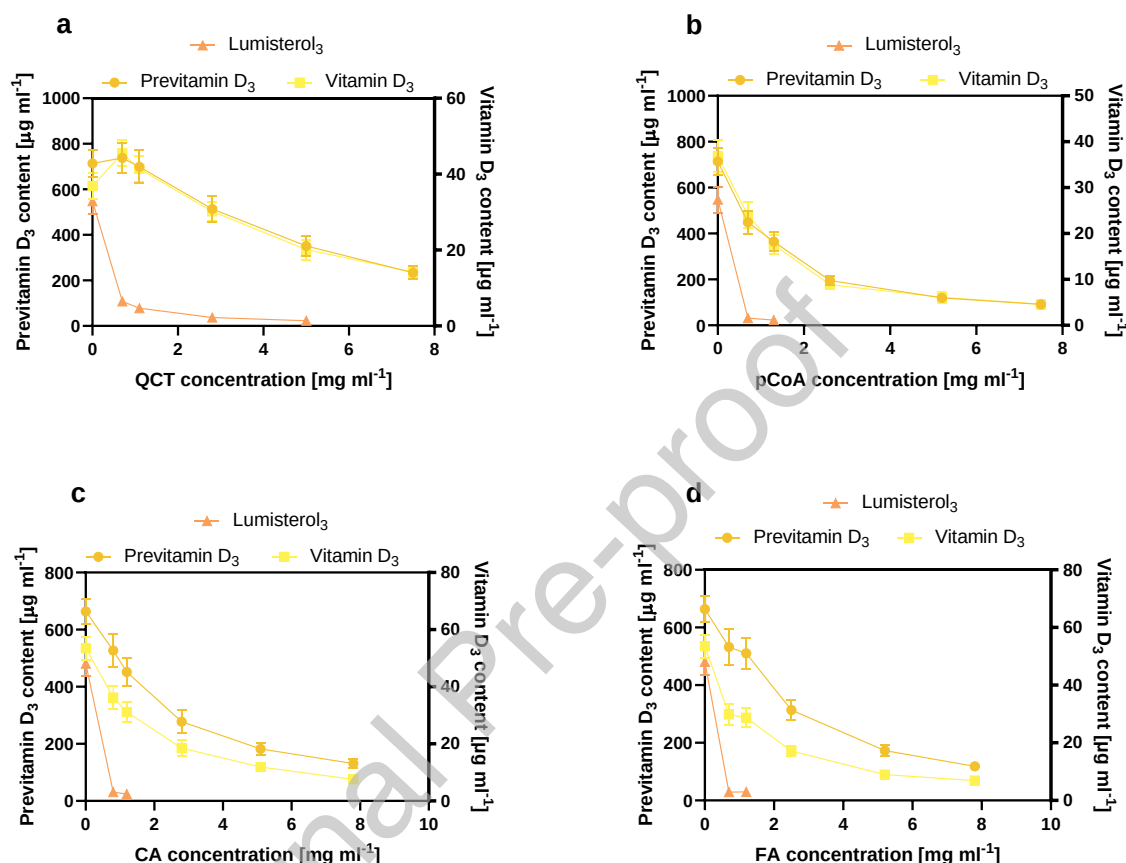


Fig. 8 Influence of absorbing compounds on the vitamin D₃ conversion. Content of vitamin D₃, pre-vitamin D₃ and lumisterol₃ content influenced by: (a), quercetin (QCT); (b), *p*-coumaric acid (pCoA); (c), caffeic acid (CA); (d), ferulic acid (FA). Points represent mean $\pm$ SEM. Correlation analysis can be found in Table S3.

3.3.2 Influence of antioxidative compounds

To investigate the influence of antioxidants on the vitamin D₃ conversion, different antioxidative compounds were added to the *in vitro* system. In the first experiment, three different antioxidative compounds (AC, DHA and GSH) were added and their effects compared to a control with no added antioxidative compounds but the same 7-DHC concentration. In a second experiment, gallic acid was added at five different concentrations and a zero-concentration was used as a control.

Firstly, the yield of vitamin D₃ increased with the addition of AC and particularly with a combination of AC+GSH (Figure 9a). Previtamin D₃ content was decreased by GSH and DHA additions compared to the control (Figure 9b).

