Journal of Photochemistry and Photobiology 24 (2024) 100253

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology

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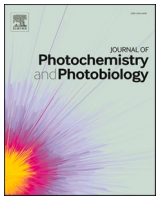

An interplay of light and temperature: Vitamin D_3 formation *in vitro*, a model for *in vivo* plant studies

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ARTICLE INFO

Keywords:
UVB
Lumisterol
Tachysterol
Cholecalciferol
Photochemical reactions
Model system

ABSTRACT

Vitamin D deficiency is a global issue that requires attention, given its essential functions in the human body. The synthesis of vitamin D_3 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 D_3 can be acquired through diet. Given that most vitamin D sources are animal-based, the discovery of vitamin D_3 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 D_3 conversion reaction. The formation of previtamin D_3 from 7-dehydrocholesterol (7-DHC) was demonstrated to be dependent on UVC and UVB light, while the subsequent formation of vitamin D_3 from previtamin D_3 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 D_3 will underpin future strategies aimed at optimising vitamin D_3 content in crop species.

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 D_3 (cholecalciferol) and vitamin D_2 (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 D_2 which is lacking in vitamin D_3. These structures originate from different precursor molecules. Vitamin D_3 is synthesised from 7-dehydrocholesterol (7-DHC), whereas vitamin D_2 is synthesised from ergosterol [3].

Ultraviolet (UV) light is essential for the conversion of vitamin D_3 precursor molecules into vitamin D_3 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 D_3 to vitamin D_3 [6].

In humans, the synthesis of vitamin D_3 occurs when UVB light from the sun reaches the epidermal layer of the skin, inducing the synthesis of vitamin D_3 [7]. Following the conversion of 7-DHC to vitamin D_3, 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

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https://doi.org/10.1016/j.jpap.2024.100253

Available online 15 October 2024

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 D_3.

Vitamin D_3 can also be obtained from animal-based foods including fatty fish, eggs, and cod liver oil, which all have a high content. Additionally, vitamin D_2 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 D_3, vitamin D_5 (sitocalciferol) and vitamin D_7 (campecalciferol) [11]. So far, especially plants of the *Solanaceae* family, such as tomatoes, have been shown to contain vitamin D derivates [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 D_3 [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 D_3 content following UV treatment.

The synthesis of vitamin D_3 from 7-DHC in humans is relatively well understood [7,13]; however, the location of vitamin D_3 synthesis in plants, and its potential function, remain poorly understood [6]. It is also largely unknown how vitamin D_3 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 D_3 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 D_3 conversion reaction can be divided into two distinct phases. The initial step is the B-ring-opening reaction of 7-DHC to previtamin D_3, which is photochemically driven by UV radiation (Fig. 1) [16]. Subsequently, the [1, 7]-sigmatropic hydrogen shift of previtamin D_3 to vitamin D_3 is thermally driven

