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Generation of previtamin D3 from tachysterol3: a novel approach for producing vitamin D3 in the winter

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GENERATION OF PREVITAMIN D3 FROM TACHYSTEROL3: A NOVEL APPROACH FOR PRODUCING VITAMIN D3 IN THE WINTER

by

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DEDICATION

I would like to dedicate this work to

Robert Maloney.

ACKNOWLEDGMENTS

I want to thank Dr. Holick and Dr. Chen
for being patient with me throughout this whole process
and for giving me this great opportunity to try something new
and grow as an individual.

I would also like to thank my friends and family
for helping to see me through these past few years.

GENERATION OF PREVITAMIN D3 FROM TACHYSTEROL3: A NOVEL APPROACH FOR PRODUCING VITAMIN D3 IN THE WINTER

KOSTAS ANDREO

ABSTRACT

Solar ultraviolet-B (UVB) radiation is capable of converting 7-dehydrocholesterol (7-DHC) to previtamin D_3 ($preD_3$), which undergoes thermal isomerization to produce vitamin D_3. Further ultraviolet irradiation of $preD_3$ will produce other photoproducts, including $lumisterol_3$, $tachysterol_3$, and 7-DHC. Continued exposure to UVB results in a photoequilibrium of these photoproducts. During the winter months, people living at latitudes greater than 32° north or south are incapable of converting cutaneous 7-DHC to $preD_3$. Because an increased zenith angle creates a longer path-length for UVB radiation to traverse through the atmosphere, ozone can absorb a much greater proportion of this radiation. Given the absorption spectrum of $tachysterol_3$ which absorbs UV radiation up to 340nm, it was hypothesized that winter sunlight which contains UV radiation between 315nm and 340nm would be able to convert $tachysterol_3$ to $preD_3$. Each hour between sunrise and sunset, ampules containing 50μg/mL $tachysterol_3$, $lumisterol_3$, and 7-DHC in 100% ethanol were exposed to solar radiation. These samples were chromatographed on a normal phase chromatographic column. Results revealed that $tachysterol_3$ was efficiently converted to $preD_3$ from sunrise to sunset, whereas as 7-DHC and $lumisterol_3$ were not. Exposure of $tachysterol_3$ to sunlight throughout the day revealed that $tachysterol_3$ began converting to $preD_3$ at sunrise at 8am and the peak conversion

occurred between 10:00 and 13:00. PreD_3 was generated from tachysterol$_3$ until sunset. No preD_3 was observed when 7-DHC or lumisterol$_3$ were exposed at the same time. From this data, it is feasible to use tachysterol$_3$ to produce preD_3 in a topical preparation during winter.

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LIST OF ABBREVIATIONS

1,25(OH)D	1,25-Hydroxyvitamin D
25(OH)D	25-Hydroxyvitamin D
7-DHC	7-dehydrocholesterol
cZc	Cis,Z,cis
cZt	Cis,Z,trans
CV	Coefficient of Variance
HPLC	High pressure liquid chromatography
Hr	Hour
µg	Microgram
µL	Microliter
mL	Milliliter
MED	Minimal Erythemal Dose
Nm	Nanometer
Ps	Picoseconds
$preD_3$	Previtamin D3
RANKL	Receptor activator of nuclear factor-κB ligand
SD	Standard Deviation
tZc	Trans,Z,cis
UV	Ultraviolet
UVA	Ultraviolet-A
UVB	Ultraviolet-B

UVC	Ultraviolet-C

BACKGROUND

History

The evidence for the importance of vitamin D first began to emerge in the early 1800s, when the Polish physician Sniadecki suggested that exposure to sunlight may act as a viable treatment for rickets, a disease characterized by general muscle weakness and gross bone deformities[1-5]. This disease first began to plague particularly children in urban communities of the industrialized world. In the United Kingdom, a committee of the British Medical Association would also find that rickets, while common in urban communities, was rarely seen in rural areas of Britain[1,5]. Palm would further reaffirm Sniadecki's suggestions citing evidence that children in third-world countries, where sunshine was abundant, did not suffer from rickets as did the children in the urban communities of the developing world[1,3,4,5]. He would be the first to form recommendations stressing the benefits of exposure to sunshine. Despite this early understanding of sunshine's beneficial effects, the prevalence of rickets continued to increase throughout the industrialized world as a result of both environmental and lifestyle factors.

Nearly a century from Sniadecki's first recommendations would pass before Mellanby demonstrated that he could induce rickets in puppies using a diet consisting of only oatmeal and then subsequently cure them of their ailments by feeding them cod-liver oil. From this finding, Mellanby theorized that a fat-soluble compound must be responsible for curing this disease. Although at first it was believed that the compound responsible was vitamin A, McCollum's group would later demonstrate that even after

vitamin A was destroyed, cod-liver oil was still able to cure rickets and thus named this new compound vitamin D[1,5]. Eventually scientist would discover the lack of vitamin D as being a significant contributor towards the pathogenesis of rickets and elucidate its ability to be manipulated to cure and prevent this disease[1,4].

Huldschinsky reported, in 1919, that exposure to radiation from a mercury arc lamp had the potential to not only cure, but prevent this disease. By exposing just the arms of rachitic children and noting significant systemic improvements, Huldschinsky deduced that a compound was being formed within the skin, entering the circulation, and effecting change within the whole body[1,3,4,5]. Soon after, Huldschinsky's findings would be confirmed by Hess and Unger in 1921, when they noticed improvements in children after exposing them to the sunlight from the roof of a New York City hospital[1,4,5]. Simultaneously in the 1920's, Steenbock and Black, as well as Hess and Weinstock, were able to demonstrate improvement of rachitic rodents by consumption of various foods that had been exposed to ultraviolet-B radiation[1,3,4,5].

During the 1930's, the United States' government had already been regulating the fortification of table salt with iodine in the treatment of goiter. Not long after the causal relationship between rickets and vitamin D deficiency had been established, the Food and Drug Administration began promoting the fortification of milk. With this influence, dairies, under governmental regulations, began fortifying their products by either irradiating milk that had ergosterol added to it with ultraviolet radiation or feeding their cattle UV-irradiated yeast[1,6]. Once ergosterol had been discovered and cheaply recovered from yeast as the prohormone of vitamin D_2, the dairies then opted for the more

economical option of adding vitamin D_2 directly to their products. In addition to fortified-food, corporations began supplementing personal hygiene products with vitamin D. In 1932, the Children's Bureau of the United States Department of Labor published a brochure recommending sun exposure for children. Between advances in medical treatments, such as UV-lamps, and these governmental agencies' remarkable job in advocating for the fortification of food as well as promoting sensible sun exposure, the prevalence of rickets decreased and the rickets epidemic had been eradicated within several years[1-4,6].

Unfortunately, vitamin D became the scapegoat for a 1950's outbreak of hypercalcemia in the United Kingdom. The Royal College of Physicians, citing similarities in studies of neonatal rodents whose mothers received high doses of vitamin D, concluded that the outbreak must have been caused by the intoxication of vitamin D, particularly from the over-fortification of food products. This unproven assumption had then led to legislation banning the fortification of food and personal products with vitamin D. From this fear, this ban then quickly spread throughout Europe and remains, for the most part, in effect today with several exceptions. Despite this singular outbreak**,** vitamin D fortification of milk within the United States has carried on for decades without any evidence of intoxicating and harming people. In addition, the children of this outbreak had also shown characteristics similar to those seen in children with William's syndrome, a vitamin D hypersensitivity disorder[2,4]. Whether or not this outbreak was due to an error in the fortification process or to other confounding factors inherent to the situation remains to be proven. Given strong evidence that a tremendous amount of

vitamin D is needed to produce any signs of dangerous intoxication, the benefits of vitamin D fortification may have been overshadowed by irrational fears[4].

Photobiochemistry of Vitamin D

Nature of Ultraviolet Radiation

Although ultraviolet radiation accounts for only about 8-9% of the radiation that reaches the earth, it is considered the most biologically active portion of the electromagnetic spectrum[7,8,9]. The sun emits three different types of ultraviolet radiation, ultraviolet-A (UVA), ultraviolet-B (UVB), and ultraviolet-C (UVC), each encompassing a broad range of wavelengths between 320-400nm, 280-315nm, and 200-280nm, respectively[7,9,10]. Once this radiation reaches the earth, it is reflected, absorbed, or scattered upon collisions with the various particles in the atmosphere, including trace gases, ozone, as well as both anthropogenic and non-anthropogenic aerosols[8]. The earth's atmosphere, particularly the ozone within it, scatters and absorbs all ultraviolet (UV) radiation below 290nm, preventing all UVC and about a quarter of all UVB radiation from reaching the Earth's surface[7,8]. Once the remaining UV radiation reaches the epidermis, it is once again reflected, scattered and absorbed by proteins, nucleic acids, lipids and other structures found within the skin. UVB radiation is scattered within the epidermis and superficial dermis, while the longer wavelengths of UVA radiation passes deeper into the dermis, where it is scattered and absorbed as well[7,9].

Photo-induced Ring-Opening of 7-Dehydrocholesterol

As solar ultraviolet radiation (290-315nm) is absorbed by the conjugated diene in 7-dehydrocholesterol (7-DHC), it excites the *π-system,* allowing the provitamin to undergo a π-π* transition and produce an excited intermediary state[11]. Utilizing human skin, McLaughlin, Anderson, and Holick produced an action spectrum demonstrating the optimal wavelengths for previtamin D production were between 295 and 300 nm (Figure 1)[12]. Exposing human skin to simulated equatorial solar radiation, they found that within 30 minutes, 15-20% of the 7-dehydrocholesterol had converted to previtamin D_3. In comparison, exposure of skin to only 295-nm narrow band radiation converted a maximal 55-66% of 7-dehydrocholesterol to previtamin D_3, while also establishing a photostationary state with tachysterol$_3$, lumisterol$_3$ and 7-dehydrocholesterol, accounting for 20-30%, 5-10%, and 2-5% of the total composition respectively. The action spectrum they provided coincides with Bunker and Harris' finding that 297nm radiation most effectively cures rickets in rats and Kobayashi and Yasumara's finding that 295nm radiation produced the most previtamin D_2 from ergosterol[12,13].

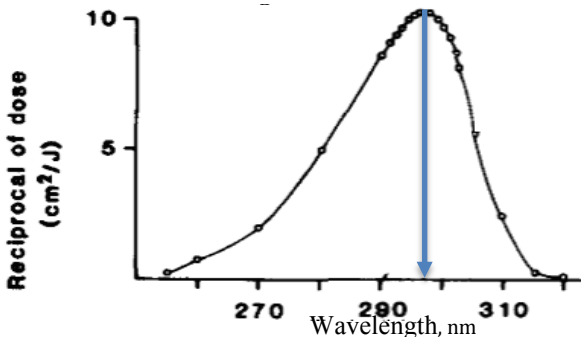


Figure 1 Action Spectrum of 7-Dehydrocholesterol Conversion to Previtamin D_3. Produced by McLaughlin, Anderson and Holick, this action spectrum shows that the optimal wavelength for 7-DHC ring opening and Previtamin D_3 production lies between 295 and 300 nm, as indicated by the arrow. No previtamin D_3 conversion is seen at wavelengths greater than 320nm. Adapted from (12).

Since the C10 methyl group predisposes 7-DHC to increased torsion, as indicated by the puckering seen within the B-ring, the addition of energy with the associated changes in bond lengths and angles allows the 9-10 sigma bond to undergo cleavage upon absorption of UVB radiation[13,14]. Rapid reshuffling of the electrons within the *π-system* then isomerizes the 5,7-diene into a highly active triene. In a recent publication, Redwood, Bayda, and Saltiel demonstrated that ring opening of 7-dehydrocholesterol produces three conformations of previtamin D_3 when suspended in a 5:5:2 mixture of diethyl ether, isopentane, and ethyl alcohol at 77K, including trans,Z,cis (tZc), cis,Z,cis (cZc), and cis,Z,trans (cZt). Of these three conformers, they found the tZc conformation to be the major product formed after ring opening of 7-DHC with cZc and cZt provided only minor contributions[15].

Focusing on the kinetics of ring-opening, Fuss et al. have found that the formation of the primary conformer of previtamin D_3, s-cis,Z,s-cis, appears within 5.2 picoseconds and is independent of temperature, concluding that the reaction must not have an activation energy requirement[12]. Once formed, the primary conformer of previtamin D_3, cZc, experiences repulsive steric forces between the C-ring and the methyl group on the 19-C, resulting in a cis-to-trans isomerization and thus producing the tZc conformation[12,14]. Evaluating the surface energies of many different conformations of previtamin D_3 using a theoretical model, Dauben and Funhoff found numerous minima consisting of variations of the cZc and tZc conformers, differing only in degrees of helicity[16]. They also found the tZc conformer to be the global minimum, which supports Redwood, Bayda and Saltiel's finding that the tZc conformer is the preferred conformer

and major product formed[14,16]. Despite cZc being the primary conformer produced from 7-dehydrocholeserol, thermally induced cis-to-trans isomerization begins shortly after ring opening[14,16]. Using Ultrafast Spectroscopy, Fuss and colleagues found that when the cZc conformer is suspended in ethanol, it isomerizes within 125ps to the more stable tZc conformer at room temperature[12]. They postulate an activation entropy must act as a barrier to account for this prolonged time[12]. In addition, they have also found that the rate of this isomerization does depend on temperature as well as viscosity of the medium, postulating that isomerization may occur as a one bond flip around a single bond as opposed to a hula-twist mechanism suggested by others within the field[12,15].

Isomerization of Previtamin D_3 to Vitamin D_3

Previtamin D_3 in the cZc conformation will undergo a thermally-dependent 1,7-sigmatropic hydrogen shift to produce vitamin D_3. Although being the most stable within organic solvents, the tZc conformation of previtamin D_3 cannot undergo this hydride shift and thus it cannot isomerize to vitamin D_3. Comparing the in vitro and in vivo production of vitamin D_3, Tian et al. found that when suspended in an organic solvent at 37º centigrade, 50% of previtamin D_3 converted to vitamin D_3 within 30 hours whereas the same amount of conversion occurred within 2.5 hours in human skin samples[17]. Holick, Tian and Allen found a similar correlation in skin samples from iguanas, a cold-blooded animal[17]. Using a liposome model, Holick and Tian were able to mimic cutaneous production of vitamin D_3[19]. Given that only the cZc conformer isomerizes to vitamin D_3, they were able to conclude that the lipid bilayer of cell membranes must stabilize this

conformer in vivo by hydrophobic and hydrophilic forces to optimize the transformation of cZc-previtamin D_3 to vitamin D_3 (Figure 2). Evaluating the membrane's effect on the rate of vitamin D_3 production, they found an inverse relationship between vitamin D_3 production and increasing cell membrane fluidity. By disrupting the amphipathic interactions among the hydrophobic tails, they demonstrated a decreased stabilization of the cZc conformer and thus a decreased production of vitamin D_3[19]. The amphipathic stabilization allows previtamin D_3 an optimal environment for the unfavorable cZc conformation to undergo the thermally-induced hydride shift and thus significantly increasing the rate of vitamin D_3 synthesis. As of yet no evidence has been found to suggest an enzymatic influence on this isomerization.