Secondly, the effects of gallic acid (GA) on previtamin D₃ and vitamin D₃ differed. While the content of previtamin D₃ showed a decrease with increasing GA concentrations, the yield of previtamin D₃ remained consistent across all concentrations (Fig 9c, Fig S9a). This was also demonstrated by a negative correlation between previtamin D₃ content and GA concentration, and by the absence of a correlation between previtamin D₃ yield and GA (Table S4). This can be explained by changes in the converted 7-DHC, which displayed a negative correlation with the GA concentration (Figure S9b, Table S4). In contrast, no correlation was observed between vitamin D₃ content and GA concentrations (Fig. S9c, Table S4). Conversely, an increase in vitamin D₃ yield was noted with increasing GA concentrations (Fig. S9a). Similarly, lumisterol₃ and tachysterol₃ showed a GA concentration-dependent change in content. However, lumisterol₃ content decreased with increasing GA (Figure 9d), whereas tachysterol₃ increased with increasing GA. Digging deeper, the yield of vitamin D₃ and previtamin D₃ was influenced in different ways (Figure S9a).

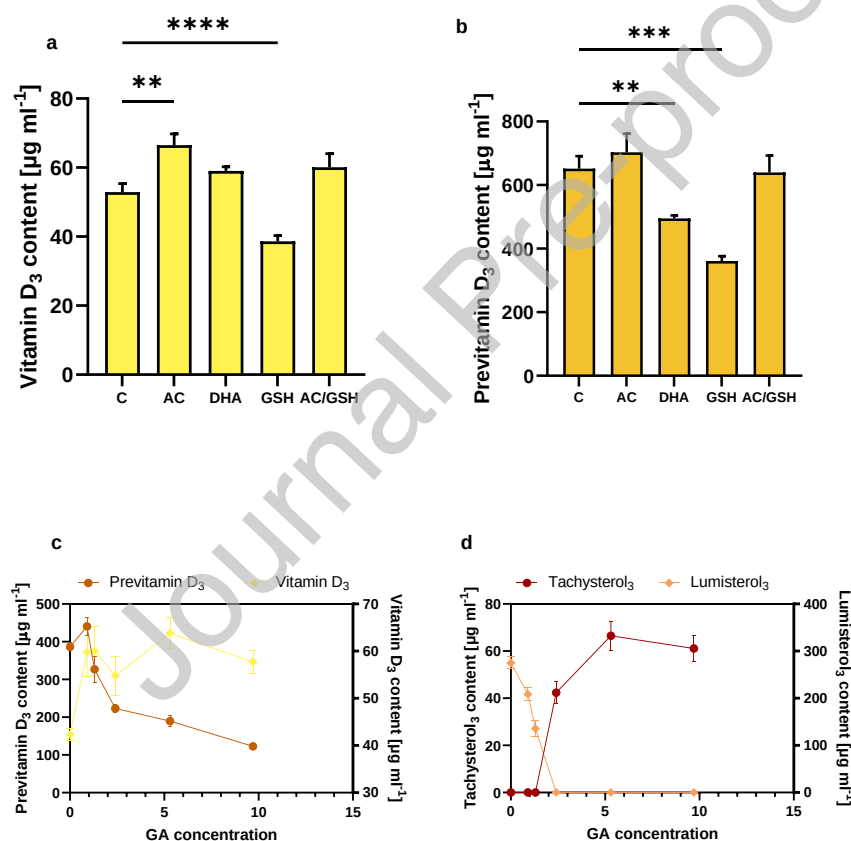


Fig. 9 Influence of antioxidative compounds on the vitamin D₃ conversion. Influence of different antioxidative compounds on (a), vitamin D₃ content, and, (b), previtamin D₃ content. Influence of different concentrated gallic acid on (c), vitamin D₃ and previtamin D₃ content and (d), tachysterol₃ and lumisterol₃ content. (a), (b), bars represent mean $\pm$ SEM, asterisks indicate significant differences to the control (1-way ANOVA followed by a post-hoc Dunnett's test, $p \leq 0.05$); (c), (d), points represent mean $\pm$ SEM. Correlation analysis for

(c), and (d) can be found in Table S4. AC, ascorbic acid; DHA, dehydroascorbic acid; GSH, glutathione (reduced); GA, gallic acid.

4. Discussion

The aim of this study was to investigate the effect of various factors on the kinetics of vitamin D₃ conversion in an *in vitro* system, as a model to gain insight into *in vivo* plant studies. The initial step involved investigating, the influence of UV and PAR light. Secondly, the influence of temperature and thirdly, the influence of plant matrix compounds was investigated. In accordance with the literature, it was demonstrated that the initial reaction from 7-DHC to previtamin D₃ is a UV-driven reaction, while the subsequent reaction from previtamin D₃ to vitamin D₃ is a thermal-driven reaction [16].