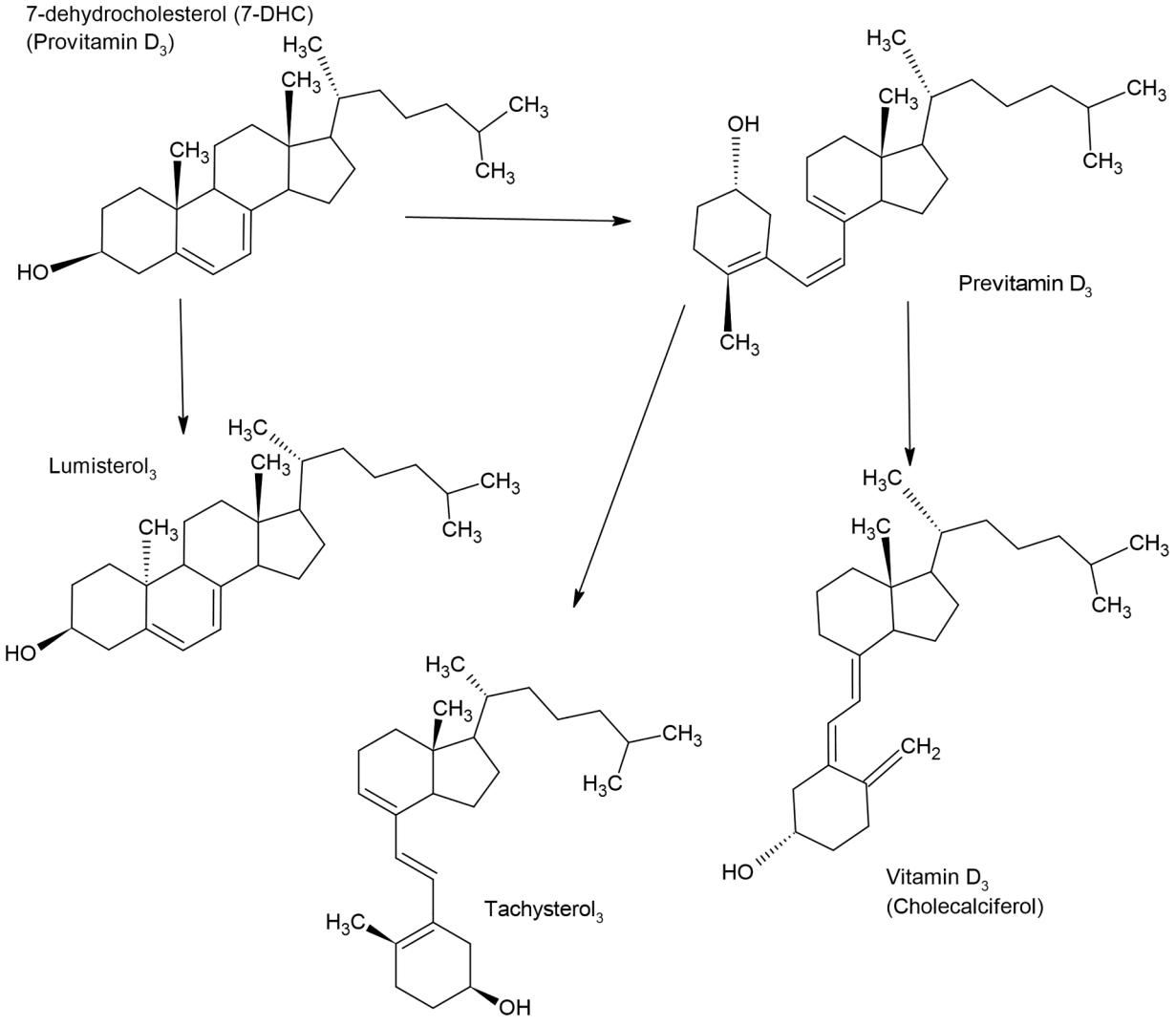

Fig. 1. Vitamin D_3 conversion reaction adapted from Okamura *et al.*, (1993) [16].

[16]. The yield of vitamin D_3 is contingent upon the efficiency of both steps of the conversion process. Once a balance between previtamin D_3 and vitamin D_3 is reached, the content of vitamin D_3 is stable. Furthermore, the occurrence of reaction by-products cannot be ruled out. 7-DHC can be photochemically converted to lumisterol$_3$ and previtamin D_3 can be thermally converted to tachysterol$_3$, which are the *trans*- and *cis*-isomers of 7-DHC and previtamin D_3, respectively [16,17]. The quantity of by-products generated can influence the yield of vitamin D_3. These photo- and thermochemical conversion reactions are critical in determining vitamin D_3 content yet have only been characterised for human skin. It is currently unknown how these reactions determine *in planta* vitamin D_3 accumulation.

The aim of this study was to investigate the kinetics of vitamin D_3 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 D_3 biosynthesis reaction provides novel insights that can be used as a starting point to understand vitamin D_3 synthesis and accumulation in plants.

2. Material and methods

2.1. Chemicals

L-ascorbic acid (99 %), caffeic acid, cholecalciferol (vitamin D_3), *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). Tachysterol$_3$ (80 %) and lumisterol$_3$ ($\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 D_3 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 D_3 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 2 h, 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 μmol m^{-2} s^{-1}; UVAB, 165 μmol m^{-2} s^{-1}; UVA, 155 μmol m^{-2} s^{-1}; and noUV, 160 μmol m^{-2} 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 °C for NB and 23.8 $\pm$ 0.5 °C for BB.

2.2.4. Influence of temperature post UV exposure

To study the influence of the temperature on the conversion of previtamin D_3 to vitamin D_3, 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 0 h, 1 h, 4 h, 6 h, 24 h, 48 h, 72 h 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 °C) and the unheated control (29.2 $\pm$ 1.3 °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 D_3 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 D_3 and vitamin D_3 were measured before and after UV exposure.

2.2.7. Influence of antioxidative compounds

To study the influence of plant antioxidant compounds on the vitamin D_3 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 2 h at 12 mW m^{-2} (87 kJ m^{-2}). The 7-DHC, previtamin D_3 and vitamin D_3 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 % H_2O) 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 D_3 and pre-vitamin D_3 at 265 nm. Identification and quantification were based on an external calibration with authentic standards of 7-DHC, vitamin D_3, tachysterol$_3$ and lumisterol$_3$ (Fig. S3).

2.4. 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 ± SEM, unless otherwise stated.

3. Results

3.1. Influence of light on the vitamin D_3 conversion reaction

3.1.1. Influence of UV dose

The influence of the UV dose (intensity × duration) on the vitamin D_3 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 D_3 content demonstrated a more complex dose-response curve (Fig. 2). Previtamin D_3 and vitamin D_3 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 D_3 and previtamin D_3 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 D_3 content was shown to be lowered by high doses of UV exposure (Fig. S4). Lumisterol$_3$ content increased at UV doses up to 164 kJ m^{-2} and then decreased rapidly, whereas tachysterol$_3$ 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 D_3 content, the most efficient UV dose was found to be between 82 kJ m^{-2} and 151 kJ m^{-2}.

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 D_3. To investigate the influence of the UV spectrum on the vitamin D_3 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 D_3 and vitamin D_3 (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 D_3 and vitamin D_3 contents. UVA and noUV treatments showed almost no pre- and vitamin D_3 content, while UVA showed a significantly lower 7-DHC content than noUV.

In summary, the highest content of pre- and vitamin D_3 can be found when no filters were applied (UVABC). UVA and noUV treatments proved to be ineffective for vitamin D_3 conversion.

3.1.3. Influence of background PAR light

To explore the influence of background PAR light on vitamin D_3 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 D_3 was very low under UVA and noUV treatments, no significant differences were observed between -/+ PAR. Under UVABC and UVAB treatments, higher previtamin D_3 contents were found in -PAR and this was matched by a higher vitamin D_3 content under UVABC (Fig. 4c, d). The lumisterol$_3$ content was increased under additional PAR light at UVAB (Fig. 4b).

In summary, additional PAR has an influence on the vitamin D_3 conversion in such a way that more 7-DHC is converted in the presence of PAR, while effects on pre- and vitamin D_3 content are modest.

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 D_3 and vitamin D_3 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 D_3, 21 % into previtamin D_3, 17 % into lumisterol$_3$ and 58 % into unknown reaction products. The reaction under NB UV converted only 1 % of 7-DHC into vitamin D_3, 4 % into previtamin D_3, 10 % into lumisterol$_3$, but into 85 % of unknown reaction products (Fig. 5e).