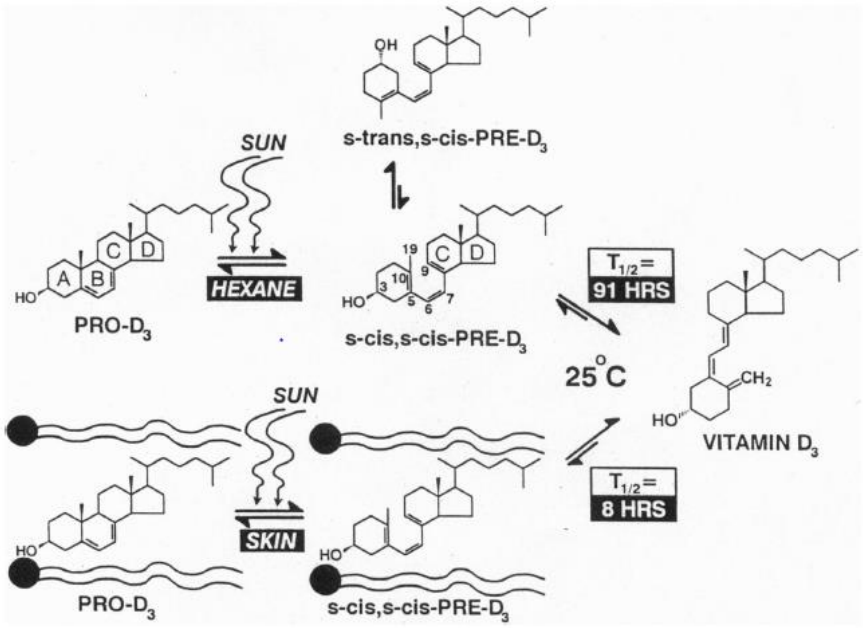


Figure 2 Comparison of the in Vitro and in Vivo Conversion of Previtamin D_3 to Vitamin D_3. In vitro, the cZc conformer undergoes an equilibrium with the tZc conformer, which cannot convert to vitamin D, and thus prolongs the time to convert half of the previtamin D to vitamin D. In vivo, the polar heads and hydrophobic tails of the lipid bilayer are capable of stabilizing the cZc conformer of previtamin D, greatly reducing the time to produce vitamin D. Obtained from (17).

Photoisomers of Previtamin D_3 and the Photostationary State

The process of previtamin D_3 isomerizing to vitamin D_3 must compete with further ultraviolet irradiation of all previtamin D_3 conformations. This irradiation forces previtamin D_3 into an excited state that could lead to the reversible and irreversible formation of other photoproducts, including tachysterol$_3$ and lumisterol$_3$, as well as several toxisterols and suprasterols[14]. Given enough radiative energy, these compounds will eventually reach a dynamic photoequilibrium, or photostationary state, where despite any added radiation, no changes in their relative concentrations will occur (Figure 3). Not shown in the figure, are the irreversible conversions of previtamin D_3 and tachysterol$_3$ to several toxisterols and suprasterols.

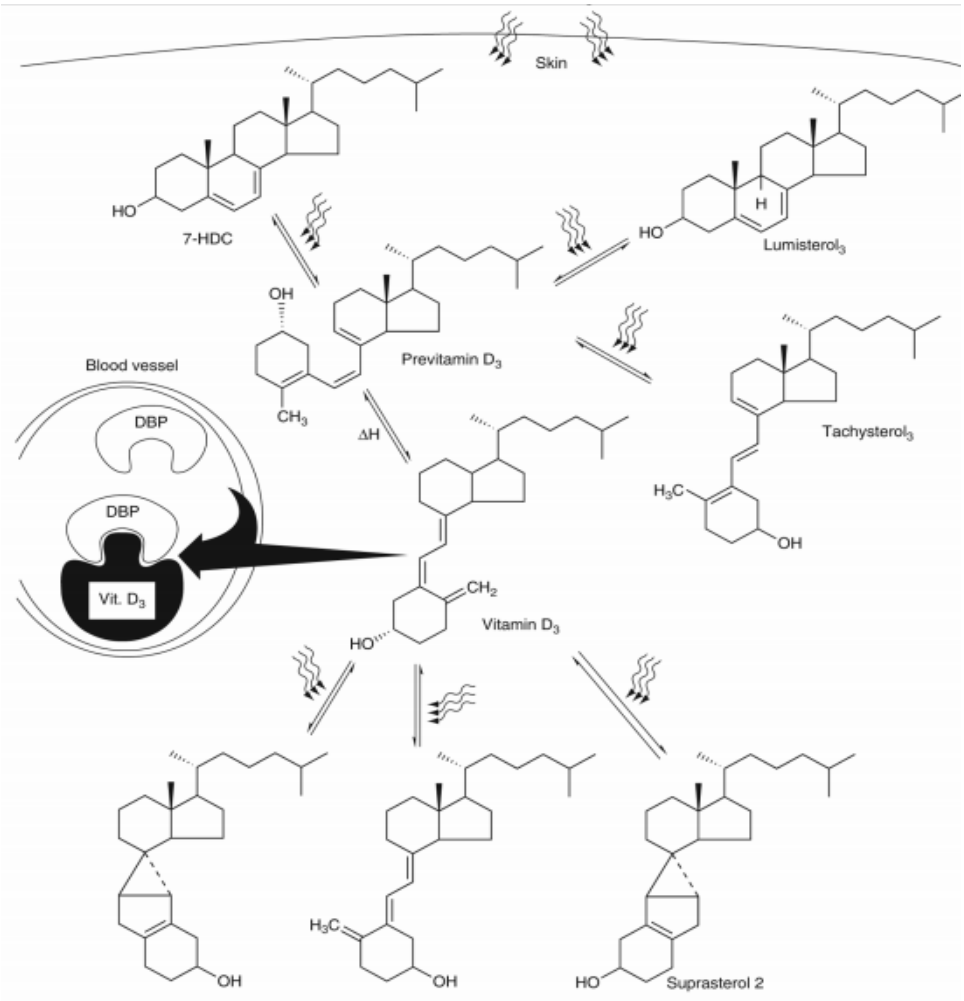


Figure 3 Depiction of the photochemical and thermal conversions involving vitamin D and its isomers. The curved lines represent ultraviolet radiation. Obtained from (12).

As depicted in the absorption spectra below, each of the major photoproducts has a unique absorption range (Figure 4)[4]. Both lumisterol$_3$ and 7-DHC can only effectively absorb UV radiation with wavelengths up 315nm. In contrast, previtamin D_3 can absorb ultraviolet radiation with wavelengths up to 325nm, while tachysterol$_3$ absorbs wavelengths up to 340nm. In theory, absorption of electromagnetic radiation may excite these isomers to undergo isomerization, just as absorption of wavelengths up to 315nm

allows 7-DHC to undergo ring-opening. This constant pattern of excitation and isomerization between the isomers is what allows them to reach the photostationary state.

However, during the winter months at latitudes greater than 32º North and South, atmospheric ozone absorbs UVB radiation to the extent that very little, if any, ultraviolet radiation below 315nm reaches the earth's surface. This is due to the tilt of the earth creating a longer path for ultraviolet radiation to cross before reaching the Earth's surface. A longer path length allows ozone to absorb more UVB radiation. Since 7-DHC is incapable of absorbing radiation above 315nm, it cannot undergo ring-opening to previtamin D_3 in these conditions and thus the body cannot produce vitamin D_3. In contrast, tachysterol$_3$ can absorb ultraviolet radiation above 315nm and therefore it may be possible for it undergo isomerization to previtamin D_3 when exposed to winter sunlight. In addition, radiation with wavelengths greater than 315nm reach the earth's surface in much larger quantities than radiation with shorter wavelengths. Lastly, although these action spectra may tell us which wavelengths these isomers can absorb, they do not provide any information on whether this radiation will excite these isomers to undergo isomerization.

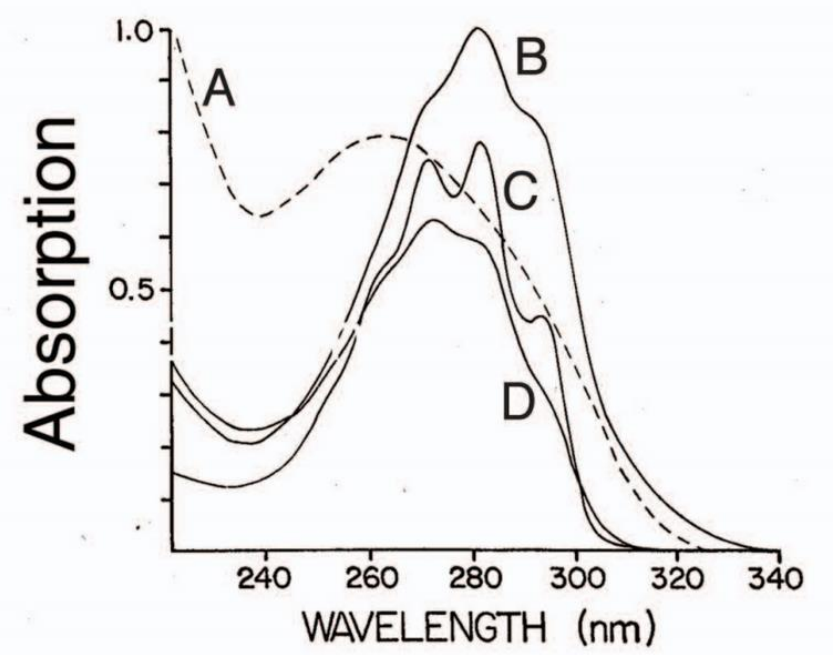


Figure 4 Absorption Spectra of Photoproducts of UV Irradiation. (A) Absorption Spectrum of Previtamin D_3. (B) Absorption Spectrum of Tachysterol$_3$ (C) Absorption Spectrum of 7-dehydrocholesterol. (D) Absorption Spectrum of Lumisterol$_3$. Obtained from (4).

Studies have shown that the specific wavelength composition of the irradiation may significantly affect the production of previtamin D_3 as well as the formation of the various other photoisomers. Fub and Lochbrunner have demonstrated in 1997, the wavelength dependence of the quantum yields for Z-to-E isomerization forming tachysterol$_3$ and ring closure leading to either 7-dehydrocholesterol or lumisterol$_3$[20]. The quantum yield for Z-to-E isomerization of the central double bond resulted in tachysterol$_3$ formation and it was at its highest yield when previtamin D_3 was irradiated with wavelengths shorter than 300nm. In contrast, wavelengths greater than 300nm favored ring-closure, producing lumisterol$_3$ or its diastereomer 7-dehydrocholesterol at a higher quantum yield[20].

A proposed model to explain this occurrence states that this wavelength-dependence is a result of the selective excitation of specific ground state conformers of previtamin D. The cZc-previtamin D_3 conformer which tends to absorb ultraviolet radiation with longer wavelengths, will favor ring closure to form either 7-dehydrocholesterol or lumisterol$_3$. In contrast, the tZc conformer, which tends to absorb shorter wavelengths, will favor the Z-to-E isomerization to tachysterol$_3$ upon ultraviolet irradiation[15,20]. Other models describing the wavelength-dependence phenomenon have also been postulated. In another model, one of the isomerizations must overcome a barrier before proceeding to the isomerization. For example, the preference for Z-to-E isomerization of previtamin D_3 to tachysterol$_3$ at shorter wavelengths is due to these wavelengths having higher energy than longer wavelengths. Thus, the shorter wavelengths allow this compound to overcome the barrier and undergo this isomerization. Longer wavelengths, which have a lower energy, do not give previtamin D_3 enough energy to overcome this barrier, so ring-closure resulting in lumisterol$_3$ or 7-dehydrocholesterol formation is preferred over Z-to-E isomerization[20,21].

Photolytic Isomerization of Vitamin D

Irradiation of vitamin D_3 excites it and photolyzes it to one of several products, including 5,6-trans-vitamin D_3, suprasterol 1, and suprasterol 2 (Figure 5). Although no biological functions have been found for these compounds, formation of these irreversible isomers may serve to decrease the effective concentration of vitamin D_3 in the skin. Webb et al. found that irradiating skin samples with topically applied vitamin D_3

to June sunlight for three hours resulted in an 80% reduction of vitamin D_3. In a comparable methanol model, they found that exposure to sunlight in January resulted in 30% reduction after just one hour of exposure, while a three hour exposure resulted in a 45% reduction[22].

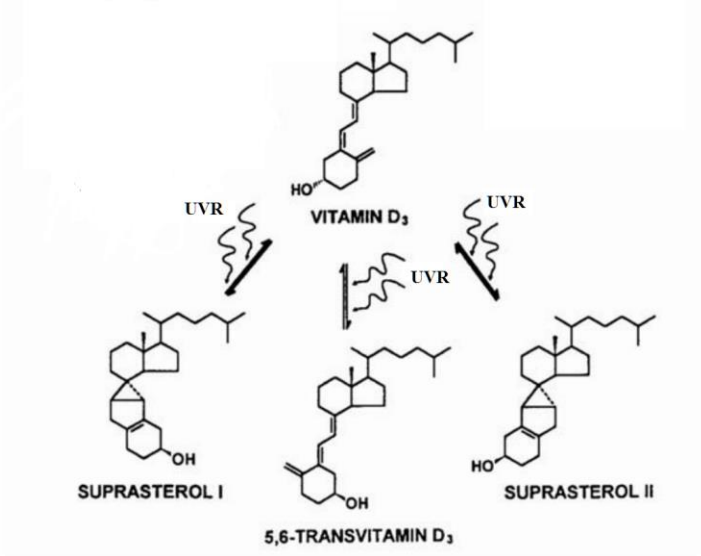


Figure 5 Depiction of the Photodegradation of Vitamin D_3. Adapted from (13).

Factors Influencing the Cutaneous Production of Vitamin D_3

Decreases in the cutaneous synthesis of vitamin D_3 have been observed as a result of various exogenous and endogenous factors. Of these, the changing of the seasons produces a dramatic effect in populations living greater than 32 degrees North and South of the equator, as discussed earlier[8,9,23]. As the tilt of the Earth and the associated zenith angle of a particular location increases, the ultraviolet radiation from the sun must traverse a longer path length to cross the ozone layer, thus leading to increased levels of UV-B absorption by the ozone in the atmosphere. This decreased UV-B radiation

ultimately leads to a reduction or the complete absence of previtamin D_3 production during the winter months (figure 6a and 6b). At latitudes greater than 30° in the northern hemisphere, the amount of vitamin D produced from 7-dehydrocholesterol is nonexistent, such as in Boston, MA and Edmonton, Canada during the months between November and April. This effect is also seen in the southern hemisphere, but during the months between April and October. In addition, as distance increases from the Earth's equator, larger latitudes will experience an earlier and more drastic change in this trend, again due to radiation traversing an even greater path-length through ozone and other atmospheric particles[8,9,23].

A similar trend is seen when observing the amount of vitamin D produced throughout the day. In the early hours of dawn and the late hours of dusk, the solar zenith angle is more obtuse to any arbitrary point. At solar noon, the zenith angle becomes more perpendicular to this arbitrary point, resulting in the shortest distance between the sun and the point for that particular day. This shorter path length allows for more direct ultraviolet-B radiation to reach the Earth's surface while avoiding as much ozone as possible and thus promoting more conversion of 7-dehydrocholesterol to previtamin D_3 (figure 6c and 6d). In addition, as the earth tilts, the latitudes furthest from the sun experience shorter amounts of sunlight per day, resulting in a smaller window for the total amount of daylight within a day as well as a smaller window for producing previtamin D_3. Again, latitudes further from the equator experience a more pronounced change than closer latitudes due to the greater amount of ozone that the radiation must traverse through to reach the surface[8,9,23].