4.1 Influence of light

The first investigation indicated the presence of a complex dose-response curve, with UV light exerting distinctive effects on vitamin D₃ accumulation at low doses, in comparison to high doses. A narrow range of efficacious doses was identified. The complex dose-response is likely caused by competing UV effects. Firstly, the reaction requires a certain amount of activation energy, which must be reached. Lower doses are unable to reach the requisite energy level, rendering them insufficient. Secondly, at higher doses, UV radiation results in a depletion of vitamin D₃ (Fig. S5). The photodegradation of vitamin D₃ was reported for sunlight, and the main products of photolysis were identified as suprasterols and *trans*-vitamin D₃ [18]. Furthermore, the formation of lumisterol₃ and tachysterol₃ was observed to be dependent on UV dose. This is consistent with previous literature, which demonstrated the formation of lumisterol₂ and tachysterol₂ during UV radiation [17]. The formation of lumisterol₃ was observed to occur at lower UV doses, with the extent of this occurrence dependent on the 7-DHC content. This indicates that lumisterol₃ is derived from 7-DHC. At the higher UV doses, tachysterol₃ is formed from previtamin D₃. However, it is also possible that tachysterol₃ is formed from lumisterol₃.

In terms of spectral responses, the maximal yield was achieved in the absence of any filter, which means that samples were exposed to a UVA, UVB and the small amount of UVC emitted by UVB tubes. The cellulose acetate filter is designed to filter-out UV light with a wavelength below 300 nm, which results in a reduction in the availability of shorter wavelength UV light [19]. As the small amount of UVC corresponds to the maximal absorbance of 7-DHC around 270-280 nm [20], filtering out these wavelengths with a cellulose acetate filter results in a *de facto* reduction in the efficacy of the conversion of 7-DHC to previtamin D₃. In plant photobiology the cellulose acetate filter is typically employed to exclude UVC, and hence deleterious effects on plants. Thus, there is a conflict-of-interest between induction of vitamin D₃, and prevention of plant stress. Nevertheless, recent studies have demonstrated the potential of UVC radiation in plant cultivation, particularly for enhancing stress tolerance and reducing pest infestation [21, 22].

UVB in the presence of the cellulose acetate filter also effectively mediates conversion of 7-DHC to vitamin D₃. However, the results clearly demonstrated that UVA (in the presence of a mylar filter) exhibited a markedly inefficient performance, resulting in a 300-fold lower vitamin D₃ content relative to the corresponding UVABC treatment.

A comparison of the UV spectra also revealed that broadband UVB spectra were more efficacious than narrowband spectra in driving formation of vitamin D₃. As equal intensities were used, this difference can be attributed to the differing wavelengths. The TL01 lamp exhibits a peak intensity at 315 nm and lacks UV light below 300 nm, whereas the broadband lamp emits irradiance below 300 nm (Fig. S1). Thus, these data are consistent with those obtained through the use filters, which shows the relative effectiveness of shorter UV wavelengths in driving vitamin D₃ formation. However, it is noteworthy that the use of the narrowband lamp resulted in a higher content of lumisterol₃ relative to previtamin D₃. This suggests that at higher UV wavelengths, corresponding to lower energy, the reaction is favoured in the direction of lumisterol₃ rather than previtamin D₃, i.e. a higher ratio between lumisterol₃ and previtamin D₃. This finding is consistent with the data observed using different UV spectra, i.e. UVAB yields a lumisterol₃/previtamin D₃ ratio of 1:2.5 while in the absence of any filter the lumisterol₃/previtamin D₃ ratio is 1:4 (Table S5). When additional PAR is used the lumisterol₃/previtamin D₃ ratio is even smaller, 1:1.2 and 1:1.4 for UVAB and no filter, respectively. This means that the lower the energy the more lumisterol₃ relative to previtamin D₃ is formed, which suggest that higher energy/shorter wavelength are favourable to a higher previtamin D₃ content. Under a narrowband UV source, the lumisterol₃ content is lower compared to under a broadband UV source. This is due to a higher yield of all vitamin D₃ related compounds under a broadband lamp. This leads to the hypothesis that a narrowband UVC lamp, ideally with an emission peak at 275 nm the absorption maximum of 7-DHC, could result in a higher yield of previtamin D₃, due to a decrease in the formation of lumisterol₃. The use of shorter wavelengths and narrowband UV lamps, for example to increase disease resistance or enhance metabolite profile [23], is an emerging field that is expected to undergo significant development in the near future. The utilisation of those narrowband UV LED lamps with an emission wavelength of approximately 270 to 280 nm represents a promising avenue of research on vitamin D₃ metabolism.