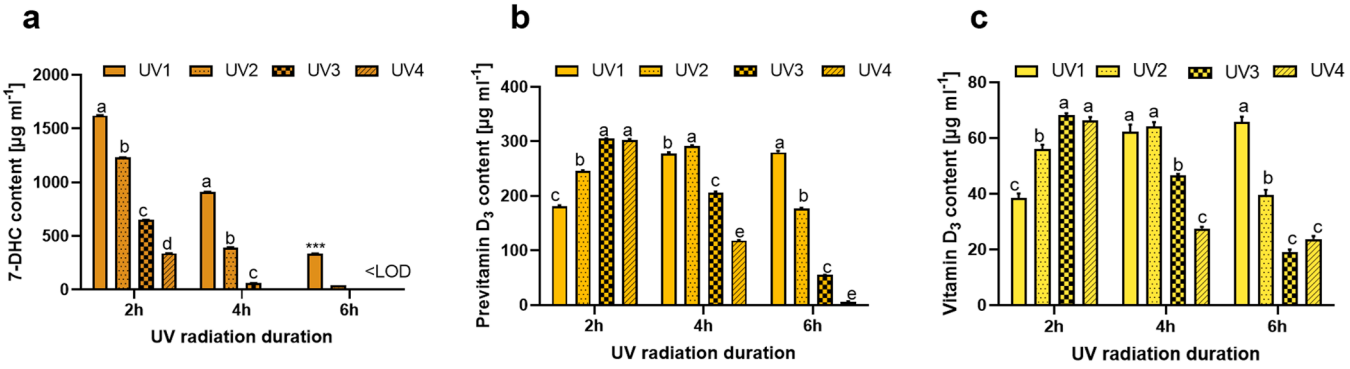

Fig. 2. Influence of UV dose on the vitamin D_3 conversion reaction. (a), 7-DHC content; (b), previtamin D_3 content; (c), vitamin D_3 content. Bars represent mean ± SEM, $n = 5$; letters indicate significant differences between the different intensities at one irradiation duration in alphabetical order from highest to lowest (1-way ANOVA followed by a post hoc Tukey's test, $p \leq 0.05$, ***≤0.001). UV doses (UV1, UV2, UV3 and UV4) are listed in Table 1.

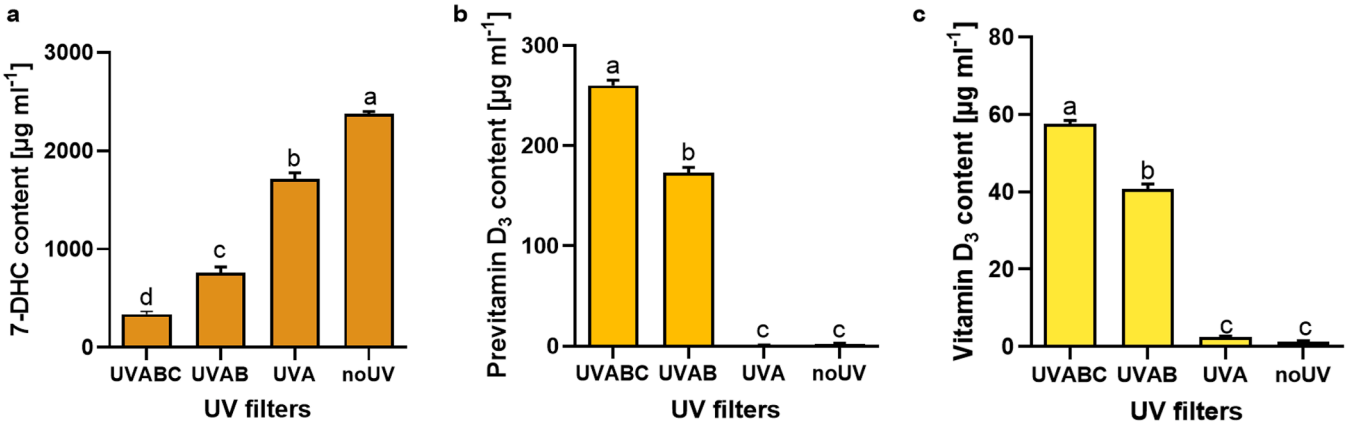

Fig. 3. Influence of UV filters that alter the UV lamp spectrum. (a), 7-DHC content; (b), previtamin D_3 content; (c), vitamin D_3 content. Bars represent mean ± SEM, $n = 5$; letters indicate significant differences between the filters in alphabetical order from highest to lowest (1-way ANOVA followed 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}.

In conclusion, the conversion of vitamin D_3 from 7-DHC was less effective under NB UV light than under BB UV light.

3.2. *Influence of temperature on the conversion reaction*

3.2.1. *Effect of temperature post UV radiation on the conversion of previtamin D_3 to vitamin D_3*

To test the influence of temperature on the conversion of previtamin D_3 to vitamin D_3, previously UV-exposed 7-DHC samples were incubated at different temperatures for a period of up to 144 h. The results showed that the previtamin D_3 content decreased with increasing temperature and time, corresponding to the increase in vitamin D_3 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 D_3 was reached after 48 h The greatest differences in vitamin D_3 content were found between 4 °C and 20 °C, with up to 3.5-fold higher vitamin D_3 content at 20 °C.

In summary, a higher temperature increases the conversion rate of previtamin D_3 to vitamin D_3 (Table S1).

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 D_3 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, lumisterol$_3$ and previtamin D_3 contents either during the UV treatment or the subsequent incubation period (Fig. 7a, b, c). However, the vitamin D_3 content was increased by 4-fold after UV exposure in a heated water bath (39 °C) (Fig. 7d, before IC). In comparison, the increase of vitamin D_3 content when samples were incubated at 40 °C after the UV-treatment, was 2-fold (Fig. 7d, 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 D_3 content during UV exposure.