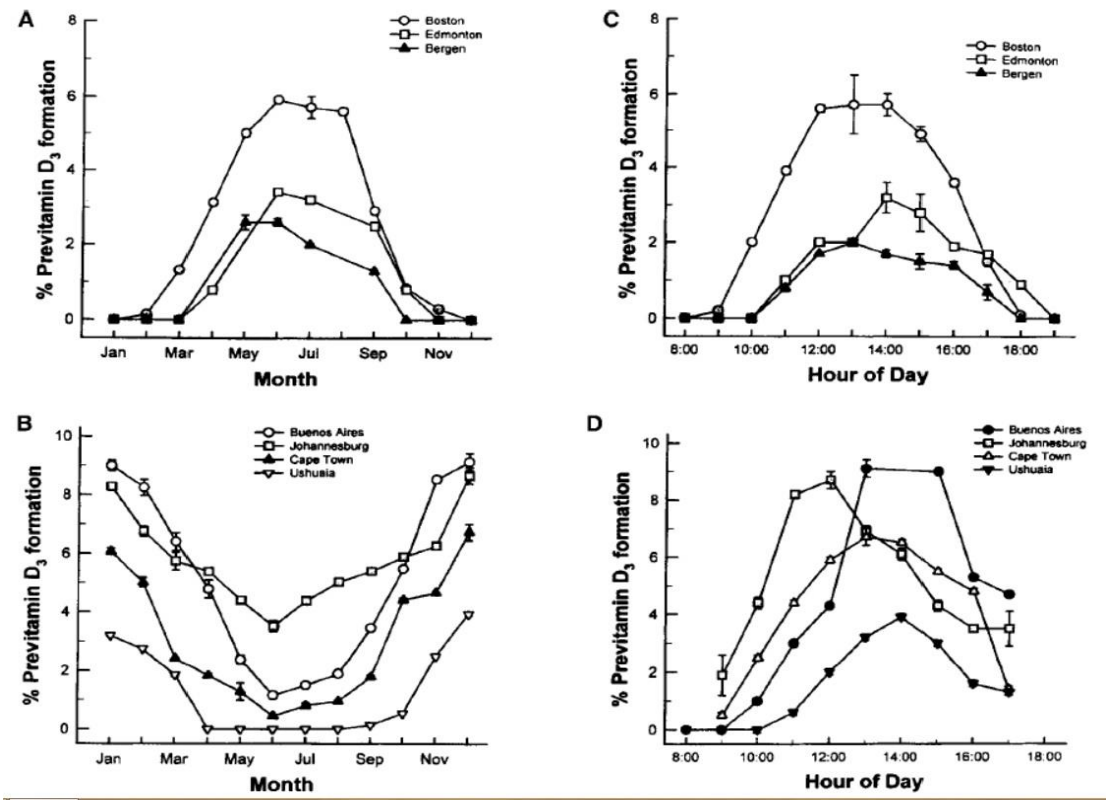


Figure 6 Changes in Previtamin D_3 Production. (A) Depiction of the yearly change of previtamin D production within the Northern Hemisphere. (B) Depiction of the yearly change of previtamin D production in the Southern Hemisphere. (C) Depiction of the daily variation within the Northern Hemisphere at a time when vitamin D production is most optimal. (D) Depiction of the daily change in previtamin D production in the southern hemisphere when production is most optimal. Obtained from (4).

In addition to the zenith angle changes during the course of a year or day, melanin pigmentation of skin, pollution released into the air, cloud coverage, reflection off of surfaces, and elevation may also influence the production of previtamin D[24,25]. A higher concentration of melanin pigmentation within keratinocytes is known to absorb broadband ultraviolet radiation, thus interfering with formation of previtamin D_3. Despite this hindrance, a person with highly pigmented skin is still capable of producing adequate amounts previtamin D_3 and but must devote more time to sun exposure or rely on dietary supplementation as a vitamin D source[24,25]. Similar to ozone absorption, pollution in the

air is capable of absorbing UV-B radiation and hindering production of previtamin D_3. As discussed previously, pollution released from coal-burning during the industrial revolution contributed to the epidemic of rickets within the cities. Concentrated NO_2 and other aerosols have been shown to attenuate both direct and diffuse UV-B radiation[8].

Depending on size, shape and thickness, clouds may either attenuate or enhance UV-B irradiance at the Earth's surface. Thin cirrus clouds may allow UV radiation to pass through without any significant impedance. Thick clouds on an overcast day may completely block UV-B irradiation from reaching the Earth's surface. Under partly cloudy conditions though, clouds may provide diffuse irradiation in addition to the direct irradiation from the sun[8].

Highly reflective surfaces and higher elevations may contribute to the total irradiation and promote production of previtamin D_3[26]. The proportion of reflected light, known as albedo, contributes to the total diffuse and direct irradiation. Typical surfaces may reflect up to 4% of the incident radiation striking them, whereas water may reflect between 5-8%. Surprisingly clean, freshly fallen snow has been found to reflect up to 80% of incident radiation, but this measure decreases as the snow begins to either crystalize or melt[8,9]. A larger amount of solar radiation has been observed at higher elevations, as compared to locations at sea-level. In part, UV-radiation does not have to traverse as great of a path length at higher elevations compared to locations at sea-level, so the inverse-square law of electromagnetic radiation has less of an influence on the total irradiance. In addition, the UV-radiation has not been subjected to the same degree of scattering or absorption from particles in the atmosphere at this higher elevation, while

the surrounding clouds may also provide a higher albedo due to scattering contributing to diffuse radiation, especially at higher elevations[8,9].

Metabolism, Regulation and Physiology of Vitamin D_3

Once vitamin D has been synthesized in the skin, it is released into the circulation through the dermal capillary bed. Vitamin D-binding protein then picks up the vitamin and carries the nascent compound to the liver to be hydroxylated by D-25-hydroxylase. If the vitamin is absorbed through the diet, then it is incorporated into a chylomicron and enters the venous circulation by way of the lymphatic system and reaches the liver just the same. In either case, the 25-hydroxyvitamin D (25(OH)D) is then carried to the kidneys to be further activated by another hydroxylation from 25(OH)D-1-alpha-hydroxylase[5,27-30]. This activated form then returns to the circulation to effect changes all throughout the skeletal system and body, as well as to promote self-regulation through feedback mechanisms, just as any hormone would (Figure 7). In addition to the activation of 25-hydroxyvitamin D by the kidneys, it has been established that various other tissues throughout the body possess the capability of producing 25(OH)D-1-alpha-hydroxylase and locally stimulating the production of 1,25-dihydroxyvitamin D (1,25(OH)D)[2].

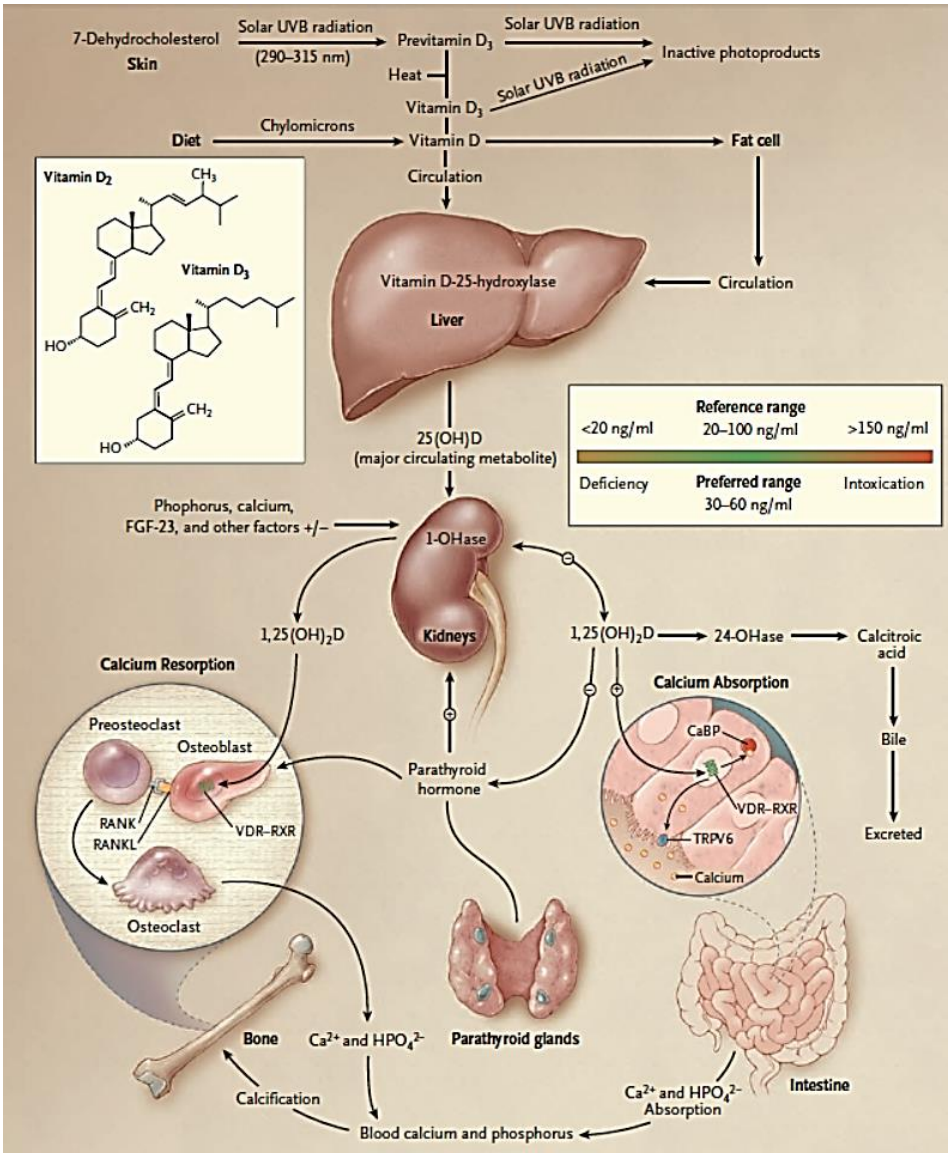


Figure 7 Metabolism of Vitamin D_3 within the Human Body. Obtained from (2).

Many factors influence the expression of 25(OH)D-1-alpha-hydroxylase, of which calcium and phosphorous are the most critical. Activated 1,25-dihydroxyvitamin D regulates its own synthesis by decreasing the concentration of parathyroid hormone, known to stimulate the production of 1,25-dihydroxyvitamin D through upregulation of 25(OH)D-1-alpha-hydroxylase[28]. In addition, this hormone also provides direct negative

feedback to the enzyme responsible for its production. 1,25-dihydroxyvitamin D also promotes its own degradation by inducing the synthesis of 25-hydroxyvitamin D-24-hydroxylase, which adds an additional hydroxyl group to both 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D. These deactivated hormones are then metabolized and the byproducts of this catabolism are then excreted through the bile system[28].

Once activated, vitamin D is known to predominantly take its effect of regulating the serum concentration of calcium, Ca^{++}, through the regulation of bone metabolism, intestinal calcium absorption and renal tubule reabsorption. Within the intestines, vitamin D increases the absorption of calcium from 10-15% to 30-40%, by increasing the expression of the epithelial calcium channel and calbindin 9K[28,31,32]. In addition to calcium, vitamin D may also increase the absorption of phosphorous. Whether or not activated vitamin D has any direct effect on calcium and phosphorous reabsorption within the nephrons remains controversial and to be elucidated[27]. Within the skeletal system, vitamin D increases the expression of receptor activator of nuclear factor-κB ligand (RANKL), which is known to induce maturation of preosteoclasts to osteoclasts[28]. Vitamin D is also known to have associations with other calcium-related and non-calcium-related processes, which may have comprehensive implications to health (Figure 8). Although these associations are striking, they need to be investigated further to better establish how, and if, vitamin D plays a critical factor in these diseases.

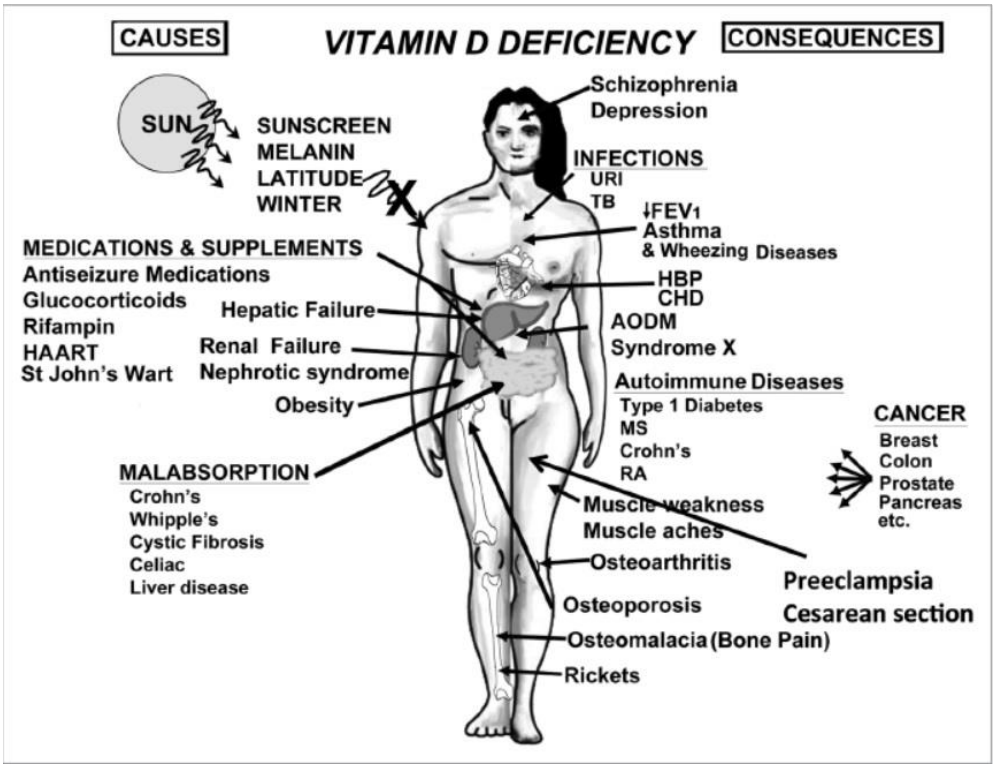


Figure 8 Consequences of Vitamin D Deficiency. Obtained from (4).

SPECIFIC AIMS

This research project was performed during Boston's fall and winter months, where it has been established that the increased atmospheric absorption of UVB radiation reduces or prevents the synthesis of previtamin D_3 from 7-dehydrocholesterol[14]. Irradiation of tachysterol$_3$ was conducted during the months of October and March, during which less than 1 percent of previtamin D_3 can be produced from solar radiation (Figure 6). The goal of this research project was to determine the feasibility of irradiating tachysterol$_3$ with fall and winter sunlight to determine if it could be converted to previtamin D_3 at a time when 7-DHC cannot. Given the fact that tachysterol can absorb UV radiation up to 340nm, it was hypothesized that it can absorb the UV radiation between 300 and 340nm, and undergo isomerization to previtamin D (figure 9), whereas 7-DHC can only absorb UVB radiation up to 315nm and therefore cannot be converted to previtamin D_3 due to its low quantum efficiency at wavelengths greater than 300nm. Lumisterol was also irradiated but since it can only absorb wavelengths up to 315nm and has a low quantum efficiency as well, it was hypothesized that this isomer would not undergo any isomerization either. 7-dehydrocholesterol was used as a control during this project.