Additional PAR light resulted in a reduction in the efficacy of the vitamin D₃ conversion process, accompanied by an increase in the quantity of by-products. This indicates that a greater quantity of energy in the visible wavelength range is responsible for the generation of by-products rather than vitamin D₃. In detail, 7-DHC is converted to lumisterol₃ rather than to previtamin D₃. In conclusion, the additional PAR results in a higher conversion of 7-DHC, but not in previtamin D₃ content.

4.2 Influence of temperature

It was demonstrated that a higher temperature following UV treatment resulted in a greater conversion of previtamin D₃ to vitamin D₃. This finding aligns with literature [24], which indicates that this stage of the reaction is temperature-dependent. It can be shown that the reaction follows the RTG rule, which states that with a 10°C increase in temperature, the reaction rate doubles. The reaction rate appears to be slowing down over time, which suggests that an equilibrium may be reached. An equilibrium is reached more rapidly at

higher temperatures than at lower temperatures. In the context of human skin or plant biology, the equilibrium of the reaction in question will most likely not be reached, given that vitamin D₃ is immediately bound to the vitamin D binding proteins (in humans) or glycosides and esters (in plants) [6, 24]. Tian et al. (2018) demonstrated that the conversion reaction from previtamin D₃ to vitamin D₃ in the human skin occurs at a faster rate than in a hexane model system [24]. The authors postulate that this is caused by the presence of highly ordered phospholipids in the human skin. Furthermore, an elevated temperature during UV treatment leads to an increased reaction rate and thus vitamin D₃ yield with unchanged content of lumisterol₃. In the human skin, the body temperature is approximately 36°C, which is high in comparison to the typical temperature of plants or mushrooms undergoing UV treatment. Further studies should be conducted to investigate the influence of temperature on vitamin D₃ conversion in poikilothermous plants, temperatures of which may vary widely.

4.3 Influence of plant matrix compounds

In considering the vitamin D₃ conversion reaction in plants, it is important to recognise that exogenous and endogenous factors combine to determine the reaction rate. In the case of the required UVB dose, both endogenous and exogenous factors interact closely. Seasonal variations in solar UVB can drive UV-acclimation responses that can, in turn, limit penetration of UV into plant tissues [25]. The main absorbing compounds in plants are derivatives of the phenylpropanoid pathway, including flavonoids and phenolic acids. For the purpose of this study, one flavonoid and three hydroxy-cinnamic acids were selected. It was demonstrated that the UV-absorbing compounds result in a reduction in the conversion of 7-DHC to previtamin D₃, with this effect being concentration-dependent. A correlation with the absorption maximum of the compounds was also noted, showing that UV-absorbing pigments are more effective in decreasing 7-DHC conversion (*p*-coumaric acid > ferulic acid > caffeic acid > quercetin) if their absorption maximum matches the absorption maximum of 7-DHC (275 nm).

In plants, flavonoids are accumulated in the vacuoles of leaf epidermal cells in order to protect underlying plant cells and tissues against UV radiation [26]. However, the precise location of vitamin D₃ synthesis within the plant cell remains unclear. Given that vitamin D₃ is linked to cholesterol biosynthesis, with 7-DHC acting as a precursor for cholesterol, it is possible that vitamin D₃ synthesis may also occur where cholesterol biosynthesis takes place. The DWARF5 enzyme, which converts 7-DHC to cholesterol, was shown to be active in the endoplasmic reticulum in *Arabidopsis thaliana* [27]. However, phytosterol biosynthesis takes place also in the plasma membrane and the cholesterol and phytosterol biosynthesis were shown to be linked and cholesterol is also present in the plasma membrane [27–29]. In that instance, the utilisation of a UV-absorbing compound would serve to influence the synthesis of vitamin D₃, given that a reduced quantity of UV light is reaching 7-DHC molecules. As pre-treatment with red or far-red light has been demonstrated to reduce the content of anthocyanins, chlorogenic acid and flavonoid compounds in lettuce [30]. It is conceivable that a pre-treatment with far/far-red light followed by UV light treatment could enhance the efficacy of the vitamin D₃ conversion reaction. Further research is needed to unravel the precise location of the vitamin D₃ conversion reaction.