3.3. *Influence of plant matrix compounds on the conversion of vitamin D_3*

3.3.1. *Influence of UV-absorbing compounds*

To explore the influence of UV-absorbing compounds on the vitamin D_3 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 D_3 content (Fig. 8). The addition of quercetin increased the pre- and vitamin D_3 content at the lowest concentration but decreased the pre- and vitamin D_3 content at the three highest concentrations compared to the control (Fig. 8a). *p*-Coumaric, caffeic, and ferulic acid decreased the pre- and vitamin D_3 content at all concentrations (Fig. 8b, c, d). Correlation analysis revealed a negative

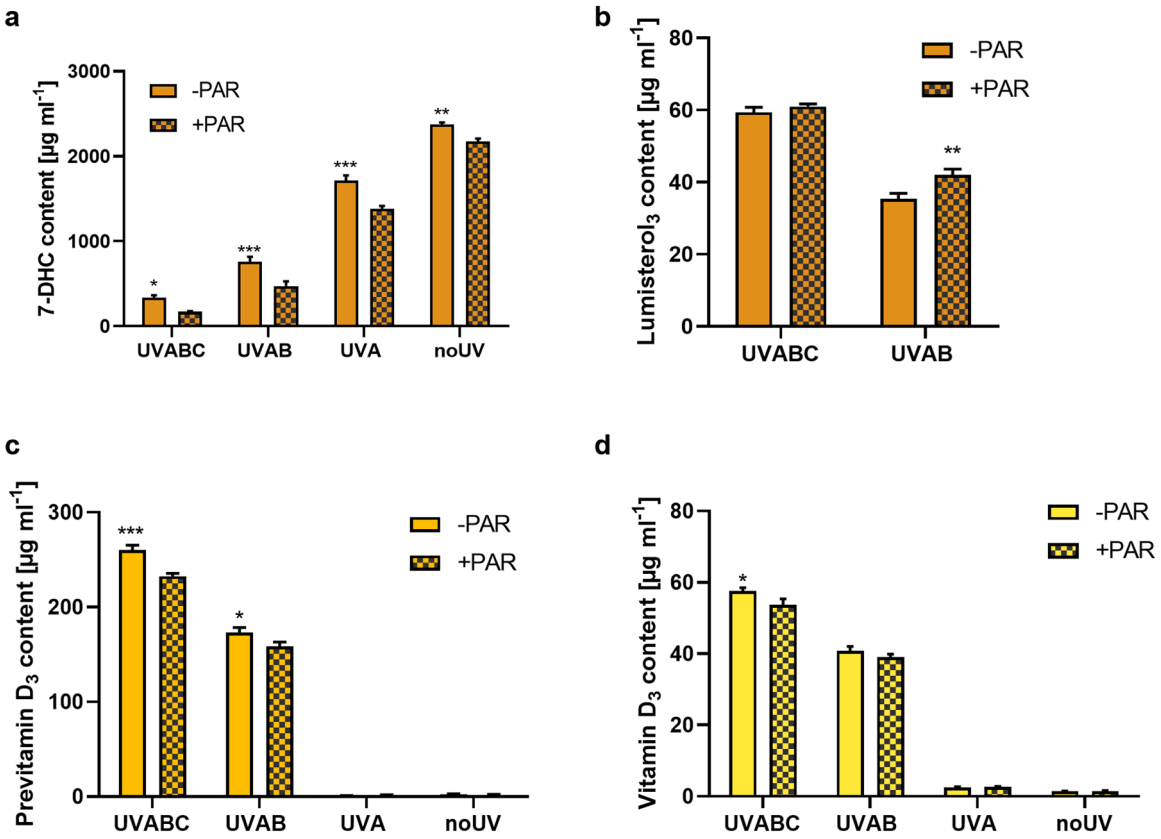

Fig. 4. Influence of PAR background light on vitamin D_3 conversion reaction with different UV blocking filters. (a), 7-DHC content; (b), lumisterol$_3$ content; (c), previtamin D_3 content; (d), vitamin D_3 content. Bars represent means ± SEM, $n = 5$. Asterisks indicate significant differences between with and without background PAR light (2-way ANOVA followed by a post hoc Šidák correction, *≤0.05, **≤0.01, ***≤0.001). UVABC, no filter; UVAB (cellulose acetate filter/CA); UVA (mylar filter/MY); noUV, (UV-blocking filter).

correlation between the concentration of the UV-absorbing compounds and the pre- and vitamin D_3 content (Table S3). Thus, a higher concentration of UV-absorbing compounds leads to a lower vitamin D_3 content. The lumisterol$_3$ content was reduced compared to the control for all additions of UV-absorbing compounds. For *p*-coumaric, caffeic and ferulic acid additions, lumisterol$_3$ was formed only at the two highest concentrations. When quercetin was added, lumisterol$_3$ was also formed at intermediate concentrations.

In summary, UV-absorbing compounds influence the vitamin D_3 conversion depending on their concentration in the *in vitro* system.

3.3.2. *Influence of antioxidative compounds*

To investigate the influence of antioxidants on the vitamin D_3 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 D_3 increased with the addition of AC and particularly with a combination of AC+GSH (Fig. 9a). Previtamin D_3 content was decreased by GSH and DHA additions compared to the control (Fig. 9b).

Secondly, the effects of gallic acid (GA) on previtamin D_3 and vitamin D_3 differed. While the content of previtamin D_3 showed a decrease with increasing GA concentrations, the yield of previtamin D_3 remained consistent across all concentrations (Fig 9c, Fig S9a). This was also demonstrated by a negative correlation between previtamin D_3 content and GA concentration, and by the absence of a correlation between previtamin D_3 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 D_3 content and GA concentrations (Fig. S9c, Table S4). Conversely, an increase in vitamin D_3 yield was noted with increasing GA concentrations (Fig. S9a). Similarly, lumisterol$_3$ and tachysterol$_3$ showed a GA concentration-dependent change in content. However, lumisterol$_3$ content decreased with increasing GA (Fig. 9d), whereas tachysterol$_3$ increased with increasing GA. Digging deeper, the yield of vitamin D_3 and previtamin D_3 was influenced in different ways (Figure S9a).