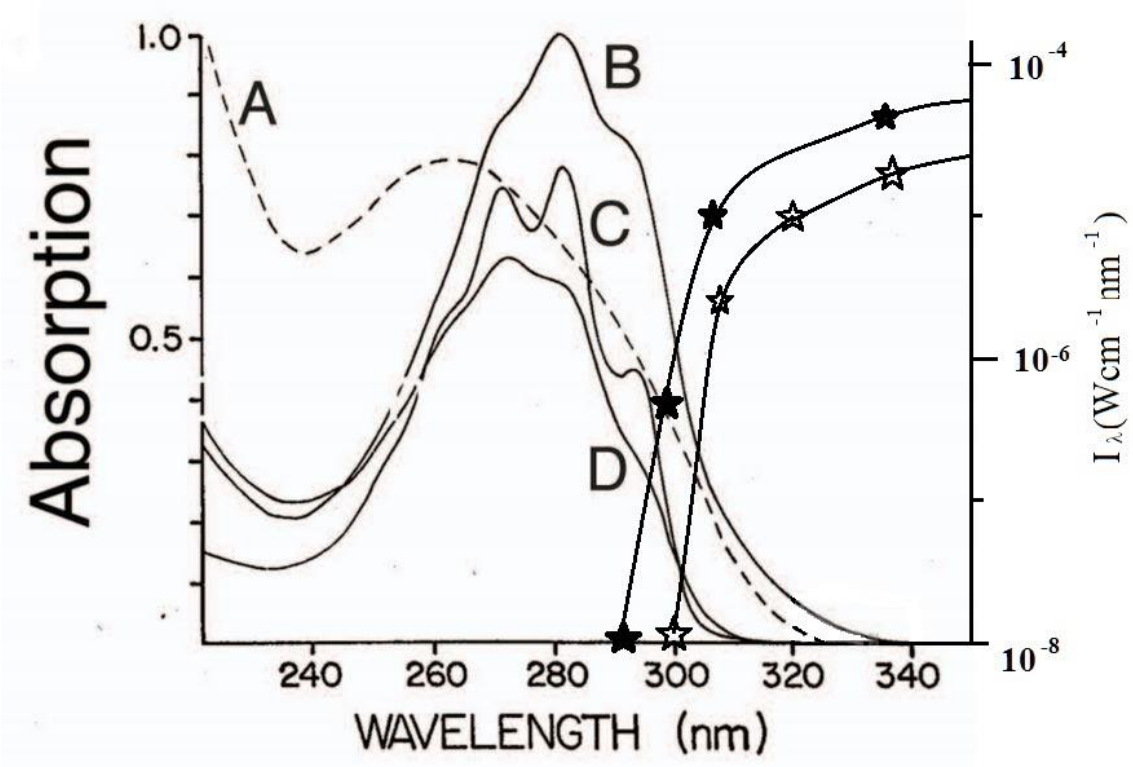


Figure 9 Absorption Spectra of the Vitamin D Field. Wavelengths of photoisomers: (A) Previtamin D (B) Tachysterol (C) 7-dehydrocholesterol (D) Lumisterol. The shaded region of the spectra indicates the target wavelengths of this study, including the tail ends of tachysterol$_3$ and previtamin D_3. The Y-axis on the left indicates the intensity of ultraviolet radiation as a function of wavelength. The line with the shaded in stars indicates solar UV radiation during the month of June, whereas the hollow stars indicate ultraviolet radiation during the month of January. Adapted from (4) with data from (22).

METHODS

Stock Solutions

7-Dehydrocholesterol

A 4 gram sample of 7-dehydrocholesterol (7-DHC) powder was weighed on a balance to 3 decimal places, reading 4.052 grams. The dry sample was then dissolved in 400mL of 100% ethanol to produce a solution with a concentration of 10mg/mL. A 1mL aliquot of this 7-DHC solution was then withdrawn and diluted to 50 μg/mL by adding 19mL of 100% ethanol, to create the stock solution of the sample to be irradiated. The 50 μg/mL solution was measured using a Hitachi U-3900 UV-spectrophotometer to determine the optical density to use in calculating the concentration using the formula: concentration (μg/mL) = optical density × molecular mass × extinction coefficient^{-1}. Once the concentration was determined, it was either diluted or dried and resuspended with 100% ethanol to ensure a concentration of 50 μg/mL. This stock solution was used to create the samples of 7-dehydrocholesterol that acted as the controls during this study.

Lumisterol$_3$ and Tachysterol$_3$

To produce stock solutions of lumisterol$_3$ and tachysterol$_3$, a 5mL sample of the 10mg/mL 7-dehydrocholesterol solution was irradiated in a petri dish with a quartz top under a KBD Inc. Sperti UV-lamp manufactured in Crescent Springs, Kentucky for thirty to forty-five minutes at a distance of 5 cm (Figure 10). This sample was rested on a block of PolarPack Foam Brick by Tegrant Corporation during the irradiation process to reduce the conversion of previtamin D$_3$ to vitamin D$_3$. The products of this irradiation were then

separated and collected using high pressure liquid chromatography (HPLC). Using a Zorbax CN column from Agilent Inc. in Santa Clara, CA, tachysterol$_3$ was separated from vitamin D$_3$. A Zorbax RX-SIL column also from Agilent Inc. was used in separating lumisterol$_3$ and previtamin D$_3$, since the CN column is incapable of separating previtamin D$_3$ from lumisterol$_3$. An Agilient 1100 Series High-Performance Liquid Chromatography System with a variable wavelength UV detector was used to carry out the analyses and separation (Figure 11). Tachysterol$_3$ was recovered by using an eluent of 0.3% isopropanol in n-hexane at a flow rate of 1.5mL/min on the CN column, whereas separation of lumisterol$_3$ and previtamin D$_3$ was performed using an eluent of 0.8% isopropanol in n-hexane with the RX-SIL column.

Figure 10 Synthesis of Tachysterol and Lumisterol. (Left) 7-Dehydrocholesterol was irradiated under the Sperti UV-Lamp by KBD, Inc. for 45 minutes over an ice pack to reduce conversion of previtamin D$_3$ to vitamin D$_3$. (Right) The Sperti lamp used in preparing the stock solutions.

Figure 11 The HPLC setup for isolation, identification, and quantification of products within the vitamin D field: (A) the main unit consisting of the autosampler, pump, degasser, and variable wavelength detector. (B) Zorbax CN column used to separate vitamin D_3 from tachysterol$_3$. (C) Zorbax RX-Sil column used to separate previtamin D_3 from lumisterol$_3$.

The stock solutions of lumistrol and tachysterol were measured by a Hitachi U-3900 UV-spectrophotometer to determine their optical densities which was used to calculate concentrations again using: concentration (μg/mL) = optical density × molecular mass × extinction coefficient^{-1}. Once the concentrations were determined, they were diluted with 100% ethanol to ensure a concentration of 50 μg/mL, to create the

tachysterol$_3$ and lumisterol$_3$ samples that were irradiated. This procedure was repeated as needed, whenever the stored stock solution had run out.

Preparing Samples for Irradiation

7-Dehydrocholesterol ampules were prepared by diluting 400uL of the 50ug/mL 7-dehydrocholesterol solution by 3.6mL of 100% ethanol to produce a 4mL solution with a concentration of 5ug/mL. From this 4mL solution, 200 uL were then dispensed into 2mL clear borosilicate ampules from Wheaton Science, producing 20 ampules of 7-dehydrocholesterol samples. Each sample was sealed with parafilm, labeled, and stored in a freezer at negative 20 degrees centigrade until ready for irradiation. A similar process was performed using lumisterol$_3$ and tachysterol$_3$ stock solutions. Samples were created as needed the night before use and any samples that were left-over were stored in the freezer until needed.

Irradiation Protocol

The weekly forecast was screened every Sunday to determine if any days during the same week would provide the best conditions for irradiation of samples. Best conditions for irradiation were considered a forecast of at least “Sunny” and preferably “Clear” by the National Weather Service and the National Oceanic and Atmospheric Association. These preferable forecasts would translate to a sky cover of at most thirty percent or less, according to the National Weather Service. The forecast was screened once more on the night before each day predicted as suitable for sample irradiation.

Samples were then placed on the roof for irradiation. Starting at sun-rise and lasting until sun-set, each sample was irradiated for roughly an hour. If the temperature of the day was predicted to reach a high above 32 degrees Fahrenheit, then the samples were left on a block of ice to reduce the conversion of previtamin D_3 to vitamin D_3 over the course of irradiation. A solarmeter from Solartech Inc. in Glenside, PA was used at sun-rise, sun-set and each hour to gauge the density of solar UV radiation, measured in minimal erythemal dose per hour, MED/hr (Figure 12), which is equivalent to $20mJ/cm^2$. According to the specifications provided by Solartech Inc., this digital UV meter is capable of detecting UV radiation within the 200-400nm range with an accuracy of plus or minus ten percent according to the National Institute of Standards and Technology[33]. The measurement was taken by pointing the receiver directly at the sun to gauge both direct and diffuse irradiation. This reading was recorded in a spreadsheet along with the amount of sky coverage over the course of the same hour that the sample was left out for irradiation. All data was obtained from the National Oceanic and Atmospheric Administration and the National Weather Service. After irradiation the samples were stored within a freezer until they were ready for analyzed.

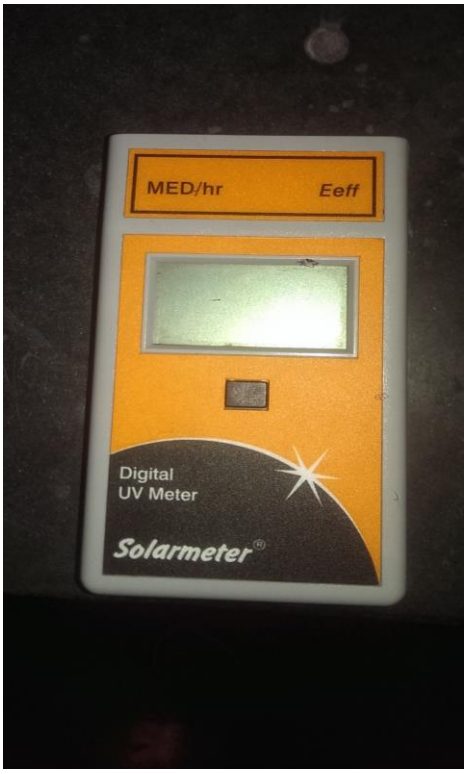


Figure 12 Solarmeter used to gauge the MED/hr during each day of the study

A time course to gauge when the irradiation of tachyssterol$_3$ would peak was also established by irradiating six tachysterol$_3$ samples at noon and collecting one every ten minutes for the hour. In addition to allowing the sun to irradiate the samples at various hours during a sunny or clear day, a comparison was also made between cloudy and clear days to determine whether or not cloud coverage could have a significant impact on the conversion of tachysterol$_3$.

High-Performance Liquid Chromatography Analysis

After the samples were irradiated, they were prepared for analysis on the Agilient 1100 Series High-Performance Liquid Chromatography System. The irradiated samples were analyzed with normal phase high-performance liquid chromatography in tandem with a variable wavelength UV detector.

The irradiated samples were removed from the freezer, transferred to a disposable culture tube, and dried down with a constant stream of nitrogen gas. The ampules originally containing the sample were rinsed with 0.5mL of ethanol and the rinse was added to the sample. Once dried, the sample was then resuspended in 140uL of the HPLC eluent used for running the analysis and transferred to a microvial with a glass sleeve for automated injection into the HPLC system.

The eluent used for analysis was a 0.8% mixture of isopropanol in hexane in conjunction with the Zorbax RX-SIL column from Agilent. The samples were processed through the system at a flow rate of 1.5mL/min for 15minutes. Once the components of each sample had cleared the column, they passed through the variable wavelength detector in tandem with the column that detects wavelengths of radiation of 280nm, 265nm, 230nm, 220nm and 210nm. An additional 2.5 minutes were used in between samples to ascertain that the sample had been completely cleared from the column before the next sample was injected.

Analysis of Raw Data

To analyze the data obtained from the HPLC, two sets of identifying data were checked. Each chromatographic peak was identified by the comparing the ultraviolet spectrum produced from the variable wavelength detector to a reference standard (Figure 13). The time at which each compound passed through the column, as indicated by the peak was also referenced to standard chromatograms (Figure 14).

After identification of each compound, their respective peaks were integrated and these areas were recorded within the spreadsheet containing the MED and sky coverage recorded as the samples were being irradiated. The integrations of lumisterol$_3$, tachysterol$_3$ and 7-dehydrocholesterol were measured using the 280nm wavelength since their absorption spectra are most pronounced at this wavelength of radiation. In contrast, the integrations of previtamin D_3 and any vitamin D_3 were measured using the 265nm wavelength.

The data retrieved from the solarmeter was converted from MED to irradiation density (mJ/cm^2), using a conversion factor of 20, as derived in Title 21 of the U.S. Food and Drug Administration's Code of Federal Regulations[36].

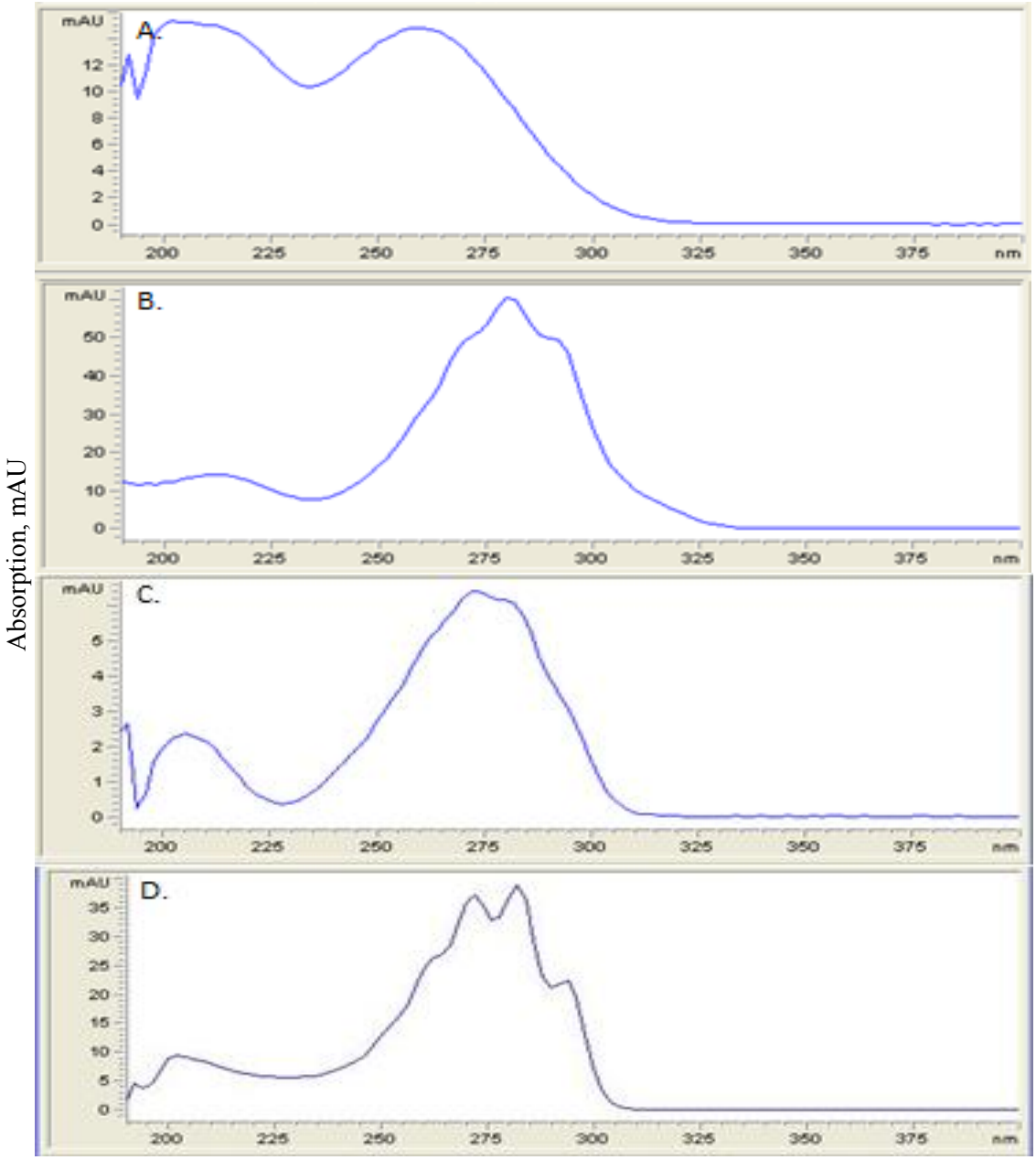


Figure 13 Absorption Spectra of the Photoproducts: (A) Previtamin D_3 (B) Tachysterol$_3$ (C) Lumisterol$_3$ and (D) 7-Dehydrocholesterol. These were used as references when determining the products produced after irradiation.