In addition to the aforementioned UV-absorbing compounds, antioxidants represent a significant group of plant compounds that influence chemical reactions. Ascorbic acid and dehydroascorbic acid, glutathione and gallic acid are among the most prominent antioxidants in plants. Consequently, we sought to investigate their influence on vitamin D₃ production. Interestingly, ascorbic acid and glutathione had opposing effects on vitamin D₃ accumulation, despite both being reducing compounds. However, glutathione had the same stimulatory effect on vitamin D₃ content as dehydroascorbic acid, which is the oxidized form of ascorbic acid. The combination of ascorbic acid and glutathione was found to balance their influence. Ascorbic acid is able to catalyse a sigmatropic H-shift [31], which is necessary for the conversion of previtamin D₃ to vitamin D₃. Gallic acid demonstrated the capacity to exert both UV-absorbing and antioxidative effects. As an absorbing compound (260 nm) it inhibits the conversion of 7-DHC to previtamin D₃. As an antioxidant, it was found to favour the reaction of previtamin D₃ to vitamin D₃.

5. Conclusion

One advantage of *in vitro* studies is that they are not as constrained by technical and/or ethical restrictions as studies on plants, humans and/or human cell lines. The objective of this study was to characterise the vitamin D₃ conversion reaction under *in vitro* conditions to gain insight in these reactions in plants, and to provide leads for future research in food applications. It was demonstrated that a specific quantity of energy is necessary to initiate the conversion reaction of 7-DHC to vitamin D₃. Furthermore, it was shown that shorter wavelengths facilitate this reaction, while the addition of extra PAR light has the effect of reducing conversion efficiency. It was demonstrated that an evaluated temperature is conducive to the reaction, which could be employed as either a pre- or post-harvest treatment. These data emphasise the regulatory complexity of the conversion of 7-DHC to vitamin D₃. It is likely that this reaction is even more complex *in vivo*, where complex relationships between phenylpropanoids, UVB penetration and the vitamin D₃ content in plants can occur.

Competing Interest

The authors declare that they have no conflict of interest.

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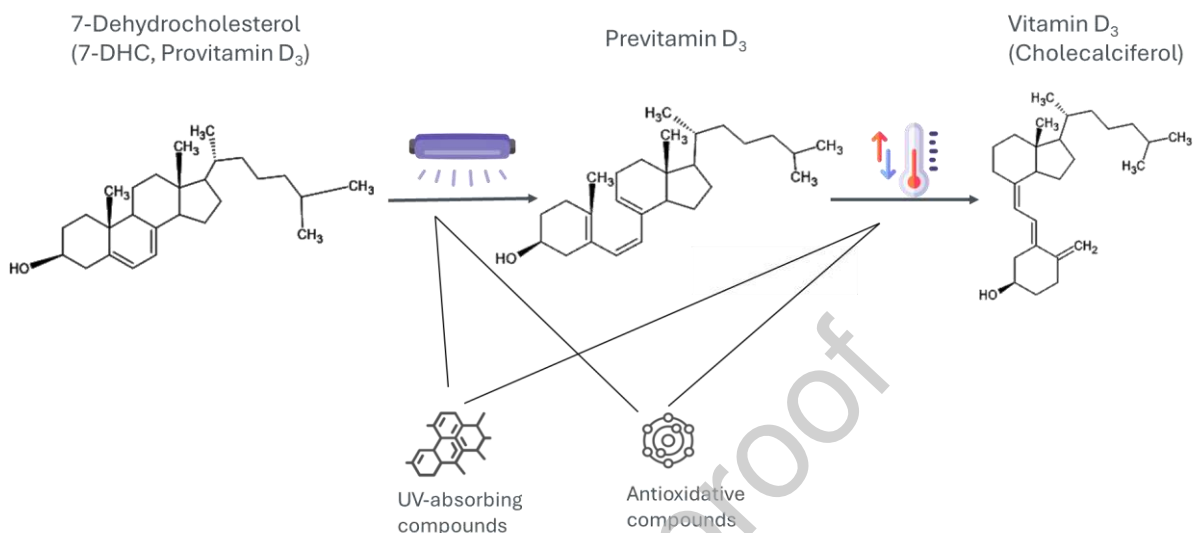
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Graphical Abstract



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