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 D_3 is a UV-driven reaction, while the subsequent reaction from previtamin D_3 to vitamin D_3 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 D_3 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

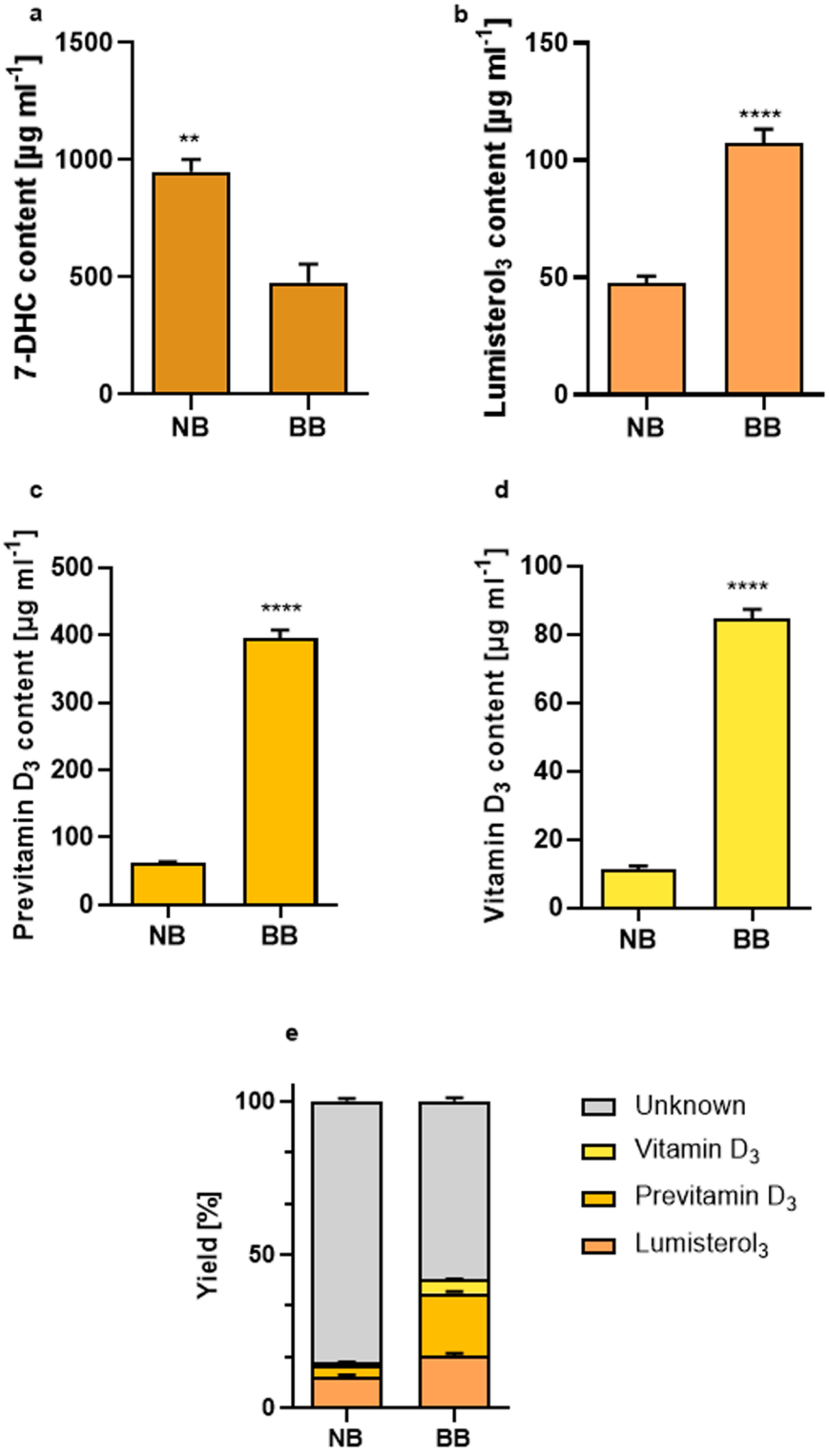

Fig. 5. Effect of narrowband and broadband UV lamps on the vitamin D_3 conversion reaction. (a), 7-DHC content; (b), lumisterol$_3$ content; (c), previtamin D_3 content; (d), vitamin D_3 content; (e), reaction yield. Bars represent mean ± SEM, $n = 5$. Asterisks indicate significant differences between narrowband and broadband lamps (unpaired *t*-test, two-tailed, **≤0.01, ****≤0.0001).

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 D_3 (Fig. S5). The photodegradation of vitamin D_3 was reported for sunlight, and the main products of photolysis were identified as suprasterols and *trans*-vitamin D_3 [18]. Furthermore, the formation of lumisterol$_3$ and tachysterol$_3$ was observed to be dependent on UV dose. This is consistent with previous literature, which demonstrated the formation of lumisterol$_2$ and tachysterol$_2$ during UV radiation [17]. The formation of lumisterol$_3$ was observed to occur at lower UV doses, with the extent of this occurrence dependent on the 7-DHC content. This indicates that lumisterol$_3$ is derived from 7-DHC. At the