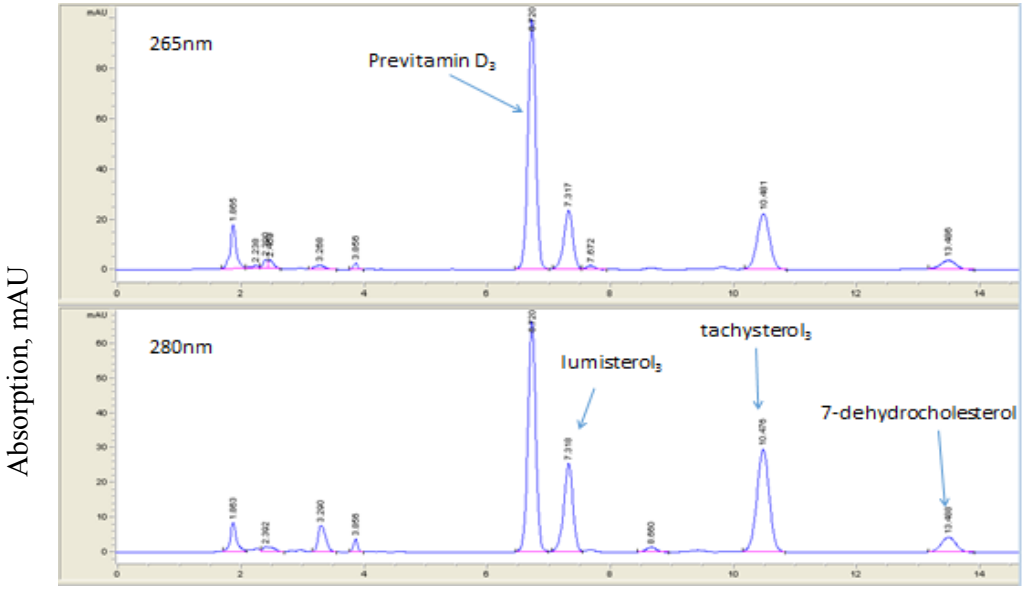


Figure 14 Labeled Sample Chromatogram of Irradiated Tachysterol$_3$. These chromatograms were used as references when analyzing the HPLC results after irradiating tachysterol$_3$, lumisterol$_3$, and 7-dehydrocholesterol.

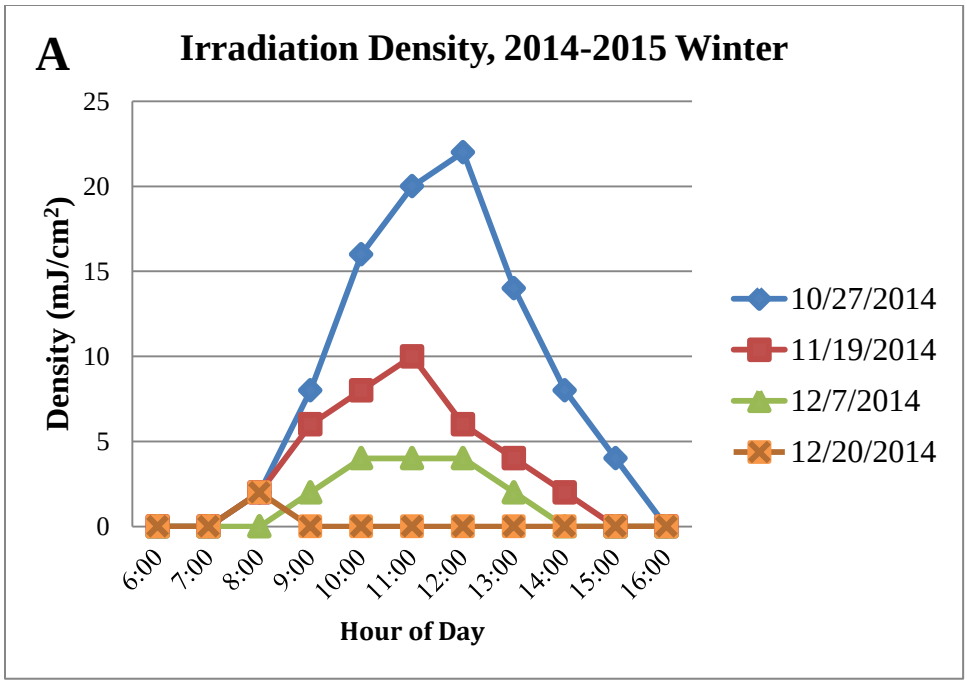
Quantification

The measurements of the areas under the curves obtained from integration are then converted to mass using conversion factors obtained from a previously created standard curve for 7-dehydrocholesterol, previtamin D$_3$, tachysterol$_3$, and lumisterol$_3$. From these conversions, the mass of each compound was calculated. Since these compounds are all isomers and contain the same molecular formula, direct comparison of mass would be equivalent to comparison of the amount in moles of each compound. This data was used to make the final analysis and draw conclusions.

RESULTS

Monthly Change in Irradiation Density

The irradiation density, measured as MED/hr and converted to equivalent values in mJ/cm^2, was logged to demonstrate the effect of season on the amount of radiation passing through the atmosphere (Figure 15). A peak of 22 mJ/cm^2 was reached on Octorber 27, 2014, followed by 10 mJ/cm^2, 4 mJ/cm^2 and 2 mJ/cm^2 on November 19, December 7, and December 20, 2014, respectively. By January 17, 2015, the radiation density increased to 8 mJ/cm^2, followed by 12 mJ/cm^2 and 18 mJ/cm^2 on February 13 and 28, respectively.



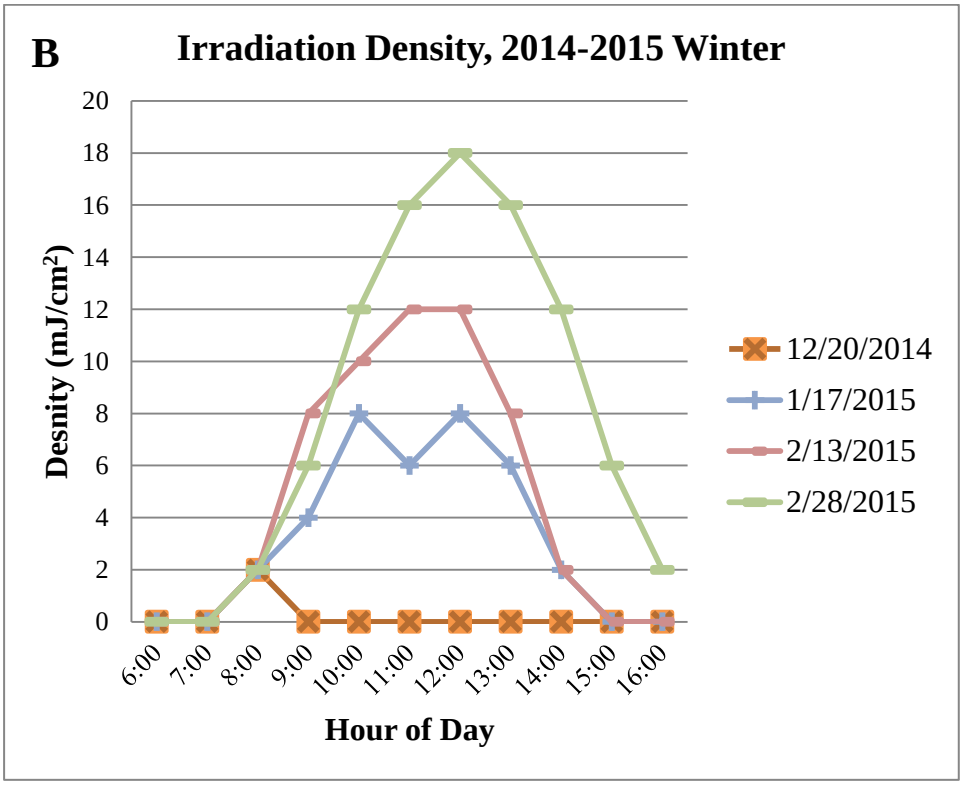


Figure 15 Irradiation Density of 2014-2015 Boston Winter. (A) Density of Irradiation during the first half of the winter. (B) Density of Irradiation during the second half of winter.

Tachysterol$_3$ Conversion to Previtamin D$_3$ Each Month

High-performance liquid chromatography of the tachysterol$_3$ samples demonstrated conversion to previtamin D$_3$ during each month that tachysterol$_3$ was irradiated. From the project's start on 10/27/2014 until the winter solstice, 12/20/2014, a progressive decrease in the maximal conversion of tachysterol$_3$ to previtamin D$_3$ was observed (Figure 15a). On October 27, a maximum of 60% of tachysterol$_3$ converted to previtamin D$_3$, followed by 58% and 44% on November 19 and December 7, respectively. By the winter solstice on December 20, conversion to previtamin D reached a maximum of 18%. After the winter solstice until the last day of data collection, 2/28/2015, a progressive increase in the conversion of tachysterol$_3$ to previtamin D$_3$ was observed (Figure 15b). On January 17 and February 13, 42% and 44% of the original tachysterol$_3$ converted to previtamin D$_3$, whereas 66% converted on February 28.

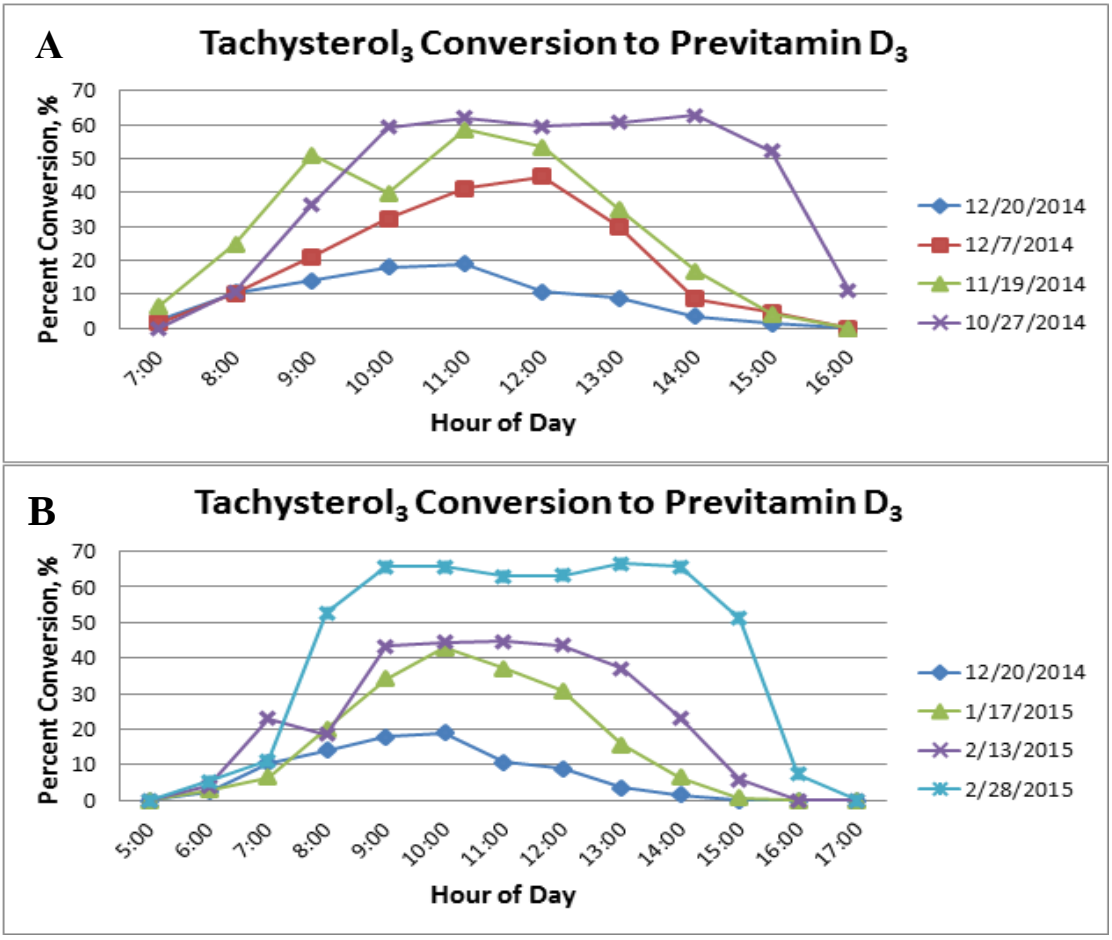


Figure 16 Tachysterol$_3$ Conversion to Previtamin D$_3$. (A) Conversion of tachysterol to previtamin D: 10/27/2014 (purple); 11/19/2014 (green); 12/7/2014 (red); 12/20/2014 (blue). NOTE: Due to daylight savings time, the data from 10/27/2014 is skewed one hour to the right and each data point should be considered one hour back. (B) Conversion of Tachysterol to Previtamin D: 12/20/2014 (blue) 1/17/2015 (green); 2/13/2015 (purple); 2/28/2015 (cyan)

Within the first hour after sun-rise, from 7:00 to 8:00, on October 27, 2014, 2.1% of tachysterol$_3$ had converted to previtamin D$_3$ (Figure 16). Conversion of tachysterol$_3$ to previtamin D$_3$ was observed throughout the day, plateauing at 60% between the hours of 11:00 and 14:00 (11:00am and 2:00pm). The conversion reached a peak of 62.8% at 14:00 before beginning a rapid decline. Within the last hour before sunset, 11.3% of the

tachysterol$_3$ had converted to previtamin D$_3$. In addition to the conversion of tachysterol$_3$ to previtamin D$_3$, lumisterol$_3$ and 7-dehydrocholesterol were also observed from irradiating tachysterol$_3$ on this day.

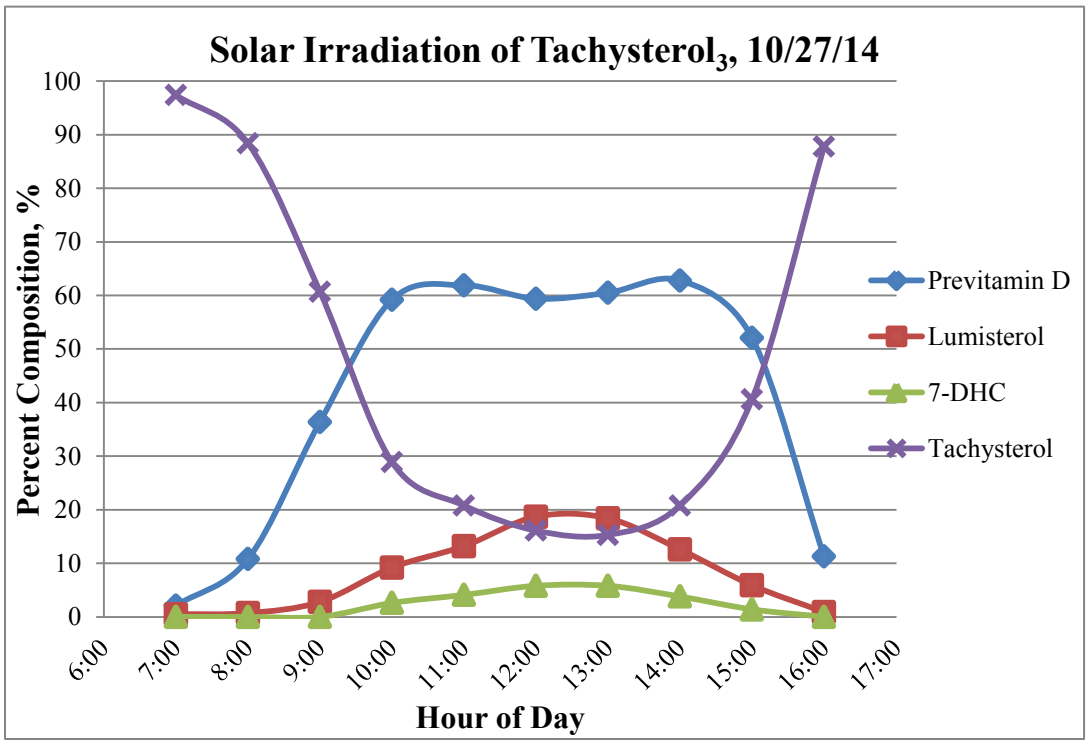


Figure 17 Solar Irradiation of Tachysterol$_3$ on October 27, 2014

On November 19, 2014, conversion of tachysterol$_3$ to previtamin D$_3$ was seen throughout the whole day. Within the first hour of sun-rise at 7:00, 6.5% of tachysterol$_3$ converted to previtamin D$_3$. Conversion reached a peak of 58.4% at 11:00 (Figure 17). During the last hour before sunset, 4.3& of tachysterol$_3$ had converted to previtamin D$_3$. A drop in tachysterol$_3$ conversion was seen at 10:00, possibly due to momentary shadowing from neighboring buildings, as there was no significant cloud coverage according to the National Weather Service. Consistent with all other data, conversion of tachysterol$_3$ to previtamin D$_3$ occurred throughout the whole day. As with the prior

irradiation day, small amounts of lumisterol$_3$ and 7-DHC also appeared. Starting at 8:00 and ending at 14:00, lumisterol peaked at 8.2%. Between the hours of 11:00 and 12:00, 7-DHC peaked at 2.0%.

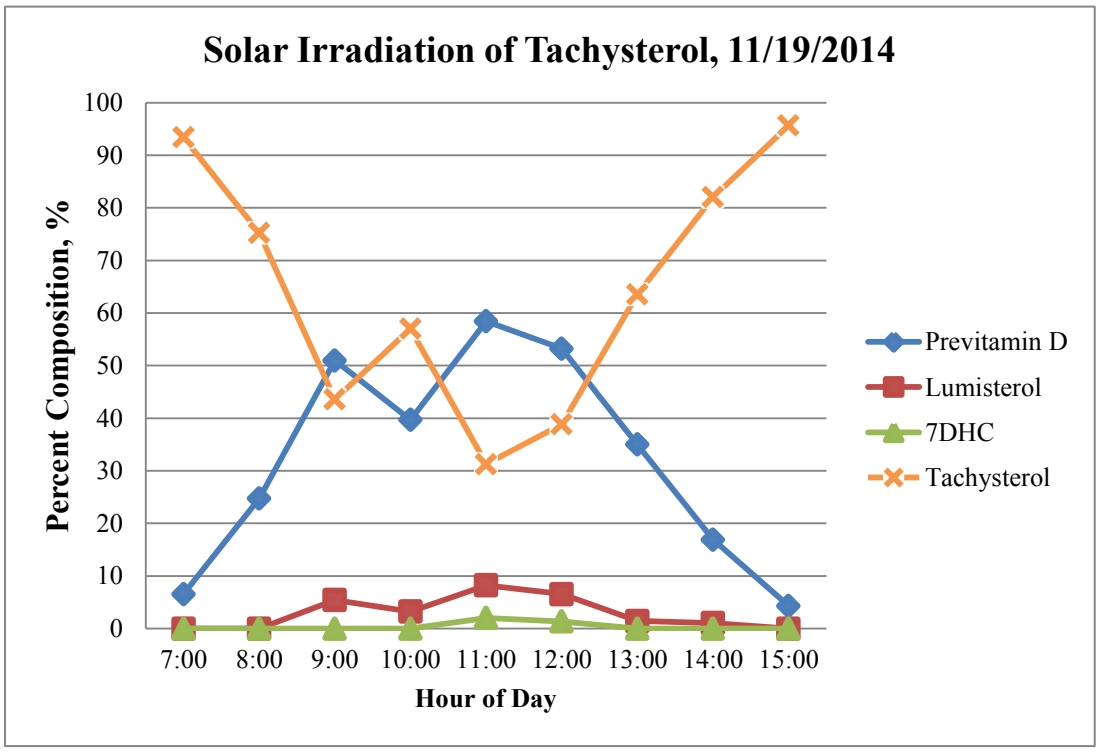


Figure 18 Solar Irradiation of Tachysterol$_3$ on November 19, 2014

On December 7, 2014, tachysterol$_3$ conversion to previtamin D$_3$ was also seen throughout the whole day (Figure 18). There was no plateau in conversion from tachysterol$_3$ to previtamin D$_3$, but conversion peaked at 44.7% at 12:00. Lumisterol$_3$ production was seen between 11:00 and 13:00, peaking at 3.9% at 12:00. No production of 7-dehydrocholesterol was detected by the HPLC.

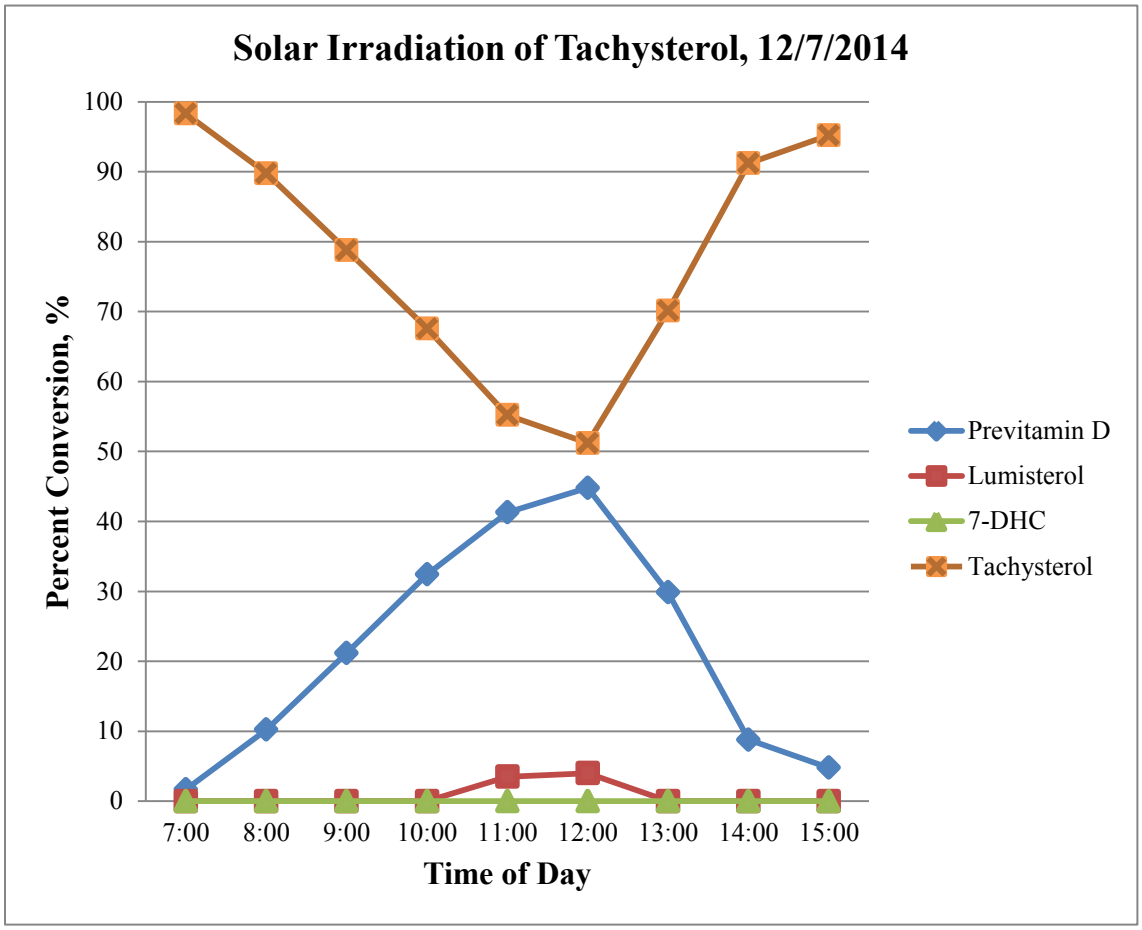


Figure 19 Solar Irradiation of Tachysterol$_3$ on December 7, 2014

Within the first hour after sun-rise on December 20, 2014, just 2.5% of tachysterol$_3$ had converted to previtamin D$_3$ (Figure 19). Although no plateau was observed a maximum of 18.9% of the tachysterol$_3$ converted to previtamin D$_3$ between the hours of 11:00am and 12:00pm. Fairly minimal lumisterol$_3$ was observed, while no 7-

dehydrocholesterol was detected by the UV detector.

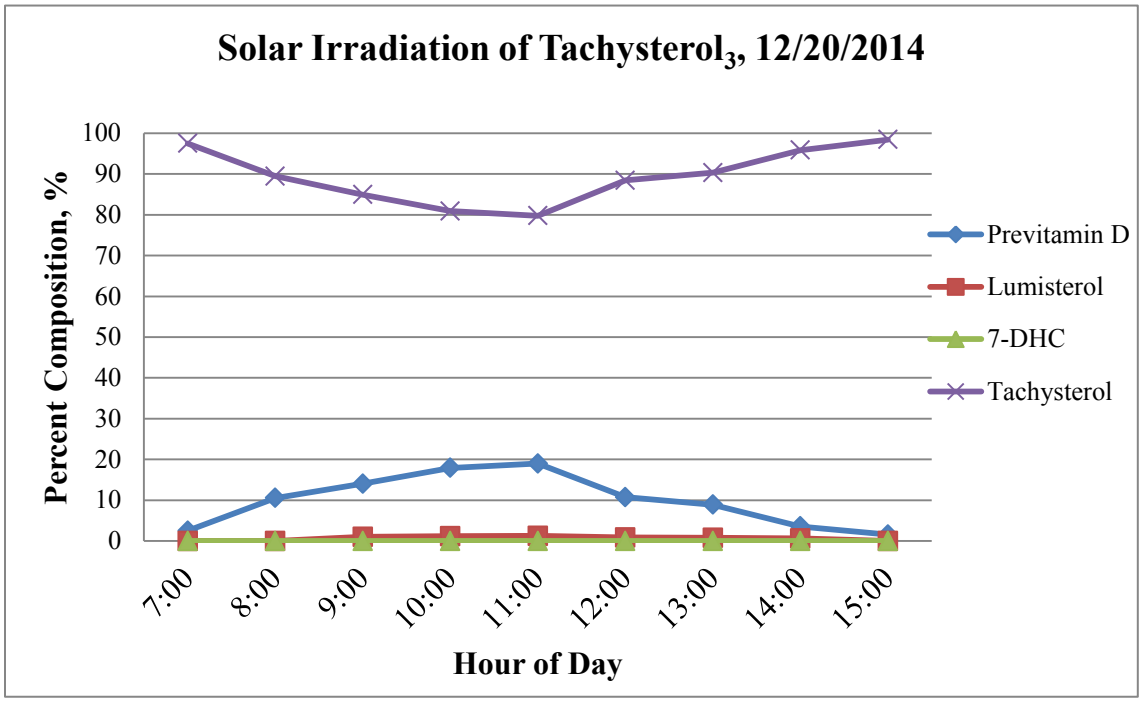


Figure 20 Solar Irradiation of Tachysterol$_3$ on December 20, 2014

On December 27, 2014, conversion of tachysterol$_3$ to previtamin D$_3$ occurred throughout the day, starting at 1.8% during the hour after sunrise, peaking at 47.1% at 12:00, and finishing at 0.6% during the hour before sunset at 15:00 (Figure 20). Trace amounts of lumisterol$_3$ were seen throughout the day, peaking at 5.2% at 12:00. No 7-dehydrocholesterol was detected. The drop in previtamin D must be noted at 11:00, which may be caused by an artifact casting a shadow on the samples. Cloud coverage was

reported at 14% by the National Weather Service.

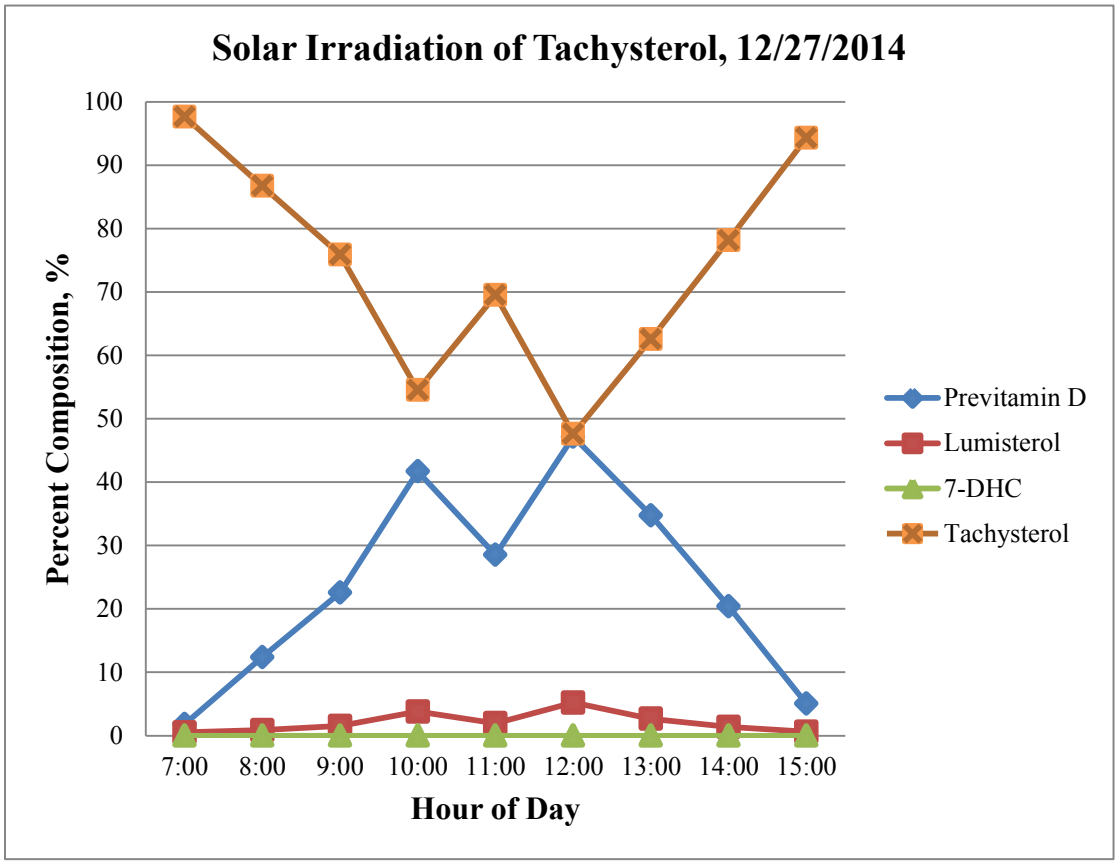


Figure 21 Solar Irradiation of Tachysterol$_3$ on December 27, 2014

Irradiation of tachysterol$_3$ on January 17, 2015 demonstrated conversion of tachysterol$_3$ to previtamin D$_3$ throughout the whole day, starting at 2.9% during the hour after sunrise, peaking at 42.9% at 11:00, and finishing the hour just before sunset at 0.8% (Figure 21). Lumisterol$_3$ was observed between 10:00 and 13:00, peaking at 5.4% between the hours of 11:00 and 12:00. No conversion to 7-dehydrocholesterol was detected.