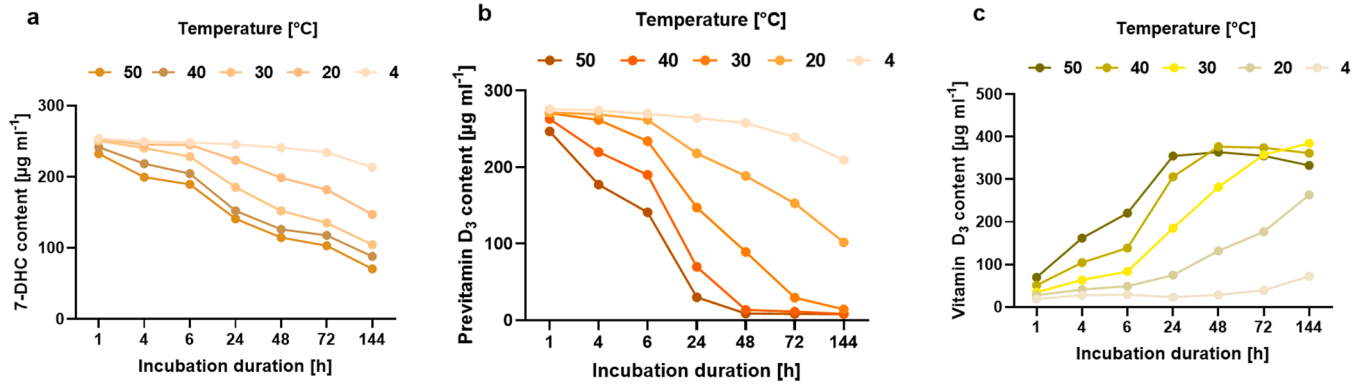

Fig. 6. Influence of temperature on conversion of previtamin D_3 to vitamin D_3. (a), 7-DHC; (b), previtamin D_3; (c), vitamin D_3 contents. Points represent mean ± SEM.

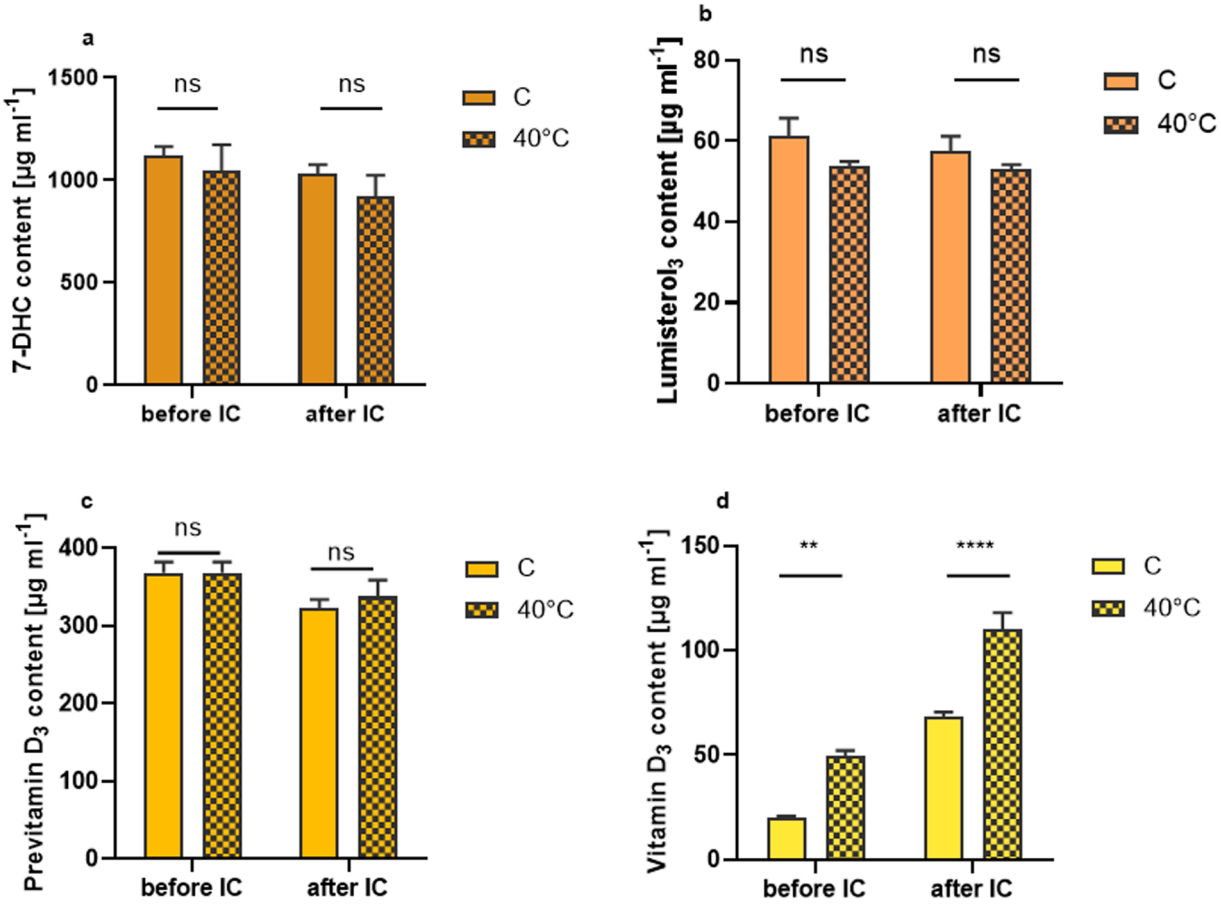

Fig. 7. Influence of temperature on the conversion reaction during UV exposure (before IC) and following an incubation period (after IC). (a), 7-DHC content; (b), lumisterol$_3$ content; (c), previtamin D_3 content; (d), vitamin D_3 content. Bars represent mean ± SEM, $n = 5$. Asterisks indicate significant differences between control and 40 °C (2-way ANOVA followed by a post-hoc Tukey's test, **≤0.01, ***≤0.001).

higher UV doses, tachysterol$_3$ is formed from previtamin D_3. However, it is also possible that tachysterol$_3$ is formed from lumisterol$_3$.