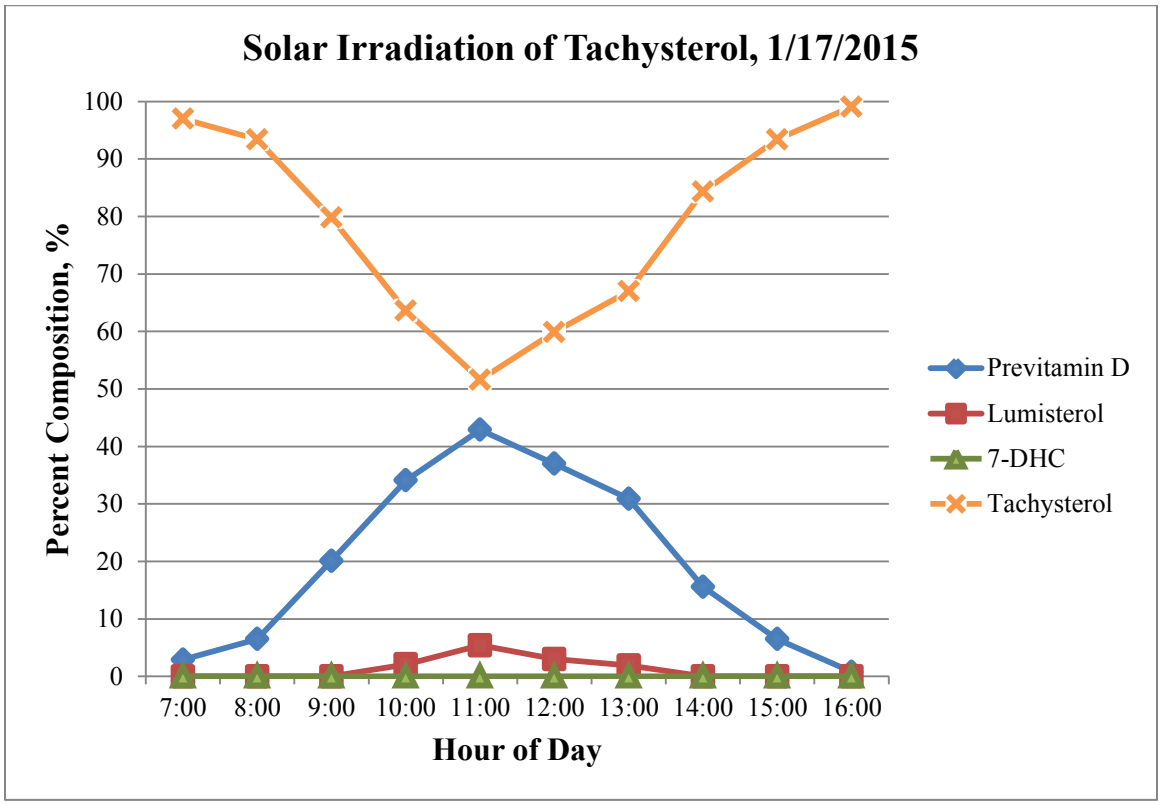


Figure 22 Solar Irradiation ofTachysterol$_3$ on January 17, 2015

Irradiation of tachysterol$_3$ on February 13, 2015 demonstrated conversion throughout the day (Figure 22). Within an hour of sunrise at 7:00 4.1% of tachysterol$_3$ converted to previtamin D$_3$. A plateau in conversion was detected between the hours of

10:00 and 13:00, reaching a maximum conversion at 44.6% at 12:00. Lumisterol$_3$ was produced from the irradiation throughout the day, peaking at 22.6% at 12:00. No 7-dehydrocholesterol was detected throughout the day. The drop in conversion at 9:00 may be due to a neighboring structure casting a shadow on the samples.

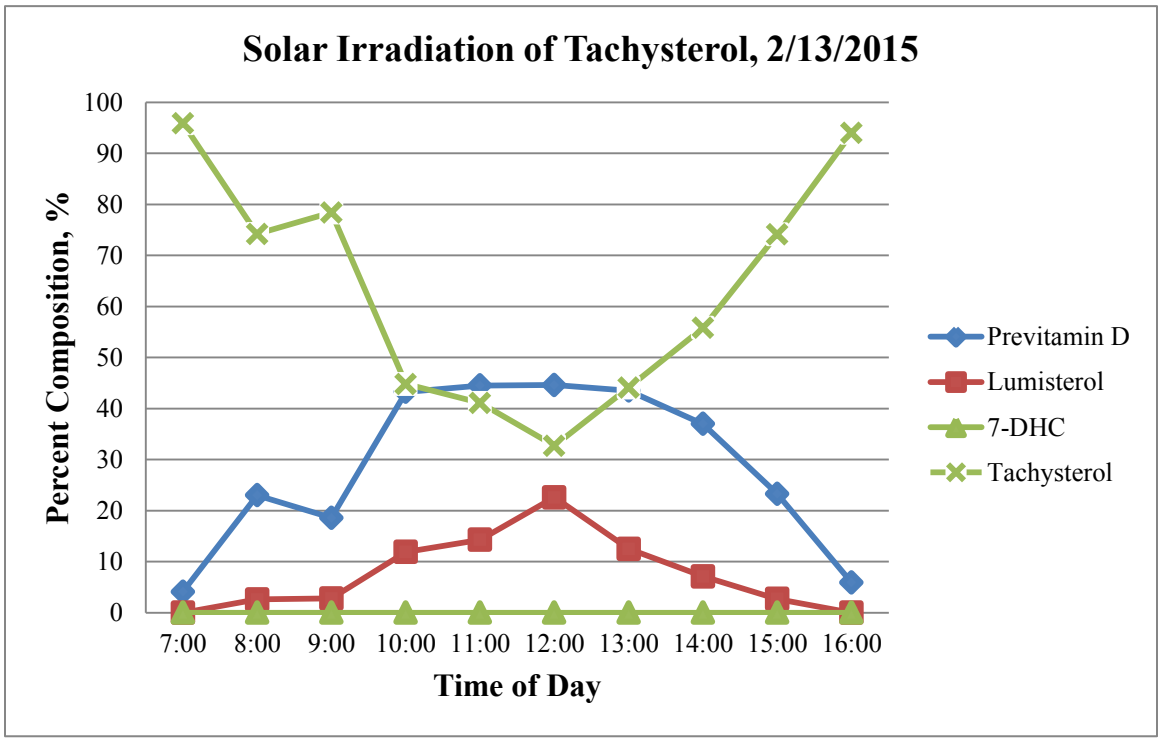


Figure 23 Solar Irradiation of Tachysterol$_3$ on February 13, 2015

Within the first hour on February 28, 2015, 5.3% of tachysterol$_3$ converted to previtamin D$_3$ (Figure 23). Conversion of tachysterol$_3$ to previtamin D$_3$ was observed throughout the day, plateauing between the hours of 9:00 and 14:00 (9:00am and 2:00pm). A maximum of 66.5% of tachysterol$_3$ converted to previtamin D$_3$ during the hour between 13:00 and 14:00. Production of lumisterol$_3$ was also observed, peaking at

20.% at 12:00. No production of 7-dehydrocholesterol was observed by the HPLC.

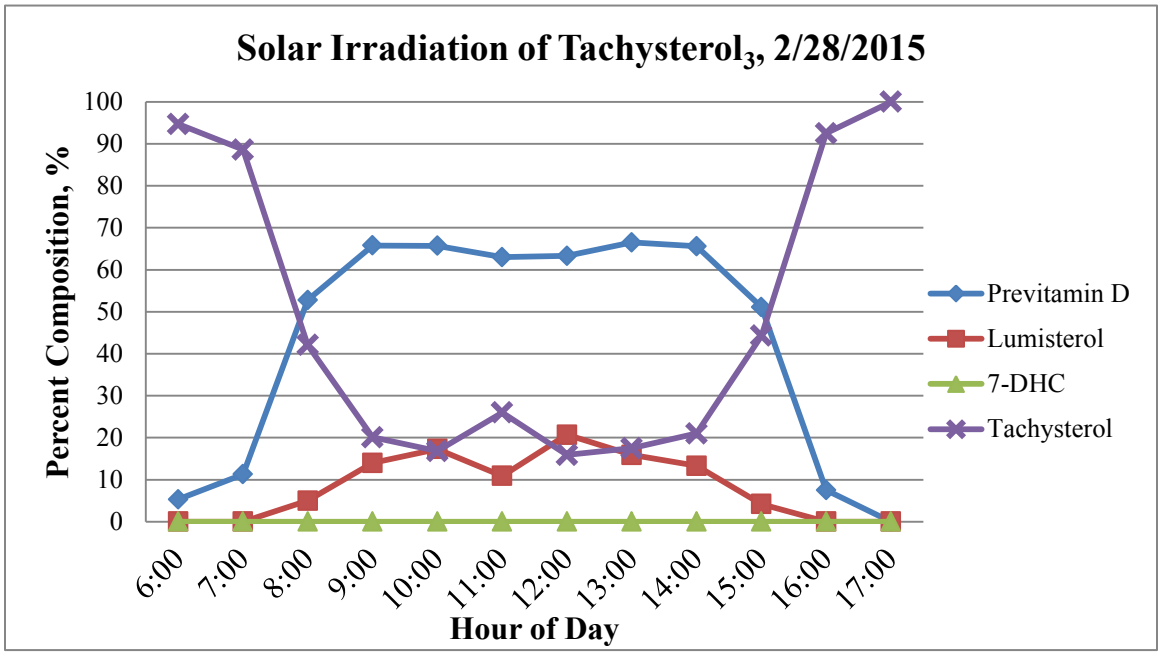


Figure 24 Solar Irradiation of Tachysterol$_3$ on February 28, 2015

7-Dehydrocholesterol Conversion to Previtamin D_3

Throughout the course of this study, no 7-dehydrocholesterol converted to previtamin D_3 until the last day, 2/28/2015. During the hour between 13:00 and 14:00, 2.8% of 7-dehydrocholesterol converted to previtamin D_3, whereas 1.7% of the original 7-dehydrocholesterol converted during the hour between 14:00 and 15:00.

Lumisterol$_3$ Conversion to Previtamin D_3

Throughout the course of this study, no conversion of lumisterol$_3$ was detected by the HPLC.

Time Course

A time-course for the conversion of tachysterol$_3$ to previtamin D$_3$ at noon in March was determined (Figure 25). In the first twenty minutes, 52% of all the tachysterol$_3$ was converted to only previtamin D$_3$. After the initial 20 minutes, the amount of lumisterol$_3$ began to gradually increase until the full hour and did not plateau at 60 minutes. No isomerization to 7-dehydrocholesterol was seen during this hour. Conversion to previtamin D$_3$ continued to increase during the first 30 minutes and then reached a plateau of 65% at 40 minutes of irradiation.

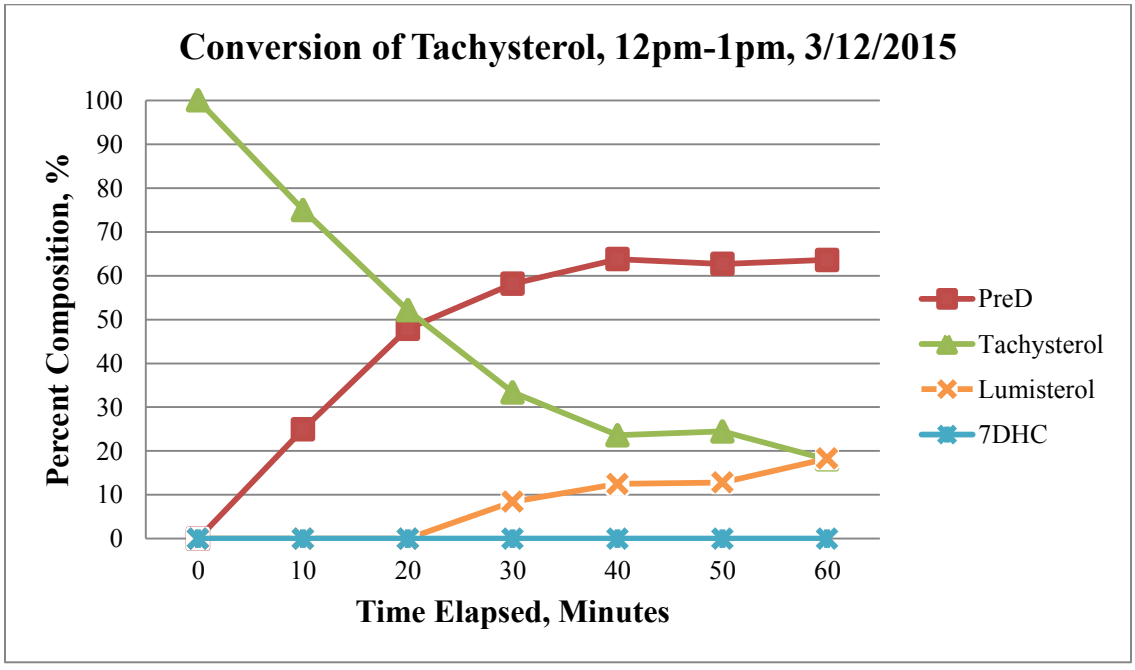


Figure 25 Time course of Solar Irradiation of Tachysterol$_3$ on March 12, 2015. This time course was run during the hour between 12:00 and 13:00, or between 12:00pm and 1:00pm EST.

Triplicate Measures

The triplicate measurements were obtained on March 12, 2015 (figure 24). The mean and standard errors are provided in (Table 1), as well as the coefficient of variation.

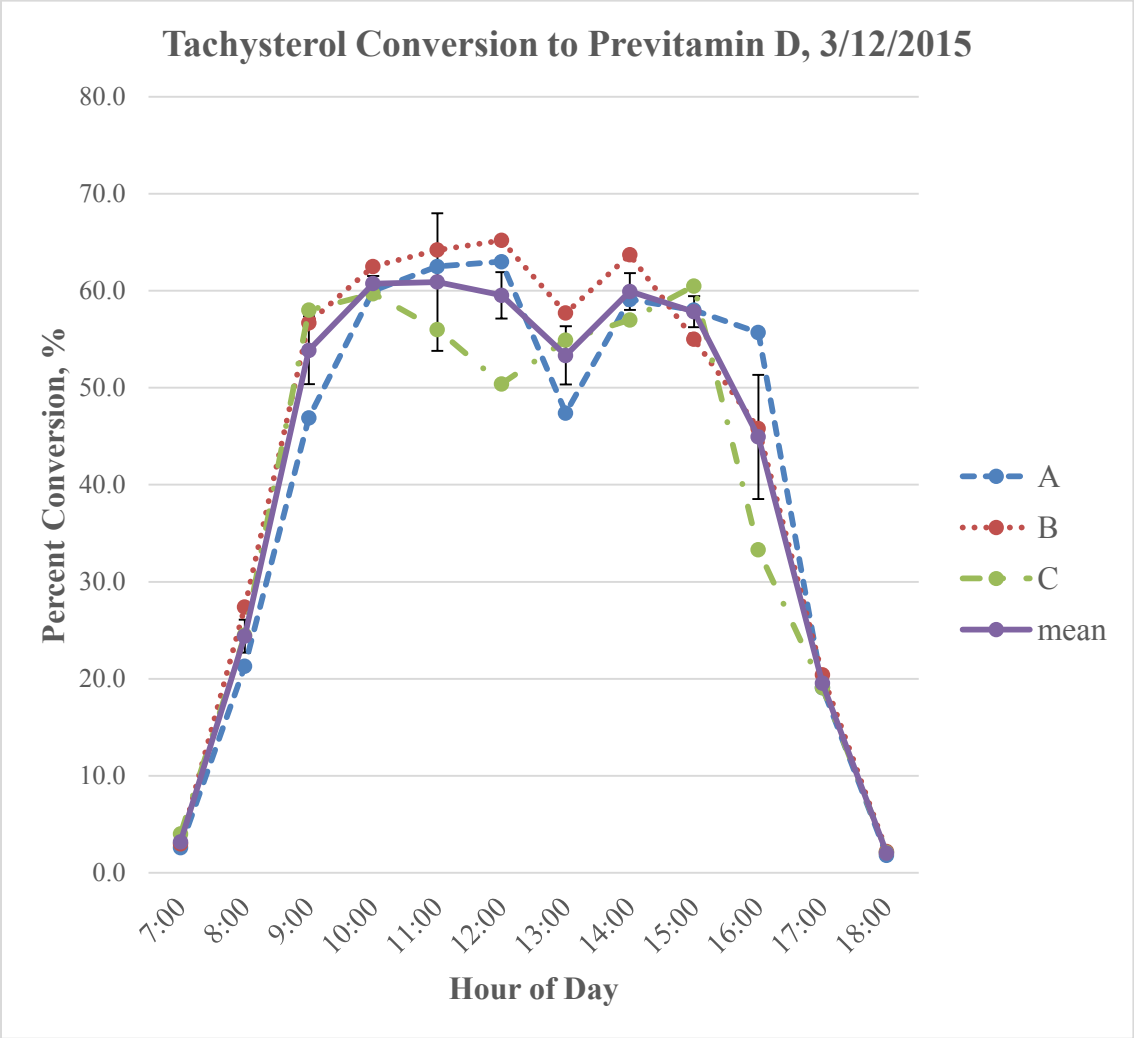


Figure 26 Triplicate Measurements of Tachysterol$_3$ Irradiation on March 12, 2015.

Table 1 Triplicate Measurements and Mean ± Standard Error of Irradiated Tachysterol$_3$ obtained on March 12, 2015

Hour	A	B	C	Mean ± SE	CV
7:00	2.6	3.0	4.0	3.2 ± 0.4	22.5
8:00	21.3	27.4	24.5	24.4 ± 1.7	12.5
9:00	46.9	56.7	58.0	53.9 ± 3.5	11.3
10:00	60.0	62.5	59.7	60.7 ± 0.8	2.5
11:00	62.5	64.2	56.0	60.9 ± 7.1	7.1
12:00	63.0	65.2	50.4	59.5 ± 2.4	13.4
13:00	47.4	57.7	54.9	53.3 ± 3.0	10.0
14:00	59.1	63.7	57.0	59.9 ± 1.9	5.7
15:00	58.0	55.0	60.5	57.8 ± 1.6	4.8
16:00	55.7	45.8	33.3	44.9 ± 6.4	13.0
17:00	19.1	20.4	19.1	19.5 ± 0.4	3.8
18:00	1.8	2.2	2.1	2.0 ± 0.1	10.2
				Avg CV =	10.7

Influence of Cloud Cover

The comparison between the sky conditions showed little to no variation in the amount of previtamin D_3, lumisterol$_3$, and 7-dehydrocholesterol produced from tachysterol$_3$. Average sky coverage was obtained from the National Weather Service, through http://www.noaa.gov/ while photographic evidence is also provided below (Figure 26a). An hour long exposure of tachysterol$_3$ to solar radiation at 11:30 during an overcast day on November 13, 2014, resulted in 53.9% conversion to previtamin D_3, which was comparable to exposure during a clear day, on November 19, 2014 (Figure 26b). During the hour on November 13, 53.9%, 5.7% and 1.4% of tachsterol$_3$ converted to previtamin D_3, lumisterol$_3$, and 7-dehydrocholesterol, respectively. On November 19, 58.4% and 53.2% of tachysterol$_3$ converted to previtamin D_3 during the hours between 11:00 and 12:00 and 12:00 and 13:00, respectively.

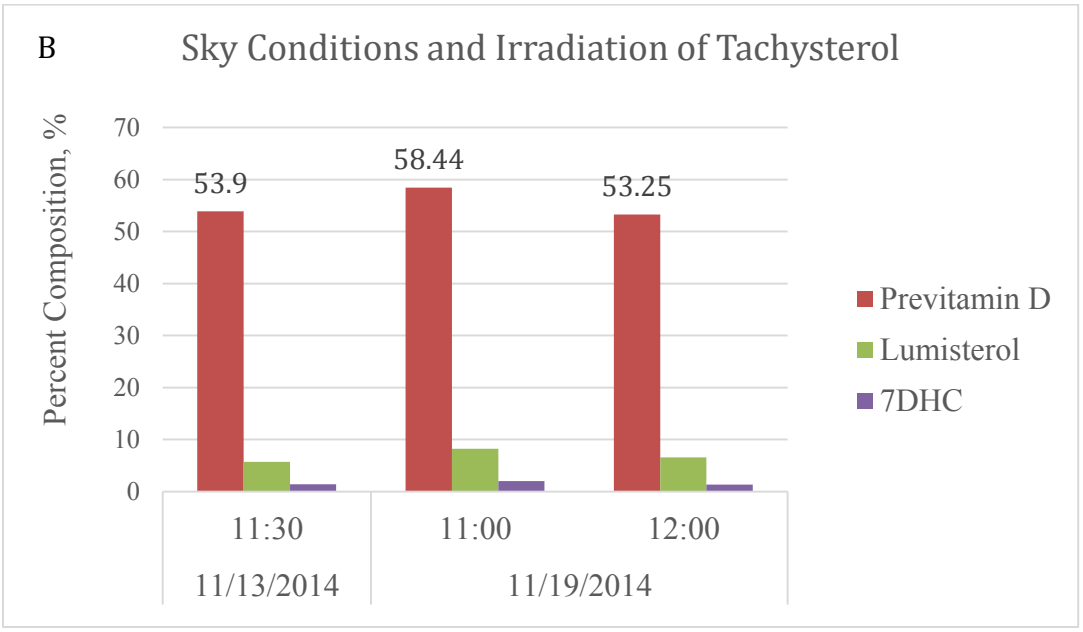


Figure 27 Effect of cloud cover on conversion of tachysterol$_3$.to previtamin D$_3$, lumisterol, and 7-DHC (A) Photographs taken in real time to document the effect of cloud cover on conversion of tachysterol$_3$. The photograph on the left was taken at 11:30 on November 13, 2014 when sky coverage with clouds was at 100%, according to the National Weather Service. The photograph to the right was taken at 11:00 on November 19, 2014. (B) During the hour on November 13, 53.9% of tachsterol$_3$ converted to previtamin D$_3$, compared to 58.4% and 53.2% conversion on November 19, between the hours of 11:00 and 12:00 and 12:00 and 13:00, respectively.

DISCUSSION

Significance of Vitamin D

Vitamin D is known to regulate calcium and phosphorus homeostasis in the human body, thus maintaining optimal extracellular calcium concentrations for nervous and muscular system functioning as well as proper bone mineralization. In addition, vitamin D has been associated with the regulation of numerous proteins and cell processes through the vitamin D receptor's actions in gene regulation. More recently vitamin D has been shown to have an influence on many other diseases throughout the lifespan. As discussed earlier, vitamin D deficiency was an epidemic problem during the industrial revolution, resulting in bone deformities in youth and osteomalacia in adults. Although the emergence of fortified foods and recommendations for sensible sun exposure reduced the grave effects of this epidemic, vitamin D deficiency and insufficiency have recently begun to resurface due to changing lifestyle factors, including an increase in sun avoidance.

Historically, the primary source of vitamin D for humans is through the cutaneous conversion of 7-dehydrocholesterol to previtamin D_3. Although an extremely high dose of vitamin D is required to cause toxicity, cutaneous synthesis of vitamin D has also been shown to safeguard against toxicity through the light-induced conversion of previtamin D_3 to lumisterol$_3$ and tachysterol$_3$, as well as the formation of numerous toxisterols and suprasterols.

Findings of the Study

Although the photochemistry of 7-dehydrocholesterol and previtamin D has been studied extensively, the effect of solar radiation on tachysterol$_3$ and lumisterol$_3$ has not been evaluated. The goal of this study was to determine if winter sunlight at 42ºN, which is incapable of producing previtamin D_3 from 7-dehydrocholesterol, could convert tachysterol$_3$ to previtamin D_3. The data that was retrieved from this study proves that the solar energy tachysterol$_3$ absorbs from 315 to 340nm radiation light excites it enough to induce it to isomerize to previtamin D_3.

It has been previously demonstrated that during Boston's winter months, the total amount of ultraviolet radiation, including the UVB range, declines resulting in no production of previtamin D_3 from 7-dehydrocholesterol. The data from this study's control, the 7-DHC samples, confirmed that when 7-DHC was exposed to fall and winter sunlight in Boston, MA it was not converted to previtamin D_3. This is due to the increasing solar zenith angle as the hemisphere tilts further from the sun during the earth's revolution. The increased angle results in a longer path length for UVB to cross the atmosphere, allowing ozone to absorb much greater UVB radiation. In contrast, ozone does not efficiently absorb UVA radiation, therefore more UVA radiation passes through the atmosphere. In addition to absorption, both ultraviolet A and B radiation are always susceptible to scattering and reflection from particles in the atmosphere and thus a proportion of the direct radiation may either reflect back out of the atmosphere or scatter into the diffuse radiation.

However, when tachysterol$_3$ was exposed to winter sunlight it was effectively converted to previtamin D_3 throughout the course of the winter. Conversion of

tachysterol$_3$ was observed from sunrise to sunset and reached a plateau at 65% around midday on both October 27, 2014 and February 28, 2015. Even on December 20, 2014, when the zenith angle was at its most obtuse and the path-length is at its greatest, roughly twenty percent of the original tachysterol$_3$ had converted to previtamin D$_3$. In addition to previtamin D$_3$, production of lumisterol$_3$ and 7-dehydrocholesterol was also observed, indicating the possibility that a photoequilibrium could be achieved. For these two compounds to appear, previtamin D$_3$ must be absorbing UV radiation greater than 315nm to undergo ring-closure. According to the absorption spectra discussed earlier (Figure 4), the tail end of previtamin D$_3$'s absorption spectrum extends to 325nm, therefore these UVA photons were able to excite previtamin D$_3$ with enough energy to undergo ring-closure.

The time course produced over the several months show that conversion of tachysterol$_3$ to previtamin D$_3$ begins as soon as the sun rises and plateaus at roughly 65% of the composition. Although the conversion to previtamin D$_3$ reached a plateau within 40 minutes, the time of day, the tilt of the earth, and the latitude of the location will greatly influence the amount of radiation and its wavelength composition reaching the surface. Therefore these factors will greatly influence the amount of time it will take for this plateau to occur. A more controlled study with constant radiation is needed to determine how much energy is required for photoequilibrium to be reached and the percent composition of each isomer.

The preliminary results have shown that even on a cloud covered day, tachysterol$_3$ was effectively converted to previtamin D$_3$. When comparing the overcast sky conditions

with 100% cloud cover to the clear conditions with just 2% cloud cover, an equivalent amount of previtamin D_3 was produced. The irradiations were performed as soon as possible within the same week to minimize any effect from the solar zenith angle. It is known that UVA radiation passes through clouds more efficiently when compared to UVB radiation. Unfortunately, there were no measures to control against any diffuse radiation that could have influenced the total irradiation.

The findings of this study support the hypothesis that tachysterol can be converted to previtamin D after absorbing UV radiaition in the 315-340nm range. Currently, people living greater than 32º in latitude from the equator must rely on supplementation to maintain their vitamin D status during the winter months. This supplementation usually takes the form of fortified foods or pills. Unfortunately, people suffering from fat malabsorption syndromes, such as Crohn's disease or cystic fibrosis, are incapable of effectively absorbing this fat-soluble vitamin from their diet. This data supports incorporating tachysterol in a topical preparation as an alternative form of supplementation that may be used during the winter for everyone, including those suffering from the aforementioned fat malabsorption syndromes. During this summer, this preparation may not only be used as a supplement, but may also serve as a functional sunscreen as well. In addition to tachysterol$_3$, the newly formed previtamin D_3, lumisterol$_3$, and 7-dehydrocholesterol may absorb ultraviolet radiation before entering the skin and thus preventing these photons from damaging DNA and causing oxidative stress.

Limitations

Although this study may serve as a proof-of-concept for tachysterol's capacity to absorb UVA radiation and isomerize to previtamin D, due to the design of this study, several limitations still need to be addressed. First, better control of confounding factors must be implemented. Although Boston, MA, USA experiences relatively little pollution in the United State, not accounting for this factor may underestimate the conversion of tachysterol$_3$ to previtamin D$_3$. The amount of ozone within the atmosphere was not accounted for either and would have the same effect as pollution. In addition, due to the lack of access to a more open location for irradiation, we could not control for any reflection that may have occurred off the surroundings or shadows cast onto the samples by neighboring buildings or structures.

Future Directions

In addition to addressing the limitations of this study, it is also important to identify any possibilities to further develop this theory. Reproducibility must be demonstrated at other latitudes, while the influence of other environmental conditions must be addressed. Given its absence within the literature, establishing an action spectrum for tachysterol$_3$ will help better inform us on how this compound will interact with ultraviolet radiation at different points in the year and at different environmental conditions. It will also provide us with a model that could be used to formulate hypotheses for clinical applications.

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List of Abbreviated Journal Titles

Am J Clin Nutr	American Journal of Clinical Nutrition
Ann New York Acad of Sci	Annals of New York Academy of Science
Arch Biochem Biophys	Archives of Biochemistry and Biophysics
Dermatol Clin	Clinics in Dermatology
J Biol Chem	Journal of Biological Chemistry
J Bone Miner Res	Journal of Bone and Mineral Research
J Cell Biochem	Journal of Cellular Biochemistry
J Chromatogr A	Journal of Chromatograph A
J Clin Endocrinol Metab	Journal of Clinical Endocrinology and Metabolism
J Clin Invest	Journal of Clinical Investigation
J Nutr	Journal of Nutrition
J Org Chem	Journal of Organic Chemistry
J Phys Chem	Journal of Physical Chemistry
J Phys Chem Lett	Journal of Physical Chemistry Letters
Mayo Clin Proc	Mayo Clinic Proceedings
Mol Aspects of Med	Molecular Aspects of Medicine
N Engl J Med	New England Journal of Medicine
Proc Natl Acad Sci	Proceedings of the National Academy of Sciences
Pure & Appl Chem	Pure and Applied Chemistry

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