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 D_3. 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 D_3, 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 D_3. However, the results clearly demonstrated that UVA (in the presence of a mylar filter)

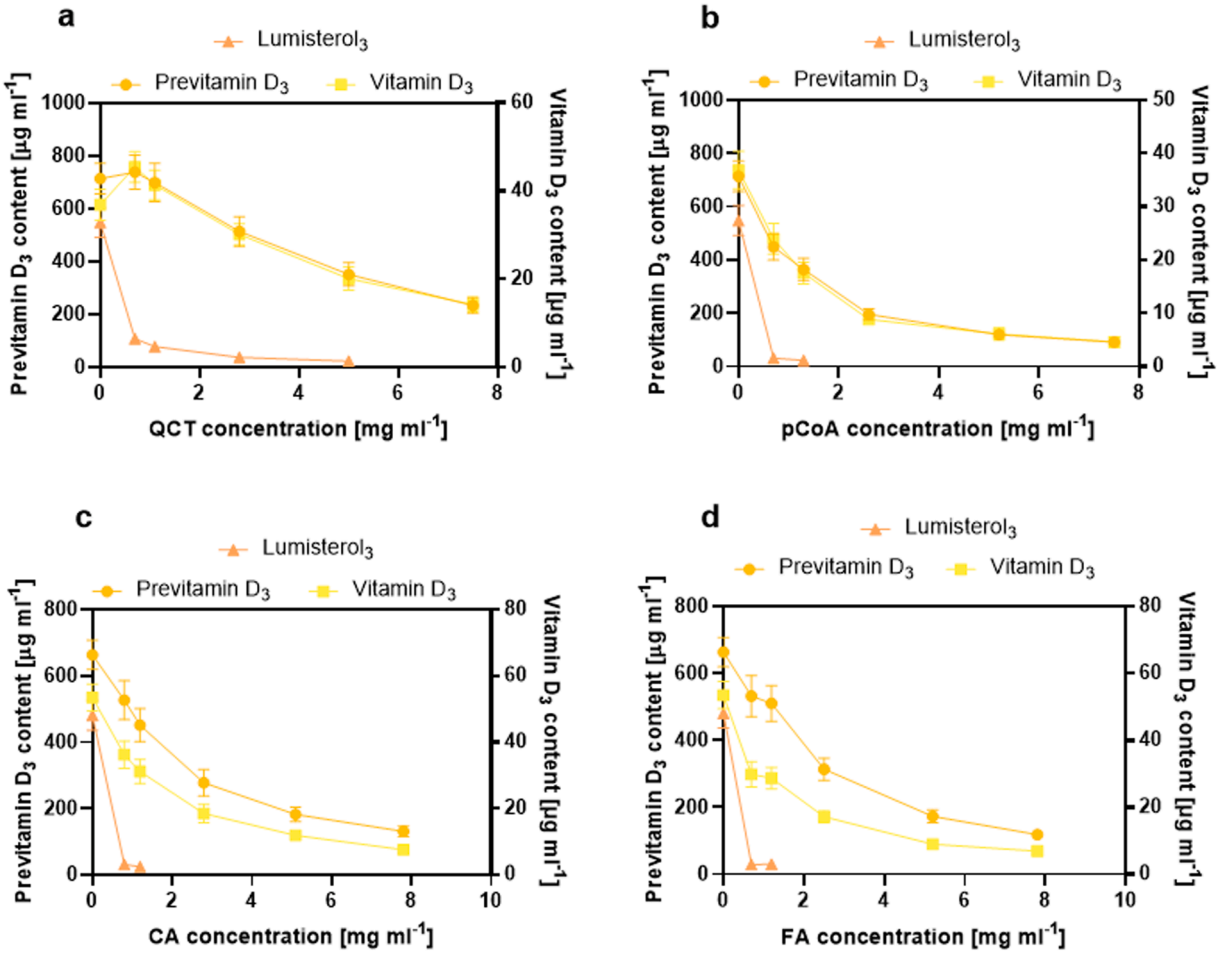

Fig. 8. Influence of absorbing compounds on the vitamin D_3 conversion. Content of vitamin D_3, pre-vitamin D_3 and lumisterol$_3$ content influenced by: (a), quercetin (QCT); (b), *p*-coumaric acid (pCoA); (c), caffeic acid (CA); (d), ferulic acid (FA). Points represent mean ± SEM. Correlation analysis can be found in Table S3.

exhibited a markedly inefficient performance, resulting in a 300-fold lower vitamin D_3 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 D_3. 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 D_3 formation. However, it is noteworthy that the use of the narrowband lamp resulted in a higher content of lumisterol$_3$ relative to previtamin D_3. This suggests that at higher UV wavelengths, corresponding to lower energy, the reaction is favoured in the direction of lumisterol$_3$ rather than previtamin D_3, *i.e.* a higher ratio between lumisterol$_3$ and previtamin D_3. This finding is consistent with the data observed using different UV spectra, *i.e.* UVAB yields a lumisterol$_3$/previtamin D_3 ratio of 1:2.5 while in the absence of any filter the lumisterol$_3$/previtamin D_3 ratio is 1:4 (Table S5). When additional PAR is used the lumisterol$_3$/previtamin D_3 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 lumisterol$_3$ relative to previtamin D_3 is formed, which suggest that higher energy/shorter wavelength are favourable to a higher previtamin D_3 content. Under a narrowband UV source, the lumisterol$_3$ content is lower compared to under a broadband UV source. This is due to a higher yield of all vitamin D_3 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 D_3, due to a decrease in the formation of lumisterol$_3$. 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 D_3 metabolism.

Additional PAR light resulted in a reduction in the efficacy of the vitamin D_3 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 D_3. In detail, 7-DHC is converted to lumisterol$_3$ rather than to previtamin D_3. In conclusion, the additional PAR results in a higher conversion of 7-DHC, but not in previtamin D_3 content.

4.2. *Influence of temperature*

It was demonstrated that a higher temperature following UV treatment resulted in a greater conversion of previtamin D_3 to vitamin D_3. 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

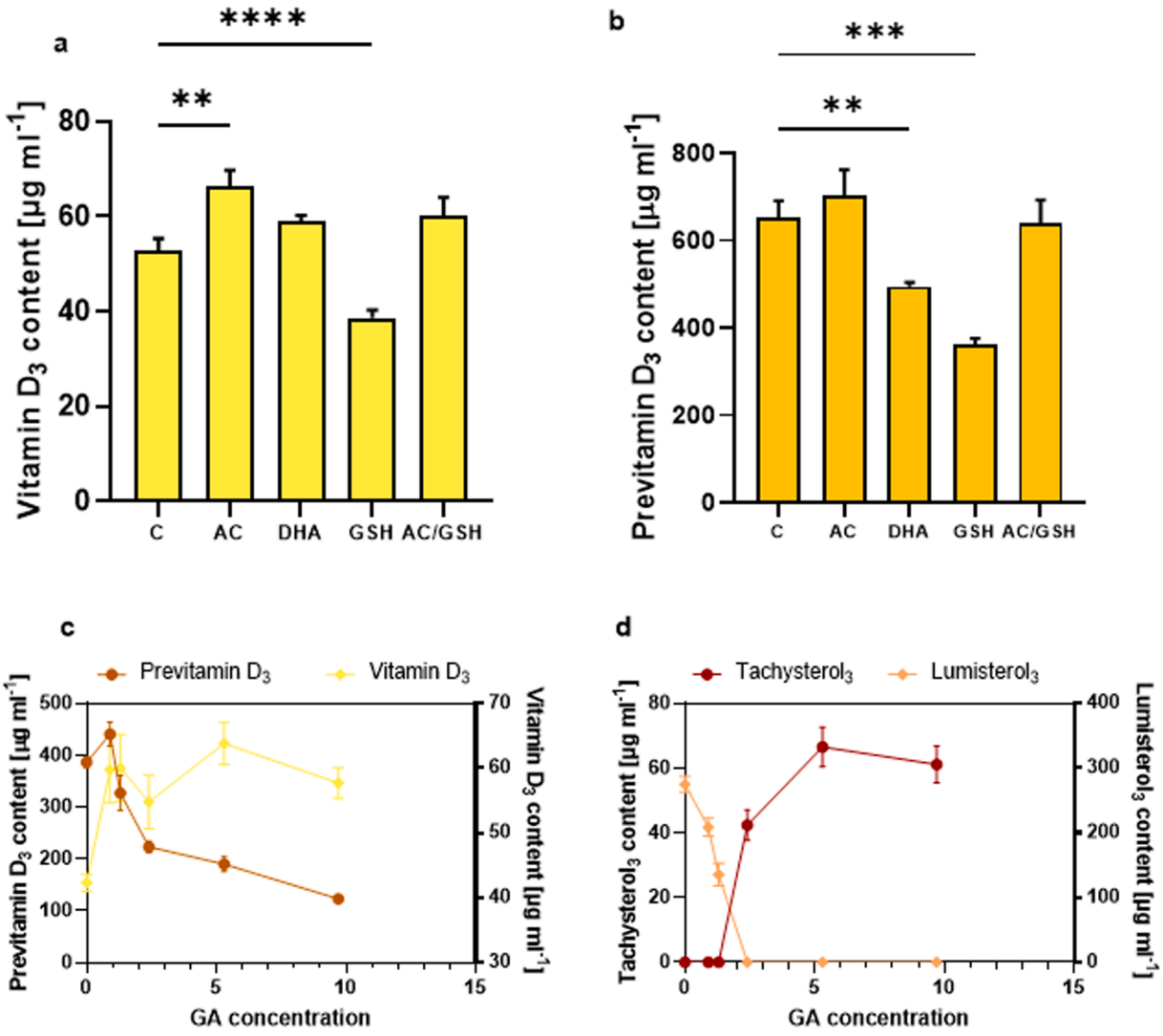

Fig. 9. Influence of antioxidative compounds on the vitamin D_3 conversion. Influence of different antioxidative compounds on (a), vitamin D_3 content, and, (b), previtamin D_3 content. Influence of different concentrated gallic acid on (c), vitamin D_3 and previtamin D_3 content and (d), tachysterol$_3$ and lumisterol$_3$ content. (a), (b), bars represent mean ± 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 ± 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.

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 D_3 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 D_3 to vitamin D_3 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 D_3 yield with unchanged content of lumisterol$_3$. 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 D_3 conversion in poikilothermous plants, temperatures of which may vary widely.

4.3. *Influence of plant matrix compounds*

In considering the vitamin D_3 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 D_3, 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 D_3 synthesis within the plant cell remains unclear. Given that vitamin D_3 is linked to cholesterol biosynthesis, with 7-DHC acting as a precursor for

cholesterol, it is possible that vitamin D_3 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 D_3, 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 D_3 conversion reaction. Further research is needed to unravel the precise location of the vitamin D_3 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 D_3 production. Interestingly, ascorbic acid and glutathione had opposing effects on vitamin D_3 accumulation, despite both being reducing compounds. However, glutathione had the same stimulatory effect on vitamin D_3 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 D_3 to vitamin D_3. 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 D_3. As an antioxidant, it was found to favour the reaction of previtamin D_3 to vitamin D_3.

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 D_3 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 D_3. 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 D_3. It is likely that this reaction is even more complex *in vivo*, where complex relationships between phenylpropanoids, UVB penetration and the vitamin D_3 content in plants can occur.

CRediT authorship contribution statement

Maria Fitzner: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Natalie Cunningham:** Data curation, Formal analysis, Writing – review & editing, Funding acquisition. **Marcel AK Jansen:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

The financial support of Science Foundation Ireland (grant 16/IA/4418) and Irish Research Council (grant GOIPG/2023/4071) is gratefully acknowledged.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jpap.2024.100253.

Data availability

Data will be made available on request.

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